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Singh et al. Scr Med 2023 Mar;54(1):1-7. DOI:10.5937/scriptamed54-42288

EDITORIAL

The Twelve Fundamental Dimensions of a High Quality Indo-Mediterranean Diet

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Abstract

High-quality Indo-Mediterranean foods are characterised with unrefined, unprocessed foods, whole grains such as dry millets and beans and porridge. Vegetables such as leaves and gourds, nuts and fruits such as apples, grapes, papaya, guava, etc are major components of this diet. It seems that healthy vegetable oils such as olive, mustard, rape seed oil and protein sources that are beneficial; beans, pulses, cottage cheese are crucial in this diet. Among animal sources, white meats; such as sea foods should also be part of this diet. Vegetables and spices with medicinal properties; gourds, turmeric, fenugreek, coriander and cumin may also be called high quality nutraceutical foods, respectively. There is a need to find out traditional foods from all other countries which may be protective and healthy. There is evidence that calories have tremendous role in weight gain and weight loss. It seems that emphasis on food quality is crucial for prevention of oxidative stress and inflammation in the adipocytes, which predispose obesity and risk of cardiovascular diseases (CVDs) and diabetes. There are gaps in knowledge about the qualities of traditional foods, which prompt authors to present this communication.

Key words: Flavonoids; Inflammation; Chronic diseases; Dietary guidelines; Antioxidants.

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Introduction

It has been established in the Indian Ayurveda (Charak Samhita, Chap 5, Sutra 12,500 BCE), that green beans and porridge are super foods to achieve health and wellness. These foods are important component of Indo-Mediterranean diet.[1] Green beans are source of specific carotenoids that are known to inhibit oxidative stress and prevent chronic diseases such as cardiovascular diseases (CVDs), macular degeneration and improve the microbiota in the gut. Green bean consist of carotenoids like lutein and zeaxanthin which help to prevent oxidative stress on the inner workings of eyes. Green beans are richer in vitamins E and K, vitamin B5, calcium and sodium. Wheat porridge is also rich in flavonoids which have anti-inflammatory effects.[2] The wheat grain contains ellagic acid, ferulic acid, chlorogenic acid, syringic acid, vanillic acid, p-coumaric acid, caffeic acid and gallic acid that were the most abundant phenolic acids. Other flavonoids in wheat (luteolin, chlorogenic acid, caffeic acid and apigenin) could predict inhibition percentage by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay, suggesting a possible role

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in the cellular defence against oxidative stress in wheat.[2] This article aims to highlight the role of the twelve functional dimensions of the Indo-Mediterranean foods which may be determinant of beneficial effects of high quality diets.

High-quality Indo-Mediterranean foods are chara-cterised with unrefined, unprocessed foods, whole grains such as dry millets and beans and porridge, vegetables such as leaves and gourds, nuts such as walnuts and almonds, fruits such as apples, grapes, papaya, guava, healthy vegetable oils such as mustard, rape seed oil or olive oil and healthy sources of proteins; pulses, beans, cottage cheese, white meats, fish and other sea foods.[1-3] Vegetables and spices with medicinal properties such as gourds, turmeric and millets, may also be called high quality nutraceutical foods, respectively. These foods may be also recommended in the modified, Healthy Eating Plate.[3] It seems that guidelines on dietary intakes have changed during the last decade due to better accuracy in research in finding out suitable foods that may be consumed to maintain health and ideal body weight without obesity and central obesity, with minimal inflammation in the body tissues.[1-5] Cohort studies indicate that energy intake appears to have significant role in weight gain and weight loss. However, attention should be on food quality, which is crucial for inhibition of oxidative stress and inflammation in the adipocytes, that predispose obesity due to various pro-inflammatory qualities of foods.[3, 4]

Changing Dietary Guidelines in Countries

The Healthy Eating Plate as a guide for creating healthy balanced meals; whether served on a plate or packed in a lunch box has been advocated.[3] The International College of Cardiology and International College of Nutrition advise to modify existing guidelines of National Cholesterol Education Program by advising more of whole grains such as millets and spices and lower intake of fatty acids blended with rape seed or mustard and olive oil, similar to Japanese, Mediterranean and traditional Indian diets.[1, 2] The World Health Organization (WHO) advises that any healthful diet should be comprised of healthy fat (30 % of calories) and rest from proteins (12-15 %) and complex carbohydrates.[1-3] This means eating about 50 to 80 grams of fat daily for health promotion providing, calories and fat for requirement of physiology and metabolism. Since human body alters approximately 10 % of fatty acids in the gluconeogenesis, rest of the amount of fat is used for physiological functions in the tissues. Among Asians, the fatty acids intake varies between 15-25 % in the traditional diets. The Japanese have the longest life expectancy, therefore, some experts have advised lower intake of fat.[1, 2] The fat intake is also lower (20-30 % / day) among Koreans, Chinese and people living in South Asia, showing no poor health.[1] It has been proposed in 1996 that the limits of waist circumference and body mass index for the identification central obesity and obesity, should be lower for populations living in Asia.[1] Thus, the guideline for consumption of fat for Asians are lower.[1, 2] Therefore, most of the health agencies have proposed food based guidelines.[3-6]

There are variations in the biologic effects of saturated fatty acids (SFA), depending upon quality that may be modified via food matrix and content of carbohydrate in the foods.[6] It seems that foods, such as dark chocolate and whole-fat dairy, although rich in fat, but may not predispose greater risk of diabetes and CVD, possibly due to diversity of foods and slow absorption rate. There is a scarcity of evidence about upper limits on the consumption of SF in the diets that can cause decline in CVD and all-cause mortality. However, cohort studies and randomised trials reported that diet quality may have a role in the health promotion as well as in disease prevention.[6-10] There is a significant reduction in cardiovascular events and all causes of mortality in all the controlled trials, using Mediterranean style diets as intervention.[11-14]

It seems that high quality foods with high nutrient density with greater diversity of foods and low glycaemic index are important characteristics of the Indo-Mediterranean style diets. This diet also contain lower saturated fat, trans fat, sugar and salt, but high PUFA including omega-3 fatty acids and flavonoids with other foods known to activate release of nitric oxide, that may be protective against diabetes and CVDs, as well as other NCDs.[6-14] Interestingly, the role of Indo-Mediterranean style diets in the prevention of CVDs, has also been reported in a meta-analysis of studies.[11] The intervention and control groups in these trials, were compared for behavioural risk factors, food intakes, fatty acid intake and on ratio of polyunsaturated fatty acid (PUFA)/ flavonoid intake respectively in the two groups (n = 1,446 vs 1,320). The results revealed marked beneficial effects of Indo-Mediterranean style

Table 1: The twelve qualities of the high quality Indo-Mediterranean diet [23]

Qualities of foods	Examples of foods
1. Slowly absorbed foods	Nuts, vegetables, whole grains
2. Food diversity	Whole grains, beans, vegetables
3. High nutrient density	Nuts, vegetables, whole grains
4. No trans fat	Grilled foods, boiled foods
5. No/low sugar refined	Use dates, apples, papaya, oranges, raisins
6. Low salt	Fruits, vegetables, nuts
7. Moderate fat	Nuts, pulses, beans, green leaves
8. High fibre	Vegetables, whole grains, fruits
9. Beneficial effects on gut microbiota	Vegetables, whole grains, fruits
10. Non per-oxidised foods	Fresh foods, without frying
11. Spices, 15-30 g/day	Turmeric, cumin, coriander, fenugreek, etc
12. Foods requiring mastication, day time eating	Whole grains like porridge, nuts, fruits, snacks with millets

foods and nutrients on arrhythmias and heart failure.[11] It seems that a large body of evidence has shown that a diet rich in healthy plant based foods and with fewer animal source foods; fish other sea foods and poultry; up to five servings of plant sources of food per week, can confer both improved health and environmental benefits.

It seems that Earth has been considered as "Mother Earth", in Mesopotamia and Indus Velley ancient civilisations (7000 BCE). Hence, Earth should be protected from change in climate that can occur due to unbalanced use of natural resources for human development. It is the quality of food intake and functional food production which would determine the effects of The Paris Agreement and Sustainable Development Goals (SDGs), as well as the safety of farming with reference to how much food is wasted or lost and how much earth is saved without environmental degradation.[4] The EAT-Lancet Commission has made scientific targets sustainable food systems and for healthy diets.[4] Interestingly, achieving functional foods, for 10 billion population, by 2050 from sustainable food systems is possible, if and only if, there is a significant decrease in the animal source of foods, specifically in G20 countries. There is an urgent need of universal increase in healthy plant-based foods and possibly sea foods in our diets. It is possible that the Planetary Health Diet, based on plant foods and sea foods may optimise health and nutrition, while reducing environmental degradation.[4] The patterns of food intake in most of the G20 countries have differences; hence, country based guidelines are not successful, in bringing food systems to reduce global warming to 1.5 °C.[1, 2, 5-7]

The world's population is facing double burden of diseases due to undernutrition as well as over-nutrition. There are 820 million people having food scarcity and consuming unhealthy foods. It seems that there is an urgent need to shift dietary patterns towards healthful traditional foods with sustainable functional crops to alter the trends of unhealthy global diets.[15] It is possible that health education of public as well as governments on Indo-Mediterranean foods and Japanese foods with reference to traditional foods in the concerned country are important steps for prevention of the risk of NCDs.[15] It may be prudent to be selective on healthful foods which are known to have the Twelve Best Qualities of High Quality Indo-Mediterranean Foods (Table 1).

Diversity of Foods in the Indo-Mediterranean Diet

It is possible that the relation between quality of foods, characterised with diversity of foods and nutrient adequacy with overall status of health and development of diseases are graded based on food quality scores.[1-4, 16] A simple count of intake of foods or food groups over a given reference period can determine food diversity, giving due emphasis on traditional diets. Epidemiological studies have shown a consistent positive association between diversity of foods and growth

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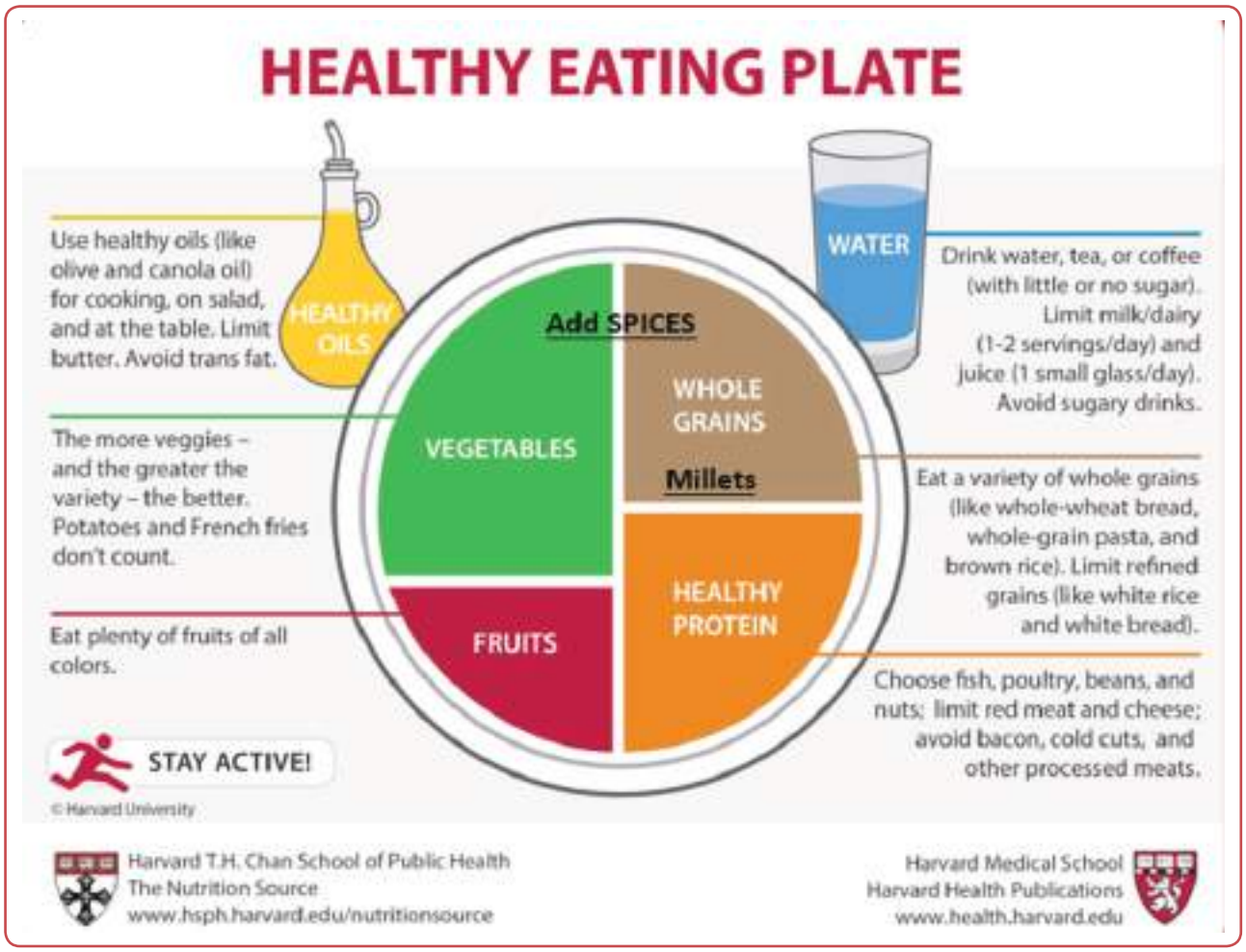

Figure 1: The best diet, quality counts. Harvard TH Chan School of Public Health[3]

of infants and children. Further evidence from a multi-country analysis suggests that diversity of foods at household-level is strongly associated with social class and availability of total energy from food quantity may be an indicator of food security.[16] It seems that contribution of nutrients such as iron, calcium and protein from animal foods has sufficient nutrient adequacy. In addition, Indo-Mediterranean plant foods can provide all the Twelve Qualities of High Quality Foods which may be adequate for the growth of infant and children as well as for prevention of chronic diseases in later adult life.[11-14] The growth of infant and children with emphasis on peri-conception and perinatal factors and socioeconomic status of the family and community are known to influence food consumption as well as food production.

Some experts have suggested The Healthy Eating Plate, which appears to be an interesting approach for providing healthful diets with diversity of foods to communities, which may include traditional foods of the concerned community and country (Figure 1).[3]

Glycaemic Index

In many developing countries, grain-based carbohydrates may constitute approximately 60 % of total energy consumption compared with 42 % for Caucasians. The intake of refined carbohydrate diets, causes hyperglycaemia and insulin response, which may contribute to insulin resistance.[1, 2] Poor quality and refined carbohydrates are quickly digested and absorbed, thereby giving rise to high blood glucose and insulin induced spikes with pro-inflammatory cytokines. Observational studies have shown that the consumption of low glycaemic index (GI) foods is associated with a lower risk of type 2 diabetes mellitus, significantly less insulin resistance and a lower prevalence of the metabolic syndrome.[1-4] The concept of GI may provide a useful marker to select the most appropriate carbohydrate-containing foods or for the maintenance of health and the treatment of several diseases.[17] Eating a high carbohydrate, rapidly absorbed foods may be associated with rapid increase in triglycerides, free fatty acids, with increase in nuclear factor kb

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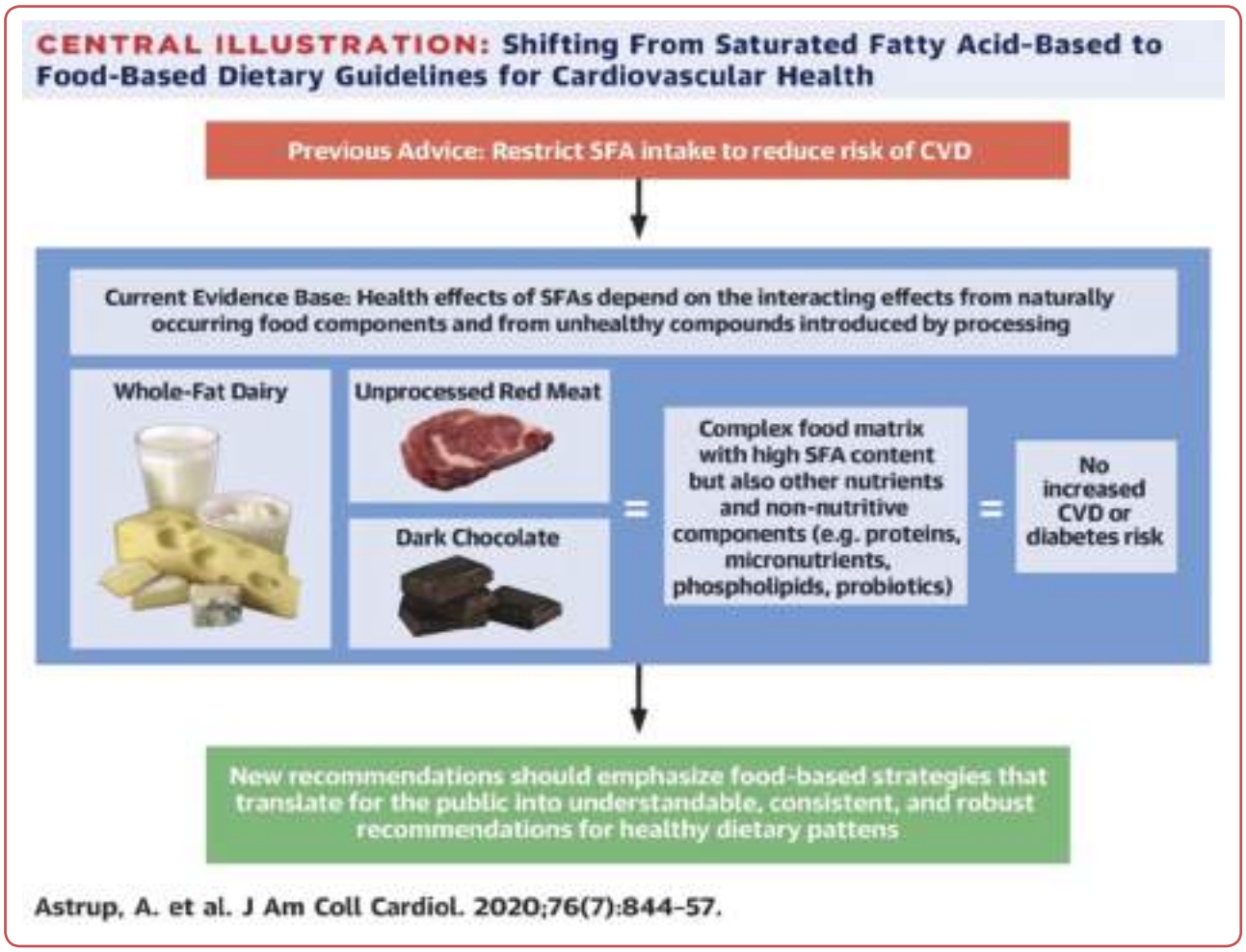

Figure 2: Effects of fatty acid intake on cardiovascular diseases[5]

(NFbkB0), a transcriptional factor regulating the activity of at least 125 genes, most of which are pro-inflammatory. High GI foods also increase pro-inflammatory transcription factors; activating protein-1 (AP-1) and early growth response protein-1 (Egr-1) which are markers of dysfunction of endothelial cells and adipocyte.[17]

The GI is a numerical figure to show the ability of a highly absorbed food to increase blood glucose level. It is expressed as a percentage of the incremental area under the glycaemic response curve (AUC) produced by a food portion with 50 g carbohydrate compared with the AUC shown by a standard reference food of 50 g glucose or sugar or white bread.[17] If the rate of absorption of carbohydrate is slow, into the blood, there would be lesser rise in blood glucose level with the lesser GI value. A GI value ≤ 55 is low and a GI value 56–69 inclusive is medium and the value of GI of ≥ 70 is considered high, where glucose GI = 100.[17] It is possible that food-based intervention using GI could be an important tool in the management of diabetes. The foods that have low GI such as whole, grains, nuts and vegetables may reduce insulin demand and lipid concentrations, causing improvement in blood glucose and reduction in body weight. The intake of such foods may prevent glycaemia induced cardiovascular complications. It is known that traditional foods in most Asian and some of the European and African countries, have lower GI. However, all kinds of fast foods, including some snack foods, such as chips, crackers, tater tots and French fries, some spreads, such as margarine spreads or peanut butter and cookies have higher GI. The GI data of Asian, African and East European countries are scarce.[17]

Dietary Fat

Most of the developed countries, follow the limits given by American guideline for total fat intake, which is 30 % of total calories intake including

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10 % energy from saturated fat.[4-7] The limit for intake of trans fat in foods is up to 3 % in most guidelines. The limit is higher in countries dependent on ready prepared foods provided by the industry.[1-4] There are several options made available for eliminating or reducing trans fats, saturated fat and salt in the food-supply chain. It explores how such policies could contribute to decreasing the disease burden caused by intake of industrially produced trans fats in the WHO European Region. Despite some evidence of no adverse effects, the advice to limit intake of saturated fat is persisting, due to its low glycaemic index and diversity of foods (Figure 2).[5, 7]

Diet and Gut Microbiota

It seems that some vegetarian plant foods rich in whole grains, nuts, vegetables and fruits as well as microalgae may produce beneficial effects on gut microbiota, whereas meat intake may cause harmful effects.[18] It is also proposed that eating a flexitarian diet; containing excess of plant foods along with moderation in meat may not be unhealthy, which could be a healthy approach for health promotion with improved SDGs.[4, 5, 18-20] The shape of gut microbiota may be improved via healthy diet and lifestyle factors that are crucial for the development of microbiome.[18-21] In a cohort of 441 Colombians, microbial diversity was greater in subjects with greater consumption of nutrients from sources of plant-food.[18] The consumption of food groups and nutrients correlated with structure of the microbiota. The communities of microbes, producers of short-chain fatty acid (SCFA) were more common in the microbiota of subjects eating diets rich in fibre and plant-foods, such as beans, vegetables and fruits.[18] Surprisingly, an inflammatory microbiota composed of putrefactive microorganisms and bile-tolerant and along with opportunistic pathogens thrived in subjects eating diets of animal-food sources and of ultra-processed foods and lower in fibre.[18] It is possible to conclude that diet is strongly linked with the gut microbes and emphasise connections between health and microbiota.

Diets for Healthy Life Expectancy and Sustainability

It seems that the diets for good health in future and improved life expectancy are determined by sustainability and health effects of diets and foods consumption.[4-7, 18-20] It is proposed that dietary transitions to Indo-Mediterranean foods can reduce, food-related greenhouse gas emissions and provide improved distribution of these emissions within planetary boundaries.[4, 15] These efforts are likely to address all forms of undernutrition as well as over-nutrition. The countries such as India, China and Indonesia that have dense population, appear to have food intake patterns, that is crucial to protect health and the planet. This state of food pattern may be due to scarce modernisation and continuation of traditional food intake pattern and traditional lifestyle with greater occupational physical activity.[1, 2] However, this situation may change due to attack by advertising food industry.[4, 15] The role of diet qualities have been well emphasised,[20, 21] because these diets can modulate immune function which is a worldwide problem.[22-24] It is proposed that there are clear opportunities for all health agencies, to reduce greenhouse gas emissions and realise the health and related economic benefits of shifting toward more healthy traditional and sustainable Indo-Mediterranean type of diets, to serve the Sustainable Development Goals of the United Nations Organisation (UNO).

Conclusion

It is possible that the twelve protective features of a high quality diet may be crucial, for healthiness and healthy life expectancy. Functional food diversity from traditional food sources, in particular with dry whole grains such as millets and beans, vegetables, nuts, fruits, spices, with no trans-fat and little salt and low glycaemic index are important for health promotion and disease prevention.

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None.

Conflict of interest

None.

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ORIGINAL ARTICLE

Antioxidative Potential of Pomegranate Peel Extract: *In Vitro* and *In Vivo* Studies

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Abstract

Background/Aim: Due to the numerous beneficial effects of pomegranate that can be explained through its antioxidative effects, the aim of this study was to determine the antioxidant potential of pomegranate peel extract (PoPEx) prepared from pomegranate that was harvested in the south-east region of Herzegovina (Bosnia and Herzegovina), through *in vitro* and *in vivo* studies.
Methods: In PoPEx total phenols, flavonoids, flavonols, flavan-3-ols and anthocyanins content was determined, as well as several antioxidative assays, including 2,2 diphenyl-1-picrylhydrazyl assay (DPPH), 2,2'-azino bis(3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS), iron (III) - 2,4,6-tripyridyl-S-triazine complex assay (FRAP), reduction of copper(II) ions (CUPRAC) assay, Briggs-Rauscher oscillatory reactions, neutralisation of OH radicals and lipid peroxidation assay. *In vivo* studies were performed by administrating 100 mg/kg of body weight of PoPEx to the rats by gavage for 7 days, after which the rats were euthanised and prooxidative parameters (thiobabrituric acid reactive substances - TBARS as an index of lipid peroxidation, nitrites - NO_2, hydrogen peroxide - H_2O_2 and superoxide anion radical O_2^-) were determined in plasma, as well as antioxidative parameters (superoxide dismutase - SOD, reduced glutathione - GSH and catalase - CAT) in erythrocyte lysates.
Results: High content of phenolic compounds was found in PoPEx, which resulted in high antioxidative potential in all *in vitro* tests performed. *In vivo* study showed that PoPEx administration caused a significant decrease in TBARS, NO_2^-, as well as an increase in reduced glutathione ($p < 0.05$) in comparison to the control group, while H_2O_2 and O_2^* showed a lowering trend and SOD and CAT showed an increasing trend in PoPEx group, but without statistical significance.
Conclusion: PoPEx demonstrated high antioxidative capacity measured *in vitro* and *in vivo* and can be potentially used as a supplement treatment in the prevention of various inflammatory conditions.

Key words: Pomegranate peel extract; Antioxidative capacity; Phenolic compounds.

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Introduction

Pomegranate (*Punica granatum* L.) is a fruit-bearing tree that is cultivated and consumed worldwide. It is used in folk medicine and has scientifically proven beneficial effects in diseases such as diabetes mellitus type 2,[1] cardiovascular diseases,[2, 3] inflammatory diseases[4] and even cancer.[5] Knowing that non-communicable diseases are the leading cause of morbidity and mortali-

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ty worldwide,[6] there is a high interest in finding potential agents that could be used in the prevention and/or treatment of these diseases. The main active compounds in the pomegranate are different polyphenolic antioxidants such as anthocyanins, tannins and flavonoids.[7] Although pomegranate is mostly consumed in the form of juice produced from the aerial parts of the plant, more than 40 % of the fruit is its peel, which is usually considered as waist.[8] However, studies have shown that the pomegranate peel has 48 different polyphenolic compounds,[9] that have been proven to have high antioxidative capacity.[2, 7, 10] Gallic and ellagic acid, ellagitannin, punicalin, punicagalin, anthocyanins, flavonoids and other phenols, are found in abundance in pomegranate peel extracts (PoPEx) and have been shown to exhibit various biologically beneficial properties such as hypoglycaemic, lipid-lowering, anti-inflammatory, antihypertensive, antimicrobial and antioxidant effects.[11-14]

Therefore, the aim of this study was to investigate the antioxidative potential of a PoPEx produced from pomegranate plants collected in the south-east Herzegovina region using various *in vitro* techniques and an *in vivo* study.

Methods

Materials

Fruits of the pomegranate (*P. granatum*) were harvested in the south-east Herzegovina region (the Republic of Srpska, Bosnia and Herzegovina).

Preparation of PoPEx dry extract

The fruits were washed by hand after which the peel was removed from the fruits and dried for 4-6 days and grounded into fine powder. The pomegranate peel dry extract was prepared using 70 % (v/v) ethanol as a solvent and the method of triple percolation was performed, followed by evaporation of the solvent using a vacuum oven.

Liquid chromatography – mass spectroscopy (LC-MS) chemical analysis

The LC-MS analysis of PoPEx was performed with an Agilent Technologies HPLC 1260 Infinity system coupled with a single quadrupole mass detector (Singlequad MS detector 6130). Compounds were separated on Zorbax SB Aq-C18 column (3.0 150 mm; 3.5 m) at a temperature adjusted to 25 ºC. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B) and a gradient elution was applied at a flow rate of 0.3 mL/min. The following gradient program was utilised: 0–30 minutes from 10 % to 25 % B, 30–35 minutes from 25 % to 70 % B and 35–40 minutes from 70 % B to 10 % B. The detection wavelengths were 280 and 350 nm and the range of m/z was 50–2000. The electrospray ionisation method was used to ionise the sample at a pressure of 40 psi, a temperature of 350 ºC, and a flow rate of 10 L/min of nitrogen. Signals from deprotonated molecules and fragmented ions were acquired in full-scan mode at voltages of 100 V and 250 V.

Total phenols, flavonoids, flavonols, flavan-3-ols and anthocyanins determination

For the determination of total phenols, flavonoids, flavonols and *in vitro* antioxidative tests, different concentrations of ethanolic (80 % v/v) solution of PoPEx were used. Total phenols were determined using the spectrophotometric method of Folin-Ciocalteu,[15] by measuring the absorbance at 765 nm and the results were expressed as gallic acid equivalent per dry weight (mgGAE/g_{DW}). Flavonoids were determined spectrophotometrically at 420 nm[16] and expressed as quercetin equivalents per dry weight (mgQc/g_{DW}). Flavonols were determined according to Kumaran and Karunakaran[17] spectrophotometrically at 510 nm and expressed as quercetin equivalents (mgQc/g_{DW}). The method of Revilla et al[18] was used for the determination of total flavan-3-ols. For the determination of total and monomeric anthocyanins, "single" pH method and pH differential methods were used.[19] The content of total and monomeric anthocyanins is expressed as mg cyanidin-3-glucoside (C3G)/g_{DW}.

In vitro determination of antioxidant potential

(1) 2,2 diphenyl-1-picrylhydrazyl (DPPH) method

The antioxidant capacity of the examined samples of PoPEx was determined by measuring its antioxidant capacity to reduce DPPH radicals.[20, 21] $DPPH^{\cdot}$ is a stable free radical that has the ability to delocalise a free electron over the entire molecule. This is the reason why dimerization of $DPPH^{\cdot}$ does not occur, as it happens with most free radicals and because of which the purple coloration of the solution oc-

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curs with the absorption maximum at around 520 nm. The reaction of DPPH$^{\cdot}$ with a hydrogen donor creates a yellow-coloured reduced form of the radical (diphenyl picrylhydrazine) and the consequence is the loss of purple coloration. Absorption decrease is linearly dependent on antioxidant concentration.

(2) 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method

The antioxidant capacity of the tested samples was determined by measuring their efficiency in reduction of ABTS$^{\cdot}$+ radicals. ABTS is a compound that upon oxidation with potassium peroxodisulphate ($K_2S_2O_8$) gives a dark-blue cation radical ABTS$^{\cdot}$+, with an absorption maximum at 734 nm. The resulting ABTS$^{\cdot}$+ cation radical reacts with antioxidants, decolourising them and the decrease in colour intensity is proportional to the amount of antioxidants present.[22]

(3) Ferric reducing antioxidant power (FRAP) method

The FRAP method is based on the ability of antioxidants to reduce the iron (III) - 2,4,6-tripyridyl-S-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ in the acidic medium.[23] FRAP values were calculated by measuring the increase in absorbance at 593 nm and comparing them with a standard solution of coloured ions, or a standard solution of antioxidants (Trolox).

(4) Cupric reducing antioxidant capacity (CUPRAC) method

The CUPRAC method[24] is based on monitoring the reduction of copper(II) ions, which with neocuproine (2,9-dimethyl-1,10-phenanthroline), in a neutral environment, builds colourless bis (neocuproine) copper(II)) chelate complex, Cu(II)-Nc. In the presence of a reducing agent, Cu(I)-Nc is formed, an orange-red complex compound that shows an absorbance maximum at 450 nm.

(5) Briggs-Rauscher reactions

The mechanism of Briggs-Rauscher oscillatory reactions is quite complex and takes place through two processes, one of which is radical and the other non-radical.[25] Briefly, the Briggs-Rauscher reaction represents the oxidation of malonic acid in the presence of hydrogen peroxide and iodate in an acidic medium, which is catalysed by manganese ions. In doing so, various reaction intermediates are created. The main intermediates in the reactions are the iodide ion, the oxoiodine species HOI, HOIO and IO• and the hydrogen peroxide radical HOO•. The addition of various antioxidants to the reaction mixture leads to the "neutralisation" of free radicals, synthesised in the reactions, which disrupts the kinetics of the reactions. Oscillations in Briggs-Rauscher reactions were monitored potentiometrically by measuring the potential of the tested mixture using a platinum electrode and a reference electrode $Ag/AgCl/Cl_{sat}$. (+197 mV compared to saturated hydrogen electrode SHE). The results were expressed as mmol Trolox/g extract.

(6) Neutralisation of OH radicals

The method is based on measuring the degree of degradation of 2-deoxy-D-ribose under the influence of the hydroxyl radical generated in the Fenton reaction. The resulting reactive hydroxyl radical in the presence of 2-deoxy D-ribose and oxygen builds malondialdehyde, which is determined in the thiobarbituric acid (TBA) test. In doing so, a pink coloured complex with maximum absorption at a wavelength of 530 nm is formed.[26] The original method was modified because it was found that organic solvents, especially ethanol, significantly increase the degree of inhibition of OH radicals.[27, 28]

(7) Lipid peroxidation

As the primary products of lipid peroxidation, unstable hydroperoxides are formed, which break down to give secondary compounds, one of which is malondialdehyde. TBA was used for its determination. Two molecules of TBA and one molecule of MDA participated in this reaction, resulting in a pink complex with an absorption maximum at around 530 nm.[29]

In vivo studies of antioxidant potential

(1) Experimental animals

For the in vivo studies of antioxidant potential, male Wistar rats (n = 11) with body weight (bw) of 250 ± 25 g were used. The study was conducted at the Centre for Biomedical Research of the Faculty of Medicine, University of Banja Luka. The laboratory animals were kept in standard laboratory conditions with ambient temperature of 21 ± 2 ^{0}C, humidity of 55 ± 5 % and 12/12 h light/dark cycle with water and food access ad libitum. The study

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was approved by the Ethics Committee for the Protection and Welfare of Experimental Animals in Biomedical Research, Faculty of Medicine, University of Banja Luka (Decision No 18/1.51/21-7; 9th March 2021).

(2) Experimental design

The animals were divided into two groups:
1. Control (C) group (n = 5) which received 0.5 % Na-carboxymethyl cellulose water solution (1 mL/kg) for 7 days by gastric gavage and
2. P100 group (n = 6) which received 100 mg/kg PoPEx for 7 days by gastric gavage.

At the end of experiment, on eighth day the animals were anesthetised with a combination of ketamine (30 mg/kg bw) and xylazine (5 mg/kg bw) intraperitoneally (ip) and euthanised. Blood was collected into 3.2 % Na-citrate tubes, after which plasma was separated by centrifugation at 3,000 rpm for 10 minutes and the erythrocytes were lysed using deionised water. Plasma was used for the determination of O_2^-, H_2O_2, NO_2^-, TBARS and erythrocyte lysates were used the determination of SOD and catalase CAT activity as well as for the amount of total reduced glutathione (GSH).

(3) Determination of superoxide anion radical (O_2^-)

The determination of the amount of superoxide anion radical (O_2^-) in plasma was based on the reaction of O_2^- with nitro tetrazolium blue (Nitro Blue Tetrazolium - NBT) to nitroformazan blue.[30] Spectrophotometrically measurement was performed at the wavelength of maximum absorption λmax = 550 nm.

(4) Determination of hydrogen peroxide (H_2O_2)

The determination of the amount of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red using hydrogen peroxide in peroxidase from horse radish enzyme-catalysed reaction (Horse Radish Peroxidase - HRPO). This reaction resulted in the formation of a compound whose maximum absorption was λmax = 610 nm.[31]

(5) Determination of lipid peroxidation index (TBARS)

The lipid peroxidation index, as one of the parameters of oxidative stress, was determined indirectly through the products of the lipid peroxidation reaction with thiobarbituric acid. The level of TBARS in plasma was determined spectrophotometrically at 530 nm.[32]

(6) Determination of nitrites (NO_2^-)

Biochemically, this method was based on the use of Griess-reagent, which with nitrites builds a diazo-complex that gives a purple colour. After colour stabilisation at room temperature for 5-10 minutes the concentration of released nitrites were measured spectrophotometrically at wavelength of λ = 550 nm.[33]

(7) Determination of superoxide dismutase (SOD) activity

Superoxide dismutase was determined by the adrenaline method according to Beutler. The measurement was carried out spectrophotometrically at the wavelength of λ = 470 nm.[34]

(8) Determination of catalase (CAT) activity

Catalase was determined by measuring the decrease in absorbance for 1 minute at 240 nm of the reaction mixture that was comprised of the sample and 10 mM hydrogen peroxide.[35]

(9) Determination of reduced glutathione (GSH)

The activity of reduced glutathione, was measured in erythrocyte lysate using a spectrophotometric method. This method was based on the reaction of oxidation of glutathione with 5.5-dithio-bis-6.2-nitrobenzoic acid, according to the method of Beutler.[36]

Statistical analysis

In vitro experiments were performed in 3 parallel repetitions and the results were expressed as mean value ± standard deviation. IC50 values were calculated by linear regression analysis using Origin 6.0 software. All *in vivo* results were expressed as mean ± standard deviation (STDEV) and statistical program SPSS 18.0 was used for the analysis. Statistical significance was set up at $p < 0.05$.

Results

Qualitative and quantitative analyses

The LC-MS analysis of the PoPEx identified 14 major compounds, including bioactive compounds such as punicalin, punicalagin and tellimagran-

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Table 1: Compounds identified in pomegranate peel ethanol extract by LC-MS method

RT (280 nm)	λmax	MW	[M – H] – (m/z) (100 V)	MS data (m/z) (250V)	Compound name
3.321	258, 376	482	481	275, 301	HHDP-hexoside
3.777	260, 380	782	781	601, 721	Punicalin isomer (α or β) (4,6-gallagyl-glucose)
3.961	260, 380	782	781	601, 721	Punicalin isomer (α or β)
4.982	260, 380	784	783	481, 301	Penduculagin I isomer (bis-HHDP-hexoside)
6.072	258, 378	1416	933	783, 633	bis-(HHDP-galloylglucose)-pentose
6.767	256, 376	952	951	933, 301	galloyl-HHDP-DHHDP-hexoside (granatin B)
8.285	258, 380	1084	1083	781, 601	Punicalagin isomer 1 (HHDP-gallagyl-glicoside)
11.129	258, 380	1084	1083	781, 601	Punicalagin isomer 2 (HHDP-gallagyl-glucoside)
12.537	220, 266	1086	1085	783, 633, 451, 301	Digalloyl-gallagyl-hexoside
13.542	230, 278	1568	1567	935, 783, 633	Sanguiin H-10 isomer (digalloyl triHHDP-dihexoside)
14.222	266, 362	634	633	463, 301	Galloyl - HHDP hexoside
15.226	218, 274	786	785	755, 301	Tellimagrandin I
17.489	252, 304, 362	464	463	301, 463	Quercetin-hexoside
25.513	252, 308, 368	628	627	301	Ellagic acid derivate

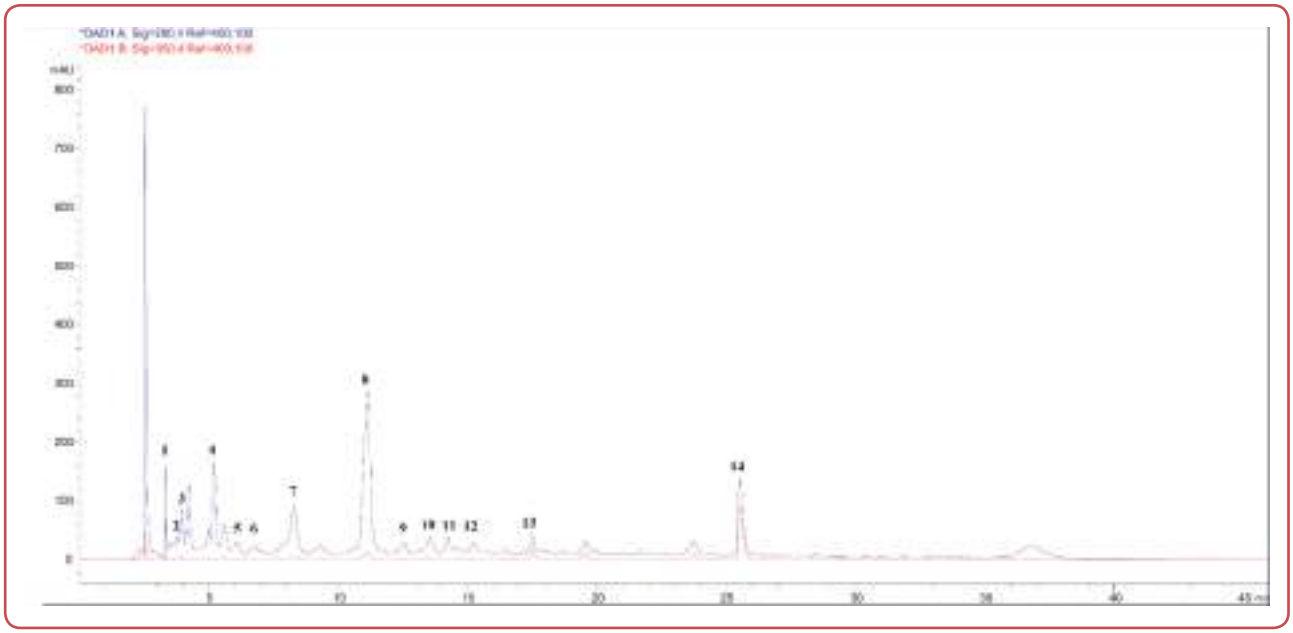

Figure 1: Chromatogram of PoPEx (5 mg/mL) analysed by the LC–MS

PoPEx: pomegranate peel extract; The LC-MS analysis of the PoPEx identified 14 major compounds, including bioactive compounds such as punicalin, punicalagin and tellimagrandin I. Punicalagin was the most prevalent compound.

din I. According to the results, punicalagin was the most prevalent compound (Table 1 and Figure 1).

The content of total phenols in the samples was extremely high and amounted to 621.13 mg GAE/g_{DW} sample. Total flavonoids were relatively high, amounting to 63.3 mg Qc/g_{DW}. A high content of total flavonols was found in the sample and it was 90.84 mg Qc/g_{DW}. In the sample, a relatively low content of flavan-3-ol was found compared to other phenols, only 71.44 μg CAT/g_{DW}. In this sample, the content of total monomeric anthocyanins was 0.55 mg C3G /g_{DW}. On the other hand, the content of total anthocyanins, polymerised (degraded) and monomeric, was 6.25 mg C3G /g_{DW} (Table 2).

Table 2: Content of total phenols, flavonoids, flavonols, total and monomeric anthocyanins

Compounds	Content
Total phenols (mgGAE/g_{DW})	621.13 ± 32.33
Flavonols (mgQc/g_{DW})	90.84 ± 3.56
Flavan-3-ols (μg CAT/g_{DW})	71.44 ± 7.80
Flavonoids (mgQc/g_{DW})	63.30 ± 0.43
Total anthocyanins (mg C3G/g_{DW})	6.25 ± 0.01
Monomeric anthocyanins (mg C3G/g_{DW})	0.55 ± 0.02

In vitro studies of antioxidant potential

In vitro measured antioxidative capacity of PoPEx, with Trolox used as a reference antioxidant, showed significantly better capacity in neutralising OH radical ($p < 0.05$), while the other tests showed slightly lower potency in comparison to Trolox (Table 3).

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Table 3: Antioxidative capacity of PoPEx (mean ± standard deviation)

Reaction	PoPeX (mmol Trolox/g_{DW}Ex)	IC_{50} µg/mL	IC_{50} Trolox µg/mL	mmol Fe/g	mmol Fe/g trolox
B-R	0.815 ± 0.074	-	-		
DPPH	2.142 ± 0.037	13.260 ± 0.110	7.110 ± 0.190		
ABTS	2.735 ± 0.137	3.606 ± 0.233	2.469 ± 0.106		
CUPRAC	2.891 ± 0.167	-	-		
FRAP	3.422 ± 0.044	-	-	7.137 ± 0.057	8.532 ± 0.095
Lipid peroxidation	3.945 ± 0.079	19.130 ± 0.240	18.890 ± 4.380		
OH	5.020 ± 0.090	49.920 ± 0.930	62.690 ± 2.040		

PoPEx: pomegranate peel extract; BR: Briggs-Rauscher reactions; DPPH: 2,2 diphenyl-1-picrylhydrazyl assay; ABTS: 2,2'-azino bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; CUPRAC: Cupric reducing antioxidant capacity assay; FRAP: ferric reducing antioxidant potency assay;

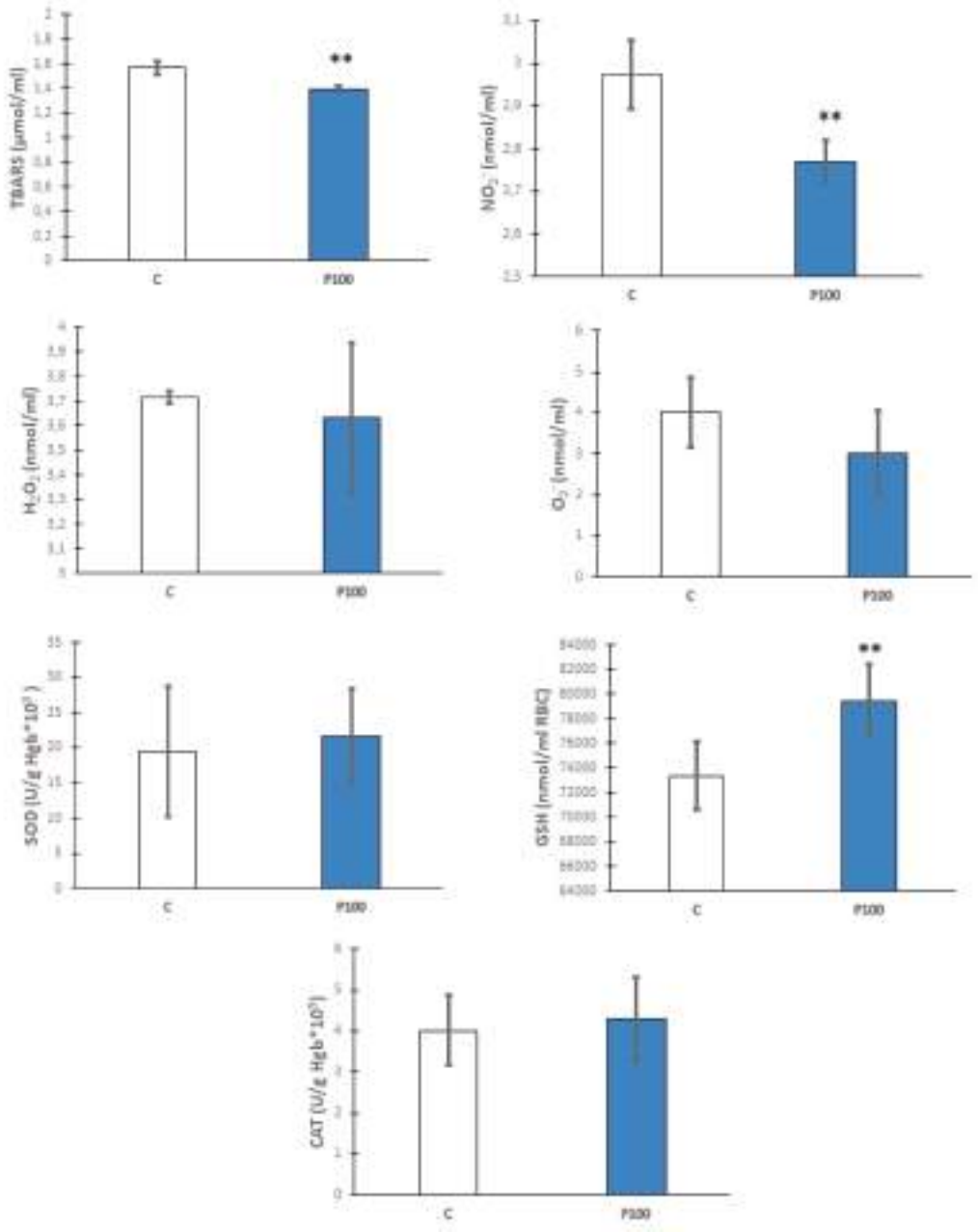

Figure 2: Effects of pomegranate peel extract on prooxidant and antioxidant parameters

*TBARS-thiobabrituric acid reactive substances, NO_2^- - nitrites, H_2O_2 – hydrogen peroxide and O_2^- - superoxide anion radical and antioxidative parameters: SOD – superoxide dismutase, GSH- total reduced glutathione and CAT - catalase. C-control group, P100- pomegranate peel extract 100 mg/kg bw group. Data are presented as means ± standard deviation. **-statistically significant difference (t-test, α = 0.05) vs control group;*

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In vivo studies of antioxidant potential

After 7 days of PoPEx administration, oxidative markers (TBARS and NO_2^-) were significantly lowered in the PoPEx group compared to the control, while GSH was significantly increased ($p < 0.05$). Other two oxidative parameters (H_2O_2 and $O_2^{\cdot}$) showed a lowering trend in the values measured after PoPEx administration, as well as two antioxidative parameters (SOD and CAT) which showed an increasing trend in PoPEx group, but without statistical significance (Figure 2).

Discussion

There is a large number of studies that investigated the content of total phenols in extracts of pomegranate peel.[37] The data show that the content of total phenols ranged from 53.65 to 841.5 mg GAE/g_{DW} sample. The results of presented study are well above the average and are close to the results obtained for ethyl acetate extracts of Italian pomegranate peel, which were 3.75 mmol GAE/g_{DW} = 638 mg GAE/g_{DW}.[38] The content of total flavonoids was higher than the content found in ethanol extracts of pomegranate peels in Turkey, which ranged from 12.83-17.27 mg Qc/g_{DW}[39] and lower than the content of samples from Mauritania where a value of 180.1 mg Qc/g_{DW} was found in the methanol extract.[40] Content of flavonols in the samples was significantly higher than the total flavonols in the ethanol extract of blackberry pomace, which showed significant antioxidant and antimicrobial effects and where the values ranged from 2.53–6.39 mgQc/g_{FW}.[41] Low level of flavan-3-ols is not surprising because a high level of tannin (14.15 %) was found in the sample, so probably a large part of flavan-3-ol was used for the synthesis of these compounds. The presence of total monomeric anthocyanins in different pomegranate genotypes, from different geographical latitudes and in different solvents varies in a wide range from 0.68-102.2 mg C3G /g_{DW}.[37] Significant differences in values of monomeric and total anthocyanins in the samples indicate that the anthocyanin content is significantly higher in the polymerised, biologically less valuable form. Such a large range in contents of total phenols, flavonoids, flavonols, total and monomeric anthocyanins, is an obvious consequence of the diversity of samples considering the geographical and climatic area, genotype, degree of maturity, method of extraction and especially the type of solvent used for the extraction of these compounds.

Due to the complexity of oxidative processes, multiple methods are required to determine antioxidant capacity; otherwise, the obtained results cannot be accurately interpreted and confirmed.[42] The results showed that the extract had a very low antioxidant capacity in relation to Briggs Rausher's oscillatory reactions. The value was only 0.815 mmolTr/g of extract and it was significantly lower than the capacity of wild and tame blackberry pomace extracts, which ranged from 17-28 μgTr/μg of extract.[43] Briggs-Rauscher's oscillatory reactions take place at low pH, close to the pH in the stomach, where the basic free radical that initiates the oscillatory reaction system is the hydroperoxyl radical HOO·. From the results, it could be concluded that pomegranate peel extract has a weak antioxidant effect at low pH values and on the hydroperoxyl radical.

The antioxidant capacity of the 70 % ethanol PoPEx against the stable DPPH radical was lower than that of the synthetic antioxidant Trolox. The values were 2.142 mmol Tr/g, or if the value is expressed as IC50, 13.26 μg/mL while Trolox had an IC50 of 7.11 μg/mL. The obtained values indicate that the antioxidant capacity of the extract against the stable DPPH radical is higher than the capacity of ethanol extract of pomegranate by other authors who found the following values: 1.71 - 2.0 mmol Tr/g,[44] 225 μmolTr/g_{dw},[45] IC50 = 69-91 μg/mL.[39] On the other hand, some authors found a higher antioxidant capacity of ethanol extract of pomegranate IC50 = 4.9 μg/mL.[46] Besides, some authors tested pomegranate extracts in different solvents and obtained significantly different results. When pure methanol was used as a solvent, a higher antioxidant capacity was obtained (IC50 = 8.3 μg/mL;[47] IC50 = 8.73 μg/mL[48]), whereas other solvents yielded significantly lower antioxidant capacity, as observed in the study of different varieties of pomegranate in Tunisia (IC50 = 56-65 μg/mL).[39] When 70 % methanol was used, the antioxidant capacity of pomegranate peel was slightly different, IC50 = 14.67.[48]

The obtained values for the antioxidant capacity against the stable ABTS radical were 2.735 mmol Tr/g, ie IC50 = 3.606 μg/mL, therefore the extract showed a weaker effect than Trolox (IC50 = 2.469 μg/mL) and from some literature values 2.8-4.41 mmol Tr /g.[44] On the other hand, the values were significantly higher than those in the literature for extracts in ethanol IC50 = 5.013 μg/mL,[38] as well as for some extracts in methanol (6.5-8.5 mmolTr/100g).[49] The highest antioxidant capac-

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ity was shown by the extract of pomegranate in ethyl acetate; IC50 = 1.725 μg/mL.[38]

The FRAP results indicated a lower antioxidant capacity than Trolox, as the values were 3.42 mmol Tr/g or 7.157 ± 0.057 mmol Fe/g, compared to 8.532 ± 0.095. Also, the results for ethanol extracts were lower than those reported in the literature, which were 12.4 mmol Fe/g dw;[39] 4.05-6.64 mmolTr/g.[44] On the other hand, some other authors found lower values of 21.24-21.5 mmol Tr/100g_{dw} for the methanol extract,[50] 9.07 mmol/100 g,[51] 82.11 mmol/100 g.[52]

Due to the favourable redox potential in a neutral medium, the CUPRAC method is preferable for simulating physiologically significant redox reactions of antioxidant compounds, such as serum antioxidants. The values determined by the CUPRAC method (2.89 mmol Tr/g) indicate that the antioxidant capacity of pomegranate peel extract is weaker than that of Trolox. They were also lower than the literature values, which were 3756 μmolTr/g_{dw}.[45]

The values for the inhibition of lipid peroxidation were 3.945 mmol Tr/g, ie IC50 = 19.13 μg/mL, which was slightly lower than the capacity of the standard antioxidant Trolox, which showed IC50 = 18.89 μg/mL. Also, from the results, it can be seen that the ethanol extract of pomegranate showed a better capacity to inhibit lipid peroxidation than the extract in methanol, which showed only IC50 = 32.4 μg/mL.[47]

PoPEx showed significant antioxidant capacity against the OH radical and was 5.020 mmol Tr/g, ie IC50 = 49.92 μg/mL. These values were lower, ie the extract showed a better antioxidant capacity than the standard antioxidant Trolox, which had an IC50 value of 62.69 μg/mL. The antioxidant capacity against the OH radical of extracts in different solvents was tested and the following values were obtained: IC50 = 322; 126; 13.6; 54.9; 289 μg/mL: hexane, ethyl acetate, methanol, 70 % methanol, water, respectively.[48] By comparison, it can be seen that the extract showed better results, except in the case of the extract in pure methanol. Based on the results, it can be concluded that pomegranate extract can significantly inhibit the formation of OH radicals.

The antioxidative capacity of PoPEx was further investigated in *in vivo* conditions. A seven-day consumption of PoPEx (100 mg/kg) lead to a significant decrease of the index of lipid peroxidation (TBARS) and plasma NO_2^-, as well as a decrease of other two free radicals – superoxide anion radical and hydrogen peroxide, when compared to the control group, but without statistical significance. These results suggest that PoPEx acts as a free radical scavenger. Besides, PoPEx consumption also led to an increase of the levels of the two antioxidative enzymes (CAT, SOD) and GSH. The SOD acts through conversion of superoxide radicals into hydrogen peroxide, that is afterwards converted to molecular oxygen and water via the activity of CAT.[53] GSH, on the other hand, led to the reduction of the hydrogen peroxide radicals. Knowing that the levels of these two enzymes and GSH are decreased in the presence of free radicals, these findings are in concordance with the hypothesis that PoPEx acts as a free radical scavenger. The cornerstone of the antioxidative capacity of the PoPEx is considered to be its high polyphenolic content. This was also shown in previous studies that investigated free radical scavenging capacity of the PoPEx.[54] The main mechanism through which polyphenols scavenge free radicals is considered to be donation of hydrogen atoms.[55]

Conclusion

From the results, it can be seen that PoPEx shows significant antioxidant activity both, *in vitro* and *in vivo*. Besides, different values were obtained for the antioxidant capacity against different radicals, depending on the method used. The values for the antioxidant capacity are arranged in the following order sequence: B-R < DPPH < ABTS < CUPRAC < FRAP < Lipid peroxidation < OH. Such high values of antioxidant capacity measured *in vitro*, as well as the significant decrease of TBARS and NO_2 radical on the one hand and an increase in levels of GSH measured *in vivo* on the other hand are the result of a high content of total phenols, flavonoids, flavonols and monomeric anthocyanins with high radical scavenging potential of PoPEx.

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None.

Conflict of interest

None.

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ORIGINAL ARTICLE

Epidemiological and Clinical Characteristics of Patients with Healthcare - Associated *Clostridioides Difficile* Infection Before and During the COVID-19 Pandemic

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Abstract

Background/Aim: Diarrhoea that occurs as a result of the presence of *Clostridium difficile* (reclassified as *Clostridioides difficile*) is usually manifested as a hospital infection, usually after antibiotic treatment. The study aim was to assess the incidence, characteristics and outcomes of hospitalised patients with healthcare – associated *Clostridioides difficile* infection (HA - CDI) before and during the COVID-19 pandemic.
Methods: This retrospective cohort study included patients older than 18, who met the HA - CDI case definition. The CDI diagnosis was made by demonstrating toxins A and B in stool samples using an immunochromatographic assay test and polymerase chain reaction (PCR).
Results: The incidence of HA - CDI has significantly decreased from the pre-COVID-19 period to the COVID-19 period (11.04 per 10,000 vs 6.49 per 10,000, $p < 0.001$). Before establishing the HA - CDI diagnosis, 41.4 % of patients used one antibiotic, 25.9 % used two and 11.2 % were treated with three or more antibiotics. Almost one half of the applied antibiotics were from the group that represents high risk for the development of HA - CDI. Multivariable logistic regression analysis showed that older age (OR = 3.4; 95 % CI = 0.9-12.4; $p = 0.038$) and complicated disease course (OR = 11.8; 95 % CI = 2.6-53.6; $p \leq 0.001$) were associated with a higher risk of death.
Conclusion: The incidence of HA - CDI has decreased during the observed period of the COVID-19 pandemic, however, no clear connection between the impact of the pandemic and incidence reduction was found. Due to unfavourable outcome of the treatment of HA - CDI patients during COVID-19 pandemic, the rational use of antibiotics is necessary.

Key words: *Clostridioides difficile* infections; COVID-19 pandemic; Risk factors; Disease outbreak.

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Introduction

Clostridium difficile (*Clostridioides*, according to the latest classification from 2016) is one of the leading causes of infections associated with healthcare (nosocomial infection), most often affecting the elderly and hospitalised. Colon infection with the Gram-positive bacterium *C difficile* (CDI) can be life-threatening and almost 20 % of patients are colonised with this bacterium during hospitalisation and more than 30 % of them develop diarrhoea.[1-3] After the spread of highly virulent strains, mostly ribotype 027 (ribotype 027, North American pulsed-field gel electrophoresis type 1 or restriction endonuclease analysis group BI, NAP1/027/B1), an increase in the number of severe cases was noticed, as shown by studies from Europe and of North America.[4,

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[5] Another problem is the recurrence of infection that occurs in 25 % (6 ± 42 %) of all CDI cases, endangering the health of patients even more.[6] These infections in hospitalised patients lead to prolonged hospital stays, adverse outcomes and increased costs. CDI has been present in the hospital environment for more than 50 years, but in the last two decades it represents one of the growing public health problems both worldwide and in the Republic of Srpska, Bosnia and Herzegovina. The USA Centre for Disease Control and Prevention (CDC) describes it as a "threatening infection due to the possibility of the manifestation of *C difficile* highly resistant to antibiotics".[7, 8] A research of the prevalence of diseases associated with healthcare and the use of antibiotics conducted by the European Centre for Disease Prevention and Control (ECDC) in acute care hospitals found that *C difficile* was the eighth most frequently reported microorganism.[9]

The COVID-19 pandemic had a pronounced negative impact on the outcome of patient treatment. The changes in the gut microbiota and immune response disorders can affect the development of healthcare – associated *Clostridioides difficile* infection (HA – CDI) in patients suffering from COVID-19. During hospitalisation, the majority of the infected with the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus received antibiotics, which can increase the risk of antibiotic-associated diarrhoea (AAD) and CDI. Uncontrolled use of antibiotics and disinfectants can lead to the selection of resistant strains of *C difficile* not only in hospitals but also in the community.[10, 11]

The aim of this study was to evaluate the frequency, characteristics and outcomes of HA - CDI patients treated in hospitals before and during the COVID-19 pandemic.

Methods

Study design and patients

This was a retrospective, cohort study in which all necessary variables were collected from the original laboratory and medical files of all hospitalised patients with HA - CDI from the beginning of July to the end of December 2019 (pre-COVID-19 period) and from the beginning of July to the end of December 2020 (COVID-19 period). The research was conducted at the University Clinical Centre of the Republic of Srpska (UCC RS) at the Clinic for General and Abdominal Surgery, Clinic for Internal Diseases, Clinic for Oncology, Clinic for Infectious Diseases and the Intensive Care Unit (ICU). For the purposes of the research, the consent of the Ethics Committee of UCC RS (No 01-19-612-2/19) was obtained.

A case in which symptoms appeared 48 h or later after admission to the hospital was considered a hospital CDI or if the case occurred outside the hospital within four weeks after the previous discharge from the health institution. Recurrent cases of CDI meet the case definition, with recurrent diarrhoea after the end of therapy, with a positive laboratory test, that occurred more than two and less than eight weeks after the beginning of the previous episode (regardless of the place of origin of the episode). CDI cases in which the symptoms started more than eight weeks after the onset of the previous episode were considered new CDI cases. Lethal outcome of treating patients was considered to be CDI-related when there were no other causes or it occurred within 10 days after establishing the CDI diagnosis or it was due to known CDI-related complications.

The study included all patients older than 18 years of age (total of 116 patients), who met the HA - CDI case definition: the date when the CDI symptoms started was during the study period, even if the patient was admitted before the start of the study; the patient was admitted to the hospital during the study period with signs and symptoms of CDI present at admission, even though this episode of CDI had already been diagnosed before admission (for example, in acute medical units) and these were repeated cases of CDI. The study did not include patients with CDI acquired in the community, as well as patients from outpatient clinics, for example same day surgery, haemodialysis patients and outpatients.

CDI was diagnosed by detecting toxin A and toxin B in stool samples using the VEDA LAB Toxin A+B (*C difficile*) DUO immunochromatographic test (*ZAT du Londeau - Rue de l'expansion, Cerisé - BP 181 - 61006 Alençon, France*) or using polymerase chain reaction (PCR) method for the detection of binary toxin in *C difficile* ribotype 027 (*Cefeid Xpert® C difficile BT, Röntgenvägen 5, SE-17154, Solna, Sweden*).

Variables

In order to compare the variables that could have contributed to differences in the frequency of CDI, the following patient data was obtained: age, sex (male/female), date and duration of hospitalisation, primary diagnosis, comorbidities antibiotics administered before and during hospitalisation (but before laboratory testing for *C difficile*), use of antacids, probiotics and corticosteroids during hospitalisation, previous medical history of CDI, date of laboratory testing for *C difficile*, previous admission to healthcare facilities in the last three months in relation to the occurrence of CDI (hospital or other healthcare facility, for example long-term treatment institutions, such as a nursing home, rehabilitation centre, long-term care home, etc), 24-hour-stool count, peripheral blood leukocyte count, serum creatinine and albumin value and C-reactive protein (CRP) value. To assess the severity of chronic disease and health status, the McCabe score was used, according to which patients were divided into three categories. The patient's treatment outcome was taken as the patient's status at hospital discharge or at the end of hospital follow-up.

Data and statistical analyses

For statistical data processing, the software package SPSS, version 25.0 was used with a 95 % confidence interval (CI) of statistical significance ($p < 0.05$). The incidence rate of HA - CDI was calculated as the ratio of the number of infections/10,000 patient days. Nominal variables were presented as number (percentage), whereas continuous variables were presented as mean (M) and standard deviation (SD). The Shapiro-Wilk test and histogram were used to analyse the normality of the distribution. If necessary, the Chi-square test or the Wilcoxon test for dichotomous variables and the Mann-Whitney test or Dependent t-test for constant variables were used to compare research groups. For the construction of the regression model, those variables that showed $p < 0.05$ using univariate analysis were used. Multivariable logistic regression analysis was performed to determine the combination of parameters predicting HA - CDI in patients with a fatal outcome.

Results

During the research period, a total of 116 patients with HA - CDI were treated, of which 74 (63.8 %) patients were treated in the period before COVID-19 and 42 (36.2 %) during the COVID-19 pandemic. In the second half of 2020, 17 (40.5 %) patients had COVID-19 as the primary disease for which HA - CDI patients were hospitalised. The incidence of HA - CDI between time periods is presented in Table 1. The incidence of HA - CDI has significantly decreased from the pre-COVID-19 period to the COVID-19 period (11.04 per 10,000 vs 6.49 per 10,000, $p < 0.001$). In the majority of observed clinics, there was a significant drop in the incidence during the pandemic period, except at the Clinic for Surgery, where there was a slight increase in incidence (4.56/10,000 vs 5.05/10,000) (Table 1).

Table 1: Trends in the incidence of healthcare – associated Clostridioides difficile infection (HA – CDI) in patients during study period

Variables	Pre-COVID-19 HA-CDI group n = 74		COVID-19 HA-CDI group n = 42		p-value
	Hospital inpatient (days)	New cases per 10,000 patient (days)	Hospital inpatient (days)	New cases per 10,000 patient (days)	
Internal medicine	33.994	8.53	30.486	5.90	0.041*
ICU	2.666	26.26	5.782	12.11	
General Surgery	9.069	1.10	8.718	4.59	
Oncology	13.144	5.05	9.896	4.56	
Infectiology	8.133	38.12	9.859	8.11	
Total	**67.006**	**11.04**	**64.741**	**6.49**	**< 0.001***

*HA - CDI, Healthcare - associated Clostridioides difficile infection; COVID-19, coronavirus disease 2019; ICU, Intensive care unit; *Chi-square test; p - value statistically significant (p < 0.05)*

CONTENT

Table 2: Demographic and epidemiological data, comorbidities, clinical characteristics of the patients with healthcare – associated Clostridioides difficile infection (HA – CDI) before and during COVID-19 pandemic and their differences

Variables	Pre-COVID-19 HA-CDI group n = 74	COVID-19 HA-CDI group n = 42	p-value
Male sex, n (%)	34 (45.9)	30 (71.4)	0.008 *
Women sex, n (%)	40 (54.1)	12 (28.6)	
Age in years (Median, IQR)	67 (21-75)	69 (63-74)	0.807 **
Previous hospital admission, n (%)	45 (60.8)	21 (50.0)	0.358 *
CDI case origin			0.041 *
Current hospital, n (%)	51 (68.9)	30 (71.4)	
Other hospital, n (%)	5 (6.8)	0 (0.0)	
LTCF, n (%)	6 (8.1)	0 (0.0)	
Not specified, n (%)	12 (16.2)	12 (28.6)	
Recurrent CDI, n (%)	16 (21.6)	11 (26.2)	0.464 *
Days of hospitalisation prior CDI, X ± SD	16.11 ± 12.46	16.88 ± 12.27	0.767 **
McCabe–Jackson disease classification, n (%)			0.352 *
Non-fatal	33 (44.6)	24 (57.1)	
Rapidly fatal	31 (41.9)	15 (35.7)	
Ultimately fatal	10 (13.5)	3 (7.1)	
ICU admission, n (%)	9 (9.5)	9 (16)	0.041 *
Mean duration of diarrhoea, days (range)	9.67 (5.36)	11.47 (6.46)	0.181 **
Leukocyte count ≥ 15 x 10^9/L, n (%)	25 (33.8)	17 (40.5)	0.205 **
Serum creatinine > 1.5 mg/dL, n (%)	30 (40.5)	5 (11.9)	0.001 **
Albumin, g/l, n (%)	18 (24.3)	14 (33.3)	0.297 ***
C-reactive protein ≥ 200 mg/L, n (%)	12 (16.2)	6 (14.3)	0.783 ***
Proton pump inhibitors, n (%)	49 (66.2)	38 (90.5)	0.020 *
Immunosuppressive condition, n (%)	7 (9.5)	19 (45.2)	< 0.001 *

*HA - CDI, Healthcare - associated Clostridioides difficile infection; COVID-19, coronavirus disease 2019; LTCF, long term care facility; ICU, Intensive care unit; *Chi-square test ** Wilcoxon test, *** Dependent t-test; p - value statistically significant (p < 0.05)*

Table 2 shows data on HA - CDI patients with specific characteristics during the 2020 wave of the COVID-19 pandemic and the same calendar period in 2019. A statistically significant difference (p = 0.008) in gender was observed between patients during the reporting period. Before the COVID-19 pandemic, men contracted HA - CDI significantly less frequently (45.9 %) compared to the pandemic period (71.4 %). The average age of HA - CDI patients before the study period (66.25 ± 13.12) and after (66.14 ± 12.56) was similar. During the reporting period in 2020, 17 (40.47 %) patients had COVID-19 as the primary disease for which CDI patients were hospitalised. In the ICU during the pandemic period, statistically significantly (p = 0.041) more patients were treated compared to the period before the COVID-19 pandemic (16.7 % : 9.5 %). During the surveillance period, the previous admission to healthcare institutions in the last three months in relation to the occurrence of HA - CDI was determined in 66 (56.9 %) patients and the largest number were acute care hospitals 59 (50.9 %). There were no differences in the frequency of HA - CDI symptoms present at admission, nor in the occurrence of repeated HA - CDI before and after the COVID-19 pandemic. There was no statistically significant difference in the severity of the underlying disease between the cohorts of HA - CDI patients (p = 0.352). Data analysis showed that patients with HA - CDI had a significantly higher number of leukocytes in peripheral blood, significantly elevated CRP values, lower serum albumin levels and higher serum creatinine levels compared to basal values. Table 2 also shows that proton pump inhibitors were significantly more often used in patients with HA - CDI (57.1 %) during the COVID-19 pandemic compared to patients examined before the pandemic (47.3 %) (p = 0.020). Before the pandemic, 90.5 % of patients were treated without corticosteroids, but in the observed period of the pandemic, that percentage was highly statistically significantly lower (p < 0.001) and amounted to 54.8 % (Table 2).

Drugs administered to patients before the onset of HA - CDI is showed in Table 3. Before establishing the diagnosis of HA - CDI, 48 (41.4 %) patients used one antibiotic, 30 of them (25.9 %) used two, 13 patients (11.2 %) were treated with

Table 3: Drugs administered to patients before the onset of healthcare – associated Clostridioides difficile infection (HA – CDI)

Variables	Pre-COVID-19 HA-CDI group	COVID-19 HA-CDI group	p-value
Antibiotic exposure n (%)	61 (82.4)	32 (76.2)	0.282 *
Number of received antibiotics			**0.424** *
One	33 (44.6)	15 (35.7)	
Two	17 (23.0)	13 (31.0)	
Three and more	10 (13.5)	3 (7.1)	
Beta-lactams	60 (81.1)	18 (42.8)	**0.033** **
Cephalosporins	40 (54.1)	13 (30.9)	0.008 **
Carbapenems	17 (23.0)	7 (16.7)	0.806 **
Quinolones	12 (16.2)	15 (35.7)	**0.197** **
Macrolides	2 (2.7)	8 (19.0)	0.007 **
Aminoglycosides	10 (13.5)	1 (2.4)	0.013 **
Colistin	5 (6.7)	4 (9.5)	0.594 **
Level of risk for CDI			**0.331** *
High risk	36 (48.6)	18 (42.9)	
Moderate risk	9 (12.2)	8 (19.0)	
Low risk	16 (21.6)	5 (11.9)	

*HA - CDI, Healthcare - associated Clostridioides difficile infection; COVID-19, coronavirus disease 2019; *Chi-square test; ** Mann-Whitney test; p - value statistically significant (p < 0.05)*

Table 4: Healthcare – associated Clostridioides difficile infection (HA – CDI) patient outcome

Variables	Pre-COVID-19 HA-CDI group (n = 74)	COVID-19 HA-CDI group (n = 42)	p-value
Discharged alive	62 (83.8)	28 (66.7)	
In-hospital death	12 (16.2)	14 (33.3)	0.04*
CDI-related death	5 (6.7)	9 (21.4)	

*HA - CDI, Healthcare - associated Clostridioides difficile infection; COVID-19, coronavirus disease 2019; *Chi-square test; p - value statistically significant (p < 0.05)*

three or more antibiotics, whereas 25 (21.6 %) patients did not use antibiotics in therapy. Almost half of the applied antibiotics 54 (46.6 %) were from the group representing a high risk for the occurrence of HA - CDI. Antibiotics from the group of beta-lactam antibiotics (54.97 %) were significantly more often used in patients before the COVID-19 pandemic (81.1 vs 42.8; p = 0.033). Macrolides (azithromycin) were used more often during the observed pandemic period in 2020 (2.7 vs 19.0; p = 0.007) as well as quinolones (16.2 vs 35.7; p = 0.197) (Table 3).

Mortality of HA - CDI patients before the COVID-19 pandemic was 16.2 % and during the observed pandemic period it was 33.33 %. The highest mortality rate was recorded in the ICU (71.4 %). The mean time period (in days) from establishing the laboratory diagnosis of HA - CDI to HA - CDI-related death was 12.56 ± 14.60 (Table 4).

Table 5: Risk factors for the HA - CDI-related fatal outcome

Parameter [a]	RR	95 % CI	p-value
Age > 65	3.426	0.944 - 12.437	0.038
Complicated course	11.850	2.622 - 53.642	< 0.001
Leucocytosis	3.794	1.193 - 12.066	0.024
CRP > 200 mg/L	3.635	1.143 - 11.562	0.029

[a]Reference category: HA - CDI, healthcare - associated Clostridioides difficile infection related fatal outcome; RR, risk ratio; CI, confidence interval; CRP, C-reactive protein; p-values were calculated by logistic regression analysis.

Multivariate logistic regression analysis showed that older age, complicated disease course, leucocytosis and elevated values of CRP (> 200 mg/L) were associated with a higher risk of death (Table 5).

Discussion

Surveillance of infections caused by *C difficile* is an important component of the prevention program. Understanding the determinants of HA - CDI incidence will allow for more meaningful comparisons of rates, the course and outcome of the disease for the planning of preventive programmes.[12] Treating a patient during the COVID-19 pandemic represents a challenge for hospital systems worldwide. UCC RS had to adapt to a greater influx of patients who requested healthcare, which led to the reorganisation of certain clinics. During the COVID-19 pandemic, many preventive procedures (personal protective equipment (PPE), regular use of hand sanitisers, cleaning the environment, disinfecting the objects, bio-medical waste management) were adopted to prevent the spread of microorganisms in the hospital environment.

The results of the present study showed that there was a significant decrease in the incidence rate of HA - CDI during the observed period. The studies conducted by Italian and Spanish authors showed similar results, where the incidence of HA - CDI during 2020 was significantly lower compared to the period before the COVID-19 pandemic.[13, 14] Contrary to these studies, Velev et al found a significant increase in the incidence of HA - CDI during the COVID-19 pandemic compared to the pre-pandemic period at one university hospital in Bulgaria.[15]

In the group of HA - CDI patients during the observed period in 2020, 40.5 % patients had COVID-19 as the primary disease. Departments where patients with COVID-19 were hospitalised had a higher incidence of HA - CDI than departments that were not. A study conducted at one Serbia university hospital from January 2019 to December 2021 showed that out of 547 patients with CDI, 62.3 % had COVID-19. The incidence of HA - CDI per 1000 patients-days was 1.33 in the non-COVID period and 4.53 in the COVID period.[16]

The most common risk factor for HA - CDI in the population compared to the period before and after the study was age ≥ 65 years. Numerous other studies have shown that due to frequent hospitalisations and a greater number of comorbidities, people older than 65 years of age have a higher risk of developing infections caused by *C difficile*.[17, 18] The basic mechanisms as to why CDI occurs more often and presents a more severe clinical manifestation in the elderly population, have not been sufficiently clarified. It is believed that several associated factors, such as comorbidities, polypharmacy and frequent hospitalisations may contribute to the observed outcomes. However, three possible biological factors may be critical for the development of CDI in the elderly: humoral response, innate immunity and gut microbiota.[19]

According to the study data during the surveillance period, previous admission to healthcare institutions in the last three months in relation to the occurrence of CDI was recorded in 56.9 % of patients and majority of these were acute care hospitals (50.9 %). The currently observed hospital had the highest proportion of HA - CDI cases.

In an ECDC research conducted in 20 EU/EEA countries, which included CDI surveillance in 593 hospitals in 2016, the overall results showed that in 85.6 % of hospital-associated cases, the source of CDI was the hospital where the patient was treated at that moment. Out of the total number of reported cases of CDI (3,042), in the previous 3 months, 62.6 % of cases were admitted to healthcare institutions; 87.0 % of these cases were admitted to hospital and 6.4 % to long-term care institutions.[20] On the contrary, in the report on the epidemiology of CDI in Belgian hospitals from 2019, the proportion of hospital-associated cases was 56 % and 29 % of community associated CDI (CA - CDI) and it represented an increase compared to the research conducted ten years before (22 %).[21] Similarly, Song et al observed decreasing trends for nosocomial CDI (- 0.03 % per year) and increasing trends for non-nosocomial CDI (+ 0.04 % per year).[22] Recognising the importance of these infections, in 2016, the ECDC developed a protocol and initiated active surveillance of *C difficile*-related infections. The Republic of Serbia initiated this surveillance at the end of 2018, whereas Bosnia and Herzegovina still has not. This surveillance would make it possible to estimate the frequency of infections in acute care hospitals, to compare rates with other hospitals in the country and in Europe, to assess adverse outcomes of infections, including death, to promote the introduction of diagnostic procedures with high diagnostic accuracy and to detect new ribotypes using PCR.[23]

In the present study, from the total number of HA - CDI cases, recurrent HA - CDI was recorded in 23.3 % patients. Based on the literature review

conducted by Finn et al, recurrent cases of HA - CDI occur in approximately 10-20 % of HA - CDI patients worldwide and the average recurrence rate from all included studies was 17 %. The highest rates were recorded in Canada (23.7 %), Poland (21.7 %) and the USA (20.2 %).[24] According to a study conducted in a tertiary care hospital in Romania, the recurrence rate of HA - CDI was 53.8 %.[25] On the opposite, in one study conducted in Tel Aviv, 12.7 % of patients had at least one confirmed recurrent case of HA - CDI.[26]

In the sample of patients with HA - CDI, the majority of patients had McCabe score (marker of co-morbidity in HA infection) 1 (49.1 %), while slightly fewer had score 2 (39.7 %) and score 3 had 10.3 % patients. In contrast to this data, it is notable that in the 2018 ECDC research, 13.9 % of 2,577 cases with a reported McCabe score were indicated to have had a 'rapidly fatal underlying disease', ie the patient was expected to survive for less than a year.[20]

The results of this study showed that proton pump inhibitors were significantly more frequently used in patients with CDI in the COVID-19 pandemic period of the research ($p = 0.020$). Proton pump inhibitors are most often prescribed by gastroenterologists due to the high success rate of symptom relief (such as heartburn, reflux esophagitis) and prevention of stomach ulcers and the first part of the small intestine. However, recently, there has been concern regarding the association between the use of proton pump inhibitors and several possible serious side effects, such as CDI.[27]

There have been several clinical studies that support the success of corticosteroids in patients with COVID-19 disease. A retrospective study by Wu et al from 2020, showed that the use of methylprednisolone significantly reduces the risk of death in patients with COVID-19.[28] However, other recently published research, such as the one conducted by Carlson et al from 2021 showed that patients who took corticosteroids in the last 90 days had a lower risk of CDI occurrence.[29] In one research conducted by Russian authors who examined the impact of CDI on the course of the COVID-19 disease, the use of glucocorticoids was not a predictor of death.[30]

Antibiotic therapy is the most important risk factor for the occurrence of CDI, which depends on the duration of their use, as well as the class and number of simultaneously administered antibiotics. Because of the fear of developing a bacterial infection, most patients infected with the SARS-CoV-2 virus received antibiotics during hospitalisation. According to the results of the present study, before the diagnosis of HA - CDI, 42.2 % of patients used one antibiotic, 25.9 % simultaneously used two antibiotics and 11.2 % used three or more antibiotics. Almost half of the applied antibiotics (46.6 %) were in the group representing a high risk of HA - CDI.

The COVID-19 pandemic had a negative effect on patient outcomes. Mortality from HA - CDI during the pandemic period was higher compared to the pre-pandemic period (16.2 % vs 33 %). The highest number of deaths was recorded in a period shorter than 10 days from the moment of the laboratory diagnosis of HA - CDI and in the ICU during the pandemic period of monitoring the outcome of treatment of HA - CDI patients.

Study by Filippidis et al have shown that there is a significant correlation between HA - CDI clinical failure (day 10) and mortality. These authors state that an all-cause mortality at week 8 was estimated at 15.3 %, with 50.9 % of these patients dying within 10 days from diagnosis.[31] The results of a research conducted by Maslennikov et al showed that HA - CDI was associated with an increased risk of death in COVID-19 patients, particularly after 20 days of illness onset. AAD patients with a positive test for *C difficile* infection had diarrhoea longer and more severely than those with a negative test. Unlike AAD patients with a negative test for *C difficile* infection, AAD patients with a positive test were admitted to the ICU and needed mechanical ventilation more often than patients without diarrhoea.[30]

The results of this study showed that older age, complicated course of the disease, leucocytosis and increased values of CRP (> 200 mg/L) were associated with a fatal outcome. Those predictors have also been identified in several other HA - CDI studies.[32, 33] HA - CDI patients aged over 65 are at particularly high risk for mortality. Also, high CRP values in patients with HA - CDI may be a repercussion of the presence of more serious concomitant infections of other aetiology, which puts these patients at additional risk of a poor outcome of the disease.[17]

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Strengths and limitations

To according to authors' knowledge, this is the first study in the Republic of Srpska, Bosnia and Herzegovina examining several clinical and epidemiological features of HA - CDI patients before and during the COVID-19 pandemic. The importance of the results of the current study is that they contribute towards providing a means for identifying HA - CDI patients at risk for a fatal outcome, in order to reduce their exposure to such risk factors. One of the main limitations of this study is the small sample size and the intervention was conducted in a single centre. The total consumption of antibiotics in the hospital was not measured. Findings in single tertiary hospital in the Republic of Srpska, Bosnia and Herzegovina need to be confirmed in a well-conducted prospective study involving multiple sites.

Conclusion

The incidence of HA - CDI has decreased during the observed period of the COVID-19 pandemic, however, no clear association of the impact of the pandemic on the incidence reduction was found. Patients with COVID-19 and HA - CDI coinfection were significantly with more comorbidities and hospitalised in the ICU. Almost half of the patients with HA - CDI were treated with antibiotics from the group that represents a high risk for the development of CDI, mostly from the group of beta-lactam antibiotics, followed by macrolides and quinolones. Due to the unfavourable outcome of the treatment of HA - CDI patients during the COVID-19 pandemic, the rational use of antibiotics and disinfectants is necessary to prevent the emergence of resistant strains of *C difficile* in hospitals and in the community.

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None.

Conflict of interest

None.

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ORIGINAL ARTICLE

Electronic Cigarettes with Different Nicotine Concentrations in Unflavoured Liquid Induce Oxidative Stress

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Abstract

Background/Aim: Nicotine content and flavour in electronic cigarette (e-cig) liquids have been demonstrated to cause oxidative stress in acute exposure. However, the chronic effects of using unflavoured and with or without nicotine in e-cigs liquid have not been evaluated. This *in vivo* study aims to investigate the chronic effect of e-cig exposure with unflavoured liquids at different nicotine concentrations on oxidative stress.

Methods: The 24 male Wistar rats were divided into four groups of six each. Normal, as a control group. Nic 0, Nic 6 and Nic 12 groups were exposed to unflavoured e-cig liquid for eight weeks with different nicotine concentrations: 0, 6 and 12 mg/mL, respectively. E-cig exposure in rats was conducted using an exposure instrument adjusted to real-life exposure to humans. Oxidative stress markers, including plasma, liver and lung malondialdehyde (MDA) and superoxide dismutase (SOD), as well as plasma catalase (Cat) and glutathione peroxidase (GPx) were assessed at the end of the study.

Results: Unflavoured e-cig liquids induced oxidative stress in a nicotine concentration-dependent manner, in which the nicotine content of 12 mg/mL demonstrated the greatest response. There was a significant increase in plasma, liver and lung MDA and concurrently decreased plasma and selected organs SOD, as well as plasma Cat and GPx in all nicotine concentration exposed groups compared to the Normal group.

Conclusions: Chronic unflavoured liquids in e-cig exposure at different nicotine concentrations induced oxidative stress, potentially leading to various oxidative stress-induced diseases.

Key words: E-cig; Flavour; Liquid; Nicotine; Oxidative stress.

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Introduction

Electronic cigarette (e-cig) or electronic nicotine delivery systems (ENDS) is not considered an alternative to conventional cigarette only but are also used by people who have never smoked.[1] The fact that e-cigs are easily available in online stores, social media and vape stores at low prices results in a high prevalence of e-cig use among adolescents, 6.3 % in females and 29 % in males, as reported in an epidemiological study in Jakarta, Indonesia.[2] Furthermore, Indonesia has the worst tobacco control regulations compared to other Southeast Asian countries, with no specific regulations on e-cig control at the national and regional levels.[3] It is estimated that the e-cig usage trend will continue to increase yearly.

The use of a conventional cigarette and e-cig, both acute and chronic, has been reported in several previous studies to induce oxidative stress, leading to chronic obstructive pulmonary

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disease (COPD), hepatic steatosis and various diseases.[4–7] Accumulating evidence has shown that this effect is associated with nicotine content as an independent risk factor, either without or through the combustion process in e-cig and conventional cigarettes, causing oxidative stress and inflammatory responses.[5,8,9] Oxidative stress is a state of imbalance between reactive oxygen species (ROS) free radicals and antioxidants as detoxifiers of these reactive substances.[10] ROS generated by endogenous and exogenous sources, including conventional cigarettes and e-cig, contribute to deoxyribonucleic acid (DNA) damage.[11] However, the body has a defence mechanism to counteract oxidative stress through several antioxidant enzyme defences, including superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GPx).[10, 11] Previous studies reported that antioxidant activity decreased after e-cig exposure, indicating that the antioxidant defence mechanism was inadequate to counteract excessive oxidative stress.[12, 13]

Besides the nicotine content, e-cig aerosols produced during combustion contain harmful components, including acrolein acetaldehyde and formaldehyde.[14, 15] In addition, acute e-cig exposure with commercially available flavourings in liquids was confirmed in an *in vitro* study, playing a role in inflammation and oxidative stress even in the absence of nicotine.[12] A previous *in vivo* and *in vitro* study by Lerner et al[5] demonstrated that acute exposure to e-cig with flavourings and nicotine causes an increase in ROS, contributing to the inflammatory response and oxidative stress.

The chronic effects of using e-cig without flavours and with or without nicotine have not been evaluated and studies in experimental animals are limited. This study aimed to investigate the chronic effect of exposure to e-cig with unflavoured liquids at different nicotine concentrations *in vivo* on systemic, lung and hepatic oxidative stress characterised by malondialdehyde (MDA), SOD, Cat and GPx. It was hypothesised that all e-cig exposure groups with different nicotine concentrations without flavouring induce oxidative stress.

Methods

Study design

Using the resource equation formula, the sample size calculation for four groups resulted in 4-6 rats in each group.[16] Twenty-four male Wistar rats weighing 100 ± 20 g aged 4-5 weeks old were obtained from *Laboratorium Penelitian dan Pengujian Terpadu,* Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats have housed two rats per cage at a 22 ± 2 °C temperature, 50 ± 10 % humidity and under a light/dark cycle of 12 h. Rats were given tap water and standard feed *ad libitum*. The rats were acclimatised for seven days before beginning of treatments, then randomly divided into four groups (n = 6 each):

1. Normal: a control group that did not receive any exposure,
2. Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid,
3. Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid,
4. Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.

After eight weeks of e-cig exposure treatment, rats were fasted for 12 h and continued to be injected intramuscularly with 50-75 mg/kg of *Zoletil 50* (25 mg/mL tiletamine hydrochloride and 25 mg/mL zolazepam hydrochloride) supplied by *Virbac SA* (Carros, France) for general anaesthesia. Thus, 2 mL of blood from the heart was drawn and then put in EDTA tubes and the lung and liver were collected for biochemical assay. Before the study began, ethical approval was issued by the Ethics Committee of the Faculty of Medicine of Universitas Islam Indonesia, Yogyakarta, Indonesia (No 14/Ka.Kom.Et/70/KE/VIII/2021).

E-cig liquid formulation

E-cig liquid was adapted with modification as described previously by Glynos et al,[6] formulated by mixing propylene glycol (PG) (*Dow,* Jakarta, Indonesia), vegetable glycerine (VG) (*Iniko Karya Persada,* Jakarta, Indonesia) and pure nicotine liquid (*RTS Vapes,* NC, USA). To avoid study bias caused by flavour additives, the unflavoured e-cig liquid was used. The liquid was made in 100 mL for three different nicotine concentrations (0, 6 and 12 mg/mL) without flavouring agents, as follows: 1) Nicotine of 0 mg/mL liquid was formulated by mixing 40 % PG and 60 % VG; 2) Nicotine of 6 mg/mL liquid was formulated by mixing 34 % PG, 60 % VG and 6 % pure nicotine;

3) Nicotine of 12 mg/mL liquid was formulated by mixing 28 % PG, 60 % VG and 12 % pure nicotine (percentage equals volume in 100 mL liquid).

To monitor the stability of ingredients and e-cig liquid formulation over time, the standard method of storage recommended by the supplier was followed. The e-cig liquid was stored in a tightly closed glass container at room temperature and kept in the dark. The liquid was periodically checked for changes in colour, odour, viscosity and signs of separation or precipitation. The e-cig liquid remained stable over time and did not exhibit any significant changes in its physical properties or composition.

E-cig exposure instrument and protocol

A commercially available mod e-cig with batteries and atomisers was used (*Dovpo*, Guangdong, China). Exposure to e-cigs was carried out using the semi-automatic exposure instrument, as detailed information was reported in a previous study.[17] A group of exposed rats was placed in the exposure instrument chamber according to their respective group. They were exposed to 0, 6 and 12 mg/mL of nicotine concentration in e-cig liquid for 25 cycles (1 cycle consisting of a 5-second puff, 30-second interval and 30-second exhaust) per day, adjusting for real-life exposure to humans.

In preparation for the e-cig exposure in this study, a preliminary investigation on several e-cig users to gain insight into their typical usage patterns was conducted. Investigation showed that users generally inhale for 2-3 seconds, followed by an interval of 5-10 seconds to allow the aerosol to remain in the user's respiratory tract and followed by exhaling slowly. However, to adapt this to animal experiments, some adjustments were made. To ensure that semi-automatic exposure instrument delivered enough aerosol to the chamber within sufficient time, 5-second puffs with a 30-second interval were produced. As a group of rats was in the chamber, it was necessary to ensure that they inhaled the aerosol produced by the e-cigs and were assisted by the pump to flow slowly, mimicking human exposure. Therefore, the interval was set to 30 seconds. After exposure, the chamber was cleaned for 30 seconds using a mini fan and an exhaust air pump to eliminate any residual aerosol. Finally, the 25 cycles obtained from the time it took to spend one cigarette in previous study were used and replicated for e-cig use.[17]

Plasma and tissue preparation for assay

Collected blood samples were centrifuged at 1073 × g (at 4 °C) for 10 min to obtain plasma. Thus, plasma separated and transferred to microcentrifuge tubes and stored at -25 °C until analysis within less than a week.

Liver and lung organs were stored immediately after collection at -25 °C until ready for assays within less than a week. To prepare the tissue for MDA assay, 1 g of liver and lung tissues were homogenised with phosphate-buffered saline (0.01 M, pH 7.4) and centrifuged at 10,000 × g for 10 minutes (at 4 °C). The supernatant obtained was used for MDA assay.

For SOD assay tissue preparation, 1 g of liver and lung tissues were homogenised using a solution containing 0.1 M Tris/HCl (pH 7.4), 0.5 % Triton X-100, 5 mM β-ME and 0.1 mg/mL PMSF on ice. Centrifuge the resulting homogenate at 14,000 × g for 5 minutes (at 4 °C) to obtain supernatant for SOD assay.

Biochemical assay

MDA in plasma and liver and lung tissue supernatant assays were performed using commercial reagent kits and standard protocol provided by *Elabscience* (Wuhan, Hubei, China). The MDA assessment was carried out using the colourimetric method (Catalogue No: E-BC-K025-S) based on its reaction with thiobarbituric acid-reactive substances that produces a pink chromogen, then measured with a spectrophotometer at 532 nm.

A colourimetric method was also performed to assess plasma and tissue supernatant SOD activity (Catalogue No: K335-100) at 450 nm, plasma Cat activity (No: K773-100) at 570 nm and plasma GPx activity (No: K762-100) at 340 nm using a microplate reader according to manufacturer's standard protocols and their respective commercial reagent kits provided by *BioVision* (Milpitas, California, USA).

Plasma AST and ALT for assessing liver function were performed using an optimised UV-test method with a commercial reagent kit and standard protocol provided by *DiaSys* (Holzheim, Germany).

Statistical analysis

Data analysis was performed using IBM SPSS

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26 (Chicago, IL, USA). Statistical analysis was determined using One-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All data were displayed as the mean ± standard deviation (SD). The $p < 0.05$ was considered significant.

Results

Oxidative stress in plasma

Plasma MDA levels shown in Figure 1a indicated that e-cig exposure for eight weeks was significantly increased in e-cig-exposed groups compared to the Normal group. Plasma MDA level in the nicotine variation groups was highest in Nic 12, followed by Nic 6 and Nic 0, with significant differences among the exposed group.

Contrary, compared to the normal group, different nicotine concentrations demonstrated a significant decrease in SOD, GPx and Cat activities in e-cig-exposed groups, as shown in Figure 1b-d. The lowest mean of these antioxidant activities was found in Nic 12 group, followed by the Nic 6 and Nic 0 groups, with significant differences among them by post hoc analysis except SOD activity between Nic 0 and Nic 6 groups.

Oxidative stress and function in liver

Oxidative stress in the liver was induced, reflected by high levels of MDA significantly in the groups exposed to e-cigs with liquid containing nicotine content variations compared to the Normal control group. The Nic 12 group had the highest levels of MDA, followed by the Nic 6 and Nic 0 groups, with a significant difference among exposed groups (Figure 2a).

Regarding liver SOD activity, a significant decrease in all exposed groups compared to the Normal group was observed. The lowest plasma GPx activity was in Nic 12, followed by Nic 6 and Nic 0. In the exposed groups, no significant difference between Nic 0 and Nic 6 groups, but Nic 12 group had significant differences compared to Nic 0 and Nic 6 groups (Figure 2b). Liver function was impaired after exposure to e-cig, characterised by increased levels of AST and ALT in plasma in e-cigs exposed groups compared to the Normal group. The highest levels of both liver function markers were in Nic 12, followed by the Nic 6 and Nic 0 groups, with a significant difference among them (Figure 2c-d).

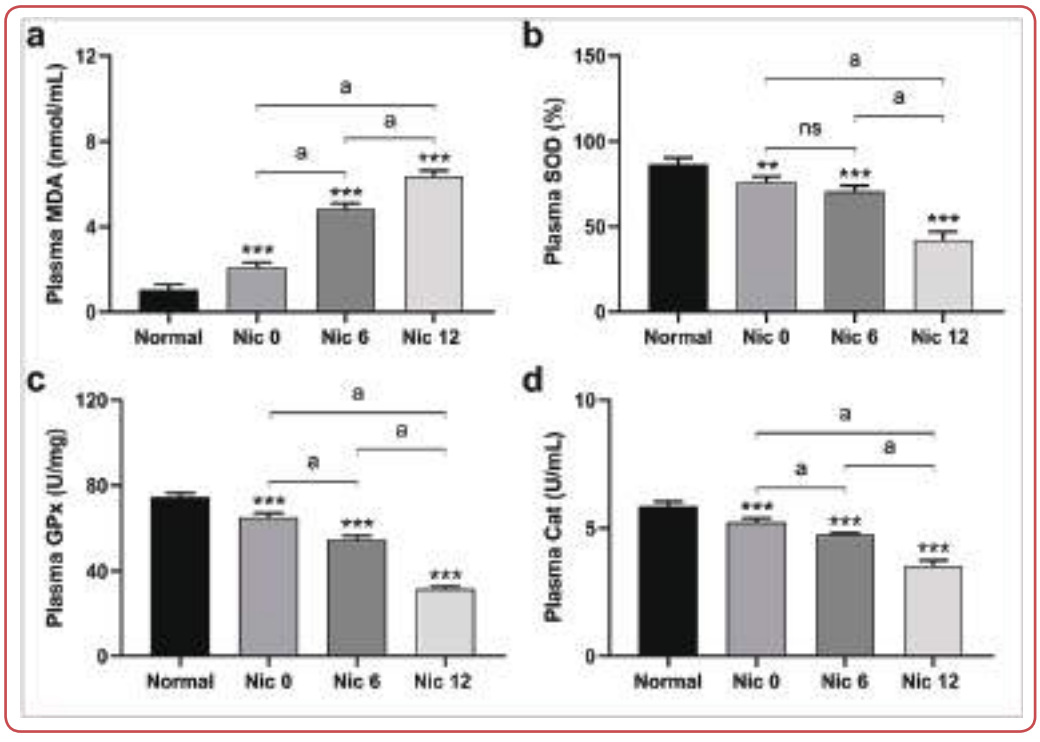

Figure 1: Plasma oxidative stress markers after e-cig exposure to different nicotine concentrations

****p < 0.001; **p < 0.01 compared to the Normal group; a = p < 0.001; ns = not significant; MDA: malondialdehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; Cat: catalase; Normal: a control group that did not receive any exposure; Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid; Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid; Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.*

CONTENT

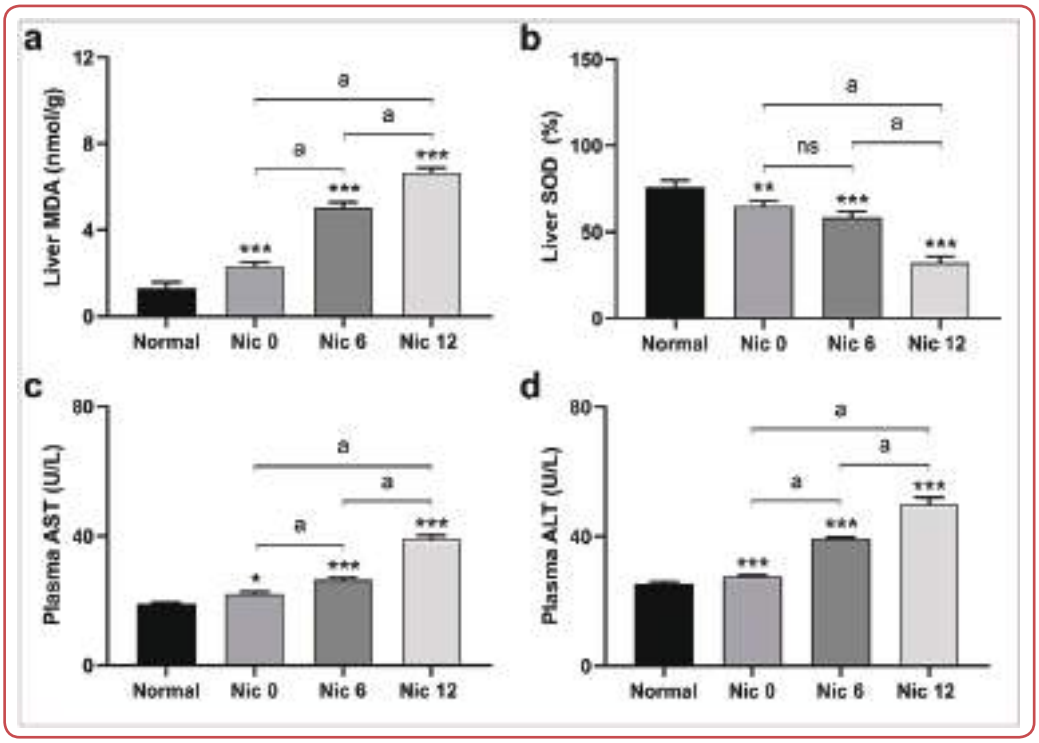

Figure 2: *Liver oxidative stress markers and function after e-cig exposure to different nicotine concentrations*

****p < 0.001; **p < 0.01 compared to the Normal group; a = p < 0.001; ns = not significant; MDA: malondialdehyde; SOD: superoxide dismutase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Normal: a control group that did not receive any exposure; Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid; Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid; Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.*

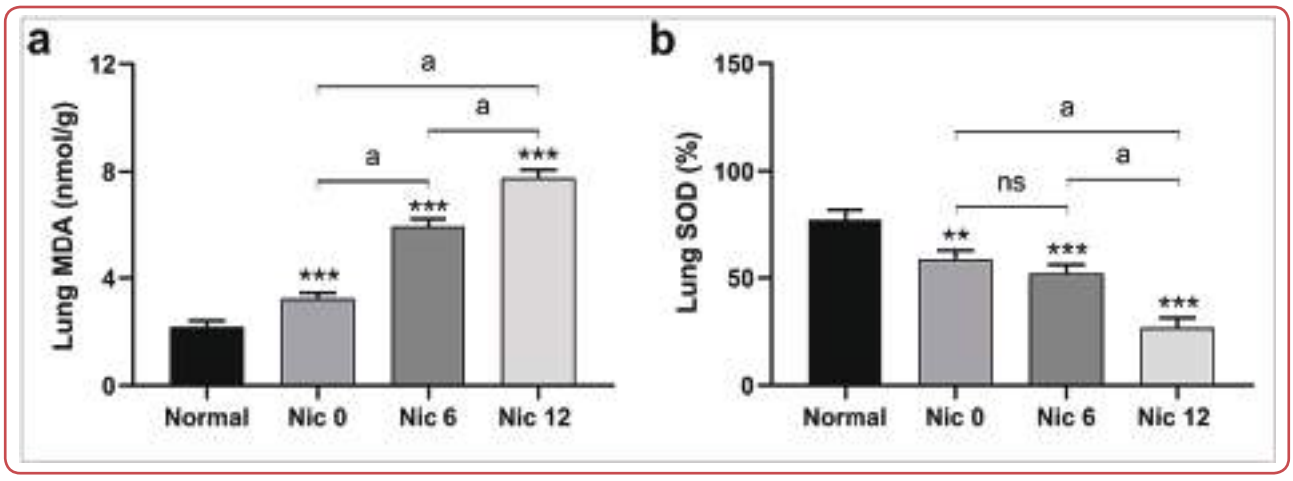

Figure 3: *Lung oxidative stress markers after e-cig exposure to different nicotine concentrations*

****p < 0.001; **p < 0.01; *p < 0.05 compared to the Normal group; a = p < 0.001; ns = not significant; MDA: malondialdehyde; SOD: superoxide dismutase; Normal: a control group that did not receive any exposure; Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid; Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid; Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.*

Oxidative stress in the lung

Variation concentration of nicotine contained in e-cig liquid caused an increase in lung MDA levels compared to the Normal control. The highest dose of nicotine in the Nic 12 group had a higher impact on increasing lung MDA levels, followed by Nic 6 and Nic 0 groups. There was a significant difference among the exposed groups (Figure 3a).

The rats treated with different nicotine concentrations in e-cig liquid demonstrated significant results compared to the Normal group. Lung SOD activity was lowest at Nic 12, followed by Nic 6 and Nic 0, with significant differences between the exposed groups (Figure 3b).

CONTENT

Discussion

A previous *in vitro* study revealed that flavourings acetoin, pentanedione, diacetyl, cinnamaldehyde, ortho-vanillin, maltol and coumarin of e-cig liquid without nicotine induce an inflammatory response with an increase in interleukin (IL)-8 in human monocytes mediated by increased ROS production.[12] E-cig is also believed to be a safer alternative to conventional cigarettes. However, the potential chronic effects of its use are not clearly understood and study on experimental animals is still limited. Therefore, this study aimed to investigate the chronic effect of e-cig exposure to unflavoured liquid on the rat's model. The different nicotine concentration in e-cig liquid without flavours was observed. The hypothesis that different levels of nicotine content, including absence (0 mg/mL), mild (6 mg/mL) and high (12 mg/mL) can cause oxidative stress was confirmed. This is evidenced by increased levels of MDA, a reactive compound of lipid peroxidation, in the plasma, liver and lung. Additionally, a decrease in the activity of antioxidant enzymes such as SOD in the plasma, liver and lung, as well as Cat and GPx in the plasma was observed. Furthermore, oxidative stress revealed in a concentration-dependent manner that the higher nicotine concentration in e-cig liquids further exacerbates oxidative stress. In addition, this study also demonstrated that PG and VG without nicotine components in e-cig liquid cause oxidative stress.

The process of generating oxidative stress either from e-cig or liquid is not fully understood. However, a previous study confirmed the formation of OX/ROS in e-cig for two reasons. Firstly, due to the activation of the liquidless heating element, replacing the new heating element has the highest OX/ROS generation compared to multi-use and when the heater is activated it produces a small amount of aerosol without the addition of liquid. Second, through the e-cig liquid vaporising process, a drop of liquid causes a fluorescence spike in oxidised dichlorofluorescein using a multi-use heat element on the fourth use.[5]

It has been reported that without the combustion process in e-cig and conventional cigarettes nicotine administration induce oxidative stress. Subcutaneous injection of nicotine alone to rats for a month has been confirmed to induce oxidative stress in a concentration-dependent manner, as reported in the previous study, which increased MDA and decreased total antioxidant capacity.[9] Simultaneously, the mean of ZO-1 immuno-positive cells decreased and vascular endothelial growth factor increased in the group with higher nicotine concentrations, indicating dysfunction of the airway epithelial barrier and increased angiogenesis.[9, 18] In another *in vivo* study, two months of intraperitoneal nicotine injection caused oxidative stress by mediating increased MDA, decreased activity of SOD, Cat, reduced glutathione (GSH), oxidised glutathione (GSSG), GPx and glutathione transferase (GST). Furthermore, an excessive inflammatory response was also observed with increased tumour necrosis factor (TNF)-α, IL-17 and nuclear factor (NF)-κB expression.[13] These findings suggest that nicotine alone independently induces oxidative stress and inflammation without combustion in e-cig and conventional cigarettes.

The current study demonstrated increased plasma, liver and lung MDA levels in all groups of rats exposed to an e-cig aerosol with different nicotine concentrations corresponding to increased nicotine concentration. AST and ALT levels also increased, indicating oxidative stress-induced liver damage. Simultaneously, the activity of plasma, liver and lung SOD, as well as plasma Cat and GPx, decreased, which indicates oxidative stress generated by chronic e-cig aerosol exposure. That is in line with a previous *in vitro* study by Lerner et al[5] that demonstrated that e-cig aerosol exposure in human airway epithelial cells (H292) increased IL-6 and IL-8 expression. Furthermore, exposure to e-cig aerosol in C57BL/6J mice increased monocyte chemoattractant protein (MCP)-1, IL-1α, IL-6 and IL-13 expression compared to the control group. A previous *in vivo* study reported that NOX-2-mediated chronic e-cig exposure induces oxidative stress that develops vascular, brain and lung damage.[8] In addition, e-cig use induces hepatic steatosis through increased oxidative stress and hepatocellular apoptosis, as well as disruptions in cholesterol and lipid metabolism and the liver's circadian system.[7] Another study reported that acute e-cig exposure induces inflammation and oxidative stress, resulting in cognitive impairment.[19] Exposure to e-cig aerosol, both with and without nicotine, has been shown to induce oxidative stress by enhancing the inflammatory response, indicating that other constituents of e-cig liquids besides nicotine may also be involved.

CONTENT

Previous studies have reported the effect of nicotine on e-cig without distinguishing whether the parameter changes are because of the nicotine, the flavours or PG and VG as the main component of e-cig liquid.[6, 20] When heated at high temperatures, PG and VG lead to carbonyl compounds formation, such as acrolein, acetaldehyde and formaldehyde.[21, 22] Carbonyl compounds have irritant properties and cause acute inhalational toxicity. Acrolein exposure has been known to induce ROS generation, cause pulmonary inflammation associated with COPD, contribute to asthma and induce p53 adduction in the development of lung cancer.[23] An *in vitro* study by Chen et al[24] in human lung epithelial BEAS-2B cells demonstrated that acetaldehyde exposure reduced lysine residues acetylation at the N-terminus of cytosolic histones H3 and H4 and could inhibit chromatin assembly. These findings indicate that defective chromatin assembly is associated with dysregulation of transcription, DNA replication and repair and genome instability. Formaldehyde also causes airway irritation and impairs pulmonary function, which is associated with asthma.[25] Meanwhile, PG and VG in e-cig liquid exposure for four weeks in mice increased bronchoalveolar lavage fluid (BALF) cellularity, induced oxidative stress, increased epithelial Muc5a production and negatively impacted lung mechanics suggesting respiratory irritation.[6] In line with presented study, exposure to e-cig aerosol with liquid containing PG and VG without nicotine causes an increase in oxidative markers in rats.

Conclusion

In summary, oxidative stress plays an essential and broad role in pathogenesis in many organ systems. This study suggests that chronic exposure to e-cig with unflavoured liquids, with or without nicotine, induces systemic as well as liver and lung organs oxidative stress in a concentration-dependent manner, which in turn, potentially leads to a variety of oxidative stress-induced diseases.

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Conflict of interest

The authors declare there is no conflict of interest. This study does not get financial support from any conventional cigarette or e-cig company.

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ORIGINAL ARTICLE

Knowledge, Attitudes and Nursing Self-Evaluation Related to Clinical Research

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Abstract

Background/Aim: Clinical research nursing is a well-known concept in Europe and other countries. The purpose of this study was to investigate the nurses' knowledge and attitudes towards clinical research and their opinions and self-evaluation about clinical research nursing and factors affecting them.
Methods: A cross sectional study was conducted at the University Clinical Centre of the Republic of Srpska (UCCRS). A questionnaire included 50 questions/statements was created in order to address the aims of the research and afterwards distributed to 120 nurses from 6 departments.
Results: Response rate was 91.6 %. Most of the respondents showed a low level of knowledge, but positive attitude related to clinical research. Nurses who participated in clinical research were confident in their competencies according to their self-evaluation.
Conclusion: Systematic approach to the additional nurses education could have a significant impact on a success of clinical research.

Key words: Clinical research nurse; Self-evaluation; Practice; Clinical centre.

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Introduction

Clinical research plays an important role in the development of health services and it can significantly influence the quality of patient care.[1-4] Clinical research is strictly regulated and should be conducted in accordance with the principles of Good Clinical Practice (GCP).[5] The availability of new treatment options or the improvement of existing treatments can only be achieved and justified through clinical research.[6] It is well known that clinical research could provide additional benefits for patients, health personals, health institutions, society and to the state economy. Despite all values and benefits, no more than 20 clinical research studies have been conducted in Bosnia and Herzegovina (B&H) annually[7] and approximately 17 of those at the University Clinical Centre of the Republic of Srpska (UCCRS).[8]

The success of clinical research depends on involvement and contribution of different profiles of health professionals, such as: physicians, nurses, pharmacists, statisticians and professional bodies like Ethics committee, Contract Research Organisations (CRO) and other participants.[6, 9]

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According to Organisation for Economic Cooperation and Development (OECD) recommendation, the education, training and infrastructure have been emphasised as key elements for success of clinical research.[10] German Scientific Council also states that qualified and experienced staff are essential at several levels for conducting the clinical research.[11]

According to European survey on national training activities in clinical research an important role in clinical research team belongs to the Clinical Research Nurse (CRN).[12] On the other hand, the clinical research nursing in Turkey is recognised as a new concept, but framework and legislation in this subject have not yet been put into practice.[6] In B&H there is no specific study for CRN and that kind of concept has not been recognised at practice level yet. By the *Ordinance on clinical trials on medicinal products and medical devices,* published by the Agency for Medicinal Products and Medical Devices of B&H, the principal investigator creates clinical research team, without obligation to include nursing stuff.[13] However, the standard operational procedures of the UCCRS Ethics Committee have clear demand for principal investigators to involve nurses into clinical research team.

There are only few studies globally which analysed nurses' knowledge, attitudes and opinions towards clinical research. To the best of authors' knowledge there are no such studies in West Balkan region including B&H.[6, 9] The purpose of this study was to investigate the nurses' knowledge and attitudes towards clinical research and their opinions and self-evaluation related to the clinical research nursing and factors affecting them.

Methods

The questionnaire-based study was designed as a cross-sectional study performed at the UCCRS from 14-18 March 2022. The study was approved by the UCCRS Ethics committee (No 01-19-94-2/22) and was conducted in accordance with the Declaration of Helsinki. The questionnaire containing 50 questions/statements was created, according to previously used tools, to address the aims of the research.[6, 14] The main researcher contacted principal nurses at the departments of: oncology, neurology, pulmonology, haematology, gastroenterology and rheumatology in order to distribute questionnaires to 120 nurses at their belonging departments. In these 6 departments most of the clinical research was conducted in the past years.

The first part of the questionnaire consisted of 8 questions related to the main characteristics of the respondents: gender, years of work experience, level of education, whether they have received education in the field of clinical research and GCP and which one, whether they were satisfied with their education in the field of clinical research, as well as whether they have participated in clinical research so far.

The second part consisted of 18 statements related to basic knowledge about clinical research, ethical concepts, etc. For each question, 3 answers were offered: *true*, *false* or *I don't know*. Each correct answer was scored with 1 point, while an incorrect answer (or *I don't know*) was scored with 0 points. A higher total score represented the higher level of knowledge about clinical research.

In the third part, respondents were offered with 8 statements related to clinical research, based on nurses' attitudes towards clinical research. Three choices were offered for each statement: *true*, *false* or *I don't know*. All statements marked with *true* were scored with 1 point and the rest with 0 points. A higher score indicated a more positive attitude towards clinical research.

In the fourth part, 3 questions were offered to assess the respondents' opinion regarding necessity of education and the position of nurses in clinical research.

Fifth part was created in order to assess the role and self-evaluation of nurses who participated in clinical research in the UCCRS.

Data were processed using IBM SPSS v 18.0 for Windows. After the normality of data distribution was determined using the Kolmogorov-Smirnov test, adequate parametric/non-parametric statistical tests were applied. Categorical data were compared by Chi-squared test and continual data with Student t-test and One-way ANOVA or Man-Whitney U-test and Kolmogorov-Smirnov test for non-parametric data. Unanswered questions were excluded from the statistics. The questions in Table 3, 4, 5 were related to knowledge regarding clinical research and the questions in

Table 6 related with attitudes about clinical research. Therefore, correlations of respondents' characteristics with their knowledge and attitudes were analysed. $P < 0.05$ was taken as the level of statistical significance.

Results

A 110 nurses contributed to this study. The response rate was 91.6 %. Majority of them were female with working experience of less than 20 years. Nearly 71 % of them finished secondary nursing school and less than 12 % finished high nursing school. The basic characteristics of the respondents are shown in Table 1.

Table 1: Basic data on respondents

Parameter	N	%
Gender		
Female	95	86.36
Male	15	13.64
Experience (years)		
1-10	50	45.45
11-20	34	30.91
21-40	25	22.73
Missing	1	0.91
Education		
Secondary nursing school	78	70.91
High nursing school	13	11.82
Nursing college	18	16.36
Missing	1	0.91

N: number of participants (total number of respondents was 110);

The largest number of respondents did not have any education related to clinical research and those who had some kind of education got it either online or by CRO. Majority of respondents were not satisfied with their knowledge in this area and just a small number of them participated in 1 or more clinical research (Table 2).

In the part of the questionnaire that refers to knowledge about clinical research related to ethical concepts and regulations, it was noticed that a significant number of the respondents showed a lower level of knowledge (Table 3).

In the part of the questionnaire that refers to the knowledge of the term volunteering in clinical research, the lowest level of knowledge was shown in connection with following statements S2, S7, S8 and S9 (Table 4).
In the part related to the design of clinical research, it is noticeable that more than half of respondents were not familiar with basic terms in clinical research such as blinding and randomisation and a high number of respondents were not sure whether a placebo can be used in clinical research (Table 5).

Correlation of knowledge and characteristics of respondents showed that there was no significant difference in relation to gender. The knowledge of

Table 2: Participation and education of respondents in clinical research

Question	Answer	N	%
Have you received training in clinical research and good clinical practice (GCP)?	Yes	23	21.90
	No	82	78.10
If so, the education was conducted by:	CRO	8	32.00
	State institutions	2	8.00
	On-line	12	48.00
	Other	3	12.00
How satisfied are you with your current education in the field of clinical research?	I am not	42	51.22
	A little	17	20.73
	Average	18	21.95
	Very much	5	4.10
How many clinical research have you participated in?	None	69	71.88
	1-3	20	20.83
	4-5	3	3.12
	6 or more	4	4.17

CRO: Contract Research Organisation; N: number of participants (total number of respondents was 110);

Table 3: Knowledge of clinical research, ethical concepts and regulations

Statement	%	Statement	%	Statement	%
S1		**S3**		**S5**	
True	89.72	True	76.85	True	60.19
False	0.00	False	6.48	False	0.00
No idea	10.28	No idea	16.67	No idea	39.81
S2		**S4**		**S6**	
True	92.52	True	75.00	True	62.96
False	3.74	False	0.00	False	0.00
No idea	3.74	No idea	25.00	No idea	37.04

S1: Clinical research is study of a drug, biological drug or medical device in humans with the intention of discovering potential beneficial effects and/or determining its safety and effectiveness.
S2: Before a new drug can become available to the public, it must be tested on humans.
S3: Clinical research cannot be initiated without the approval of the ethics committee of the institution where they are conducted, as well as without the decision of the state regulatory body.
S4: Clinical research must be conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki and must be in accordance with good clinical practice (GCP) and legal regulations.
S5: The GCP is an international ethical and scientific quality standard for the design, conduct, recording and reporting of research involving the participation of human subjects.
S6: Compliance with this GCP provides public assurance that the rights, safety and well-being of subjects are protected in research.

CONTENT

Table 4: Knowledge of voluntary concept in clinical research

Statement	%	Statement	%	Statement	%
S1		**S4**		**S7**	
True	80.55	True	89.81	True	64.81
False	0.93	False	0.93	False	0.93
No idea	18.52	No idea	9.26	No idea	34.26
S2		**S5**		**S8**	
True	69.44	True	80.56	True	55.56
False	0.00	False	0.00	False	5.56
No idea	30.56	No idea	19.44	No idea	38.89
S3		**S6**		**S9**	
True	63.89	True	87.74	True	62.96
False	0.00	False	3.77	False	2.78
No idea	36.11	No idea	8.49	No idea	34.26

S1: A volunteer is defined as a patient or healthy person participating in clinical research who must give written informed consent to participate in the research either in person or through a legally authorised representative of the participant.
S2: An informed consent form (ICF) is a document that provides a potential volunteer with detailed and comprehensible information about clinical research and must be signed by the participant or his legally authorised representative.
S3: The volunteer must sign the ICF before participating in the clinical research.
S4: The safety of clinical research participants is a high priority.
S5: Clinical research on children, pregnant and lactating women are strictly prohibited by national legislation.
S6: In clinical research, participants' personal and medical information is kept confidential.
S7: Volunteers must not be offered high fees to induce them to participate in clinical research.
S8: A participant can leave clinical research at any time without giving a reason.
S9: The costs of treatment of adverse events occurring during the clinical research are covered by the sponsor.

Table 5: Knowledge of clinical research

Statement		N	%
It is possible to use a placebo in clinical research	True	79	73.15
	False	3	2.78
	No idea	26	24.07
Blinding (single-blind, double-blind) is used in clinical research to avoid errors arising from bias	True	44	41.51
	False	2	1.89
	No idea	60	56.60
Randomisation is used in clinical research to avoid errors due to bias	True	49	46.67
	False	3	2.86
	No idea	53	50.48

nurses who have worked for less than 10 years was significantly lower compared to the other groups ($p < 0.001$) and nurses with a university degree have significantly better knowledge compared to those who completed secondary nursing school ($p < 0.001$). Nurses who had training related to clinical research showed significantly better knowledge ($p < 0.001$) (Figure 1).

In the assessment of attitudes regarding clinical research, it is worrying that half of the respondents did not have a positive attitude towards the reliability of clinical research and that only a quarter of the respondents believed that standard treatment is not always more reliable compared to the drugs being tested. A large number of respondents did not have a certain attitude related to ethics in conducting clinical research (Table 6).

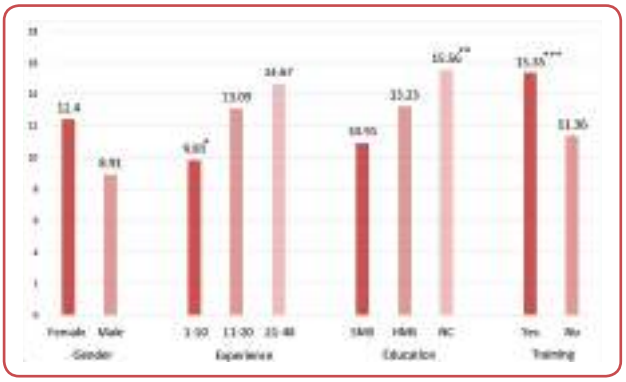

Figure 1: Average knowledge of nurses about clinical research

SMS: Secondary nursing school; HMS: high nursing school; NC: nursing college; Training: previous training in clinical research; A higher total score is represented as a higher level of knowledge about clinical research.
**The knowledge of nurses who have worked for less than 10 years was significantly lower compared to the other groups ($p < 0.001$).*
***Nurses with a university degree have significantly better knowledge compared to those who completed secondary nursing school ($p < 0.001$).*
****Nurses who had training related to clinical research showed significantly better knowledge ($p < 0.001$).*

Correlation of attitudes and characteristics of respondents shows that females had a more positive attitude compared to males ($p = 0.011$). The

Table 6: Attitudes about clinical research

Statement	N	%	Statement	N	%
S1			**S5**		
Agree	88	83.02	Agree	2	1.87
Disagree	3	2.83	Disagree	65	60.75
Neither	15	14.15	Neither	40	37.38
S2			**S6**		
Agree	101	94.39	Agree	15	14.02
Disagree	1	0.93	Disagree	29	27.10
Neither	5	4.67	Neither	63	58.88
S3			**S7**		
Agree	106	99.07	Agree	106	73.33
Disagree	0	0.00	Disagree	0	4.76
Neither	1	0.93	Neither	1	21.90
S4			**S8**		
Agree	50	46.73	Agree	98	93.33
Disagree	12	11.21	Disagree	2	1.90
Neither	45	42.06	Neither	5	4.76

S1: Clinical research improve the quality of care for patients.
S2: Clinical research are beneficial to society.
S3: Clinical research are important for the advancement of medicine.
S4: Clinical research are reliable.
S5: It is not ethical to conduct clinical research.
S6: Standard treatments are always more trustworthy than new investigational drugs.
S7: Patients can refuse to participate in clinical research without affecting their further treatment.
S8: Nurses should have more knowledge about clinical research.
Neither: neither agree nor disagree.

CONTENT

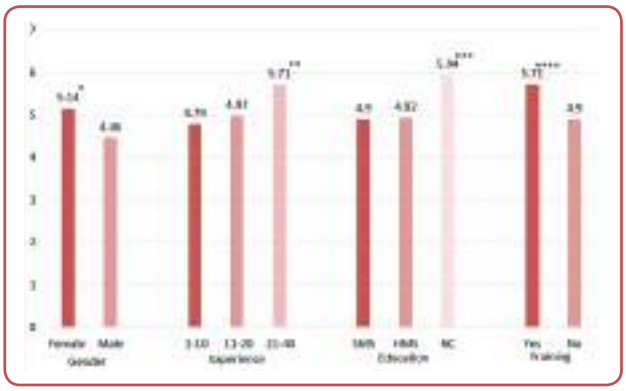

Figure 2: Average attitudes of nurses about clinical research

SMS: Secondary medical school; HMS: high medical school; NC: nursing college; Training: previous training in clinical research; A higher score indicated a more positive attitude towards clinical research.
**Females had a more positive attitude compared to males (p = 0.011).*
***The attitudes of technicians who have been working for more than 20 years were more positive than the other two groups (p = 0.005).*
****Technicians with a university degree have significantly more positive attitudes compared to both groups (p = 0.003).*
*****Technicians who had training related to clinical research show a significantly more positive attitude about clinical research (p = 0.002).*

attitudes of technicians who have been working for more than 20 years were more positive than the other two groups (p = 0.005). Technicians with a university degree had significantly more positive attitudes compared to both groups (p = 0.003). Technicians who had training related to clinical research showed a significantly more positive attitude about clinical research (p = 0.002) (Figure 2).

In the part that refers to the opinion about the place of nurses in clinical research, it was evident that more than half of the respondents believed that a new specialisation for nurses, as nurses in clinical research, should be proposed and must be a mandatory part of the nurses' education. Also, more than half of the respondents expressed their desire to participate in clinical research (Table 7).

Opinions about clinical research in relation to knowledge and attitudes are shown in Figure 3. A higher score indicated a more positive attitude

Table 7: Opinions about clinical research

Statement		N	%
Nurses who work in clinical research should be considered as a separate profession	Yes	57	53.77
	No	20	18.87
	Not sure	29	27.36
Clinical research must be part of the education of nurses	Yes	77	72.64
	No	12	11.32
	Not sure	17	16.04
Would you like to participate in a clinical research?	Yes	63	59.43
	No	29	27.36
	Not sure	14	13.21

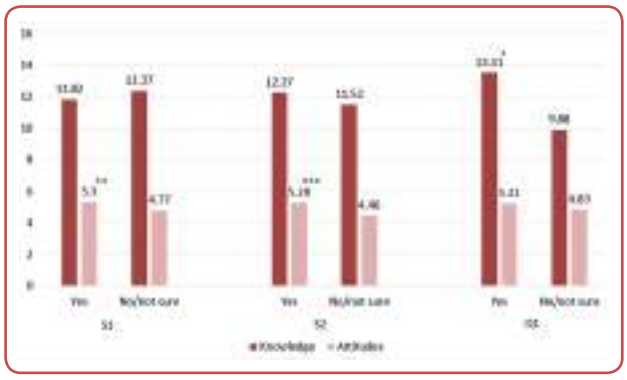

Figure 3: Opinions about clinical research related to knowledge and attitudes about clinical research

S1: Nurses who work in clinical research should be considered a separate profession.
S2: Clinical research must be part of the education of nurses.
Q1: Would you like to participate in a clinical research?
A higher total score is represented as a higher level of knowledge about clinical research. A higher score indicated a more positive attitude towards clinical research.
**Nurses with better knowledge about clinical research were more willing to participate in clinical research (p = 0.027).*
***Nurses with more positive attitudes more often thought that nurses who work in clinical research should be a separate profession ***and that clinical research should be part of nurses' education (p = 0.009 and p = 0.009, respectively).*
****Nurses with experience in clinical research.*

towards clinical research. Nurses with more positive attitudes more often thought that nurses who work in clinical research should be a separate profession and that clinical research should be part of nurses' education (p = 0.009 and p = 0.009, respectively). Nurses with better knowledge about clinical research were more willing to participate in clinical research (p = 0.027).

The fifth part of questionnaire, related to those nurses (27/110) who had some experience in clinical research, was additionally evaluated. Almost 96 % of nurses were engaged in collecting and preparing specimens from patients according to study protocol, while 52 % documented study participants' data. Only 5 of them were involved in screening of study participants and 6 participated in the informed consent process. Forty percent of them reported issues related to adverse events and 44 % collaborated with research team. Only 5 of them were engaged as educators for their research team.

Self-evaluation showed that most of the nurses were confident and very confident in their competencies to adequately explain all procedures to patients involved in clinical research (84.62 %), including the terms related to randomisation or placebo (61.5 %) and possible adverse events (73.08 %). On other hand, 65.4 % nurses felt unpleasant when placebo as comparator and 42.3 % when the new treatment was offered.

CONTENT

Discussion

A lot of nurses globally have been involved in clinical research, but small attention is dedicated to their real professional role. Presented study showed that more than half of the respondents stated low levels of satisfaction with their education in clinical research. There is a lack of studies which analyse nurses' experience and contribution in this field. Furthermore, many nurses from various fields do not understand defined role of CRN and have a perception of CRN as nurses who have a more administrative role and that they are excluded from daily care for patients.[9, 15]

In this study, it was found that most of respondents were women who had up to 10 years of experience as nurse and most of them had only secondary education in nursing school. In the Turkish study most of the respondents were also women, but with higher education (Bachelor's degree).[6] On the other hand, compared to the Turkish study there were significantly higher percentage of nurses in presented study who passed the training in clinical research and had experience in clinical research, 44 % and 85 %, respectively. Most of the nurses from study passed the training online, while other were educated by CRO and for only 8 % of them the education was organised by the different state institutions.

This study analysed knowledge of nurses about regulations in clinical research. It revealed the lack of knowledge related to ethic principles and legislations in clinical research which is congruent with literature findings.[6, 16-18] The lowest knowledge related to items linked to patients' voluntariness, were for knowledge due to: informed consent form, payments for participation in the research and "reasons" for leaving the clinical research. These results were congruent with the results that only 5 respondents from presented study were involved in screening of study participants and 6 actually participated in the inform consent process. Considering that nurses spend more time with patients than other health professionals, they were in a position to make the best assessment of patients' benefits with participation in certain clinical research.[3, 9] In contrary to this results, findings from other studies showed that nurses were quite knowledgeable on volunteering issues.[6, 17] Regulatory bodies in certain countries also provide formal trainings on appropriate procedures involved in informed consent in each specifical clinical site.[19]

Around half of the respondents in study were not familiar with basic knowledge related to placebo, blindness and randomisation in clinical research. This lack of knowledge was seen in other studies as well and may lead to staff nurses difficulties in communication with the clinical research team and patients as well.[6, 16] On the other hand, in presented study the self-evaluation of the nurses, who participated in clinical research, showed that most of them were confident in their competencies to adequately explain all procedures to patients during clinical research, including the terms like randomisation, placebo or possible adverse events.

Nurses' positive attitudes towards clinical research affect the collaboration with the clinical research team, the number of volunteers and finally hence the success of clinical research, as it was suggested by many studies.[3, 20-25] Nurses play a vital role in increasing the quality of patient care and the progress of the medical science. However, if there is no obligatory training in clinical research on national and institutional level there is a high possibility that nurses could not have positive attitudes related to clinical research, as it was found in this analyses. This result is a message to institutions to make an effort for performing more education and training in this field. On the other hand, it is encouraging that most of the nurses from presented study were willing to participate in clinical research and had awareness of needed qualification for CRN. Those nurses who had higher education and knowledge levels showed better willingness to participate in clinical research. Furthermore, women and those who had more than 20 years of work experience were more positively oriented toward clinical research in presented study.

Strengths and limitations

The strength of this study is that its subject is of practical significance, which evidently deserves further investigations. On the other hand, limitations are relatively small sample size from single centre and the questionnaire which have not been tested for reliability and validity.

CONTENT

Conclusion

Many nurses participate in clinical research but small attention is paid to defining their actual roles and evaluating their work in the process of conducting clinical research. Results from study showed that the largest number of nurses did not have education related to clinical research and most of them were not satisfied with their knowledge in this area. Nurses showed low level of knowledge in field of ethical principles and legislations, as well as in volunteering concept and design in clinical research. It is encouraging that most of nurses from study were willing to participate in clinical research and had awareness of needed additional education about clinical research. Those nurses who had higher education and knowledge levels showed better willingness to participate in clinical research. Additionally, self-evaluation of nurses who participated in clinical research showed that they were confident in many competencies related to their involvement in clinical research. It is needed to design and impose a systematic approach to the education of nurses having in mind its significant impact on a success of clinical research.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Sharma et al. Scr Med 2023 Mar;54(1):45-51. DOI:10.5937/scriptamed54-42967

ORIGINAL ARTICLE

Assessment of Adverse Drug Reactions in Oral Cancer Patients Receiving Chemotherapy Treatment at Tertiary Care Centres in North-Western India

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Abstract

Background/Aim: Pharmacovigilance in oncology is imperative as antineoplastic drugs are two-edged swords whose irrational use can pose a major health problem and a needless financial burden on the patient. The aim of this study was to study the comprehensive safety profile of anti-neoplastic drugs used for treating oral cancers.

Methods: This hospital-based prospective observational study was conducted at two premiers (a government and a private) tertiary care centres in North-Western India among newly diagnosed cases of oral cancers of both sexes between the ages of 20-70 years and requiring chemotherapy treatment. The prescribing pattern of chemotherapy drugs, associated adverse effects and potential risk factors for the development of adverse effects was studied. An adverse drug reaction (ADR) causality was assessed by the WHO-UMC algorithm and preventability by Schumock and Thornton's criteria. Univariate and multivariate logistic regression analyses were used to identify the predictors related to chemotherapy-induced adverse effects.

Results: The data concerned 188 patients, of which 64.3 % developed chemotherapy-related adverse effects. Among the prescribed anti-neoplastic drugs, a combination of 5-Fluorouracil, Cisplatin and Paclitaxel regimen was associated with the majority (91.42 %) of the adverse effects. Alopecia was the most common adverse effect noted in 26.44 % of patients, followed by nausea and anaemia in 15.7 % and 9.9 % of patients, respectively. Independent predictors of chemotherapy-related adverse effects were site (Adjusted odds ratio [AOR] = 1.95; 95 % CI 1.04 - 3.62, p = 0.03), chemotherapy and radiotherapy treatment (AOR = 5.00; 95 % CI 2.62 - 9.53, p < 0.001), combination regimen of 5-Fluorouracil, Cisplatin and Paclitaxel (AOR = 8.68; 95 % CI 2.55 - 29.48, p = 0.001), associated comorbidities (AOR = 16.68; 95 % CI 2.45 - 28.34, p < 0.001). Causality assessment revealed most adverse effects (82.64 %) to be possible.

Conclusion: The adverse effect varies with the type of regimen which is prescribed for the patient. Site of cancer, concomitant radiotherapy treatment and associated comorbidities were the identifiable risk factors for developing adverse effects. Onco-pharmacovigilance studies in the future will help to provide tailored treatment to patients and improve their quality of life.

Key words: Adverse drug reactions; Causality assessment; Chemotherapy; Oral cancers; Risk factors.

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Introduction

In developing countries like India, noncommunicable diseases are the biggest cause of premature death.[1] New cancer patients are estimated to be 1.1 million per year in India,[2] while the mortali-

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CONTENT

ty associated with it is about 0.6 million people each year.[3] Tobacco-related cancers itself are the major culprits in the mortality associated with cancer.[3] In Jaipur City located in north-western region of India, the four leading sites of cancer in males are tobacco-related cancers – lung, tongue, mouth and oesophagus, followed by prostate cancer, as reported in the National Cancer Registry Programme of the Government of India.[4]

World Health Organization (WHO) defines adverse effects[5] as "a response to a drug which is noxious and unintended and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modifications of physiological function." The main modalities used for cancer treatment include surgery, radiation, chemotherapy or immunotherapy. Cancer chemotherapy utilises either single or combination of anti-neoplastic drugs in a standardised regimen as prescribed for the management of neoplasia.[6] It is imperative to monitor adverse drug reactions (ADRs) in oncology, where pharmacotherapy is linked by high prevalence of drug-related complications due to their narrow therapeutic window.[7] Chemotherapy adversely affects the quality of life in cancer patients and may land them in a series of misfortunes that includes feelings of low mood, restricted mobility, low sexual desire, reduced social interaction and undermining capabilities at work.[8]

Although few studies pertaining to pharmacovigilance in cancer patients have been undertaken in the past, all were generally designed as snapshot studies with relatively little attention to a particular cancer and its associated risk factors in the target populations.[9-12] Therefore, the present study was planned among the oral cancer patients with the aim of providing them with tailored pharmacotherapy with fewer adverse effects and complications.

Methods

Ethical consideration

This study was approved by the institutional ethics committee of SMS Medical College (Reference No 3206 MC/EC/2017). It was conducted according to the Declaration of Helsinki. Written informed consent was obtained from the patients prior to their recruitment.

Participants and eligibility

Inclusion criteria for the study were: a) patients between 20-70 years of age b) newly admitted and confirmed cases (by histopathology) of the squamous cell carcinoma of the oral cavity and oropharynx which required treatment with chemotherapy (irrespective of adjuvant setting, neo-adjuvant setting, with or without radiotherapy, as radical definitive chemo-radiotherapy or as palliative treatment) and in any of the stage I, II, III and IV (according to AJCC of head and neck cancers). The non-inclusion criteria for the study were pregnant and lactating females, patients of psychiatric disorders, patients suffering from HIV and hepatitis B infection.

Sample size and sampling technique

Taking prevalence (P) of ADRs as 58.6 %, confidence interval (CI) as 95 % and 5 % relative precision, the sample size was worked out to be 94 participants.[13] However, a pilot study was conducted in 100 patients for a month and the final sample size for the study was calculated as 188 to compensate for any loss to follow up. For the data collection two days of the same week of the month were chosen randomly by a computer-based random number generator to collect data from both hospitals.

This was a prospective and observational study conducted among 188 patients receiving chemotherapy treatment from May 2017 to December 2017 in two leading government and private charitable tertiary care centres in North-Western India, Swai Man Singh Medical College and Bhagwan Mahaveer Cancer Centre, Jaipur. These institution catered to the needs of patients with various haematologic and solid malignancies, with an average daily volume of more than 150 patients in the outpatient department and 50-80 patients were admitted daily in the indoor facility.

Study variables

A comprehensive review of each patient's medical record was conducted from the day of chemotherapy prescription to 90 days post-start of therapy. Parameters included age, gender, diagnosis, type of tobacco consumption, site of oral cancer, chemotherapy prescribed, dose and directions, any documented follow-ups and laboratory investigations like complete blood counts and other blood investigations to access the functioning of kidney and liver. Drug-related toxicities were identified by interviewing each patient personal-

CONTENT

ly for any chemotherapy-related adverse effects during oncology/haematology clinic visits and by personal telephonic encounters. A study proforma was developed in accordance with the ADR reporting form of the Central Drugs Standard Control Organisation (CDSCO), Government of India, for collecting data regarding the patients' demographic profile, the details of drugs received during chemotherapy sessions, route, dose and number of chemotherapy cycles, any pre-existing comorbidities and any adverse effects following chemotherapy cycles. The patients were monitored throughout and interviewed personally by the nursing staff and clinical pharmacist for ADR checks at three separate points of time - 30, 60 and 90 days (2 weeks at each interval) post-initiation of the chemotherapy drug. Adverse events were classified according to WHO-UMC causality assessment scale and the severity of these were assessed by Modified Hartwig and Siegel Scale.
The chemotherapy treatment (including the drug, dose, frequency and number of cycles) for each patient was decided by the consultant medical oncologist and team in accordance with the Indian Council for Medical Research guidelines and NCCN guidelines and evidence based protocols for different stages. The clinical pharmacists assisted the oncologists and nurses with treatment and therapy plans, ensuring appropriate supportive care options for each patient and addressing any drug-related questions, including ADRs. To manage the myelosuppression observed in the patients in this study, supportive intervention with haematopoietic growth factors (granulocyte colony-stimulating factors [G-CSFs] and erythropoiesis-stimulating agents [ESAs]) and blood transfusions were given.

Study outcomes

The primary outcome of interest was the incidence and pattern of chemotherapy-related ADRs occurring within 90 days of a patient starting treatment. The secondary outcomes of interest were predicting risk factors for the development of these adverse effects.

Statistical analysis

The collected data were analysed using IBM SPSS (Statistical Package for Social Sciences) statistics for Windows, version XX.0 (International Business Machines Corporation (IBM) Corp., Armonk, N.Y., USA). At first, all the prescriptions were coded using automated generated codes to avoid any information-related bias as the prescriptions were collected from two different settings that is government and private. Descriptive statistics (ie number and range) were used to analyse patients' demographic characteristics. For risk estimate analysis, the variables with p-values of < 0.2 in the binary univariate analysis were included in the multiple binary logistic regressions to control the effect of confounding variables. $P \leq 0.05$ was considered statistically significant and the results were reported as odds ratios (ORs) with 95 % confidence intervals (CIs).

Results

Demographic characteristic of study population

One hundred eighty-eight patients who received chemotherapy alone or in combination with other treatment were enrolled for this study. Out of that number 81 patients had oral cavity cancers, while 107 had cancer of oropharynx. There was a male preponderance of oral cancers (91.4 %). The majority of the patients receiving chemotherapy treatment were in the age group of 41-50 years. Both males and females were more likely to be diagnosed in stage 3 (52.65 %) as depicted in Table 1. 19.68 % of the patients suffered from different comorbidities.

Table 1: Demographics of patients

Characteristics	Patients (N = 188)
Age (years) - mean (range)	45.6 (24-68)
Male (%)	171 (91.4 %)
Tobacco history	
Yes	167
No	21
Site	
Oral cavity	81
Oropharynx	107
Stage*	
I	6
II	27
III	99
IV	56
Co-morbidities	
Hypertension	18
Diabetes	14
Rheumatoid arthritis	3
Hypothyroidism	2

**Stage according to AJCC of head and neck cancers;*

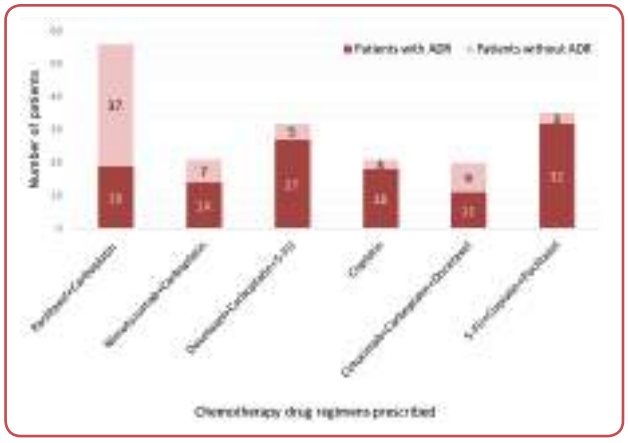

Figure 1: Prescribing pattern of chemotherapy drugs and distribution of associated adverse effects

5FU: 5- Fluorouracil;

Prescribing pattern and adverse drug reactions profile in oral cancer patients

Figure 1 shows that Paclitaxel and Carboplatin combination was the most frequently prescribed regimen for 29.8 % of the patients, while the Cetuximab, Carboplatin and Docetaxel combination was the least frequently prescribed regimen, prescribed to only 10.64 % of the patients. 64.3 % of patients developed 15 different types of ADRs, with the gastrointestinal system commonly affected (33.06 %) followed by skin (32.23 %). The 5-Fluorouracil, Cisplatin and Paclitaxel regimens were associated with majority (91.42 %) of the adverse effects. The majority (66.2 %) of the adverse effects occurred in male patients and the age group of 45-60 years was commonly implicated. Alopecia was the most common adverse effect noted in 26.44 % of patients, followed by nausea and anaemia in 15.7 % and 9.9 % of patients, respectively. 82.64 % of ADRs were classified as possible and 17.35 % as probable according to the WHO-UMC causality assessment scale as depicted in Table 2. Most reactions were mild (94.21 %) in nature and the remain were moderate (5.78 %), as assessed by Modified Hartwig and Siegel Scale. Furthermore, only 52.2 % of the drugs were prescribed by their generic names. The average number of drugs per prescription was 7.4. On an average, one anti-peptic ulcer drug was given to each patient.

Risk factors for development of adverse reactions

Risk estimates (Table 3) revealed that there was a significant association between site (adjusted odds ratio [AOR] = 1.95; 95 % CI 1.04 - 3.62, p = 0.03), chemotherapy and radiotherapy treatment (AOR = 5.00; 95 % CI 2.62 - 9.53, p < 0.001), combination regimen of 5-Fluorouracil, Cisplatin and Paclitaxel (AOR = 8.68; 95 % CI 2.55 - 29.48, p = 0.001), associated comorbidities (AOR = 16.68; 95 % CI 2.45 - 28.34, p < 0.001) and development of ADRs. However, age failed to show a statistically significant difference in risk of developing ADR.

Table 2: Pattern of adverse drug reactions (ADRs) to anticancer drugs and causality assessment of associated adverse effects (World Health Organization UMC causality assessment scale)

Regimen	ADRs	Number of patients	Causality assessment
5-Fluorouracil + Cisplatin + Paclitaxel	Alopecia	8	All Possible
	Anorexia	4	All Possible
	Dysgeusia	5	Possible: 4, Probable: 1
	Nail Discoloration	3	Possible: 2, Probable: 1
	Nausea	6	All Possible
	Anaemia	4	Possible: 3, Probable: 1
	Leukopenia	2	All Possible
	Neutropenia	2	Possible:1, Probable: 1
	Thrombocytopenia	1	All Probable
Cetuximab + Carboplatin + Docetaxel	Alopecia	2	All Possible
	Erythema (around nails)	2	All Possible
	Fever	3	Possible: 1, Probable: 2
	Mucositis	5	All Possible
Cisplatin	Alopecia	5	Possible: 4, Probable: 1
	Mucositis	4	All Possible
	Nausea	5	All Possible
	Anaemia	2	Possible: 1,Probable: 1
	Leukopenia	2	All Probable
Docetaxel + Carboplatin + 5-Fluorouracil	Alopecia	9	All Possible
	Anorexia	3	Possible: 2, Probable: 1
	Diarrhoea	2	All Possible
	Dysgeusia	1	All Possible
	Nail discoloration	1	All Probable
	Fever	3	All Possible
	Nausea	6	Possible: 5, Probable: 1
	Anaemia	2	Possible: 1, Probable: 1
Nimotuzumab + Carboplatin	Fatigue	6	All Possible
	Headache	2	Possible: 1, Probable: 1
	Nausea	2	All Possible
	Anaemia	4	All Possible
Paclitaxel + Carboplatin	Alopecia	8	Possible: 5, Probable: 3
	Anorexia	4	Possible: 3, Probable: 1
	Diarrhoea	2	Possible: 1, Probable: 1
	Erythema	1	All Possible

Table 3: Risk factors analysis of the patients experiencing adverse effects

Risk factors	AOR	Lower 95 % CI	Upper 95 % CI	p-value
Age				
Site (Oral cavity/Oropharynx)	1.95	1.04	3.62	0.03
Treatment (Chemotherapy + Radiotherapy)	5.00	2.62	9.53	< 0.001
Chemotherapy drug regimen (5-Fluorouracil + Cisplatin + Paclitaxel)	8.68	2.55	29.48	< 0.001
Comorbidity	8.33	2.45	28.34	< 0.001

AOR = Adjusted Odds Ratio; CI = Confidence Interval; p-value ≤ 0.05 was considered statistically significant;

Discussion

Identification and reporting of ADRs and associated predictors in cancer patients is crucial in developing preventive strategies and improve their quality of life. With the development of new and targeted chemotherapy drugs, there has been a new revolution in the field of onco-pharmacology, mainly based on a tailored approach to cater to the needs of specific individuals.[14] Nonetheless, clinicians can't turn a blind eye to the adverse effects antineoplastic drugs can pose. In the current study, the pattern and possible predictors of adverse effects were evaluated in 188 oral cancer patients.

Although there was a male preponderance for oral cancers in this study, which was consistent with other previous studies,[15-17] no gender difference in the development of adverse effects was found in risk estimate analysis. These findings are contradictory to a few previous studies in cancer patients, with female gender being a significant risk factor for the development of ADRs.[18, 19] The possible reason for this difference could be the inclusion criteria for this study, which is limited to oral cancer patients only, which itself is prevalent among males as compared to females. Majority of the adverse effects in the study occurred in 45–60 years age group, which can be correlated with the high age-related morbidity in this particular age group.[20]

The drug utilisation pattern in the present study revealed that the most common class of cytotoxic agents prescribed for oral cancer was Paclitaxel and Carboplatin combination. These results are also mirrored in previous studies by Murti et al.[11] Another previous study by Motghare et al reported oral Cisplatin to be the most commonly prescribed therapy for oral cancer patients, followed by 5-Fluorouracil, Paclitaxel, Carboplatin and Docetaxel.[10]

Cancer chemotherapy includes cytotoxic medicines accompanied by adjuvant and supplementary therapeutic measures to combat their adverse effects. Clinical pharmacists and nurses, being an integral part of the oncology care team, provided their counselling service to all the new patients awaiting their first-cycle of chemotherapy and also discussed in detail about the potential adverse effects and their possible treatment to the patients and their caregivers. Furthermore, they also developed educational materials for the patients and their caregivers in a local language that assisted better monitoring of the treatment and reporting of the treatment-related concerns, including the adverse reactions.

Proton pump inhibitors, H2 antagonists and steroids were frequently used prophylactically as well as therapeutically for the management of chemotherapy-induced nausea and vomiting caused by different drug regimens. Newer anti-emetics and neurokinin-1 receptor antagonists like aprepitant were not prescribed as frequently.[21] This could be the possible reason for nausea to be the major adverse effect noted in the study. While destroying cancer cells, chemotherapy drugs can also damage rapidly dividing cells of bone marrow, resulting in myelosuppression, thus affecting white blood cells, platelets and red blood cells.[22]

Some of the chemotherapy related adverse effects were self-remitting and did not require any treatment. Alopecia seen in the patients was acute and reversible and was noted after the first chemotherapy cycle itself. The lost hair gradually regrows starting three to six months after the last chemotherapy cycle, returning to baseline progressively.[23] Similarly, the nail changes noted in the patients were self-remitting and required no treatment.[23]

In presented study, the average number of other drugs per prescription was 7.4 while the average number of cytotoxic drugs per prescription was 2.3. This is contradictory to the findings of studies in Nepal and in Karnataka state in India, with an average number of 1.97 and 1.78 cytotoxic medications prescribed per prescription.[24, 25] The increased number of drugs per prescription is an indicator of polypharmacy practice, which is quite prevalent in cancer patients as well as they are provided with supportive treatment apart from chemotherapy.[26]

Despite recommendations to use generic names (rather than brand names), only 52.2 % of cytotoxic drugs were prescribed with generic names.[27] 71.4 % of the prescribed drugs were from the essential medicine list. These results signify irrational prescribing practices and the reason for this difference could be the two different study sites considered for the study. The patients were recruited from private as well as government tertiary care hospitals. In a government setting, all the drugs were prescribed from Essential Medicine List and by their generic names, but this was not the practice observed in the private setting.

Well planned pharmacovigilance studies in cancer patients ensured proper reporting of the ADRs in these patients. 64.4 % patients in this study developed various ADRs. This finding is slightly contrary to that of Murti et al, which showed adverse effects in 87.5 % of oral cancer patients in the Bihar region receiving chemotherapy drugs.[11] The most noticeable finding was that all the ADRs recorded in this study were collected by the active surveillance method. Drug safety methods can undergo a major overhaul if such active surveillance practices are initiated.

Only chemotherapy-related ADRs were taken into consideration for this study. The majority of the ADRs (91.42 %) were due to the combination regimens of 5-Fluorouracil, Paclitaxel and Cisplatin. Alopecia was the most common adverse effect noted in 26.44 % patients, followed by nausea and anaemia in 15.7 % and 9.9 % patients, respectively. These findings were quite similar to a study by Saini et al.[6] However, these results were in contrast to a few previous studies which reported neutropenia and constipation as the most common ADRs.[28, 29] This difference could be due to a difference in the usage pattern of different chemotherapy drugs used to treat different malignancies.

The WHO causality assessment scale indicated that 82.64 % of the reactions were "possible". The reason for lack of any certain category of ADRs could be the multiple drugs which are being prescribed to cancer patients under different drug regimens. Sometimes, other concomitant drugs in the regimen might contribute to the observed adverse effects. Furthermore, it cannot be completely ruled out that associated comorbidities and the cancer disease itself may sometimes mimic an ADR in these patients.

The strength of this study is that it highlights the potential risk factors like the site, nature of treatment (chemotherapy/radiotherapy), prescribed drug regimen and associated comorbidities for the development of ADRs in oral cancer patients being treated at government and private tertiary care settings. These risk factors should be taken into account while deciding the line of treatment for these patients. The study findings further emphasised the need for new policies and educational strategies that should be undertaken to promote rational and generic prescribing in cancer patients.

One limitation of this study was that only oral cancer patients from the specific geographical location in India were included to study the adverse effects and the potential risk factors, while other cancers were not taken into account, so the results are not generalisable for other types of cancers with different geographical distribution. Also the patients were followed for a short duration of time, so long term adverse effects could not be studied. It can be expected that in the near future, more studies will be undertaken among different cancer patients with a long follow-up period so that pharmacovigilance database can be setup for developing countries like India with diverse pharmaco-genetic variation.

Conclusion

The adverse effects following chemotherapy depends on the drug regimen chosen for a patient. Few identifiable risk factors for developing adverse effects based on this study were the site of cancer, concomitant radiotherapy treatment and associated comorbidities. Onco-pharmacovigilance studies, if undertaken, can play an important role in the better management of patients receiving chemotherapy treatment by early detection and timely management of drug-related toxicities. Furthermore, identification of the predictors can aid in improving the prescribing pattern in cancer patients, thereby decreasing hospitalisations and economic burden and improving their quality of life.

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Conflict of interest

None.

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CONTENT

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ORIGINAL ARTICLE

The Gastroprotective Role of Yellow Kepok Banana (*Musa* x *Paradisiaca* L. var. *Kepok*) Peel Extract and Influence on Markers of Oxidative Stress: Malondialdehyde and Nitric Oxide

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Abstract

Background/Aim: Flavonoids, tannins, saponins and polyphenols in yellow kepok banana (*Musa* x *paradisiaca* L. var. *kepok*) peel potentially could be a solution for peptic ulcer prevention. This study aimed to prove the efficacy of kepok banana peel extract as gastroprotective by analysing the number of gastric ulcers and markers of oxidative stress - malondialdehyde (MDA) and nitric oxide (NO).

Methods: The study was performed on 33 female Wistar rats aged 3-4 months, weighed 100-250 g. Rats were divided into 3 groups: Musa Paradisiaca Var Kepok 1 (MPVK1) treatment group, Musa Paradisiaca Var Kepok 2 (MPVK2) and control group (K). In MPVK1 kepok banana peel extract at a dose of 80 mg / 200 g body weight (BW) was given and the MPVK2 group dose was 160 mg / 200 g BW. The gastritis induction was performed by using 5 % acetylsalicylic acid at a dose of 1500 mg/kg BW. MDA examination by HPLC method, NO examination by ELISA method and macroscopic examination by counting the number of ulcers on the gastric mucosa was performed.

Results: The results showed that the lowest average MDA level, as well as the highest average NO level was in the MPVK2 group 3.27 and 286.17, respectively. The highest mean number of ulcers was in the control group 3.55. By analysing all the results it can be concluded that there is a significant difference in the average levels of MDA ($p = 0.013$), NO ($p < 0.001$) and the number of ulcers ($p < 0.001$) in the three groups.

Conclusion: Banana peel extract was proven to be effective as a gastroprotective through markers of MDA, NO and the number of ulcers in Wistar rats.

Key words: Banana peel; Malondialdehyde; Nitric oxide; Gastroprotection.

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Introduction

According to World Health Organization (WHO), the occurrence of gastritis in Indonesia is 40.8 % with quite high incidence rate in several regions in Indonesia, with a prevalence of 274,396 cases out of 238,452,952 people. Gastritis is one of the 10 most common diseases in hospitals with 33,154 cases (4.9 %).[1, 2]

Gastritis is caused by hypersecretion of hydrochloric acid and pepsin which erode the lining of

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CONTENT

the gastrointestinal mucosa.[3, 4] When the stomach is exposed to gastric mucosal destroying agents (acetylsalicylic acid) there will be a back diffusion of H^+ from the lumen into the mucosa, causing a reaction that can harm the stomach and release a large amount of pepsin. Many sodium (Na^+) and plasma proteins enter the lumen and release histamine.[5, 6] This results escalation in secretion of hydrochloric acid by parietal cells, increased capillary permeability and bleeding. In addition, it stimulate local parasympathetic system due to the increase of hydrochloric acid secretion so that venous congestion gets worse and eventually causes bleeding. If this condition is admissible to continue, superficial erosion or ulceration may occur. The process of gastritis is due to an imbalance between mucosal defences and several aggressive factors. One of them is caused by long-term consumption of non-steroid anti-inflammatory drugs (NSAIDs). The exogenous aggressive factors that cause deterioration to the gastric mucosa are in the form of inflammation, or if it is chronic inflammation it can cause bleeding and perforation.[7, 8]

The popular way to prevent the formation of peptic ulcers is by administering drugs that function as cytoprotective on the gastric mucosa. Hence, prevention efforts that have minimal side effects are needed, including the use of tropical plants in the development of phytopharmaceutical use, considering that Indonesia is rich in a variety of medicinal plants.[9–11] Flavonoid antioxidants, tannins, saponins and polyphenols have benefits as anti-inflammatory and antioxidant in hyper lipidemic DM rabbits.[12, 13]

Further, banana peel extract contains flavonoids, tannins, saponins and polyphenols. Tannins minimise gastric acid secretion and have a cytoprotective effect.[9, 11] Tannins also promote tissue reconstruction, inhibit gastric acid production, act as antioxidants and inhibit the activity of *Helicobacter pylori*. Saponins inhibit gastric acid production and lower the pH levels of gastric juices.[14] Based on this, the banana peel has potential as a gastroprotector.[15, 16] This study investigated the extract of the yellow kepok banana (*Musa paradisiaca L. var Kepok*) peel as a gastroprotective in acetylsalicylic acid-induced Wistar rats. Thus, the aim of this study was to prove the effect of kepok banana peel extract on gastritis through markers of oxidative stress (malondialdehyde (MDA), nitric oxide - NO) and the number of ulcers.

Methods

The preparation of kepok banana peel extract

The kepok banana (*Musa* x *paradisiaca* L. var. *kepok*) peel extract was prepared to refer to the banana peel extraction procedure (Copyright Document No HKI S00201809745) initiated with the preparation of the raw materials.

Extract characteristics

Kepok banana is one of the banana varieties in Indonesia. Kepok bananas consist of white kepok bananas and yellow kepok bananas. The part used to be extracted in this study is the peels. The chopped banana peels were dried in an oven at 40 °C for 24 h until they were completely dry, characterised by a texture that was easily broken by hand squeezing. Extract was provided in two doses, namely kepok banana peel extract at a dose of 80 mg / 200 g body weight (BW) with 0.3 % Sodium carboxymethyl cellulose (NaCMC) solvent, 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent and control with 0.3 % NaCMC.

Phytochemical screening extract procedures

The banana peel was washed, roughly sliced and then processed to dry by aerating. The drying process was carried out using an oven and further mashed. The extraction method used 76 % ethanol maceration.

Experimental animals

The experimental animals used in this study were Wistar rats kept in group cages sized 20 x 33 cm in the Experimental Animal Laboratory of Universitas Muhammadiyah Semarang, Indonesia. The environmental conditions of the cage were arranged at 24-26 ^{0}C, supported by sufficient ventilation. The food was provided in the form of pellets, while its drinking water was provided *ad libitum* in the cannula bottles.

Experimental model

The experimental animal samples consisted of 33 female Wistar rats aged 3-4 months weighed 100-250 g. The gastritis experimental animal model was prepared by induction using 5 % acetylsalicylic acid at a dose of 1500 mg/kg BW in one-time administration.[7]

This research was conducted for 18 days. Data collection were in the form of blood serum and counting the number of ulcers and it was carried out on the 18th day (post-test). The study began with the rats being adapted for 14 days. On the

15th day, the rats were divided into 3 groups: Musa Paradica Var Kepok 1 (MPVK1) treatment group, Musa Paradica Var Kapok 2 (MPVK2) treatment group and control group (K). The groups were divided using a random sampling technique. Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC. On the 16th day, the rats were enforced to not eat for 24 h while still being given water *ad libitum*. On the 17th day, the rats in group K were given 0.3 % NaCMC, meanwhile MPVK1 group was given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and the MPVK2 group was given a yellow kepok banana peel extract at a dose of 160 mg / 200 g BW with 0.3 % NaCMC solvent. After one hour, all rats were induced with 5 % acetylsalicylic acid at a dose of 1500 mg/kg BW. On the 18th day, the rats were anesthetised and blood was taken through the orbital sinus, to be terminated. The stomach organs were taken and washed with 0.9 % NaCl and the number of ulcers was counted. The blood serum preparations were examined for markers of oxidative stress (MDA, NO). The MDA examination by high-performance liquid chromatography (HPLC) method, NO examination by enzyme-linked immunosorbent assay (ELISA) method and the macroscopic examination by counting the number of ulcers on the gastric mucosa were performed.

Data processing

The normality of data distribution was tested and appropriate tests were used: One-way ANOVA for data with normal distribution and Kruskal-Wallis for abnormal data. The limit of the degree of significance was set at $p < 0.05$ with 80 % research power and 95 % confidence interval.

Ethical Consideration

The study was successfully accepted by Gadjah Mada University, Indonesia review board with institutional review board (IRB) decision No 00017/LPPT/VI/2021.

Results

Sample characteristics

The samples consisted of 33 female Wistar rats aged 3-4 months weighed 100-250 g. They were grouped into MPVK1, MPVK2 and K group, the minimum weight of rats in the MPVK1 group was 170, the maximum weight was 250 while the average was 207. The minimum body weight for rats in the MPVK2 group was 180, the maximum was 250 and the average was 217. At the same time, the minimum body weight for the rats in the control group (K) was 178, the maximum was 250 and the average was 210, as shown in Figure 1.

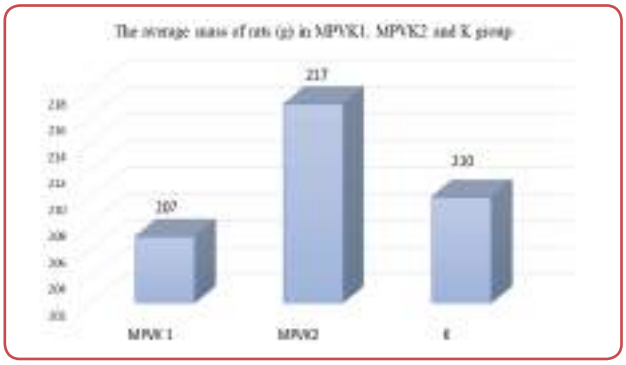


Figure 1: The average weight of Wistar rats in the MPVK 1, MPVK 2 and K group

Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC.

The minimum level of MDA in the MPVK 1 group was 1.01, while the maximum level was 7.67 and its average was 2.60, respectively. The minimum level of MDA in the MPVK2 group was 1.5, while its maximum level was 2.10 and the average was 1.81. The minimum level of MDA in the control group (K) was 2.19, the maximum level was 3.93 and the average was 3.27. The lowest mean level of MDA was in the MPVK2 group, as shown in Figure 2.

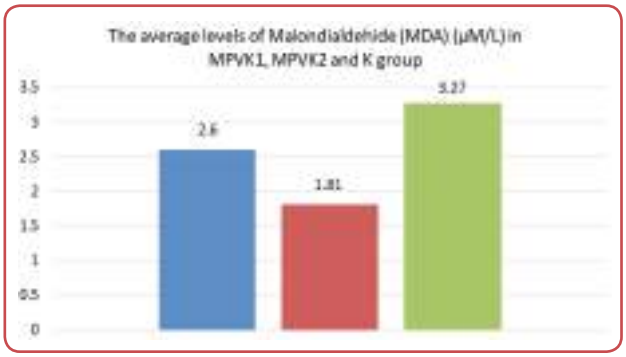


Figure 2: The average levels of malondialdehyde (MDA) of rats in MPVK1, MPVK2 and K group

Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC.

The average levels of nitric oxide (NO)

The minimum level of NO in the MPVK1 group

was 88, meanwhile the maximum level was 131.28 and the average was 112.73. The minimum level of NO in the MPVK2 group was 221.28, while the maximum level was 353.21 and the average was 286.17. The minimum level of NO in the K group was 101.95, meanwhile, the maximum level was 120.28 and the average was 111.74. The lowest average NO level was in group K, as shown in Figure 3.

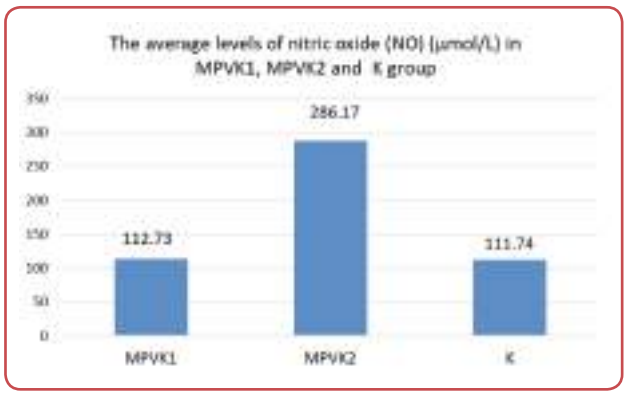


Figure 3: The average levels of nitric oxide (NO) in MPVK 1, MPVK 2 and K group

Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC.

The average number of ulcers

The minimum number of ulcers in the MPVK1 group was 0, with its maximum was 3 and the average was 0.45. The number of ulcers in the MPVK2 group was 0, a maximum of 1 and its average was 0.04. The number of ulcers in the control group (K) was 0, a maximum of 7 and its average was 3.55. The lowest mean of number ulcers was in the MPVK 2 group, as seen in Figure 4.

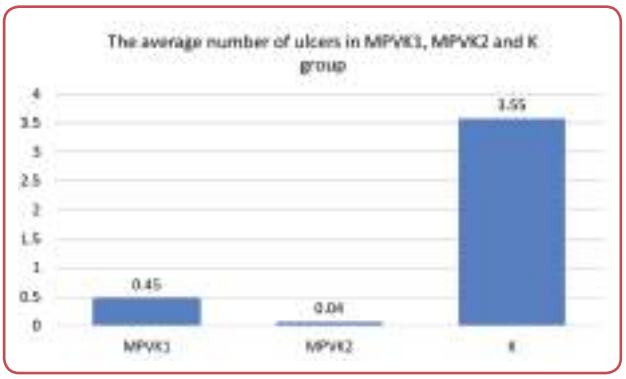


Figure 4: The average number of ulcers in the MPVK1, MPVK2 and K group

Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC.

A macroscopic picture of gastric mucosal deterioration is shown in Figure 5. The results showed that the highest average number of ulcers was found in the control group.

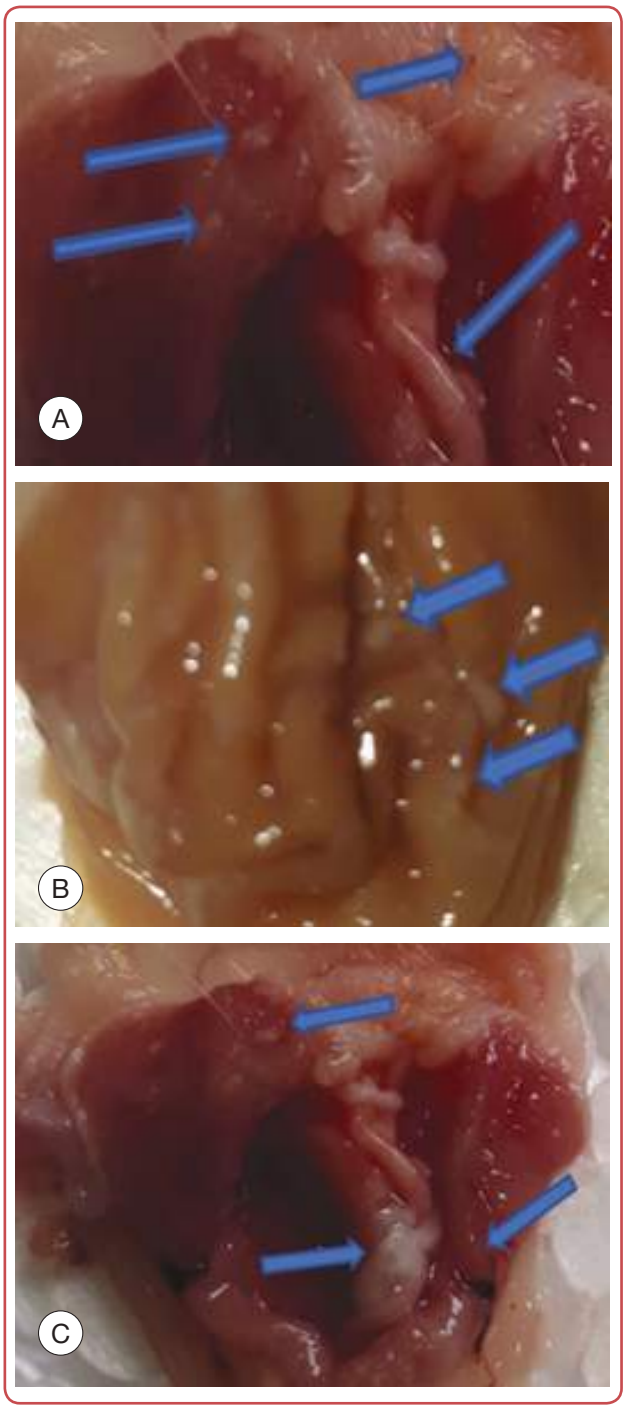
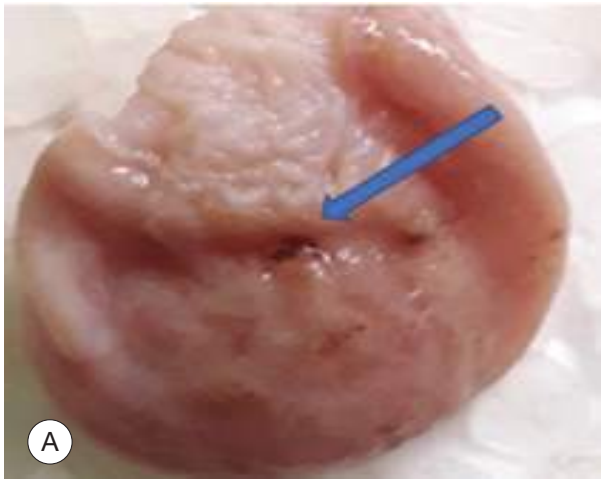


Figure 5 (A, B, C): Gastric macroscopic characteristics observed in the control group. The signs of hypoxaemia, bleeding and gastric mucosal ulcers were displayed by the blue arrows

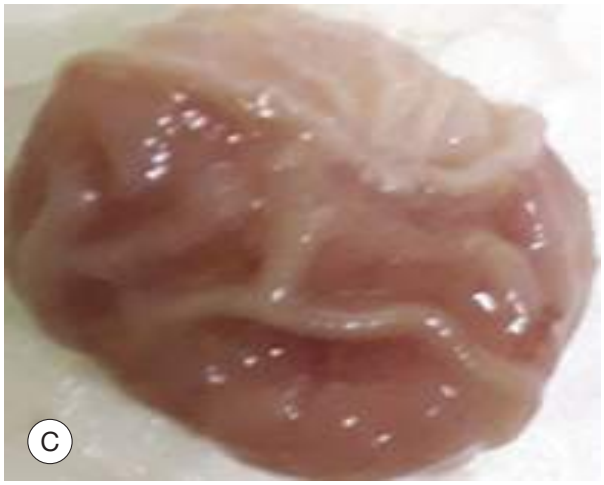
The control group rats were given 0.3 % NaCMC solvent.

The results of gastric macroscopic examination in treatment of group 1 and 2 are shown in Figure 6.



Minimal bleeding for MPVK1 group

Absence of bleeding and ulcers in MPVK2 group



Absence of bleeding and ulcers in MPVK2 group

Figure 6: *Gastric macroscopic characteristics observed in the treatment groups*

A. Macroscopic characteristics of stomach in the MPVK1 group showing minimal bleeding (pointed by the blue arrow), with fewer ulcers than that in the control group; B and C, showing macroscopic characteristics of the stomach in the MPVK2 group which did not perform bleeding and ulcers. Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given the yellow kepok banana peel extract at a dose of 160 mg / 200 g BW with 0.3 % NaCMC solvent.

Table 1 shows the results of the One-Way ANOVA test. It revealed that there were significant distinctions in the average level of MDA and NO and the number of ulcers in the 3 group of experimental rats; MPVK 1, MPVK 2 and K.

Table 1: One-Way ANOVA test results has displayed the average values of MDA and NO, accompanied by the number of ulcers in the experimental groups MPVK1 and MPVK 2 and control group K

Parameter	MPVK1	MPVK2	K	p-value
MDA	2.60	1.81	3.27	0.013
NO	112.73	286.17	111.74	< 0.001
Number of ulcers	0.45	0.04	3.55	< 0.001

MDA: malondialdehyde; NO: nitric oxide; Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC.

Treatment group 1 (MPVK1) rats were given the yellow kepok banana peel extract at a dose of 80 mg / 200 g BW with 0.3 % NaCMC solvent and treatment group 2 (MPVK2) rats were given 160 mg / 200 g BW yellow kepok banana peel extract with 0.3 % NaCMC solvent. The control group (K) was given 0.3 % NaCMC.

Discussion

An induction of free radicals and oxidative stress causes gastric mucosal deterioration.[2] Further, an imbalance of aggressive and defensive factors can cause gastric mucosal ulcers. The aggressive factor is more dominant than the defensive factor. The existence of free radicals is part of the aggressive factor.[1] An example of free radicals is NSAIDs. Acetylsalicylic acid works by blocking certain natural substances in the body to reduce pain and swelling. Acetylsalicylic acid is an irritant.[4-6] Acetylsalicylic acid causes a defect in the mucosal barrier and back diffusion of H^+ ions occurs. Histamine is stimulated to secrete more gastric acid, resulting in dilation and increased permeability of capillaries, gastric mucosal damage, acute or chronic gastritis and gastric mucosal ulcers.[16]

The experimental animal model of gastric mucosal ulcers in this study was carried out by inducing acetylsalicylic acid. 5% acetylsalicylic acid was given at a dose of 1500 mg/kg BW in all study groups. The results showed that the highest average number of ulcers was found in the control group. The minimum number of gastric ulcers in the control group (K) was 0, the maximum number of ulcers was 7 and the average ulcer was 3.55.

The detrimental effect of free radicals that cause biological damage is oxidative stress. Cells exposed to oxidative stress will activate defence mechanisms to survive.[7, 17] Lipid peroxidation itself is the result of the performance of free radicals and this parameter is the most accessible to measure.[2, 11] Lipid peroxidase can damage membrane structures, it leads changes in permeability, inhibits metabolic processes and transform ion transport as well.[11] The measurement of lipid peroxidation level is carried out by measuring the final product, one of which is MDA. The accumulation of MDA is an early indicator of the mechanism of cell and tissue deterioration. MDA as final product of the lipid peroxidation process is used as an indicator of cell deterioration in the stomach due to the oxidative stress.[9-11]

The results of this study have revealed that the minimum level of MDA in the control group (K) of 2.19 is the highest among those three groups, as well as the maximum and the average level of MDA.

Kepok and Uli banana peel extracts had increased superoxide dismutase (SOD) activity and decreased MDA levels in hypercholesterolemic rats.[18] Kepok banana peel extracts decreased MDA levels in male mice (*mus musculus*) that was exposed to cigarette smoke.[14] Kepok banana peel contains flavonoid and phenolic antioxidants, the antioxidant content of flavonoids and phenolics showed hepatoprotection in acetylsalicylic acid-induced rats.[19] Other antioxidants component in kepok banana peels are flavonoids, tannins, saponins and polyphenols. Tannins are useful in minimising gastric acid secretion and have cytoprotective effect.[5] Other benefits of tannins are the increasing tissue reconstruction, gastric acid production inhibition and the inhibition of *Helicobacter pylori* activity. Further, saponins also inhibit gastric acid production and minimise gastric fluid pH levels.[8, 16, 20]

Kepok banana peel extract reduces oxidative stress in peptic ulcers through the antioxidant pathway.[19] Prevention and treatment of gastric mucosal ulcers by exploring natural products is something that is very impressive. The rats that were given kepok banana peel extract performed the increase of protection phenomena against acetylsalicylic acid induction by elevating the antioxidant level of NO. Acetylsalicylic acid converts hydroperoxyl to hydroxy fatty acids which is culminated from lipid peroxidation of cell destruction. The release of damaging free radicals likely occurs resulting in the death of tissue cells in the stomach. The ulceration effect of superficial epithelial cells on the gastric mucosa constructs the base of gastric ulceration.[14] The kepok banana peel extract exhibits its gastroprotection by increasing mucosal defence factors by increasing the body's antioxidant levels, NO.[14, 16]

This study revealed that the rats given kepok banana peel extract had increased average level of NO antioxidants compared to the control group, namely MPVK1 (112.73) and MPVK2 (286.17). The lowest average NO level was found in the control group, that is 111.74. There is a significant distinction in the mean of NO in the three groups with $p < 0.001$. The average number of ulcers in the treatment group was lower than in the control group. Hence, the mean number of ulcers in the MPVK1 group was 0.45. The average number of ulcers in the MPVK2 group was 0.04. The mean number of ulcers in control group (K) was 3.55. Meanwhile, the lowest mean number of ulcers was found in MPVK2 group. There was a significant distinction in the mean number of ulcers in the three groups with $p < 0.001$.

Conclusion

There is a significant distinction in the mean value of MDA, NO as well as the distinction of number of ulcers among the three groups. Therefore, kepok banana peel extract has good efficacy in reducing the markers of gastric damage in Wistar rats.

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Conflict of interest

None.

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CONTENT

Pranata et al. Scr Med 2023 Mar;54(1):61-7. DOI:10.5937/scriptamed54-42230
ORIGINAL ARTICLE

A Pilot Test for Implementing Precision Healthcare Programme in Patients with Diabetes in Indonesia

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Abstract

Background/Aim: An evaluation of precision healthcare interventions among patients with diabetes in a small sample through a pilot test before being tested in a larger sample is needed. Thus, the purpose of this study was to evaluate the feasibility of a precision healthcare programme. It also assesses the programme's outcome among patients with diabetes in Indonesia.
Methods: Data were collected during December 2020. The researchers first gathered data about participant characteristics. Furthermore, the strategies of precision healthcare were implemented in sixty respondents to evaluate the feasibility and outcome of the programme in a month.
Results: The participants mentioned that they can follow all procedures of precision healthcare. However, they asked the researcher to provide a guide and monitoring book which provides safe choices information on diet, exercise, glucose monitoring and drug medication. Moreover, participants mentioned that they could complete all questionnaires but needed a company of a research assistant. The benefits of a month of precision healthcare were improved diabetes self-care activity, blood pressure and blood glucose level. However, the body weight, body mass index (BMI), triglyceride, cholesterol and triglyceride glucose index were not significantly improved.
Conclusion: A pilot test is needed to ensure the feasibility of the implementation strategy with the culture and background of diabetic patients in Indonesia. Improving diabetes self-care activity stabilised blood pressure and blood glucose during a month, so it can be assumed that precision healthcare approaches were potentially being applied in Indonesia. On the other hand, it is needed more than a month to improve body weight, BMI, triglyceride, cholesterol and triglyceride glucose index. Thus, testing the precision healthcare approach in a larger sample with long time series for patients with diabetes in Indonesia through a randomised controlled trial (RCT) is needed.

Key words: Precision health; Strategy; Personalised; Patient preference; Genetic.

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Introduction

Diabetes mellitus has become a global problem. In 2019, there were 463 million people with diabetes in the world. The number will probably increase to 700.2 million people in 2045.[1] Patients with diabetes have a significant risk of experiencing complications in the future, which are 2-3 times more at risk of developing cardiovascular disease (CVD), more than 10 times likely to experience kidney problems and even amputation.[2] Among these complications, heart disease was the

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number one killer. The total state expenditure for patients with diabetes in 2019 due to complications reached US$ 760 million and will even reach US$ 845 million in 2030.[1]

Efforts made by health professionals in the community and hospitals to prevent complications for people with diabetes were focused on health education and self-management.[3, 4] However, in reality, there are many patients with diabetes who admit that it is difficult to carry out at-home self-management. It is because health information through health education is difficult to understand and does not match each patient's preferences.[5] Ideally, the approach given to diabetic patients is more focused on an individualised approach. This approach has been known as precision healthcare in various countries.[6–9]

Precision healthcare is an approach in the nursing field that emphasises patient preferences, personal goals, characteristics and genetics to achieve better patient self-management.[10] Personalised care is one of essential element in precision health or precision medicine.[11] Meanwhile, precision healthcare for patients with diabetes prioritise each patient's preferences, personal goals, characteristics, glycaemic control and genetics.[10, 12] The precision healthcare programme for diabetic patients in Indonesia has begun to be developed. Implementation strategies and consensus on the implementation of precision healthcare have also been developed. Health professionals such as doctors, nurses and nutritionists believe that precision care will be the solution to reduce the risk of CVD complications in diabetic patients because precision healthcare can improve self-management among patients with diabetes through better glycaemic control.[10, 12]

An evaluation of precision healthcare implementation among patients with diabetes in Indonesia has never been conducted yet. Therefore, the researchers considered that this study needed to be done. Evaluation of precision healthcare in a small sample through a pilot test is necessary to ensure that the implementation strategy follows the culture and background of diabetic patients in Indonesia before being tested in a larger sample through a randomised controlled trial (RCT). Thus, the purpose of this study was to evaluate the feasibility of a precision healthcare programme. It also assesses the programme's outcome among patients with diabetes in Indonesia.

Methods

Research design

A pilot test by quasi-experiment with one group was used to test the feasibility of a precision healthcare programme. Data were collected in December 2020. Furthermore, the strategies of precision healthcare to evaluate the outcomes (diabetes self-care activity, blood pressure, blood glucose, body weight, body mass index (BMI), triglyceride, cholesterol and triglyceride glucose index) among patients with diabetes were implemented. The strategies were modified after receiving comments from respondents before testing the new design with a randomised controlled trial in the future study.

Sample and setting

Patients eligible for the pilot study were people over 20 years of age at the time of recruitment, Indonesian and willing to participate in the trial. The respondents also had no history or diagnosis of ischaemic heart disease, transient ischaemic attack (TIA), peripheral vascular disease and enduring mental health problems. Sixty participants were tested for implementation of a precision healthcare programme. All participants were recruited in a primary healthcare facility in Sumbawa City, East Java, Indonesia.

The procedure of the pilot study

The procedures were divided into pre-intervention and intervention phases. In the pre-intervention stage, the permission letter of the local government and Research Ethics Committee for Ethical Clearance and further trained research assistance for implementing precision healthcare among respondents was acquired. In addition, for the intervention phase: 1) Sixty potential participants were asked for their participation in the pilot test; 2) Diabetes self-care activity, blood pressure, blood glucose, body weight, BMI, triglyceride, cholesterol and triglyceride glucose index of participants were assessed; 3) Participants were grouped for brainstorming the difficulties on glycaemic target and specific target behaviour; 4) Difficulties experienced by participant were discussed together with health professionals then selected strategies to overcome; 5) Facilitator made a list of participant needs, then ranked participants priorities further setting a goal and writing action as detailed for achieving their glycaemic target and medication management based on their condition and preferences; 6) Based on

comments and validation of sixty participants about the precision healthcare implementation, the strategies were modified for the following study in the future; 6) After a month of precision healthcare programme implemented, the outcomes were evaluated.

Instruments

Instruments used in data collection included: the diabetes self-management questionnaire (DSMQ),[13] ISH CVD risk,[14, 15] summary of diabetes self-care activity (SDSCA),[16] diabetes quality of life (DQoL)[17] and diabetes distress scale (DDS).[18] The instruments have been tested for validity and reliability in Indonesia.

Data analysis

Research assistants collected data. Descriptive statistics such as frequency and percentage, mean, standard deviation (SD), minimum and maximum, as well as the response of respondents to follow all study processes, were used to describe an overview of respondents and the feasibility of precision healthcare strategies. Moreover, paired t-test was utilised to analyse changes of study outcomes. Data were entered into SPSS 24 for Windows and checked for accuracy and missing data.

Ethical consideration

The research protocols were approved by the intuitional review board STIKES Bina Usada Bali (study number: 232/EA/KEPK-BUB-2020).

Results

Participant characteristics

Participant characteristics, status regarding diabetes self-care and physiological value of 60 respondents were collected and reported. Respondents comfort with following precision healthcare programmes through interviews in the pilot study were noted. Respondent' feedback was used to change precision healthcare strategies for diabetes (Table 1).

Likert-type items assessed age and duration of diabetes among participants is showed in Table 2.

Respondents included in the pilot study were mostly female (80 %). The education of 40 % of respondents was college level and 80 % did not get health information from health professionals.

Table 1: Characteristics of participants in the pilot study

Variables	N = 60	%
Gender		
- Male	12	20
- Female	48	80
Ethnic		
- Sumbawa	60	100
Religion		
- Islam	60	100
Education level		
- Illiteracy	12	20
- Junior high school	12	20
- Senior high school	12	20
- College	24	40
Marital status		
- Married	60	100
Resident status		
- Live with family	60	100
Complication		
- Hypertension	60	100
Received health information		
- None	48	80
- Yes, but only health education, support groups, or diabetes coaching	12	20
Health checks to health facilities routinely		
- No	12	20
- Yes	48	80
Received emergency treatment in the last three months		
- None	60	100
Hospitalised in the previous three months		
- No	60	100
Self-management of diabetes currently		
- Oral drug	12	20
- Diet and exercise	24	40
- Diet, exercise and herbal	24	40
Tobacco smoking		
- Yes	12	20
- No	48	80

Table 2: Age of participants and duration of diabetes

Variables	N = 60
Age	
- Mean	53
- Range	47 - 61
- SD	5.523
Duration of diabetes (years)	
- Mean	5.8
- Range	2 - 10
- SD	3.347

40 % of respondents chose diet, exercise and herbal medication in self-management at home. On the other hand, 80 % of respondents were not smoking and had a CVD risk of < 10 %. An average

participant in this study was 53 years old and had suffered from diabetes for 5.8 years.

The feasibility of a precision healthcare programme among patients with diabetes

After the questionnaires were distributed and filled out independently, the respondents mentioned that it was challenging to complete them by themself because it took a long time to understand questions. Respondents claimed to be able to fill out all questionnaires if they were accompanied by a research assistant. They argued 30 minutes was more than enough for brainstorming.

Almost all participants in the pilot study asked the researcher to provide a guide and monitoring book in simple language and easy to understand. Based on these conditions, it was necessary to prepare a monitoring book completed with types of diet, training and herbal medications that were safe, according to their values and preferences. Changes and strategies of precision healthcare for patients with diabetes are shown in Table 3.

Table 3: Changes and strategies of precision healthcare for patients with diabetes

Type of changes	Strategies
Content Measurement tools	Respondents claimed they could complete all measurement tools if a research assistant accompanied them.
Monitoring and guidebook	It was necessary to prepare a monitoring book complete with types of diet, exercise and herbal medicines that are safe for them to consume when doing self-management according to their values and preferences in simple and easy language. The book should provide choices about diet, exercise, drugs and both chemical drugs and herbal medicines that are safe for them. Books needed to be discussed with experts (physicians, nurses and nutritionists). The experts referred to were those who have worked for at least five years in the field they were involved in the management of diabetic patients.
Research process The sharing of information did not flow well due to lack of familiarity with other participants.	During the brief deducting teaching, respondents were grouped into small groups first and then they introduced themselves to other members before the brainstorming session on another day.

The difference in diabetes self-care activity, blood pressure, blood glucose, body weight, BMI, triglyceride, cholesterol and triglyceride glucose index pre and post-precision healthcare programme implementation in a month are shown in Table 4.

Table 4: Diabetes self-care activity, blood pressure, blood glucose, body weight, body mass index (BMI), triglyceride, cholesterol and triglyceride glucose index difference before and after intervention

Variables	Pre-intervention (mean)	Post-intervention (mean)	Difference (mean ± SD)	p-value
Diabetes self-care activity	45.68	60.96	15.28 ± 1.30	< 0.001
Systolic blood pressure	133.10	126.88	6.22 ± 2.07	< 0.001
Diastolic blood pressure	86.09	85.43	0.66 ± 2.07	< 0.001
Blood glucose	255.19	245.60	9.59 ± 6.78	< 0.001
Body weight	57.83	59.46	1.63 ± 0.54	0.402
BMI	22.98	23.67	0.69 ± 0.35	0.671
Triglyceride	227.86	225.80	2.06 ± 5.71	0.278
Cholesterol	186.74	190.56	3.82 ± 12.97	0.393
Triglyceride glucose index	5.23	5.14	0.09 ± 0.24	0.165

Data in Table 4 show the difference in diabetes self-care activity, blood pressure, blood glucose, body weight, BMI, triglyceride, cholesterol and triglyceride glucose index before and after implementing precision healthcare. The paired differences t-test showed that the p-value of self-care activity, blood pressure and blood glucose were $p < 0.001$, meaning there was a significant difference before and after the intervention. On the other hand, body weight, BMI, triglyceride, cholesterol and triglyceride glucose index before and after the implementation of precision healthcare were not significant differences before and after intervention with $p\text{-value} > 0.05$.

Discussion

Respondents included in the pilot study were women in 80 % of cases. Following data from INFODATIN of the Ministry of Health Information Data Centre, which states that the prevalence of the population of women with diabetes in Indonesia was higher than men, even in the past five years, the increase was quite significant than in previous years.[19] Postmenopausal contributes to diabetes in women because of decreasing of producing oestrogen and progesterone hormones.[20–22] Low growth hormone causes the metabolism to drop and can result in obesity.[23, 24]

Respondents in the pilot study had hypertension complication. This data correlate with a study where diabetes was closely related to hypertension.[25] Two in three people with diabetes in Indonesia have hypertension.[19] The higher blood sugar levels can cause the formation of blockages and fatty deposits in the blood vessels. Moreover, it will increase the total amount of fluid in the body, increasing blood pressure.[26, 27] Diabetes can decrease the ability of blood vessels to stretch. As a result, it will increase blood pressure.[26]

The education of 40 % of respondents was college level and 80 % did not get health information from health professionals. However, 80 % of them routinely checked their condition in health facilities. The support of health workers is needed to improve the health quality of patients with diabetes.[28–30] If patients with diabetes regularly receive information from health workers, health behaviour and knowledge will also enhance their awareness of self-management.[31]

Furthermore, 40 % of respondents had chosen diet, exercise and herbal medication for self-management at home, however it can be assumed that not all diabetic patient populations had the same characteristics as the respondents in the study. This data was supported by a study where self-management behaviours in the Indonesian population were self-regularly taking medication, managing daily exercise activity, managing food and diet intake and monitoring blood sugar and hypo/hyperglycaemia symptoms.[32] After evaluating a culturally appropriate intervention for people with diabetes in Indonesia, using herbal medicine remains an option chosen by patients with diabetes in self-management.[5, 33] Diabetic patients in Indonesia also choose oral medication and insulin to lower their blood glucose, especially patients with complications.[34]

Moreover, 80 % of respondents were not smoking and had a CVD risk of < 10 %. It might relate to most respondents in the pilot study being women. In general, women in Indonesia do not smoke. Therefore, the risk of CVD may be lower. On the other hand, if most respondents were male with smoker characteristics, the CVD risk might undoubtedly be more significant. That statement was supported by a study that smoking has significance in the incidence of CVD in the future.[35, 36]

The mean of respondents' ages in this study was 53 years old. Data from other research that analysed the age of diabetic patients in developing countries from 1995-2015 showed mean age was 45-64 years.[37] Decreasing pancreatic β cells' function to produce insulin due to aging factors plays a significant role in diabetes incidence.[31] Self-care activity, blood pressure and blood glucose level of participants who received precision healthcare programmes were changed significantly. When education programmes support behaviour change, change is potentially effective and accurate.[38] Moreover, intervention based on the participant's preferred language and incorporated culturally sensitive dietary information and the same cultural group through precision healthcare, potentially influencing participants' engagement in behavioural changes, improved adherence and further decreased the blood sugar level, blood pressure and improved self-care activity among respondents.[10, 12, 39, 40] On the other hand, body weight, BMI, triglyceride, cholesterol and triglyceride glucose index before and after the implementation of precision healthcare were not significantly different. These variables cannot be evaluated in a short time, like a month, but need to be considered for an extended period, at least three months.

Through brainstorming activities with support groups, respondents also revealed that they got a lot of new experiences with each other. The support group makes respondents more cheerful. They felt that they had many friends who understand their feeling and difficulties.[30, 41, 42] Brainstorming with support group activities strongly correlated with decreasing diabetes distress and increasing quality of life among patients with diabetes.[43, 44]

Conclusion

It was necessary to modify the strategies of precision healthcare for diabetes. Firstly, respondents must be accompanied by a research assistant when filling out all questionnaires. Participants argued that 30 minutes was more than enough for the brainstorming process. Researchers must provide a guide and monitoring book that is in simple language, easy to understand and related to patients' values and preferences in brainstorming sessions. The book was also used to see the progress related to patients' glycaemic targets. Due to improvement in diabetes self-care activity, stabilised blood pressure and blood glucose during a month it can be assumed that precision healthcare approaches has the potential to be applied in Indonesia. It is needed more than a month to improve the body weight, BMI, triglyceride, cholesterol and triglyceride glucose index. There was no control group and small samples which were limitations of this study. Therefore, future studies with large samples with long evaluation time are needed.

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Conflict of interest

None.

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ORIGINAL ARTICLE

Outcome of Vacuum Assisted Dressing in Open Comminuted Tibial Fracture with Primary Fixation

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Abstract

Background/Aim: Wound management of the compound open tibia (Gustilo-Anderson grade 2, 3a, 3b) is complicated by a higher infection and the problem of adequate soft tissue coverage is significant. Primary wound closure is not easily advisable in these types of compound open tibial fractures. Early tissue flap or graft procedure might increase the complication rate due to temporary graft rejection and wound infections. The aim of this study was to analyse the duration required for formation of healthy granulation tissue, duration required for making wound fit for skin cover procedure and duration of hospital stay in compound open tibia fracture treated with vacuum assisted closure (VAC).
Methods: A prospective interventional study of 22 patients aged 18 to 60 years was done. After assessing the size of the wound, primary bone fixation and wound debridement were carried out as soon as possible and then VAC was applied. Assessment of VAC therapy was based on mean decreases in wound size and "modified Johner and Wruh's criteria", used for assessment of the functional outcome of tibial shaft fracture was recorded during each follow-up.
Results: Twenty two patients suffered comminuted open fractures of tibia-fibula. Primary fixation of bone were done with vacuum dressing. During follow-up, the good decrease in wound size considering vacuum dressing remedy was once 18.75 ± 18.36 cm^2 (p = 0.001). Six patients achieved excellent results according to "modified Johner and Wruh's criteria" of tibial shaft fracture.
Conclusion: This technique effectively reduced wound size, accelerated the formation of healthy granulation tissue of wound with open fracture bone and provided a better functional outcome. The VAC treatment had suggestively increased wound closure rate, decreased morbidity and costs for patients.

Key words: Vacuum Assisted closure (VAC); Gustilo-Anderson classification; Open fracture; Soft tissue injury; Road Traffic Accident; Advanced Trauma Life Support (ATLS).

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Introduction

The subcutaneous location of the anteriormedial tibial surface is responsible for the high proportion of open diaphyseal fractures. Approximately 4 % of these fractures are open.[1] Road traffic accidents (RTA) account for more than 50 % of all open fractures, with the majority of other modes being caused by falls, sports-related injuries and direct blows.[1] The type 3 open fracture discovered is a mere 60 %.[2] The high number of open fractures continues to be a difficult problem for surgeons. The initial evaluation should adhere to the Advanced Trauma Life Support (ATLS) protocol guidelines. Following initial resuscitation, the focus shifts to

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the mechanism of injury and general examination of the afflicted limb. Special consideration should be given to neuro-vascular evaluation, compartment status and the involvement of soft tissue injuries and wound contaminations.

The decision must then be made as to whether the wound should be closed by primary or secondary, suture or reconstructive surgery. These include various types of dressings, hyperbaric oxygen treatment, various antiseptic agents, skin grafts or local flaps.[3] The ultimate soft tissue covering should be applied as soon as possible, ideally during the first 72 h following trauma.[4] The therapy of tibia bone fractures with locking plates or intra-medullary inter-locking nails is well established in trauma orthopaedic surgery. However, it can be very difficult and expensive to cure soft tissue infections after surgery. Studies have suggested delaying initial wound closure[5] to avoid flap rejection, deep infections and delayed bone union. Conventional wound dressing needs a longer duration, recurrent debridement and is followed with more damage to healthy tissue and non-compliance to patients.[6]

Vacuum assisted closure (VAC) (Kinche, Concepts, Inc, San Antonio, TX, USA) treatment provides a good environment that allows for both open and closed treatment, better wound healing procedures under moist, hygienic, sterile conditions.[7] Primary closure is important temporary prevention of the exposed vital structure from bacterial contamination, as well as to induce a locally normal circulatory stimulation and proliferation of wound granulation tissues. The VAC is a new modality system for the treatment of open fracture wounds. VAC therapies offer a more sterile, comfortable environment that benefits from both open and closed treatment and ultimately speeds up the process of wound healing under moist, sterile and clean circumstances. Important characteristics of a wound include: sufficient blood flow, the absence of a deep infection and adequate debridement of the wound. Individuals who respond well to VAC treatment are those who are well-nourished and in good health overall.

Negative pressure therapy (NPWT) "soothes the wound environment, reduces wound oedema/ bacterial load, progresses tissue perfusion and stimulates granulation tissue and angiogenesis". All this progresses the possibility of primary closure of wounds and reduces the need for plastic procedures. VAC dressing is a non-invasive, dynamic-wound treatment, technique that increases blood circulation, exposes wound to local sub-atmospheric pressure, drains oedema from the extra-vascular space and enhances the growth of healthy granulation tissue.[8]

The mechanism of action of vacuum aided wound treatment is not fully understood at this time.[9] However, with this method, enhanced circulation increases wound healing. When a negative pressure of 125 mm Hg is applied to the wound, granulation tissues form faster. The number of bacteria per gram tissue decreases. After four to five days, the wound is treated using a vacuum-aided closure method.[10]

This study objective was to study the duration required for formation of healthy granulation tissue, duration required for making the wound fit for skin cover procedure and the duration of hospital stay in compound open tibia fracture treated with VAC.

Methods

This study was designed as a prospective interventional study. Purposive sampling, satisfying the inclusion criteria was performed in Karnataka Institute of Medical Sciences (KIMS), Hubli, Karnataka, India. The patient's permission was also obtained for the study. Study was performed from January 2021 to January 2022. Ethical clearance was obtained from the Ethical Committee of the institution (Karnataka Institute of Medical Sciences Ethics Committee, Decision No 728/2020-21).

Patients clinically diagnosed with open complex tibial fracture (Gustilo-Anderson grade, 2, 3a, 3b)[11] were included in the study at Karnataka Institute of Medical Science Hubli. Patients with pre-existing osteomyelitis in the bone, neurovascular deficiency in the wounded limb, tumour, paediatrics fracture and vascular injury were not included in the study. The patient were without comorbidities, such as diabetes, hypertension, coagulation problems, etc.

Follow-up period was 6 months (20-24 weeks) and included following up the average time until definitive wound closure surgery, such as split thickness skin graft (SSG), flap surgery or

Table 1: Modification of Johner and Wruh's criteria

Criteria	Excellent	Good	Fair	Poor
Non-union/infection	none	none	none	yes
Neurovascular injury	none	minimum	moderate	severe
Deformity				
Varus/valgus (°)	none	2-5	6-10	> 10
Shortening (°)	0-5 mm	6-10 mm	11-20 mm	> 20 mm
Anterior/posterior (°)	0-5 mm	6-10 mm	11-20 mm	> 20 mm
Movements				
Knee joint (%)	full	> 90 %	90-75 %	< 75 %
Ankle joint (%)	full	> 75 %	75-50 %	< 50 %
Pain	none	occasional	moderate	severe
Gait	normal	normal	mild limp	significant limp

secondary suture was achieved. All the fractures were caused by high-energy trauma and the partially poly-traumatised patients were treated according to ATLS guidelines. The complex open tibia fractures were categorised using the Gustilo-Anderson classification.

Types 2, 3A and 3B fractures were treated with radical debridement, wound cleaning with copious amounts of saline (0.9 % NaCl), peroxide and betadine solutions (topical anti-septic), skin painting with povidone-iodine, compartment decompression and primary immobilisation of the fracture with external skeletal fixation. In addition, the rate of infection, fracture union (at least 3 cortical continuities of Bridging callus) in AP / lateral X-rays, length of VAC administration and complication development were also assessed.

Application of VAC dressing

After external fixation stabilised the fracture, regular wound debridement and effective haemostasis were obtained, a standard saline dressing on the first day was applied. On the second day, sterile granufoam (black poly urethane) was cut to fit the contour of the wound and applied over it. A fenestrated tube was introduced into the foam after the wound was properly sealed with adhesive tape. A fluid collecting container and vacuum pump were attached to the fenestrated tube. The device intermittently produced suction between 100 and 125 mm Hg. Every third day, after assessing the size of the wound VAC dressings were replaced. Assessment of VAC therapy was based on mean decrease in wound size and "modified Johner and Wruh's criteria".[12] Assessment of the functional outcome of tibial shaft fracture was recorded during each follow-up (Table 1).

SPSS for Windows 20 was used for statistical analysis. The t-test and the Mann-Whitney U-test were used to evaluate parametric and non-parametric variables, while categorical data were compared using the Chi-square test. $P < 0.05$ was considered statistically significant.

Results

Patients aged from 18 to 60 years, mean age of 40.2 years. Table 2 shows basic characteristics of patients as well as type and nature of the injury.

Table 2: Characteristics of injuries and participants

Variables	N	%
Gender		
Male	14	70.00
Female	8	30.00
Leg side		
Right	15	66.68
Left	7	33.32
Nature of injury		
RTA	16	84.33
Other	6	15.67
Gustilo-Anderson grade		
2	4	18.67
3a	11	49.38
3b	7	31.95
Types of fixation		
External fixation	16	72.72
Nails	6	27.28

RTA: road traffic accident;

Table 3 shows the necessary procedures after the fixing technique until the definitive secondary procedure were required (total number of VAC dressing applications).

Table 3: Total number of vacuum assisted closure (VAC) application after primary fixation

Number of VAC dressing	N	%
Four	9	42.80
Five	7	32.40
> Five	6	24.80
Total	**22**	**100.00**

Figure 1-3 shows clinical outcome of compound open tibial fracture with primary fixation by Ilizarov, followed by VAC dressing.

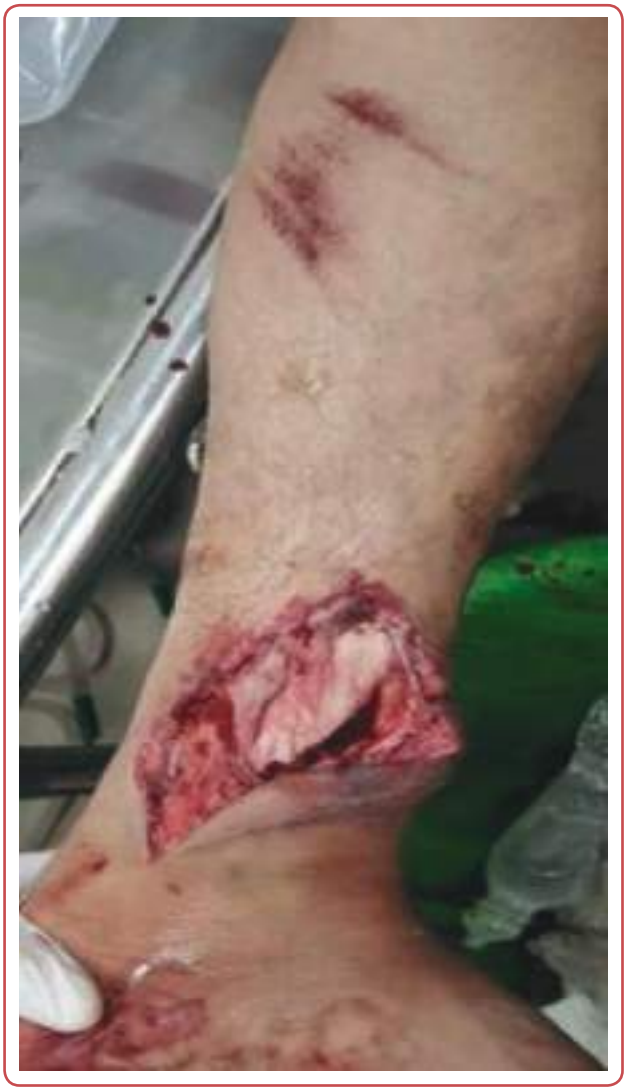

Figure 1: Compound open tibial fracture at presentation

Assessment of VAC therapy was based on mean decreases in wound size and "modified Johner and Wruh's criteria" was used and recorded for assessment of the functional outcome of tibial shaft fracture during each follow-up (Table 4 and 5).

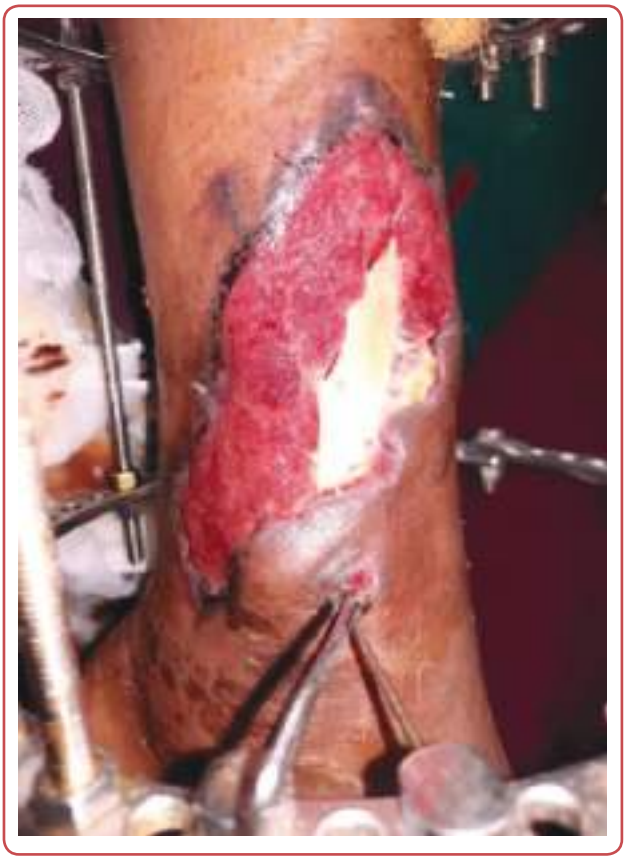

Figure 2: Compound open tibial fracture after vacuum assisted closure (VAC) dressing

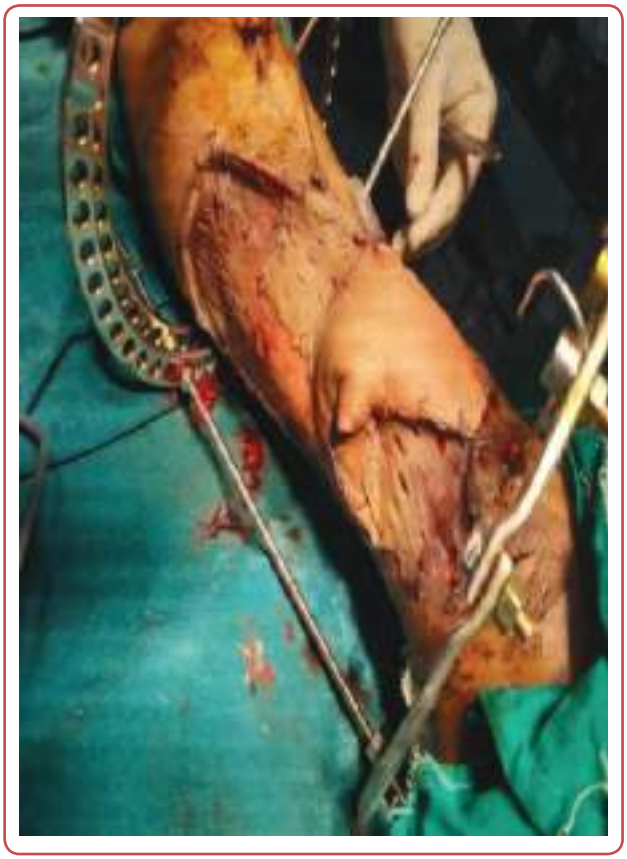

Figure 3: Compound open tibial fracture after tissue flap

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Table 4: Mean changes in the wound size after vacuum assisted closure (VAC) therapy

Value	Before (cm^3)	After (cm^3)	Reduction (cm^3)	%	p-value
Mean ± SD	48.00 ± 26.85	29.25 ± 25.80	18.75 ± 18.36	39.45	< 0.001

Before: Wound measurement before application of VAC dressing; After: Wound measurement after application of VAC dressing; Reduction: Reduction in wound measurement attained by VAC dressing; %: Percentage of reduction in wound measurement;

Following the administration of a total of 22 patients VAC dressings, surgery was performed to close the wound (Table 6).

Table 6: Definitive surgical procedure done after the end of vacuum assisted closure (VAC) application

Definitive secondary procedures	N	%
Secondary closure	2	9.10
Tissue flaps	7	31.81
Splint skin graft	12	54.54
Direct closure	1	4.55
Total	22	100.00

N: number of patients;

Patients were closely monitored for any complications. Any noted complications are presented in Table 7.

Table 7: Complications after vacuum assisted closure (VAC) therapy

Complication	N	%
Joint stiffness	6	27.27
Knee pain	13	59.09
Wound infection	2	9.09
Exposed implant	1	4.55

N: number of patients;

The average time between the trauma and the first debridement was 8-20 h (range from 2-23). The time it took to get to the union was 5.03 ± 1.58 months (range 3-8 months). The average decrease in wound dimensions was noted. The difference between pre and after VAC application was determined to be 39.45 % (20-60 %). Two of the 22 patients who had secondary closure developed a deep infection. However, one patient developed osteomyelitis and required surgical treatment. No statistically significant association was discovered between the size of the first wound and the development of infection ($p > 0.05$). There was no association discovered between the trauma type and the development of infections ($p > 0.05$).

Table 5: Assessment of functional outcome of tibial shaft fracture based on modified Johner and Wruh's criteria

Criteria	N	%
Excellent	6	27.27
Good	9	40.91
Fair	5	22.73
Poor	2	9.10

N: number of patients;

Discussion

The 4 criteria estimate the outcomes: severe soft tissue injuries, open fracture with reduced blood supply, wound contamination and fracture instability.[13] The studies deal with the management of open fractures and their delayed closure and healing. Stabilisation focuses on skeletal fixation and final wound closure. This research focuses on the wound healing process before final closure as well as temporary wound dressing. Similar to Sinha et al[14] in this study intermittent negative pressure of 100–125 mm Hg was applied. It was discovered that intermittent negative pressure was more efficient than continuous negative pressure in treating 30 open wounds using a vacuum dressing at 75-100 mm Hg.

In this study, the average time for debridement was 8 to 20 h, with a 39.45 % reduction in post-VAC treatment wound size. In Ramazan et al, the average time for trauma and surgery was 7.57 h. Wound measures taken after the most recent VAC administration revealed a mean reduction of 40.02 %.[15]

In presented investigation, the maximum time for wound healing was 8-12 days. Ghulam et al used VAC in 50 open compound tibial shaft fractures and observed that the maximum duration for wound healing was 15 to 20 days as opposed to 30 to 40 days with normal dressing.[16] The mean post-VAC treatment wound size decrease in this research was 39.45 %. The mean reduction in wound size between pre- and post-vacuum dressing was 43.06 %, according to Himanshu et al.[17] Vacuum dressing was used by Kila et al[18] to treat open wounds and they found that it reduced hospital stays and treatment costs. At institution where presented study was performed, the patient load is quite high and the availability of

operation theatre and definitive surgery like flap is delayed. Therefore, the length of the patient's hospital stay was prolonged.

This study has limitations, including the small study population from a single centre. Additionally, there was no comparison group to act as a control. Future areas of research should prospectively investigate the utilisation of VAC to increase value in the treatment of open tibia fractures, perhaps by including it as a component of standardised clinical assessment and management plans.

Conclusion

Despite the modest number of patients used in the study, the results demonstrated the establishment of healthy granulation tissue required less time. The wound could be covered definitively using techniques like SSG and flap cover more quickly. The number of wound debridement was decreased. The granulation tissue that was created was homogeneous and healthy. By creating homogeneous granulation tissue, soft tissue flaws that resulted in an unsightly and uneven surface were prevented and the defects were covered. Applying VAC dressing in the presence of an external fixator presents technical challenges, but these issues were resolved by realigning the fixator, decreasing the need for secondary intervention and reducing donor site morbidity by reducing the graft size, when the wound was required to be closed.

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Conflict of interest

None.

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ORIGINAL ARTICLE

Occupational Diseases in the Republic of Srpska from 2011-2020

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Abstract

Background/Aim: The importance of occupational diseases is due to their influence on workers' quality of life and significant socioeconomic problems for the health sector, employers and the state. Occupational diseases are generally not curable but are preventable diseases, so it is extremely important to develop a good system of detection, registration and prevention. Therefore, special attention should be paid to the occupational incidence analysis. Aim of this study was to assess the incidence of occupational diseases in the Republic of Srpska.
Methods: The research was conducted as a cross-sectional study based on the obtained data on occupational diseases in the Republic of Srpska for ten years (from 2011 to 2021).
Results: In the observed period, 12 cases of occupational diseases were registered in the Republic of Srpska and the incidence was less than 1/100,000 per year. Occupational diseases were diagnosed in: - agriculture, forestry and fishing; - construction and - healthcare and social welfare.
Conclusion: The study showed a low incidence of occupational disease and it was observed that certain industrial sectors were particularly undervalued. The current situation regarding occupational diseases in the Republic of Srpska requires an urgent response from the occupational medicine services and the state. It is necessary to initiate procedures as soon as possible to ensure uniformity in the criteria of diagnosis and records of occupational diseases and revision of the list of occupational diseases by the International Labour Organisation. In the future, it is necessary to conduct additional and more detailed research in the field of occupational diseases.

Key words: Occupational health; Employee; Occupational morbidity; Workers; Occupational medicine.

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Introduction

According to the Convention of the International Labour Organisation (ILO) from 2002, the term occupational disease includes any illness that occurs as a result of exposure to risk factors at the workplace. The main two elements of occupational diseases are the cause-and-effect relationship between exposure and specific working conditions or work activities and the fact that these diseases occur in a certain group of people with a higher frequency than in the rest of the population.[1]

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In the Republic of Srpska, the definition of occupational diseases is regulated by the Law on Pension and Disability Insurance of the Republic of Srpska[2] and according to it: "Occupational diseases are certain diseases that occurred during insurance, caused by a longer direct influence of processes and working conditions at workplaces, ie jobs that the insured performed". In addition to the Law on Pension and Disability Insurance, the field of occupational diseases in the Republic of Srpska is regulated in more detail by the Rulebook on the List of Occupational Diseases, adopted in 2018 ("Official Gazette of RS" No 84/2018). According to Article 2 of the Rulebook on the List of Occupational Diseases, occupational diseases, by the Law on Pension and Disability Insurance, are certain diseases that occurred during the course of insurance, caused by a longer direct influence of the work process and working conditions at workplaces, ie the jobs performed by the insured, such as and diseases that are known to be the result of harmful effects related to the work process or working environment and the intensity of the harmfulness and the length of the period of exposure to that harmfulness is at a level that is known to cause damage to health. Following the same Rulebook, it is determined that occupational diseases in the Republic of Srpska must be proven in the reference institution of occupational medicine and a list of occupational diseases that belongs to the category of closed lists is defined (which means that only the diseases listed in the Rulebook, 59 of them, can be verified as professional).[3] However, it should be mentioned that the list of occupational diseases in the Republic of Srpska can be considered partially open, but only for diseases caused by biological hazards, where paragraph 46 states that all infectious and parasitic diseases caused by work in activities where the risk of infection is increased with a certain diagnostic algorithm can be verified as occupational diseases.

In addition to workers' quality of life, occupational diseases also represent a significant socioeconomic problem for the health sector, employers and the state. Occupational diseases can cause a significant economic burden on the country by reducing the productivity of the working population, leading to temporary or complete incapacity for work and eventually to death.[4, 5] For example, according to data from Croatia, only costs in 2015 amounted to 900 million HRK [0.3 % of gross domestic product (GDP)], while previously estimated the annual average for the period 2002-2009 of all costs amounted to HRK 1.1 billion (0.4 % of GDP).[5-7] In the Republic of Srpska, sick leave is paid as a result of occupational disease in the amount of 100 %.[8] It must not be forgotten the costs of healthcare, which includes diagnosis, treatment and rehabilitation of workers. If there is a complete loss of working ability, the costs of early (disability) pension, that is, in case of death, are the costs of paying compensation to the family of the deceased.

Enormous importance of occupational diseases is also shown by the fact that in 1925 the ILO published the first list of occupational diseases and since then the lists have been regularly revised. In Europe, as early as 1957, it was determined that one of the basic tasks of the European Commission is the development of protective measures for occupational diseases and injuries at work. In 2003, the European Commission emphasised that in the future it is necessary to improve the list of occupational diseases, to improve data collection and adequate prevention of occupational diseases.[1] Also, according to the ILO Convention C155, all member countries should have a single registration system that would provide information and enable the creation of policies for the prevention of occupational diseases.[9] However, registers of occupational diseases differ in terms of diagnosis, criteria, records and legal regulations from country to country in the European Union.[10] That is why it is difficult, or almost impossible, to make a comparison between countries. The situation is the same with the Republic of Srpska, where the list of occupational diseases differs and the problem of data collection and processing is even more evident.

Davodi et al[4] state that because occupational diseases are generally not curable, but are preventable diseases, it is extremely important to develop a good system of detection, registration and prevention. Therefore, special attention should be paid to the analysis of the frequency and incidence of occupational diseases, as well as to the identification of the main challenges, that is, to the investigation of possibilities for solving the problems of prevention and control of occupational diseases to form guidelines and strategies for the prevention of occupational diseases. In the territory of the Republic of Srpska, no research was found that dealt with the issue of occupational diseases.

This study aimed to assess the incidence and

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trend of occupational diseases in the Republic of Srpska for ten years, from January 2011 to December 2020 and to analyse the influence of gender and the industrial sector on the occurrence of occupational diseases, to determine the most frequent occupational diseases in the analysed period and to make a proposal further measures.

Methods

The research was conducted based on the obtained data on occupational diseases in the Republic of Srpska. Data on occupational diseases from January 2011 to December 2021 were collected from the Register of Occupational Diseases of the Institute of Occupational Medicine of the Republic of Srpska (Register). This Register records occupational diseases that were diagnosed at the Institute for Occupational and Sports Medicine of the Republic of Srpska in the specified period. Diseases diagnosed out of the territory of the Republic of Srpska (Federation of Bosnia and Herzegovina, Brčko District, Republic of Serbia) were not included in the analysis.

For data analysis, the records of the Bulletin of the Statistical Office of the Republic of Srpska on employment in the Republic of Srpska in the analysed period[11] were used.

Based on data on employment and the number of occupational diseases, the incidence of occupational diseases per 100,000 employees and the trend of occupational diseases concerning gender, industry sector and diagnosis were analysed.

Descriptive statistics were used for data analysis. The Statistical Package for the Social Science (SPSS) program version 18.0 was used for statistical processing and data analysis. The results were presented tabularly and graphically.

Results

From January 2011 to December 2020 a total of 12 cases of occupational diseases were registered and recognised in the Republic of Srpska. Of these, 10 cases (83.3 %) were registered in males and 2 (16.7 %) in females. The largest number of diseases was registered among employees aged 55-64, with 5 cases (41.7 %), with exposure to work experience of 5-9 years (3 or 25 %). Table 1 shows the number and frequency of occupational diseases in the Republic of Srpska in the analysed period.

Table 1: Occupational diseases in the Republic of Srpska from 2011-2020

Parameter	N	%
Gender		
- Male	10	83.3
- Female	2	16.7
Age (years)		
- < 25	0	/
- 25-34	1	8.3
- 35-44	5	41.7
- 45-54	1	8.3
- 55-64	5	41.7
- > 65	0	/
Occupation		
A - Agriculture, forestry and fishing:	6	50.0
- Logger	4	33.3
- Tractor operator	2	16.7
F - Construction:	2	16.7
- Worker (asbestos processing)	1	8.3
- Driver	1	8.3
Q - Healthcare:	4	33.3
- Doctor of medicine	1	8.3
- Medical technician	2	16.7
- Senior laboratory technician	1	8.3
Working experience (years)		
- 10-14	1	8.3
- 15-19	2	16.7
- 20-24	1	8.3
- 35-39	2	16.7

The incidence of occupational diseases by year in the analysed period is shown in Figure 1.

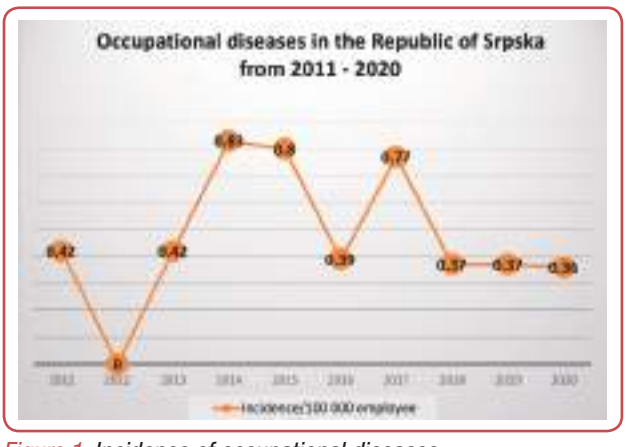

Figure 1: Incidence of occupational diseases

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Figure 2 showed the incidence of occupational diseases concerning gender per 100,000 employees in the analysed period.

Figure 2: Incidence of occupational diseases related to gender

The incidence of occupational disease concerning the industry sector is shown in Figure 3.

Figure 3: Incidence of occupational diseases concerning the industry

Table 2: Occupational diseases in an accordance to the International Classification of Diseases (ICD-10)

ICD-10	Total N (%)	Gender		Industrial sector		
		Male N (%)	Female N (%)	A N (%)	F N (%)	Q N (%)
A98.5	4 (33.3)	4 (40.0)	0	4 (66.7)	0	0
B18.1	1 (8.3)	1 (10.0)	0	0	0	1 (25.0)
C34.3	1 (8.3)	1 (10.0)	0	0	1 (50.0)	0
L23.5	2 (16.7)	0	2 (100.0)	0	0	2 (50.0)
T75.2	3 (25.0)	3 (30.0)	0	2 (33.3)	1 (50.0)	0
U07.1	1 (8.3)	1 (10.0)	0	0	0	1 (25.0)

A - Agriculture, forestry and fishing; F – Construction; Q – Healthcare; A98.5 - Haemorrhagic fever with renal syndrome; B18.1 - Chronic viral hepatitis B without delta agent; C34.3 - Malignant neoplasm: Lower lobe, bronchus or lung; L23.5 - Allergic contact dermatitis due to other chemical products; T75.2 - Effects of vibration; U07.1 - COVID-19, virus identified.

The analysis of occupational disease concerning diseases according to the International Classification of Diseases (ICD-10) is shown in Table 2.

Discussion

According to the available data, this is the first study in the Republic of Srpska that dealt with the issue of occupational diseases in country. The research aimed to analyse the incidence of occupational diseases in the Republic of Srpska, to analyse the influence of gender and industrial sector on their occurrence, as well as to determine the most frequent occupational diseases, to form further research plans and guidelines to improve the health of the working population.

The results of this research showed that the incidence of occupational diseases in the Republic of Srpska is extremely low. In the analysed ten-year period, the incidence of occupational diseases ranges from 0 - 0.83/100,000 employees, while, for example, in the Czech Republic in 2013 it was 1.4/10,000[12] and in Poland, in 2011 it was even 24.6/100,000 employees.[13] It should be noted that the comparison of the results of this study with the results of other countries is difficult due to the difference in the diagnostic criteria and the lists of occupational diseases. Also, the number of studies on occupational diseases is limited. In the research of Geharn et al, it is stated that there is a significant stagnation in publications on occupational diseases over the past 60 years compared to the increased number of publications from other branches of medicine.[14] However, the incidence of occupational diseases in the Republic of Srpska is underestimated, that is, there is a significant number of unverified occupational diseases in the country. According to data, even in the countries of the European Union, the number of occupational diseases is underestimated.[1]

Automation of the work process, which led to the improvement of working conditions or better prevention, could have contributed to the reduction of occupational morbidity; however, it is unlikely that working conditions in the Republic of Srpska are better than conditions in other countries. The important fact is that during the two thousand years healthcare has undergone certain reforms. Family medicine doctors treat the working population in primary healthcare institutions or direct them to specialists in secondary and tertiary institutions. A large number of sick working people do not direct to occupational medicine specialists. Furthermore, there is an inevitable lack of knowledge of specialists in family medicine, as well as all other specialists (internists, ENT, ophthalmologists, etc) because there is no

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basis for training in occupational medicine in any specialisation program. The lack of knowledge of occupational medicine and reduced contact with occupational medicine services contributed to the enormous and apparent reduction of occupational morbidity.

It must not be forgotten the fact that there has been significant privatisation of the industry, which has led to a decrease in employers' interest in recognising occupational diseases. Also, the workers' fear of losing their jobs certainly led to a certain extent to a reduced number of patients reporting to doctors and thus to a reduction in the number of registered occupational diseases and these assumptions are stated by other authors as well.[1]

By analysing occupational diseases concerning gender, the results showed that occupational diseases occur more often in men. The incidence of occupational diseases in males ranged from 0-1.48/100,000 employed men and in females from 0-0.97/100,000 employed women. The possible gender difference stems from the traditional division of labour and the fact that women more often do "easier" jobs, ie jobs in which risk factors are less prevalent.

A worrying fact is a result that in the ten years, occupational diseases were verified only in three industrial sectors: agriculture and forestry, construction and healthcare. Inevitably, other industrial sectors are unjustifiably underestimated, such as mining, the chemical industry, metal processing, etc. That difference in the verification of occupational diseases in healthcare could be explained by the significantly higher "health literacy" of healthcare workers compared to workers from, for example, the mining and metal-working industry. Diagnosis of diseases is also more accessible to healthcare workers. However, the fact is that this result indicates the need for urgent activation of occupational medicine services and the state to solve the problem of recognition and verification of occupational diseases. Also, in the industrial sectors where occupational diseases have been verified, the incidence is either low or there is a discrepancy in the results. For example, in agriculture and forestry, the incidence ranges from 0-24.08/100,000 workers or employees, which indicates irregular reporting and verification, as well as the need for more detailed analyses.

Analysing the data in the examined ten-year period in the territory of the Republic of Srpska, it is observed that out of the total number of occupational diseases, only one case of lung cancer was verified, while according to the data of a group of British researchers, it is estimated that more than 8 % of deaths due to cancer in men are the result of occupational exposure and of these about 20 % of cases are lung cancer.[15] Also, it should be mentioned that other malignant diseases probably remained unrecognised. It should be mentioned that in the Republic of Srpska, according to the Rulebook, there is a possibility of recognising COVID-19 as an occupational disease and this was verified in 2020 in only one case with a health worker. What about other health workers who had severe forms of illness with the development of consequences or the families of deceased health workers? This degrading information speaks in favour of the lack of basic knowledge about occupational diseases in healthcare.

The disadvantages of this research are imprecise data on occupational diseases from the Register of the Institute of Occupational and Sports Medicine, due to the possibility that some diseases are recognised outside the territory of the Republic of Srpska.

Regardless of the mentioned shortcomings, the results of this research provided insight into the trend and problems related to occupational morbidity in the Republic of Srpska. For better prevention in the future, it is necessary to revise the list of occupational diseases and harmonise it with the list and criteria of the ILO list, establish national registers and uniformly enter data by recommendations. Furthermore, one of the eternal problems is inadequate supervision and reduced contact of the sick working population with occupational medicine services, on the one hand and insufficient knowledge about occupational diseases among other health workers. Faculties, health institutions, occupational medicine institutions or other competent services should in the coming period consider the introduction of education for other medical activities on the basics of occupational medicine and the causes of occupational diseases. No less important and necessary is the health education of employers and employees to prevent the occurrence and consequences of occupational diseases.

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Conclusion

This study provided insight into the trend of occupational diseases in the Republic of Srpska in the period 2011-2020 and showed a significantly low incidence of occupational morbidity. The incidence of occupational disease was less than 1/100,000 employees per year in the research period. In addition, results showed that certain industrial sectors are particularly undervalued (for example chemical industry, mining etc). It is obvious that the current situation regarding occupational diseases in the Republic of Srpska is problematic and requires an urgent response from the occupational medicine services and the state. It is necessary to initiate procedures as soon as possible to ensure uniformity in the criteria of diagnosis and records of occupational diseases and revision of the list of occupational diseases following the list of the ILO. In the future, it is necessary to conduct additional and more detailed research in the field of occupational diseases, which would help in the formation of guidelines and strategies for the prevention of occupational diseases at all levels of healthcare.

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Conflict of interest

None.

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ORIGINAL ARTICLE

Obesity: An Important Predictor of Metabolic Syndrome

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Abstract

Background/Aim: The leading avoidable cause of mortality in the world is obesity. It modifies how the body reacts to insulin, which might result in insulin resistance and an elevated risk of type 2 diabetes. Recent investigations have revealed a link between obesity and the metabolic syndrome. Therefore, it was intended for the current study to look at the prevalence of obesity and how it relates to the metabolic syndrome.
Methods: 120 healthy males between 30-50 years of age were chosen from the general population of Kota District, Rajasthan, India and screened for obesity and divided in three groups: normal, overweight and obese. Each group comprised of forty subject. Serum was separated and run in department of biochemistry, GMC Kota. Anthropometric parameters were taken. The completely automated analyser ERBA EM 360 performed lipid profile and blood sugar analyses on serum.
Result: Obesity increased metabolic syndrome prevalence. In comparison to overweight and normal weight people, obese subjects exhibited considerably lower levels of high-density lipoprotein (HDL) cholesterol and significantly higher levels of blood glucose, triglycerides, total cholesterol and low-density lipoprotein (LDL) cholesterol. Additional coronary artery disease (CAD) risk prediction ratios, such as total cholesterol/HDL, LDL/HDL and triglycerides/HDL ratios that have gradually increased from the normal to obese group, show that obese subjects have a relatively higher risk of developing cardiovascular diseases (CVD) than do those in the overweight and normal groups.
Conclusion: One of the key elements of metabolic syndrome, which is a collection of clinical and metabolic anomalies including abdominal obesity, insulin resistance, hypertension and dyslipidaemia, is obesity. Each of these conditions raises the risk of CVD and diabetes mellitus type 2 directly. The progression towards diabetes and CVD can be prevented by lifestyle modification programmes and regular health checks (to explore the risk factors of metabolic syndrome).

Key words: Obesity; Metabolic syndrome; Lipid profile.

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Introduction

In cases of obesity, extra body fat builds up to the point that it may have a negative impact on health, reducing life expectancy and/or increasing health issues. Obesity is a leading avoidable cause of mortality globally. Most cases of obesity are assumed to be caused by a combination of excessive dietary energy consumption and a lack of physical exercise.[1] Insulin resistance may result from altered insulin responses caused by increased body fat. Fat accumulation in the subcutaneous and visceral depots is vulnerable to metabolic and cardiovascular problems because it induces a pro-inflammatory and pro-thrombotic state.[2]

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CONTENT

Metabolic syndrome (MetS) is defined as a number of clinical and metabolic problems, as abdominal obesity, insulin resistance, hypertension and dyslipidaemia, have been demonstrated to enhance the chance of developing diabetes mellitus type 2 (DMT2) and cardiovascular diseases (CVD).[3] Right now, the NCEP's ATP III and IDF definitions, which employ waist circumference as a proxy for central obesity, are the two most often used definitions. According to the NCEP ATP III definition, MetS is present if three or more of the following five criteria are met: a waist circumference greater than 40 inches for men (100 cm) and 35 inches for women (90 cm); blood pressure greater than 130/85 mmHg; a fasting TG level greater than 150 mg/dL; a fasting high-density lipoprotein (HDL) level less than 40 mg/dL for men and 50 mg/dL for women; and a fasting blood sugar level greater than 100 mg/dL. One of the most often used criteria for the MetS is the NCEP ATP III definition. Visceral obesity, atherogenic dyslipidaemia, hypertension and hyperglycaemia are all included as important characteristics. Oxidative stress and the depletion of antioxidants are essential pathogenic mechanisms in the development of obesity-associated MetS, diabetes and its consequences, according to experimental and clinical studies.[4]

Excessive fat build-up in the MetS promotes oxidative damage in adipose tissue and releases free radicals. These free radicals have a high degree of reactivity with nearby molecules including lipids, proteins and carbohydrates and they can harm cells.[5] Oxidative stress is a situation that develops when the generation of harmful free radicals outpaces the body's antioxidant defences' ability to cleanse them. Reactive oxygen species (ROS) are all free radicals that include oxygen.[6] The superoxide anion radical (O_2 •–) and the hydroxyl radical (•OH) are the ROS that are most often generated.[7] The production of O_2•– starts a chain reaction that quickly produces additional free radicals and, in the end, results in the synthesis of H_2O.[8] Antioxidants are substances that can protect bodily tissues from the harmful effects of oxidation. The production of atherosclerotic plaque requires the oxidative alteration of low-density lipoprotein (LDL), which has been associated to low plasma antioxidant levels.[9] The most significant factors may be dietary and lifestyle choices. Obesity has been linked to inadequate fruit and vegetable consumption.[10]

The goal of the this study was to determine the prevalence of obesity and how it relates to the MetS.

Methods

This study was conducted from September 2011 to August 2012 in Department of Biochemistry, Central Laboratory NMCH and MBS Hospital Kota, Rajasthan, India. For this study 120 healthy males between 30-50 years of age were selected from the general population of Kota District and accordance to recent recommendations made by the Indian Ministry of Health, who have been classified by body mass index (BMI) in 3 groups: normal, overweight and obese. Each group comprised of forty subjects.

Following anthropometric measurements were performed: body mass (kg), height (m) using a standard measuring tape, BMI, waist size (cm), hip size (cm), waist to hip ratio, heart rate (beats per minute – bpm). Serum was used for the following biochemical investigations: fasting blood glucose (mg/dL), level of serum HDL cholesterol (mg/dL), level of serum LDL cholesterol (mg/dL), level of serum total cholesterol (mg/dL), level of serum triglycerides (mg/dL), total cholesterol/HDL ratio, LDL/HDL ratio, triglyceride/HDL ratio.

Participants underwent revised NCEP ATP III criteria for MetS evaluation. New NCEP ATP III Standards:

1. Men should have a waist circumference of at least 90 cm, while women should have one at least 80 cm (according to newly released recommendations issued by the Indian Ministry of Health).
2. A triglyceride level of 150 mg/dL or more is considered to be high.
3. Low HDL ("good") cholesterol level is less than 40 mg/dL for men and less than 50 mg/dL for women.
4. Blood pressure is elevated if it is measured 130/85 mm Hg or above, or if patient is using a hypertension medication.
5. Elevated fasting glucose: 100 mg/dL or above, or patient is using a hyperglycaemia treatment.

Subjects were categorised as having MetS if they satisfied three out of these five criteria.

Excel was used to enter the data and SPSS software version 21.0 was used for analysis. Qualitative data was expressed in percentage and quantitative data expressed as mean ± standard deviation (SD).

Results

Prevalence of MetS is shown in Table 1.

Table 1: Prevalence of metabolic syndrome

Body mass	N	Metabolic syndrome	
		N	%
Normal	40	2	5.00
Overweight	40	6	15.00
Obese	40	25	62.50

N: number of subjects;

Prevalence of MetS increased with obesity (increasing BMI). As shown in the Table 1, 62.5 % obese subjects were having MetS while 15 % overweight and 5 % normal subjects had this syndrome. Comparison of anthropometric parameters of all 3 groups is shown in Table 2.

Waist circumference and the waist-to-hip ratio significantly increased along with the rise in BMI in obese people. Further, as blood pressure rose with rising BMI, waist circumference and waist/hip ratio, both systolic and diastolic blood pressure were linked to obesity ($p < 0.05$).

Table 2: Comparison of anthropometric parameters of the groups (normal, overweight and obese subjects)

Parameters	Group 1: normal	Group 2: overweight	Group 3: obese	ANOVA	
				F-score	p-value
Body mass (kg)	63.87 ± 5.33	68.75 ± 4.08	84.75 ± 8.79	116.9	< 0.001
Height (cm)	172.03 ± 5.14	169.5 ± 4.68	170.5 ± 5.36	2.51	> 0.05
BMI	21.49 ± 0.93	23.81 ± 0.48	29.2 ± 2.91	196.2	< 0.001
Waist circumference (cm)	78.02 ± 3.64	87.65 ± 2.5	100.27 ± 7.85	183.9	< 0.001
Hip circumference (cm)	82.97 ± 3.85	91.22 ± 3.56	99.1 ± 6.42	113.4	< 0.001
Waist / hip ratio	0.93 ± 0.02	0.95 ± 0.04	1.01 ± 0.04	30.02	< 0.001
Systolic BP (mm Hg)	121.74 ± 10.94	126.22 ± 7.89	127.42 ± 8.84	4.16	< 0.05
Diastolic BP (mm Hg)	80.45 ± 7.44	83.2 ± 6.89	85.05 ± 6.89	4.26	< 0.05

Values are Mean ± SD; BP: blood pressure; BMI: body mass index;

Table 3: Comparison of lipid and blood glucose parameters between the groups (normal, overweight and obese)

Parameters	Group 1: normal	Group 2: overweight	Group 3: obese	ANOVA	
				F-score	p-value
Fasting blood glucose (mg/dL)	87.3 ± 12.41	91.5 ± 10.92	102.6 ± 10.77	19.24	0.001
Triglycerides (mg/dL)	116.42 ± 19.95	117.25 ± 20.4	142.92 ± 26.46	17.98	0.001
Total cholesterol (mg/dL)	185.32 ± 32.83	199.22 ± 32.28	231.75 ± 42.46	12.83	0.001
LDL cholesterol (mg/dL)	106.17 ± 17.14	112.77 ± 20.88	133.95 ± 24.02	19.33	0.001
HDL cholesterol (mg/dL)	50.12 ± 7.31	43.92 ± 3.68	42.4 ± 4.52	22.94	0.001
Total cholesterol/HDL ratio	3.96 ± 1.37	4.64 ± 1.09	5.61 ± 1.33	17.06	0.001
LDL/HDL ratio	2.2 ± 0.63	2.6 ± 0.64	3.21 ± 0.77	22.43	0.001
TG/HDL ratio	2.41 ± 0.71	2.73 ± 0.63	3.41 ± 0.77	21.18	0.001

LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglycerides; Values are presented as Mean ± SD;

Table 4: Prevalence of components of metabolic syndrome

Parameters	Group 1: normal	Group 2: overweight	Group 3: obese
WC ≥ 90 (cm)	0	12	39
BP ≥ 130/85 (mm Hg)	7	16	19
FBG ≥ 100 (mg/dL)	10	10	24
TG ≥ 150 (mg/dL)	3	5	14
HDL < 40 (mg/dL)	2	5	11

WC: waist circumference; BP: blood pressure; FBG: fasting blood glucose; TG: triglycerides; HDL: high-density lipoprotein;

The blood sugar and lipid values of healthy, overweight and obese people are shown in the Table 3. Obesity had a significant impact on cholesterol and blood glucose levels. In comparison to overweight and normal weight people, obese subjects exhibited considerably lower levels of HDL cholesterol and significantly higher levels of blood glucose, triglycerides, total cholesterol and LDL cholesterol ($p < 0.001$). Additional CAD risk prediction ratios, such as total cholesterol/HDL, LDL/HDL and TG/HDL ratios that had grad-

ually increased from the normal to obese group, showed that obese subjects had a relatively higher risk of developing CVD than do those in the overweight and normal groups ($p < 0.001$).

Table 4 shows that the prevalence of components of MetS increased with obesity (increasing BMI).

Discussion

It is commonly acknowledged that the MetS significantly raises the chance of developing cancer, DMT2 and CVD. Obesity and insulin resistance are key elements of the MetS.[11, 12] The objective of the current study, which involved 120 participants, was to determine the prevalence of the MetS in obese individuals. Atherosclerosis, DMT2, hyperlipidaemia and hypertension are now recognised as typical illnesses associated with a sedentary lifestyle. Obesity is a frequent risk factor for several disorders. The key precursor of MetS is obesity, particularly visceral obesity, which has increased in incidence during the past ten years. Although MetS patients frequently have obesity, not all fat persons have MetS.[4, 13-16] On the other side, this condition can also manifest in lean individuals.[17] Additionally, it was shown that obese people are more likely to have MetS compared to overweight and normal subjects (63 %, 15 % and 2 %, respectively), supporting the findings of earlier researchers that though the obesity is a major component of MetS, all obese people may not have it and even lean person may develop this syndrome.

The results of the current investigation also showed that blood glucose and cholesterol levels are considerably impacted by obesity. In comparison to overweight and normal weight people, obese subjects exhibited considerably lower levels of HDL cholesterol and significantly higher levels of blood sugar, triglycerides, total cholesterol and LDL cholesterol. Additional CAD risk prediction ratios, including total cholesterol/HDL, LDL/HDL and TG/HDL ratio, showed that obese patients had a considerably higher chance of developing cardiovascular illnesses than do persons in the overweight and normal category. Adipose tissue produces more non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines and other substances in obese people, which contribute to the emergence of insulin resistance. Failure to manage blood glucose levels leads to the development of DMT2 when insulin resistance is coupled with the malfunctioning of pancreatic islet cells, the cells that release insulin. This information is encouraging research into the disease's molecular and genetic roots as well as the development of fresh methods for treating and preventing it. Increased triglycerides, lower HDL levels and an aberrant LDL composition are the hallmarks of the main dyslipidaemia brought on by obesity. Without a doubt, the dyslipidaemia connected to obesity significantly contributes to the development of atherosclerosis and CVD in obese people. It has been shown that every aspect of dyslipidaemia, including greater triglyceride levels and lower HDL levels as seen in the current study, is atherogenic.

Obesity can accelerate the atherosclerotic process by affecting endothelial function and by activating oxidative stress-related pathways.[4, 16] TNF-α, resistin, leptin and adiponectin are only a few of the physiologically active chemicals that adipocytes generate, collectively known as adipokines.[18] These adipocytokines are produced in an uncontrolled manner as a result of adipocyte malfunction and they play a role in the pathophysiology of the MetS linked to obesity. For instance, increasing insulin resistance in obesity is accompanied with increased synthesis of TNF-α from stored fat.

It is generally known that individuals with the MetS phenotype experience oxidative stress more frequently than individuals without it,[4, 14-16] even if similar findings are not seen in all research.[17] Whether oxidative stress manifests at an early stage, before complications manifest, or whether it is merely a common result of cell damage, reflecting the presence of complications, will determine whether oxidative stress is a cause or a consequence in complications associated with the MetS (probably, both). Some people think that oxidative stress is a result of MetS, which includes insulin resistance, hypertension and obesity,[4] while some contend that the MetS, which includes insulin resistance, hypertension, atherosclerosis, obesity and more, is brought on by oxidative stress.[16, 19-21] The problem is made worse by a smoking habit, an urban lifestyle, an atherogenic diet and a lack of exercise as primary dyslipidaemia associated with antioxidant depletion was observed in subjects with urban background and smokers.

Conclusion

Obesity is one of the key elements of MetS, which is a collection of clinical and metabolic disorders including abdominal obesity, insulin resistance, hypertension and dyslipidaemia, all of which raise the risk of CVD and DMT2. In the MetS, excessive fat storage promotes the formation of free radicals and oxidative damage in adipose tissue. Free radicals can play a substantial role in the onset and advancement of numerous disease processes, including CVD, cerebrovascular accidents and diabetes complications.

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Conflict of interest

None.

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CONTENT

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REVIEW ARTICLE

Secoisolariciresinol Diglucoside (SDG) from Flaxseed in the Prevention and Treatment of Diabetes Mellitus

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Abstract

This review focuses on the role of reactive oxygen species (ROS) on the development of type 1 and type 2 diabetes and its treatment with secoisolariciresinol diglucoside (SDG) isolated from flaxseed which is an antioxidant and suppresses phosphoenolpyruvate carboxykinase (PEPCK) gene expression, a rate- limiting enzyme in the gluconeogenesis in the liver. Role of ROS in the development of type 1 diabetes [diabetic prone Bio Breeding (BBdp) rats and streptozotocin-induced diabetic (STZ) rats and type 2 diabetes (Zucker diabetic fatty female rats, ZDF rats)] has been discussed. Oxidative stress has been assessed by measuring serum and pancreatic malondialdehyde (MDA), pancreatic chemiluminescence (pancreatic-CL) and oxygen radical producing activity of white blood cells (WBC-CL). Diagnosis of diabetes was made by hyperglycaemia and glucosuria. Incidence of diabetes was 100 % in SDZ rats, 72 % in BBdp rats and 100 % in ZDF rats by the age of 72 days. Development of diabetes was associated with increases in the serum and pancreatic MDA, WBC-CL and pancreatic-CL and glycated haemoglobin (HbA_1c). SDG prevented the development of diabetes by 75 % in STZ rats, by 71 % in BBdp rats and by 20 % at 72 days of age in ZDF rats. However, 80 % of the rats which did not develop diabetes by 72 days of age, developed diabetes later on, suggesting that SDG treatment delays the development of diabetes in ZDF rats. Treatment with SDG decreased the levels of serum and pancreatic MDA, WBC-CL and pancreatic-CL. In conclusion, development of type 1 and type 2 diabetes is mediated through oxidative stress and the prevention or delay in the development of diabetes with SDG could be due to its antioxidant activity and its suppressant effect on PEPCK enzyme. Lignan complex which contains 34 % to 38 % of SDG is effective in lowering serum glucose and HbA_1c in type 2 diabetes in humans.

Key words: Type 1 diabetes; Type 2 diabetes; Oxidative stress; Antioxidants; Phosphoenolpyruvate carboxykinase (PEPCK); Malondialdehyde; Pancreatic -CL; WBC-CL; Secoisolariciresinol diglucoside (SDG).

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterised by persistent hyperglycaemia caused by impaired insulin secretion and resistant to peripheral insulin action or both. There are three types of DM: type 1 DM, type 2 DM and gestational DM. Type 1 DM is characterised by autoimmune destruction of β-cells in pancreas and accounts for 5 % to 10 % of DM. Type 1 DM is commonly observed in children and adolescents. It may occur in people with any age. Type 2 DM accounts for approximately 90 % of DM. In this type of DM there is a reduction in response to insulin. Ineffec-

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tiveness of insulin in the initial stage is countered by an increase in production of insulin, but later on there is a loss of production of insulin resulting in type 2 DM. Type 2 DM is mostly observed in people older than 45 years of age. Gestational DM occurs during pregnancy. The incidence of type 1 DM is 15 per 100,000 people and prevalence is 9.5 per 10,000 in the world.[1] The incidence and prevalence respectively are 20 per 100,000 and 12.2 per 10,000 people in USA, 15 per 100,000 and 12.2 per 10,000 people in Europe and 15 per 100,000 and 6.9 per 10,000 people in Asia.[1] The prevalence of type 2 DM globally is 6059/100,000 people and projected to be 7079/100,000 people by 2030.[2] There is equal distribution of type 2 DM in male and female and the incidence peaks at about at 55 years of age.[2] Presently, the treatment of DM includes insulin, insulin secretagogues and insulin sensitising drugs, thiazolidinedione for glycaemic control. Direct and indirect cost of treatment of type 2 DM is very high.[3, 4] The discovery of cheap plant food or its constitutes would be helpful in the prevention and treatment of DM.

This review paper deals with the role of oxidative stress, defined as a shift in balance between reactive oxygen species (ROS) and antioxidants in favour of ROS in the development of DM and use of secoisolariciresinol diglucoside (SDG) isolated from flaxseed in the prevention and treatment of type 1 DM and type 2 DM. Flaxseed and its components and the mechanism of action of SDG is also described in detail.

Flaxseed and Its Components

Flaxseed contains 38 % to 45 % of its mass as oils of which 51 % to 55 % is α-linolenic acid.[5, 6] The rest of flaxseed is called flax meal which contains approximately 16.4 mg/g of SDG.[7] Most of the SDG is present in the seed coat.[8] The amount of SDG in 100 g of flaxseed is about 0.6 g to 6.0 g.[9] Flaxseed is the richest source of plant SDG.[10] The other components isolated from flaxseed are flax lignan complex which contains 34 % to 38 % of SDG, 15 % to 21 % of cinnamic acid glucoside and 9.6 % to 11.0 % of hydroxymethylglutaric acid.[11] Protein content of flaxseed by weight is about 10.5 % to 31.0 % while the fibre content is about 25 % to 28 % of which 25 % is in soluble form.[12]

Reasons SDG Could be Effective in Prevention and Treatment of Diabetes Mellitus

There are two reasons for which SDG could be effective in prevention and treatment of DM: antioxidant activity and hypoglycaemic effect due to suppression of phosphoenolpyruvate carboxykinase (PEPCK) gene expression.

1. Antioxidant activity

ROS have been implicated in the development of DM and its complication.[13, 14] Dimethylthiourea, a free radical scavenger protected the β-cells of pancreas.[15] ROS may be involved in type 2 DM because plasma levels of free radicals are positively correlated with fasting plasma insulin[16] and malondialdehyde (MDA), an indirect measure of ROS, is also elevated in type 2 DM.[17] ROS have been suggested to be a pathogenic mechanism of insulin resistance and DM.[18] Considering the above reports, antioxidants may be of value in the treatment of DM. The question arises, if SDG has antioxidant activity. Using high pressure liquid chromatography (HPLC), it has been shown that SDG scavenges hydroxyl radical (·OH) generated by photolysis of hydrogen peroxide (H_2O_2) with ultraviolet light and trapped with salicylic acid and this effect was concentration dependent.[19] This investigator also reported that SDG prevented the ·OH-induced lipid peroxidation of liver homogenate in concentration dependent manner by measuring malondealdehide.[19] The antioxidant activity of SDG and its metabolites was investigated using chemiluminescence (CL) of zymosan-activated polymorphonuclear leukocytes (PMNLs) [PMNL-CL]. Activated PMNLs generate numerous oxygen radicals [superoxide anion, H_2O_2, ·OH, singlet ($^1O_2$)].[20-22] There was a concentration-dependent reduction in PMNL-CL with SDG suggesting that SDG has antioxidant activity which was 1.27 times greater than vitamin E. The above data suggest that SDG has antioxidant activity.

2. Hypoglycaemic effect of SDG by suppressing PEPCK gene expression

Increased glucose level in DM is due to increased hepatic neoglucogenesis[23, 24] PEPCK is a rate limiting enzyme for gluconeogenesis in liver[25] and is elevated in all types of DM.[26-29] Regulation of

activity of PEPCK is controlled through gene expression.[30] SDG has been reported to suppress the PEPCK gene expression.[31] SDG could be a good antidiabetic agent. Troglitazone, a known antidiabetic agent suppresses PEPCK gene expression[32] and is an antioxidant.[33]

Reactive Oxygen Species (ROS) and Diabetes Mellitus

As mentioned earlier, ROS may be involved in the development and complication of DM.[13, 14] The common pathway of cell destruction may be due to production of cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) from activated macrophages. IL-1 and TNF-α activate macrophages[34] and polymorphonuclear leukocytes (PMNLs)[35, 36] to generate ROS which would have lethal effect on islet cells. Cytotoxicity of ROS is due to peroxidation of unsaturated fatty acids in the membrane resulting in lipid peroxidation product MDA that produces a change in cell permeability, integrity and ultimate cell death.[37, 38] Pancreatic cell death would reduce insulin levels resulting in increased serum levels of glucose.[39] It has been reported that cytokines increase the MDA content of islet cells and islet cells necrosis and lazaroid U78518E, an inhibitor of lipid peroxidation, significantly decreased the cytokine-induced increase in islet MDA and protected islet β-cells from destruction by the cytokines.[40] Considering the above data, SDG an antioxidant would prevent and/or delay the development of DM.

A. Effects of SDG on animal model of type 1 and type 2 diabetes mellitus

Beneficial effects of SDG in experimental type 1 and type 2 diabetic animals have been reported by Prasad et al.[41-43]

1. Effects of SDG in animal model of type 1 diabetes mellitus

Two models of type 1 DM have been used for this study: diabetic prone bio breeding diabetic prone rats (BBdp rats) and streptozotocin (STZ)-induced DM. Effects of SDG on these two models of type 1 DM are discussed below.

a) Effects of SDG in BBdp rats

Genetic BBdp rats develop DM spontaneously[44] and is a model of human type 1 DM (insulin-dependent DM). The incidence DM in in BBdp rats is 40 % to 70 %, more than 85 % develop DM by the age they reach 60-120 days. The objectives of the investigator for this study were to determine if SDG prevents or reduces the development of DM and if prevention /reduction in the development of DM is associated with reduction in serum glucose, serum and pancreatic MDA and antioxidant reserve of the pancreas.[42] SDG prevented the development of DM by about 71 %. Serum glucose levels were higher in BBdp rats that developed DM than those that did not develop DM. Serum glucose levels were lower in SDG treated rat that did not develop DM. Serum and pancreatic levels of MDA were elevated in BBdp rats that developed DM. Pancreatic -CL activity (antioxidant reserve) levels in untreated BBdp rats were higher than that of control group suggesting that antioxidant reserve was lower in the diabetic rats. The pancreatic -CL activity in SDG treated BBdp rats was significantly lower than that of untreated BBdp rats suggesting that antioxidant reserve was higher in SDG treated rats than untreated BBdp rats. The above data suggest that type 1 DM in BBdp rats is associated with increases in serum and pancreatic MDA and a decrease in the antioxidant reserve. Oxidative stress (levels of ROS) levels were higher in diabetic rats. These data also suggest that type 1 DM is associated with an increase in the lipid peroxidation product and a decrease in antioxidant reserve. Prevention in development of DM with SDG was associated with a reduction in serum and pancreatic MDA and an increase in the antioxidant reserve. In summary, DM in BBdp rats is mediated through ROS and that SDG prevents the development of DM through its antioxidant activity.[19] Decreased incidence of development of DM in BBdp rats with SDG could also be due to its inhibitory effect on PEPCK gene expression.[31]

b) Effects of SDG on streptozotocin-induced diabetes mellitus in rats

The purpose of this study was to investigate if streptozotocin (STZ)-induced DM is associated with increased oxidative stress (imbalance between oxidants and antioxidants in favour of oxidants)[45] and if SDG reduces/prevents the development of DM by reducing the levels of ROS.[41] Prasad et al[41] investigated the effects of SDG on streptozotocin-induced changes in serum glucose, urinary glucose, serum and pancreatic MDA, ROS producing activity of white blood cells (WBCs) chemiluminescence [WBC-CL)

and antioxidant reserve of pancreas (pancreatic -CL) in Sprague Dawley rats. They reported that STZ-induced DM (increased serum levels of glucose) was associated with an increased levels of glucose in serum and urine, MDA in serum and pancreas, pancreatic antioxidant reserve (pancreatic-CL) and WBC-CL streptozotocin produced in 100 % of rats. SDG treatment prevented the development of DM by 75 % and this was associated with decrease in the levels of glucose in urine and serum, serum and pancreatic MDA and WBC-CL and increase in pancreatic antioxidant reserve. The above data suggest STZ-induced DM is mediated through ROS and prevention of DM by SDG is because of its antioxidant property.[19] The prevention of DM with SDG could also be due to suppression of PEPCK gene expression.[31]

2. Effects of SDG on animal model of type 2 diabetes mellitus

An extensive study on the effects of SDG in the prevention of development of type 2 DM in experimental animal have been carried out by Prasad.[43] The main objectives of this study were if type 2 DM is associated with increases in oxidative stress, if SDG can prevent/reduce the development of type 2 DM and if prevention/reduction in the development of type 2 DM is associated with reduction in oxidative stress. The study was conducted on Zucker diabetic fatty (ZDF) /Gmi-fa/fa female rats, a model of human type 2 DM. DM was assessed by measuring glucose in the urine. Glucosuria started developing at the age of 64 days and all rats developed glucosuria by 72 days of age. Incidence of DM in untreated rats was 100 %. Only 20 % of the SDG-treated rats developed DM by 72 days of age suggesting that SDG treatment delayed the development of DM in 80 % of the SDG-treated rats. Blood glucose levels at the age between 72 and 101 days increased significantly compared to the levels at the age of 42 days in untreated and SDG treated ZDF rats that did develop DM, but the levels of blood glucose in SDG-treated rats (80 %) that did not develop DM, did not increase. Glycated haemoglobin (HbA_1c) was elevated in untreated but not in SDG-treated ZDF rats. Serum MDA levels were elevated in untreated and SDG-treated rats with DM but were lower in SDG-treated ZDF rats at the age 70 days that did not developed DM. The delaying of development of DM with SDG is due to its antioxidant activity. The protective effect of SDG could also be due to its suppressive effect on PEPCK gene expression.[31] There are no reports on the effects of SDG on DM in human.

The data suggest that type 2 DM is associated with increased oxidative stress and SDG delays the development of type 2 DM which is associated with decrease in oxidative stress. Also SDG delay the development of type 2 DM.

B. Flax lignan complex (FLC) on human type 2 diabetes mellitus

As mentioned above, effects of SDG in human in type 1 diabetic patients are not available in literature. However, the effects of flax lignan complex (FLC) on type 2 DM have been reported in literature. Pan et al[46] have reported that FLC in a randomised, double-blinded cross-over trial in type 2 diabetic patients, FLC reduced HbA_1c, fasting glucose, HbA_1c and insulin levels during the lignan treatment phase. The reduction in HbA_1c levels in serum was significant. It is to note that SDG content in FLC is only 34 %. It is possible that the poor effect of FLC on glucose and insulin was because of lower dose (360 mg daily) of FLC. In other double-blinded, randomised, cross-over study, Barre et al[47] have shown that FLC in the dose of 600 mg daily reduced the plasma glucose and HbA_1c significantly. In summary, flax lignan complex seems to reduce the serum/plasma levels of glucose and HbA_1c in patients with type 2 DM.

Perspectives

The above data indicate that ROS are involved in development of type 1 and type 2 DM[41-43] and that SDG isolated from flaxseed prevents the development of type 1 DM[41, 42] and delays the development of type 2 DM[43] in experimental model of DM.

Increases in the serum levels of glucose could be due to decreases in the levels of insulin. It has been reported that insulin induces suppression of gluconeogenesis by inhibiting PEPCK enzyme.[48] ROS cause pancreatic β-cell dysfunction leading to reduction in insulin in the blood.[49] Increased levels of ROS negatively affect β-cell function including insulin secretion.[50] Increase in the serum levels of glucose could also be due to increased expression of PEPCK gene.[51, 52] PEPCK is a rate limiting enzyme for gluconeogenesis in liver[25] and is elevated in all types of DM.[26-29] Decreases in serum levels of glucose with SDG could be due to its anti-oxidant activity[9, 19, 20] and its inhibitory effect on PEPCK enzyme.[31]

CONTENT

MDA is an indirect measure of levels of ROS. Increased levels of MDA could be due to increased production or reduced destruction of ROS. Glucose increases the production of ROS through autooxidation and nonenzymatic glycation of protein.[53] Several lipid soluble vitamins (Vitamin E, Vitamin A), glutathione peroxidase and glutathione are antioxidants and protects the ROS-induced tissue damage.[41] Increased levels of MDA in serum and pancreatic tissue in experimental DM could be due to increased levels of ROS.[54] Low levels of serum and pancreatic MDA with SDG treatment could be due to reduction in levels of ROS.

Pancreatic-CL measures the levels of antioxidants, glutathione peroxidase and glutathione.[47] The levels of WBC-CL which measures the generation of ROS from WBCs are elevated in streptozotocin induced DM and BBdp rats.[41, 42] The levels of WBC-CL were reduced with SDG in the streptozotocin and BBdp rats.[41, 42] Serum glucose levels were elevated in both type 1 and type 2 DM and SDG treatment reduced the serum levels of glucose.[41-43] Increases in the levels of serum glucose in DM could be due to reduction in the insulin levels because of destruction of pancreas with ROS.

The available data suggest that type 1 DM is associated with increased generation of ROS by WBCs, decrease in the antioxidant reserve of pancreas (pancreatic-CL) and an increase in the lipid peroxidation products of pancreas (pancreatic-MDA).[41, 42] Prevention/reduction in the development of type 1 DM with SDG treatment was associated with reduction in WBC-CL, pancreatic-CL and serum and pancreatic MDA.[41, 42] Type 2 DM was associated with increases in oxidative stress as suggested by increases in the serum MDA.[43] The increases in the ROS could be due to a decreases in the levels of antioxidants. The elevated levels of ROS might damage the pancreatic β-cells leading to hyperglycaemia and glycosuria. SDG treatment reduced the serum levels of glucose and glycosuria. Hundred percent of untreated type 2 diabetic rats developed DM by the age of 72 days. Eighty percent of SDG treated type 2 diabetic rats did not develop DM by the age of 72 days suggesting that SDG prevented the development of DM by the age of 72 days. However, the rats which did not develop DM by 72 days of age, developed DM by 101 days suggesting that SDG treatment delayed the development of DM. These effects of SDG may be due to its antioxidant activity and its suppressive effect on PEPCK enzyme expression.

SDG is first hydrolysed and then metabolised to secoisolaricinol (SECO), enterodiol (ED) and enterolactone (EL) by gut microflora enzymes.[55, 56] Two questions arise: if SDG does not enter the circulation then how SDG is antidiabetic through its antioxidant activity and how it inhibits the PEPCK gene expression. The antioxidant activity of SDG could be through its metabolic products (SECO, ED, EL). Prasad[20] has reported that SECO, ED and EL are antioxidants. He also reported that antioxidant potency of SECO, ED and EL and SDG were 4.86, 5.02, 4.35 and 1.27, respectively compared to vitamin E. SECO, ED and EL were respectively 3.82, 3.95 and 3.43 times more potent than SDG. The other question arises as to how SDG can affect the PEPCK gene expression in liver and kidney if it cannot enter the circulation. Prasad[31] has reported that SDG suppressed the PEPCK gene expression. In this study SDG was added in hepatic cell culture.[57] This suggests that SDG can directly act on the PEPCK gene in liver. The effects of SDG metabolites on PEPCK gene expression are not available in literatures. The possibility exists that SDG supresses PEPCK gene expression.

Very limited studies are available in literature on the effects of FLC in type 2 DM in human. Studies of pure SDG on type 1 and type 2 DM in human are nil. A well-designed, randomised, placebo-controlled, multicentre clinical trials are needed for evaluating the efficacy, long-term safety and optimal dose schedules of SDG and FLC in humans. The problem might arise because of the unavailability of pure SDG.

Conclusion

In conclusion, oxidative stress is involved in the development of animal models of human type 1 and type 2 DM. SDG could prevent / reduce the development of type 1 DM and delay the development of type 2 DM. The reduction in the development of type 1 and type 2 DM could be due to the antioxidant and suppressive effect on the PEPCK enzyme. Beneficial effects of FLC in type 2 DM may be due to the SDG content in FLC. FLC lowers the levels of serum glucose and HbA_1c in type 2 DM in humans.

CONTENT

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CURRENT TOPICS

Clinical Features and Management of Human Monkeypox

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Abstract

The COVID-19 pandemic is abating, but the threat of a new epidemic is growing due to the spread of monkeypox in non-endemic regions of the world. In 2022, there were the monkeypox outbreaks throughout Europe, in the Western Hemisphere. With the cessation of the vaccine, due to the global eradication of smallpox, outbreaks of monkeypox have become more common. Currently, there are no exact recommendations for complex treatment and alleviation of the monkeypox symptoms for infected people. Under these conditions, it is especially important to know the pathogenetic mechanisms and epidemiology of the virus for the most effective containment of its spread, especially in view of the negative experience gained in combating the COVID-19 epidemic. The purpose of this study was to summarise the known data on the epidemiology, clinical course and treatment of monkeypox, as well as an attempt to assess the possibility of a new world-spanning pandemic. A targeted search was performed on the keywords "monkeypox", "virology", "Tecovirimat", "Cidofovir", "Brincidofovir" in PubMed, in the period up to July 2022. 661 articles were reviewed, among them as reviews, original research and clinical trials. Preference was given to articles in English that dealt in most detail with cases of monkeypox infection outside the Africa and included comments on the therapy. Seventeen articles were selected and analysed, as well as links within them for additional information on the case. It was revealed that monkeypox is mainly treated with maintenance therapy and the treatment of more complex cases is based on the use of specific antiviral drugs: Tecovirimat, Cidofovir, Brincidofovir. However, there is no widespread therapeutic practice for these drugs. Little is currently known about the monkeypox virus; the transmission of infection, the animal reservoirs, the host range and the prospects for specific treatment are not fully understood. Sharing resources and data with outbreak tracking around the world will greatly facilitate the process of learning about the virus and how to deal with it effectively.

Key words: Monkeypox; Pandemic; *Poxviridae*; Tecovirimat; Cidofovir; Brincidofovir.

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Introduction

In 1958, the smallpox virus was identified for the first time in primates kept as experimental animals in Denmark.[1] The first case of human infection was reported in 1970 in the Democratic Republic of the Congo.[2] Over the past 50 years, sporadic outbreaks have been reported mainly in African countries, they amounted to some thousands. Separate outbreaks of the disease were observed during the importation of animal reservoirs outside the African continent or during the trips to endemic areas and further importation outside them.[3] It has long been feared that zoonotic poxviruses could eventually spread and occupy the ecological niche vacated

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CONTENT

by the killed smallpox.[4] Due to deforestation, population growth and the resulting expansion of living space, due to the encroachment on the habitats of animal reservoirs, as well as increased interstate relations and globalisation, the threat of the monkeypox spread has become much more real over the past 20 years.[5]

It is difficult to predict how the disease will proceed in non-endemic areas, due to the data scarcity on viral kinetics and the duration of the viral shedding. Although the virus has been given the name "monkeypox", primates are not the source of the virus and its true origin is still unknown. There are no approved and licensed treatments for this infection and maintenance care is usually assigned for alleviation of symptoms. The oral drugs Brincidofovir and Tecovirimat have approved themselves for the most severe cases.

The increase in the number of cases during the current outbreak requires updating the knowledge of this infection, in particular about prevention measures, clinical course and epidemiology, in order to prevent large-scale consequences if new outbreaks arise and spread.

The purpose of this study was to summarise the known data on the epidemiology, clinical course and treatment of monkeypox. The research objectives included the droplet spread and non-percutaneous channel of infection, the prospects for the use of specific antiviral drugs and their side effects.

Methods

A targeted search was performed on the keywords, "monkeypox" and "virology", or "Tecovirimat", or "Cidofovir", or "Brincidofovir" in PubMed, in the period up to July 2022. Articles, national recommendations, literature reviews, clinical cases were reviewed. Out of 661 articles, preference was given to reviews and clinical cases written in English, in which the mechanisms of transmission and methods of treatment were most fully disclosed. Eventually, 17 articles were selected and analysed and the accompanying references were checked for additional information on the case.

Results

The *Poxviridae* family is represented by double-stranded DNA viruses that mainly infect animals: mammals, birds, reptiles and even insects. *Poxviridae* is divided into two subfamilies: *Chordopoxvirinae* (18 genera and 52 species) and *Entomopoxvarinae* (4 genera and 30 species). Thus, according to taxonomy, the monkeypox virus belongs to the *Poxviridae* family, the *Chordopoxvirinae* subfamily and the *Ortopoxvirus* genus. Orthopoxviruses are large (140-450 nm) viruses with a genome consisting of approximately 200-500 thousand base pairs.[6] The infectious virus replication cycle can be initiated both by a mature virion and by a cell-free virion that is still enveloped. Glycosaminoglycans, which are located on the cells of all mammals, play the greatest role in binding virions to the cell membrane; however, all receptor sites and their role in interaction with the virus have not been fully characterised (Figure 1).[7]

The monkeypox is endemic in tropical regions of West and Central Africa, especially in the Central African Republic, the Republic of Côte d'Ivoire, Democratic Republic of the Congo, Liberia, Nigeria and Sierra Leone.[4] Cases outside endemic countries are usually associated with international travel or importation of infected animals.[8] It should be noted that there are two strains of monkeypox, the West African, which is less lethal and infectious and the much more dangerous Central African strain.[5]

As a result of the smallpox immunisation program being discontinued 40 years ago, a great part of the population does not have the cross-immunity from monkeypox that was previously provided by the smallpox vaccine.[9] From 1970 to the present, there has been an increase in the incidence in endemic regions, which initiates sporadic outbreaks of the disease in non-endemic countries (Table 1 and 2). Separate outbreaks were even found in Singapore[10] and Israel. Currently, outbreaks have been reported in Italy,[11] Germany,[12] the UK, South America, the Middle East, Canada and the USA.[13]

Due to the large size of viruses such as monkey smallpox, it is more difficult to break through the host's defences, by having to pass through gap junctions. The large size contributes to the early development of the host's immune response and to survive, orthopoxviruses secrete immunomodulatory proteins, which are divided

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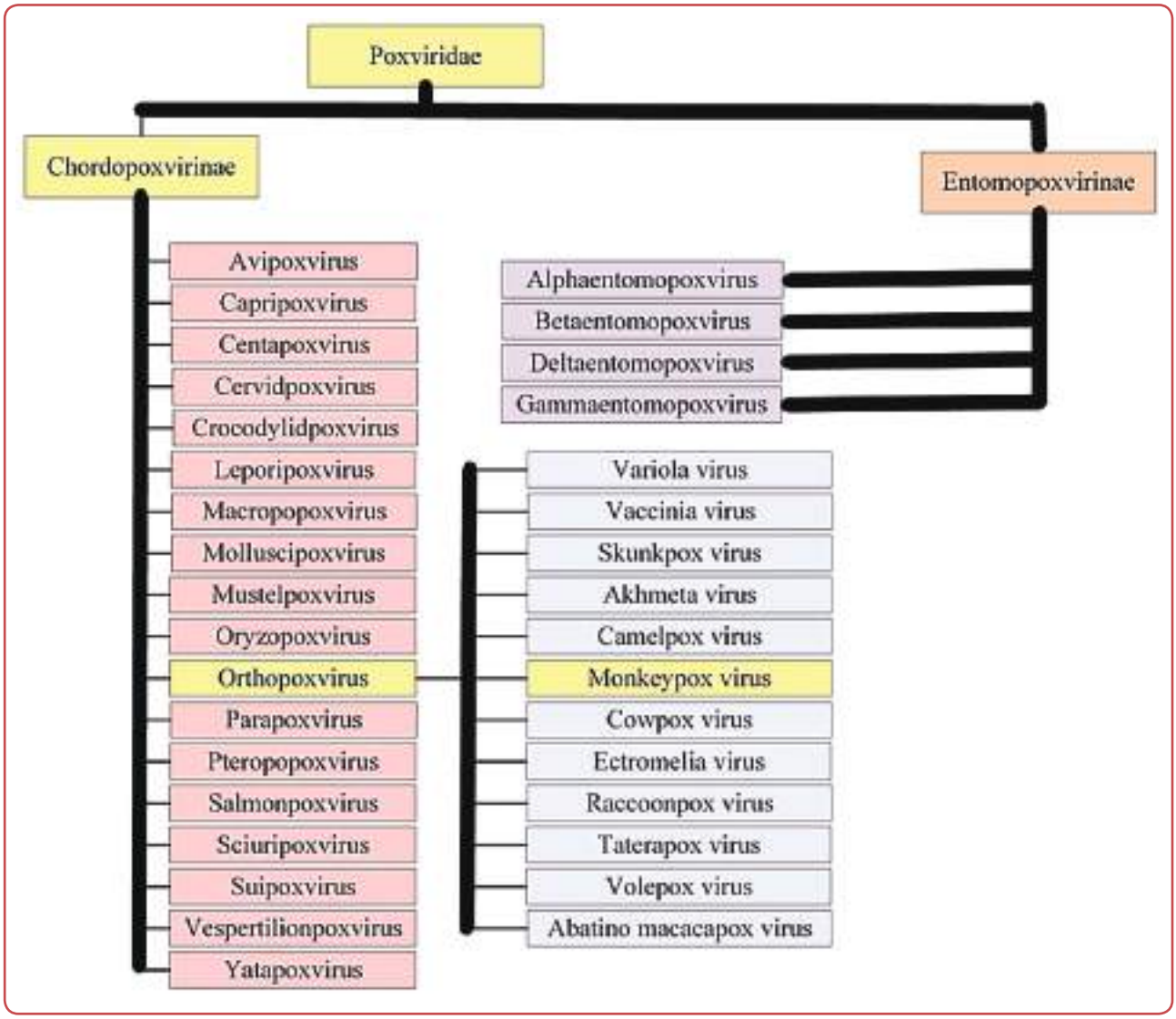

Figure 1: Taxonomy and classification of monkeypox within the family

into two groups - working inside the cell and outside it.[15] The listed proteins, virokines, mimic the activity of cytokines, chemokines and growth factors, thereby allowing the virus to create optimal conditions for the replication and spread of the virus in the body (Figure 2).[15]

The replication cycle of the monkeypox virus is the same as other poxviruses. Both mature and immature virions have an outer membrane that, under the influence of virokines, will bind to glycosaminoglycans or laminin of the host cell. Then, under the influence of additional 12 transmembrane proteins, the viral particle fuses with the affected cell. It is worth noting that mature virions are much more stable than immature ones and mediate animal-to-human transmission, while immature virions are specifically specialised to exit the intact cell and spread within the host.[16]

Table 1: Monkeypox prevalence in endemic countries[14]

Country	Period						Total
	1970-1979	1980-1989	1990-1999	2000-2009	2010-2019	2020-2021	
Central African Republic	-	8	-	-	61	-	69
Democratic Republic of the Congo	38	343	511	10027	18788	7374	37801
Republic of the Congo	-	-	-	73	24	-	97

Table 2: Monkeypox prevalence in non-endemic countries[14]

Country	Period						Total
	1970-1979	1980-1989	1990-1999	2000-2009	2010-2019	2020-2021	
United Kingdom	-	-	-	-	4	-	4
USA	-	-	-	47	-	2	49

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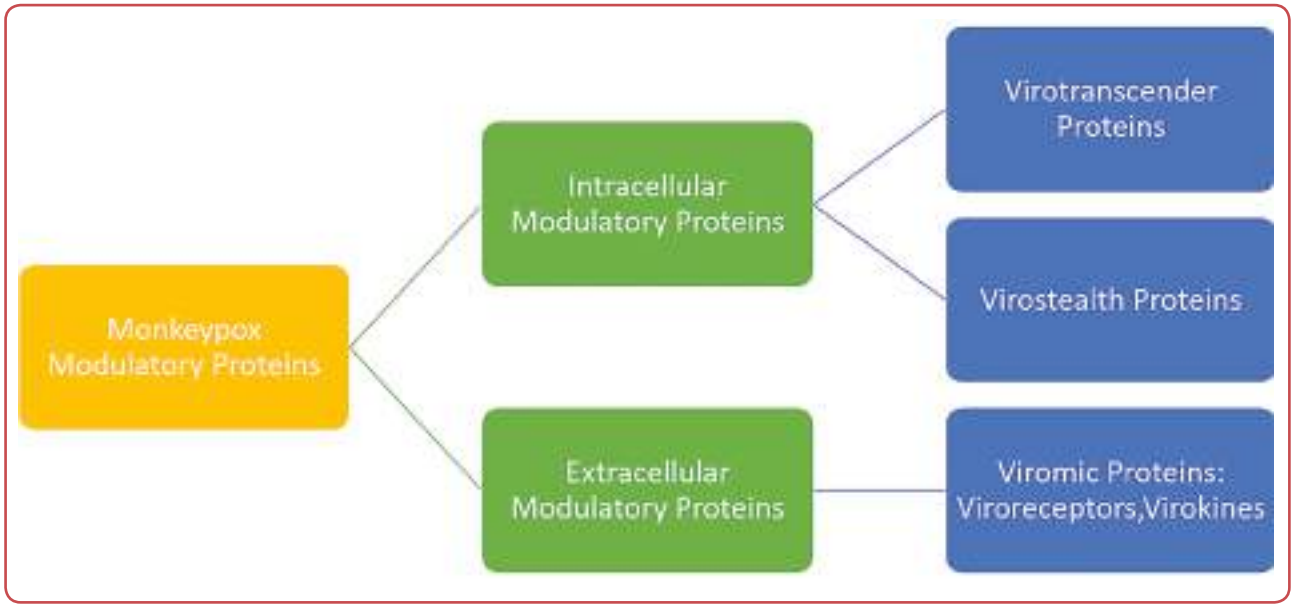

Figure 2: Classification of viral modulatory proteins

Upon entering the cell, the virus forms new structures for replication, known as Guarnieri bodies, commonly called "factories". Each "factory" is a compact DNA structure, surrounded by membranes, which were transformed by the virus from the rough endoplasmic reticulum. As the viral DNA replicates, Guarnieri bodies enlarge, viral mRNAs and host cell translation factors increase in them that promote the rupture of endoplasmic reticulum membranes and the breakthrough of immature virions outside the affected cell (Figure 3).[17]

The monkeypox virus was first identified in Denmark, in a colony of macaques imported for experiments, but animal reservoirs and transmission mechanisms remain unknown. It has been proven that in intermediate hosts the virus can be transmitted from one animal to another and subsequently to humans.[1] It is likely that the reservoir is one or more species of rodents or squirrels found in the forests of Central Africa.[18]

The monkey smallpox virus is thought to have

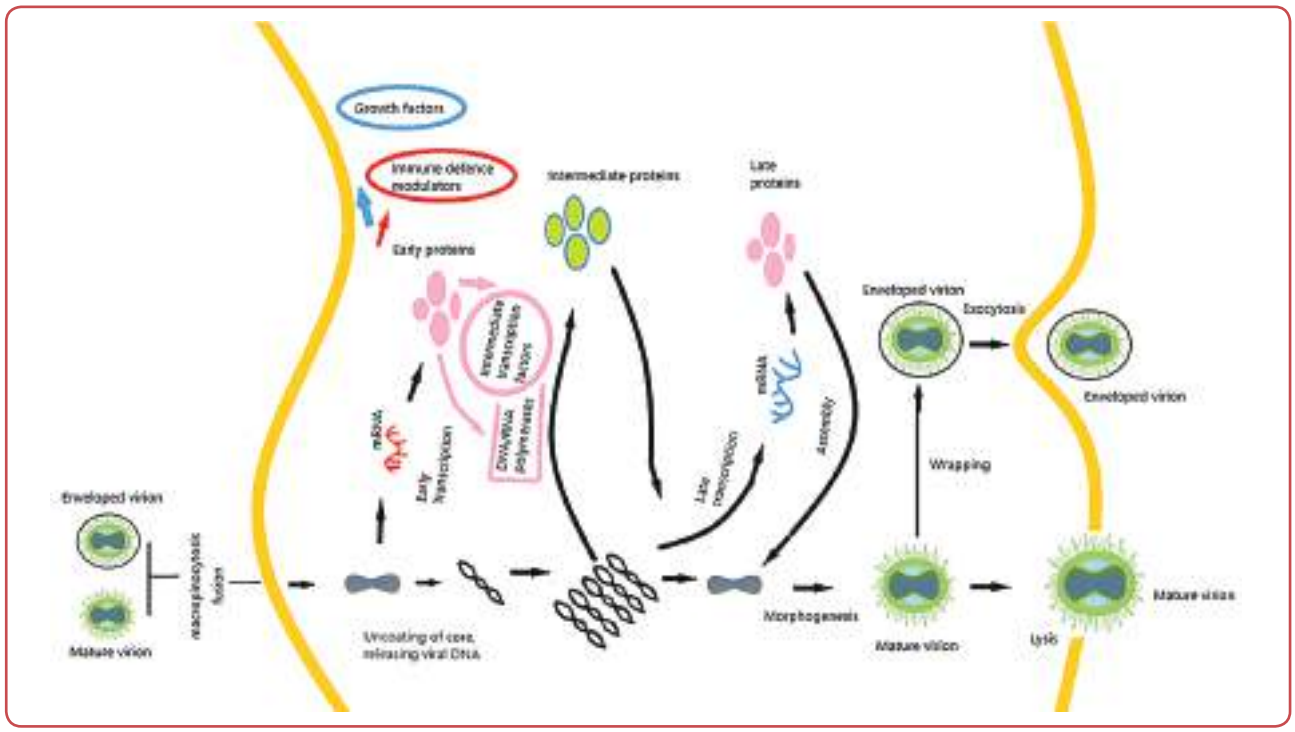

Figure 3: Illustration of the Monkeypox virus life cycle

several transmission mechanisms; in any case, they are associated with direct contact with infected animals or humans. The main monkeypox transmission mechanism is still unknown, but it is assumed that the virus is transmitted with fluids, for example, saliva, by contaminated surfaces and by direct contact, with animal faeces, with sores on the body, mucous membranes (Figure 4).[4]

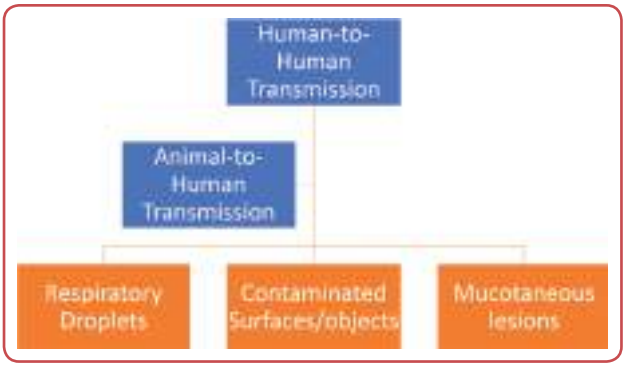

Figure 4: Human-to-human and animal-to-human transmission routes

Although human-to-human transmission is much less common than animal-to-human transmission, it usually occurs by airborne transmission through prolonged contact with an infected person.[19] Amid the current monkeypox epidemic, the disease has been found to be more common in men who have sex with men. According to the World Health Organization, it is not yet known if monkeypox is sexually transmitted or not, but transmission can be attributed to close contact.[15]

Monkeypox virus follows the same transmission as smallpox, beginning with exposure to the mucosa of the oropharynx or respiratory tract of the host. After entry, the virus replicates in the place of invasion and then spreads to the local lymph nodes. With an increase in viral load, the secondary viremia sets in - with the spread of virions to distant lymph nodes and internal organs. The incubation period is usually 7 to 14 days with a maximum of 21 days.[20]

There are no clinical aspects during the incubation period; the infected person is not contagious to others. The symptoms and clinical manifestations of monkeypox occur in the prodromal stage, when the infection spreads from the lymphoid organs to the skin and tertiary organs such as the lungs, eyes, gastrointestinal tract, etc during the course of secondary viremia. It is during the prodromal period that a person is considered most contagious. This is largely due to the occurrence of mucocutaneous lesions and lymphadenopathy, which stand out among other, less specific symptoms.

General, nonspecific symptoms begin to develop one to two weeks after human infection with monkeypox virus.[21] During the prodromal stage, nonspecific symptoms appear: fever, lymphadenopathy, myalgia. Due to its nonspecific nature, an infected person may attribute these symptoms to seasonal flu or the common cold. A signature of the disease during this period, which may alert the patient, is the synchronous enlargement of the maxillary, cervical and inguinal lymph nodes.[15] In some patients, these symptoms may be mild or not present at all.[20] In typical cases, the fever often subsides the next day or within three days of the breaking-out. The rash first appears on the face and quickly spreads throughout the body from the centre to the periphery.[22] The rash usually has a well-defined shape. By nature, it is a disseminated blistering-pustular rash.[23] The rash itself goes through several stages: the stage of enanthem, macule, papule, then vesicle and pustule, after which they flake and scab over. Once the crusted lesions slide off, the human is no longer considered contagious.[24]

A certain discomfort to the patient is caused by gastrointestinal symptoms that occur by the second week of the disease. These are vomiting and diarrhoea, they contribute to significant dehydration in the infected person. The most serious complication of monkeypox is infection of the cornea. The development of concomitant eye infections can lead to the formation of scars on the cornea and irreversible loss of vision. The tendency to more severe course, with the development of more complications, is noted in patients who are not vaccinated against smallpox (74 %); vaccinated patients are significantly less likely to get complications (39.5 %).[22] After the elimination of smallpox, vaccination of the population was not carried out. Thanks to cross-immunity, people who were vaccinated against smallpox before the 1970s are much less likely to get complications from monkeypox infection. Separately, it is worth considering the phenomena of sepsis and septic shock, which are associated with an excessive immune response.[15] However, monkeypox is a self-limited viral disease and very rarely causes lifelong complications.

Although bronchopneumonia is a complication of monkeypox infection, it occurs more frequently in persons co-infected with influenza virus.[15]

CONTENT

It has been shown that lung disease in non-human primates in the range of infectious doses leads to the development of focal lung tissue necrosis, diffuse lung consolidation and peracute bronchopneumonia.[23]

The clinical treatment of monkeypox is based on maintenance therapy, which includes maintaining fluid balance, haemodynamic support, respiratory support, treatment of associated skin and mucosal infections, usage of lubricants and topical antibiotics to prevent eye damage.[23, 25] There are currently no specific drugs approved for the treatment of monkeypox. However, a number of antiviral drugs have shown their effectiveness. Such drugs are Cidofovir, Tecovirimat, Brincidofovir.[26]

Table 3: Methods of laboratory diagnostics

Method	Point of application
Culture-based	Possibility of obtaining a pure culture of the virus for final verification. Orthopoxviruses form characteristic bodies on chorioallantoic membranes.
Electron microscopy	In the negative, a clear image of Paschen's corpuscles is obtained. The method is used to identify viral particles in samples.
Immunohistochemistry	Used to identify antigens in samples. Used as an exclusion method when identifying other suspicious agents.
Real time PCR	The method detects viral DNA and can identify active infections using material taken from the patient.
Test for IgM	Used to assess recent exposure to orthopox-virus and in suspected patients previously vaccinated against smallpox.
Test for IgG	Used to assess the duration of exposure to orthopoxvirus, including vaccination.
Tetracore Orthopox Bio-Threat Alert	Test for the detection of antigens to orthopox-virus. Can quickly identify an active case using patient material taken on sight, does not require specific skills.

For the monkeypox virus detection (Table 3), it is necessary to combine laboratory diagnostic methods,takeintoaccountclinicalsymptomatology and epidemiological anamnesis and the patient's vaccination history. Samples for detection of the virus can be blood, urine, swab from the upper air passages, scrapings with discharge from papules. Traditional diagnostic methods, such as culture-based, immunohistochemistry, microscopic examination, require appropriate qualifications and can only be carried out using specialised equipment in a modern laboratory.[27] For retrospective study analysis, it is recommended to use immunotechnique and serological diagnostic methods based on the detection of antibodies to orthopoxviruses, since they have cross-reactivity. These methods should be actively applied in endemic areas and areas where there have been outbreaks of monkeypox. At the same time, it should be noted that the presence of acute phase immunoglobulins - IgM to orthopoxvirus virions and not IgG indicating a long-term infection, is revealed in the samples. The presence of the latter in the probe is nonspecific because it may indicate lifetime exposure of the virus, or vaccination.[28] The real-time polymerase chain reaction (PCR) method, which has the highest sensitivity, seems to be the most optimal for early diagnosis at the moment. However, this method can also be used in the presence of a highly technologically equipped laboratory. In conditions of limited resources, the method is not applicable.[29] Unfortunately, the main burden on the use of resources is the arrangement of conditions for the collection and storage of samples, so there was a need for tests for detection in the field with minimal training. In this regard, a test (*Tetracore Orthopox BioThreat Alert*®) was developed in 2003.[30]

Cidofovir was approved by the Food and Drug Administration (FDA) in 1996 for the treatment of patients with retinitis caused by Cytomegalovirus (CMV), patients with acquired immunodeficiency syndrome. Cidofovir has broad antiviral activity against viruses from different families, including herpes viruses, adenoviruses and poxviruses. This drug was used as part of a treatment regimen for a 28-month-old boy with refractory atopic dermatitis who got severe eczema *vaccinatum* after contact with a smallpox-vaccinated father.[31] The child survived and there were no long-term after-effectiveness.

Tecovirimat is the first antiviral drug indicated for the treatment of smallpox in adults and children weighing at least 3 kg. It was approved by the FDA in 2018 and in January 2022, it was recognised as effective by the European Medicines Agency. It has been used in several case reports to treat disseminated and ocular cowpox[32, 33] and cowpox infections as part of a multidrug regimen.[34] Tecovirimat was used to treat a patient with imported monkeypox in the United States in 2021.[8] In a recent case series, 1 out of 7 patients received Tecovirimat for 2 weeks. No side effects were noted, but a shorter duration of viremia was achieved.[35] In a report of the first 17 patients with confirmed monkeypox in the US during the ongoing 2022 outbreak, 1 patient took Tecovirimat.[36]

Table 4: Comparative characteristics of antiviral drugs used against monkeypox

Name	Routes of administration	Mode of action	Side effects	Safety precautions	Contraindications
Tecovirimat	Orally, intravenously. Single dose. Adults: 600 mg. Children weighing 13 kg to less than 25 kg: 200 mg. Children weighing 25 kg to less than 40 kg: 400 mg. Children weighing 40 kg or more: 600 mg. Taken 2 times a day for 14 days.	Inhibitor of the orthopoxvirus VP37 envelope wrapping protein	Headache, nausea, abdominal pain, vomiting. Infusion site reactions may occur with the intravenous form	Dose adjustment of Tecovirimat is not necessary while treating patients with a kidney or liver disease when taken orally. When administered intravenously, it should not be given to patients with severe renal insufficiency.	No
Brincidofovir	Adults weighing 48 kg or more – 200 mg once a week for 2 doses. Adults and children weighing 10 kg to less than 48 kg – 4 mg per kg of body weight once a week for 2 doses. Children weighing less than 10 kg – 6 mg per kg of body weight once a week for 2 doses.	Phosphorylated to the active metabolite, Cidofovir diphosphate, which selectively inhibits viral DNA synthesis mediated by orthopoxvirus DNA polymerase	Diarrhoea, nausea, vomiting and abdominal pain	Not recommended for pregnant and lactating women (before treatment, a pregnancy test should be performed in women of childbearing age). May cause an increase in the activity of transaminases and bilirubin in serum.	No
Cidofovir	5 mg/kg once weekly for 2 consecutive weeks, then 5 mg/kg intravenously once every two weeks	It undergoes cellular phosphorylation, then selectively inhibits viral DNA synthesis mediated by orthopoxvirus DNA polymerase.	Decreased serum bicarbonate, proteinuria, neutropenia, infection, ocular hypotony, iritis, uveitis, nephrotoxicity, fever	Dose adjustment required depending on renal function	Must not be initiated in patients with serum creatinine > 1.5 mg / dL, calculated creatinine clearance (Clcr) ≤ 55 mL / minute, or urine protein concentration ≥ 100 mg / dL; with allergic reactions

Brincidofovir has been approved for the treatment of smallpox in the US since June 2021.[37] Brincidofovir, when administered orally, performs better than intravenous Cidofovir, as it has less nephrotoxicity. The drug has approved itself for patients with adenovirus,[38] CMV infection[39] and poxvirus infections. Brincidofovir was used in combination therapy in a patient with acute myeloid leukaemia and developed smallpox due to a recent vaccine after induction chemotherapy. In addition to other drugs, the patient took 6 doses of Brincidofovir.[34] The drug has also been used in a 17-year-old patient who got a fatal cowpox infection after a kidney transplant.[40] In May 2022, the clinical management of 7 monkeypox patients in the UK was described. In this case series, 3 patients received Brincidofovir. However, due to an increase in hepatic enzymes (a side effect of the drug), treatment had to be discontinued.[35]

The FDA approved immunoglobulin against cowpox in 2005 for the treatment of complications of cowpox vaccination.[41] This intravenous drug has appeared relatively recently. Its predecessor was intended for intramuscular administration and therefore had less effectiveness.[42] An FDA-approved intravenous form of immunoglobulin has been used in several reports, such as in a patient with inflammatory bowel disease and developed infection after exposure to a recombinant antirabic vaccine based on the vaccinia virus. Immunoglobulin has also been used to treat two patients with developed symptoms of cowpox after contact with a man who had had the virus transmitted sexually from a recently vaccinated sexual partner.[43]

Discussion

The neglect of monkeypox in humans is mostly benign and moderate, with a tendency for the virus to self-limit. The introduction of antiviral therapy into the treatment course should be considered in cases of especially severe disease, eye-lesion, oral or perineum lesions, in patients

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with a risk of the disease becoming severe (ie, immunocompromised persons, children under the age of 8, pregnant and breastfeeding women, patients with atopic dermatitis or other active skin conditions).[24]

Currently, there is the greatest clinical experience with Tecovirimat, therefore it is preferred. Ideally, the treatment of monkeypox should be carried out in the context of clinical trials, this is necessary to obtain long-term evidence that could provide information about the prospects for such treatment and the opportunity of adjusting it in process based on the evidence base. Therefore, clinicians are encouraged to coordinate management programs and approaches with infectious disease experts and public health authorities.
The mode of virus transmission, its tendency to self-limit, suggests that contact tracing is crucial in the fight against the monkeypox spread. For identification of the circle of contacts and potentially infected persons, it is necessary to question the patient in detail. Emphasis should be made on the nature of contacts: their duration, degree of closeness (whether it was face-to-face conversation or direct (including sexual), physical contact or contact with contaminated surfaces (bedding, shared objects). It must not be forgotten the possibility of animal-to-human transmission: if the patient had contact with a pet, the pet should be isolated for 21 days.

In order to prevent the infection spread in a medical facility, everyone who had contact with the patient (staff, hospital roommates, visitors) should be identified. If someone has been in contact with a person with monkeypox, attention should be paid to the presence of symptoms such as fever, rigor, rash and lymphadenopathy occurring within 21 days of the last contact. The asymptomatic course of the incubation and prodromal period has been repeatedly reported, therefore, everyone who has been in contact with the patient should be considered potentially infected.[19]

On 14 May 2022, 2 cases of monkeypox infection were reported in the UK in one family; patients have never been in endemic areas. Thousands of cases have been reported in many countries in Europe, South America, the Middle East, Canada and the United States. The virus mainly affected men between the ages of 25 and 35, mostly among those who self-identify as homosexual or bisexual.[13] It is not completely known whether the virus is transmitted sexually in a traditional sense, or whether close contact with an infected person plays a big role, although small amounts of the virus have been isolated from the semen of patients in Italy[11] and Germany.[12]

The ongoing global outbreak of monkeypox is one of the largest in history. The transmission chains are occurring in many non-endemic countries. This fact indicates that the virus transmission, due to the length of the incubation period, remained unnoticed for a long time. This contributed to the creation of significant clusters of infected people, who, even in the prodromal period, due to the nonspecificity of symptoms, did not arouse suspicion among clinicians. This situation allows us to draw a parallel with the barely abated COVID-19 pandemic, also a zoonotic virus that spread in a similar way. It seems unlikely that the monkeypox virus could cause a global epidemic on the scale of the COVID-19 pandemic. However, the severity of the looming threat should not be underestimated. A long incubation period, the scarcity of the arsenal of drugs for adequate and timely treatment, blind spots in the understanding of the virus transmission mechanisms in the population make the threat real.

Studying the trends and characteristics of the current outbreak will be key to identifying the tools needed to contain it. Implementing screening in healthcare facilities and maintaining a high level of suspicion will help identify new cases and determine the extent of the outbreak. Early isolation of suspected and confirmed cases of infected persons, as well as close monitoring and vaccination of their relatives and high-risk healthcare workers as needed, will help break the chains of the virus transmission. Monkeypox has a wide host range, which gives it the potential to enter new ecological niches and if the current outbreak drags on, there are significant concerns about the emergence of new endemic areas outside of Africa.

Conclusion

Thus, little is currently known about the nature of the monkeypox virus. The exact transmission mechanism, reservoir animal, host

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range and specific treatment prospects have many blind spots. Hope is that in time it will be possible to shed light on them.

The current outbreak of monkeypox is the largest seen in recent decades. It is important to prevent the emergence of a new pandemic, so it is needed to act quickly and decisively. Lessons should be learned from recent epidemics and available resources and information should be shared as early as possible. Monkeypox is becoming a global public health problem. The time has come to approach this problem globally and eradicate the infection not only in rich countries, but also in endemic regions. Only in this way can we protect ourselves and future generations from this dangerous disease.

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Conflict of interest

None.

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CASE REPORT

Postoperative Necrotising Fasciitis of the Lower Limb as an Unexpected Complication of Vascular Surgery Procedure - Case Report

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Abstract

Necrotising fasciitis is a rare and severe disease, acute infection, often life-threatening, characterised by rapid and progressive spread through the subcutaneous tissue and superficial fascia. It can occur on any part of the body, mostly affecting the perineum, limbs and abdominal wall. A 76-year-old male patient with the development of lower limb necrotising fasciitis after a vascular procedure femoral-popliteal bypass, performed due to chronic ischaemia is presented. Patient previously had several different vascular procedures on other blood vessels, with diabetes and cardiovascular disease as leading comorbidities. Treatment included urgent surgical necrotomy, with all measures of conservative treatment. During the treatment, there was no need for a new vascular procedure, arterial flow was preserved. It is necessary to constantly remind surgeons about this rare but life-threatening disease so that they can recognise it in time and adequately treat it.

Key words: Necrotising fasciitis; Severe infection; Lower limb; Urgent surgery.

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Introduction

Necrotising fasciitis (NF) is a rare and severe soft tissue infection that is characterised by rapid and progressive spread through the subcutaneous tissue and along the fascia, often manifesting with a severe clinical picture that can lead to shock and sepsis with multiorgan failure and death.[1] The incidence of this infection is 1–4/100.000 persons per year.[2] Even though knowledge about NF has been growing lately, as well as the availability of better treatment with more available drugs of the newer generation, mortality remains relatively high and, according to data from the literature, reaches up to 70 %.[1-4]

According to the literature, NF occurs more often in men and older patients usually have a poorer disease outcome and more severe clinical picture, probably due to more frequent comorbidities. The most common comorbidities in patients with NF are diabetes mellitus, obesity and hypertension.[1-4] The disease usually involves the anterior abdominal wall, the scrotum and perineum and the lower limb but it can occur on any other part of the body.[5, 6]

Due to the rapid progression, aggressive surgical debridement of the infected area as early as pos-

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sible along with other supportive measures is the gold standard in the treatment of this disease.

Here is presented a rare case of NF of the lower extremity after femoral-popliteal bypass, in a 76-year-old patient. According to the author's knowledge, there are very few published papers about NF of the lower extremities after vascular procedures.

Case History

The patient's consent to publish the case report was obtained. This article was planned in compliance with the Patient Rights Directive and ethical rules by considering the principles of the Helsinki Declaration.

A 76-year-old man, a former smoker, was admitted to the surgery department of the General Hospital Novi Pazar as a continuation of treatment after the femoral-popliteal bypass of the right leg was performed in a tertiary health institution. Among the comorbidities present were diabetes mellitus, heart weakness and chronic obstructive pulmonary disease, along with a penicillin allergy. Patient also had a history of performed carotid artery surgery on both sides and coronary angioplasty 9 years ago. Three months before this operation, an endovascular procedure (percutaneous transluminal angioplasty (PTA) and stenting *a. iliacae externae lat. sin.*) was performed on the left leg due to ischaemic changes in the foot caused by atherosclerosis of the main blood vessels. Two weeks after that intervention, due to ischaemic pain in the left leg and occlusion of the deep and superficial femoral artery of the left leg and the impossibility of revascularisation of the extremity, an above-knee amputation of the left leg was performed. The postoperative course was normal and the wound healed *per primam intentionem*.

One month after the amputation of the left leg, interdigital ulcerations appeared on the right foot (between the 3rd and 4th toe) and the initial gangrene of the 5th toe of the right foot. Due to the revascularisation of the right leg, a femoral-popliteal bypass was performed in a tertiary health institution. The early postoperative course was normal and on the 4th postoperative day, he was transferred to our institution for a continuation of treatment. On the second day of hospitalisation, the patient was febrile, with a body temperature of up to 39.5 °C. Increase of inflammatory parameters was present (Table 1), local swelling, redness, pain and tissue fluctuation below the skin on the right upper leg. The patient was in poor general condition, septic, languid, with present dyspnoea, tachycardia and arrhythmia. Pulses on *a. dorsalis pedis* and *a. tibialis posterior* were present.

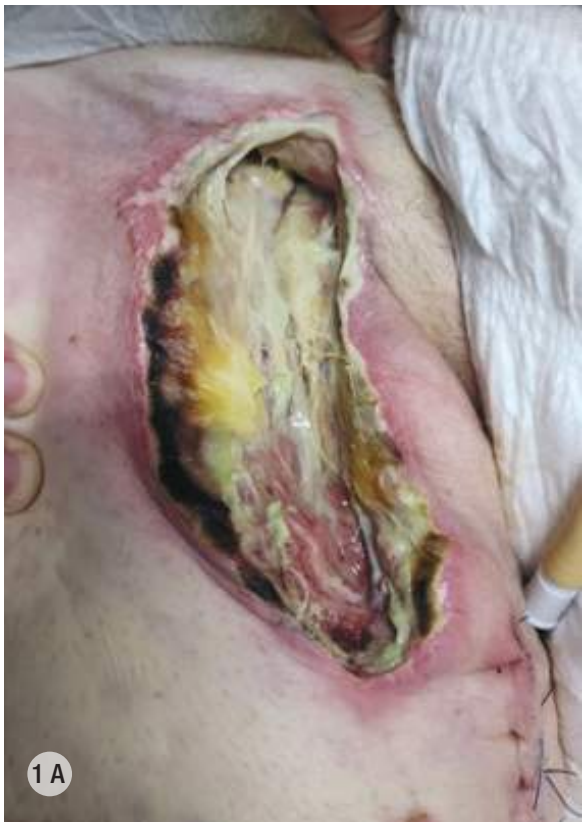
Table 1: Laboratory parameters during the hospitalisation

Parameter	Day of hospitalisation		
	2nd	5th	8th
WBC (x 10^9/L)	21.00	19.00	12.00
RBC (x 10^{12}/L)	2.43	2.88	3.12
HGB (g/L)	101.00	109.00	119.00
Creatinine (μmol/L)	174.00	161.00	101.00
Na^+ (mmol/L)	137.00	136.00	138.00
CRP (mg/L)	306.00	285.00	77.00
Procalcitonin (ng/mL)	2.10	-	0.23
Glucose (mmol/L)	23.00	19.00	12.00

WBC - white blood cells; RBC - Red blood cells; HGB - Haemoglobin; CRP - C-reactive protein; Na^+ - sodium;

The operative wound on the right leg, above and below the knee, was opened and a large amount of green-brown liquid with an unpleasant smell was evacuated. Signs of NF of the right upper leg, with the spread of the infection upwards towards the groin and downwards across the fascia of the right leg were present (Figure 1A and 1B). Extensive debridement of the affected tissue was performed. Laboratory Risk Indicator for Necrotising Fasciitis (LRINEC) score was 10.

Treatment with antibiotics (Ceftriaxone, Gentamicin and Metronidazole), infusion solutions, analgesics, Nadroparin Calcium and other supportive therapy was initiated. The patient did not respond to the prescribed therapy as it was expected, so the treatment with reserve antibiotics (Vancomycin and Meropenem) was continued. Forty-eight hours after the first surgical intervention, repeated necrotomy surgical reintervention was performed. Diarrhoea occurred, which subsides in 2 days with symptomatic therapy. *Clostridioides difficile* toxins A and B, as well as other pathological substrates, were not isolated in the stool sample. Cardiac decompensation and the development of pulmonary oedema and pneumonia also develop. Wounds were treated daily with antiseptic solutions with regular dressings. The prescribed therapy resulted in a decrease in



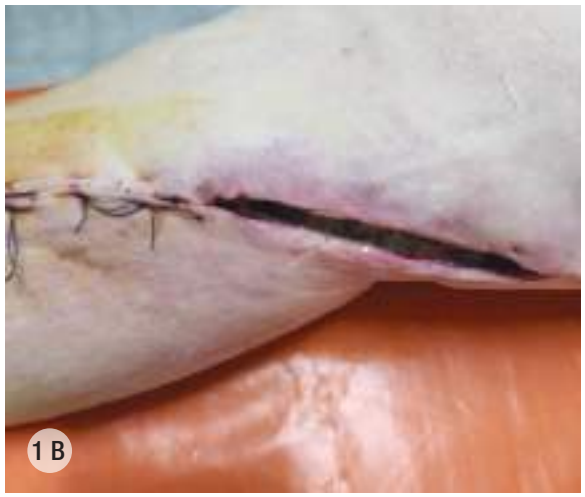


Figure 1: Postoperative lower extremity necrotising fasciitis

biochemical inflammatory parameters. *Staphylococcus aureus*, sensitive to the applied antibiotics, was isolated from the wound swab. There was no need for new surgical interventions. During hospitalisation, the patient was treated by a multidisciplinary team (pulmonologist, cardiologist, surgeon and infectious disease specialist). Due to the comorbidities present, the patient was not treated with hyperbaric oxygen therapy (HBOT).

The patient responded well to the prescribed therapy (Figure 2). There was no ultrasound and

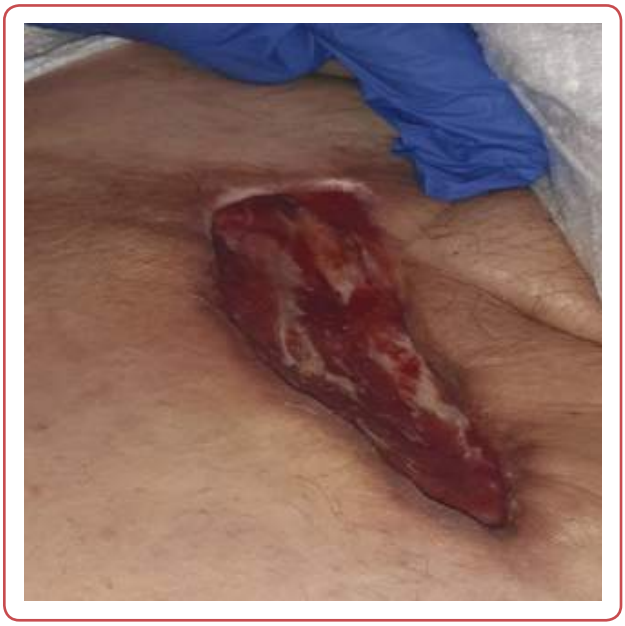


Figure 2: Condition after a month of treatment of postoperative necrotising fasciitis

clinically verified graft occlusion, the flow was satisfactory. After 48 days of hospitalisation, the patient was discharged with good general condition and satisfactory local findings with advice for outpatient wound dressing and follow-up by a general surgeon and a vascular surgeon under the supervision of a cardiologist and pulmonologist.

Discussion

NF belongs to the group of aggressive skin and soft tissue infections, usually spreading along the fascial plane, which is less vascularised, while the tissues above initially appear healthy, which can delay diagnosis and surgical intervention. Secondarily, the infection can spread to the subcutaneous tissue, skin and muscles. NF represents a rare, but often life-threatening infection and requires prompt surgical and medical treatment. In the literature, there are different data on the frequency of NF, probably due to the lack of large studies and usually published smaller case series. In the United States NF affects about 0.4 in every 100,000 people per year[7] and an incidence goes up to 15 cases per 100,000 population in some areas of the world,[7-9] but due to underreporting, the number is certainly higher. According to Das et al and Bodansky et al, there is an increase in the frequency of NF in their countries.[10, 11] The incidence in children is 0.08 to 0.13 per 100,000 per year, with a mortality of 10 %.[12] Literature

data indicate that mortality in adults ranges up to over 75 %.[1, 5] NF occurs slightly more often in males than in females. Studies show that patient survival was significantly associated with younger age. NF is more likely to occur in middle-aged patients as well as those older than 50, they have a worse prognosis, especially if they have accompanying comorbidities.[5, 13] The study of Czymeket et al stated that the female gender is a risk factor associated with higher mortality, but this claim was not confirmed in other studies.[14]

Based on the causative agent of the disease, NF can be classified as Type 1-polymicrobial (caused by anaerobic and aerobic microorganisms, more prevalent in older adults with chronic diseases), Type 2 – monomicrobial, Type III is caused by *vibrio* species and Type IV is caused by fungal infection.[15] Literature data show the prevalence of monomicrobial NF from 60–80 %.[5] Tsai et al state that the infections have a more rapid and fulminant form if they are caused by Gram-negative microorganisms.[16, 17]

Skin or mucous membrane damage and various surgical procedures are some of the factors that put patients at higher risk for NF. Necrotising fasciitis can occur post-surgery, any invasive or even a minor procedure.[7] The disease usually involves the perineum, the scrotum and the anterior abdominal wall. Lower extremity NF is a limb and life-threatening condition with published mortality rates from 10-30 %. The amputation rate of 20.4 % in the study of Park et al and 23.5 % in the study of Irmak et al was similar to the rates in other studies.[15, 18-21]

Presented patient was elderly, in his 8th decade of life. The infection occurred after a surgical intervention and the incision on the skin was the likely site of infection entry. *S aureus* was isolated from the wound swab.

The diagnosis of NF was made primarily by clinical examination. Usually, symptoms are local pain, malaise, fever, hypotension and poor general condition with local erythema, tissue swelling, ecchymosis changes to the skin, subcutaneous emphysema and crepitations and skin necrosis. What needs to be paid special attention to, is that the finding on the surface of the skin, which is visible from the outside, often does not correspond to the true stage of the infection that spreads under the skin.[1-21]

There are multiple predisposing factors for NF. Diabetes mellitus is a dominant comorbid disease for NF in most studies. Other comorbidities such as arterial hypertension, peripheral arterial disease, systemic disorders, chronic renal failure, cardiovascular disease, immunosuppression, alcoholism and local trauma also have an important role in the development of NF.[5, 10, 11, 22] In addition to all the complications caused by diabetes mellitus, the literature data shows a higher incidence of peripheral arterial disease and poor prognosis in the diabetic patient population. Arterial revascularisation in patients with critical limb ischaemia is a limb-saving procedure. Literature data show that more than 50 % of patients with NF have at least one predisposing comorbidity and the most common comorbidity factor was diabetes mellitus.[1]

In presented case, the general symptomatology and local signs of the infection coincide with the data from other studies. There are no laboratory parameters specific only to the diagnosis of NF. As it was mentioned, a diagnosis was based on a clinical presentation. Laboratory tests and imaging had a role in the prediction of the severity of infection and treatment outcome. That's why the LRINEC scoring system was developed to help clinicians screen for NF. A score of 8 or higher represents a 75 % risk of necrotising infection.[7] The LRINEC score in presented patient was 10, which places him in the group of high-risk patients.

Treatment involves urgent surgical intervention, which involves the removal of necrotic and devitalised tissue, with fluid replacement, the use of broad-spectrum antibiotics and after isolating the causative agent, targeted antibiotic therapy according to the antibiogram, as well as other substitution therapy and treatment of comorbidities and complications. Mortality can be increased several times if the primary surgical intervention is performed 24 h or more after the onset of symptoms. Several studies stated that all patients underwent 1–10 radical surgical debridements, with an average of 2.5. The role of HBOT and intravenous immunoglobulin G (IVIG) for the management of NF remains controversial.[9]

In presented case, the first surgical intervention was performed more than 24 h after the onset of symptoms and during the treatment, it had to be repeated once more. HBOT was not applied due to cardiological and pulmonary contraindications.

Conclusion

NF, although rare, is a potentially lethal disease. It is important that surgeons increase awareness about NF and not neglect the possibility of this severe infection. Prompt diagnosis, early operative debridement, appropriate supportive and causal therapy and multidisciplinary treatment are necessary for successful therapy.

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Conflict of interest

None.

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CASE REPORT

Atraumatic Isolated Bilateral Fibular Shaft Fragility Fracture: a Rare Case

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Abstract

Fragility fractures, a form of stress fracture brought on by physiological stress on weak bones are not common. It might be challenging to diagnose a fragility fracture, since a standard X-ray shows signs of fracture repair rather than the actual fracture. Here is presented a case of a young woman who has been complaining of pain in both legs for 4 months and has been unable to stand for 1 week. A further analysis revealed that patient's vitamin D levels were insufficient.

Key words: Fragility fracture; Stress fracture; *Osteomalacia*; Fibula.

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Introduction

Stress fractures are thought to be the result of continual stress on the bone rather than an acute traumatic occurrence.[1] They can arise in athletes, most likely as a result of the constant stress on bone caused by exertion. Fragility fractures develop as a result of normal stress on defective or inadequate bone. Certain areas of preference include the vertebra, pelvis, sacrum, ribs, distal end of the ulna and to a lesser extent, the proximal and distal end of tibia and fibula.[2-4] To authors' knowledge, so far, just one isolated bilateral fibula insufficiency fracture without deformity has been recorded in literature.[5] An interesting case of fragility fracture in a younger patient is presented.

Case History

A 30 year-old woman presented to the orthopaedic outpatient department (OPD) with a complaint of both leg pain for four months and has been unable to stand since last week. The pain was continuous and not relieved by rest. There was no associated history of recent trauma. She was a housewife and had no history of participating in strenuous exercise like running or playing sports. She was previously seen by a neurosurgeon and treated as neurogenic claudication for 3 weeks. Her symptoms were not revealed and she was refereed to orthopaedics department.

There were tenderness present on the anterior lateral aspect of proximal leg and varus deformity of both knee, no swelling or limitations on mobility. The patient didn't mention ever having joint discomfort.

Evidence of a transversely orientated, un-displaced fracture in the right as well as left fibular shaft is seen on the X-ray of both legs (Figure 1). Apart from the site being slightly more proximally on the left, the fractures looked to be extremely comparable. Peri-articular tibial metaphysical varus deformity and reduced lateral joint space of knee joint was found. The patient was questioned once again regarding dis-

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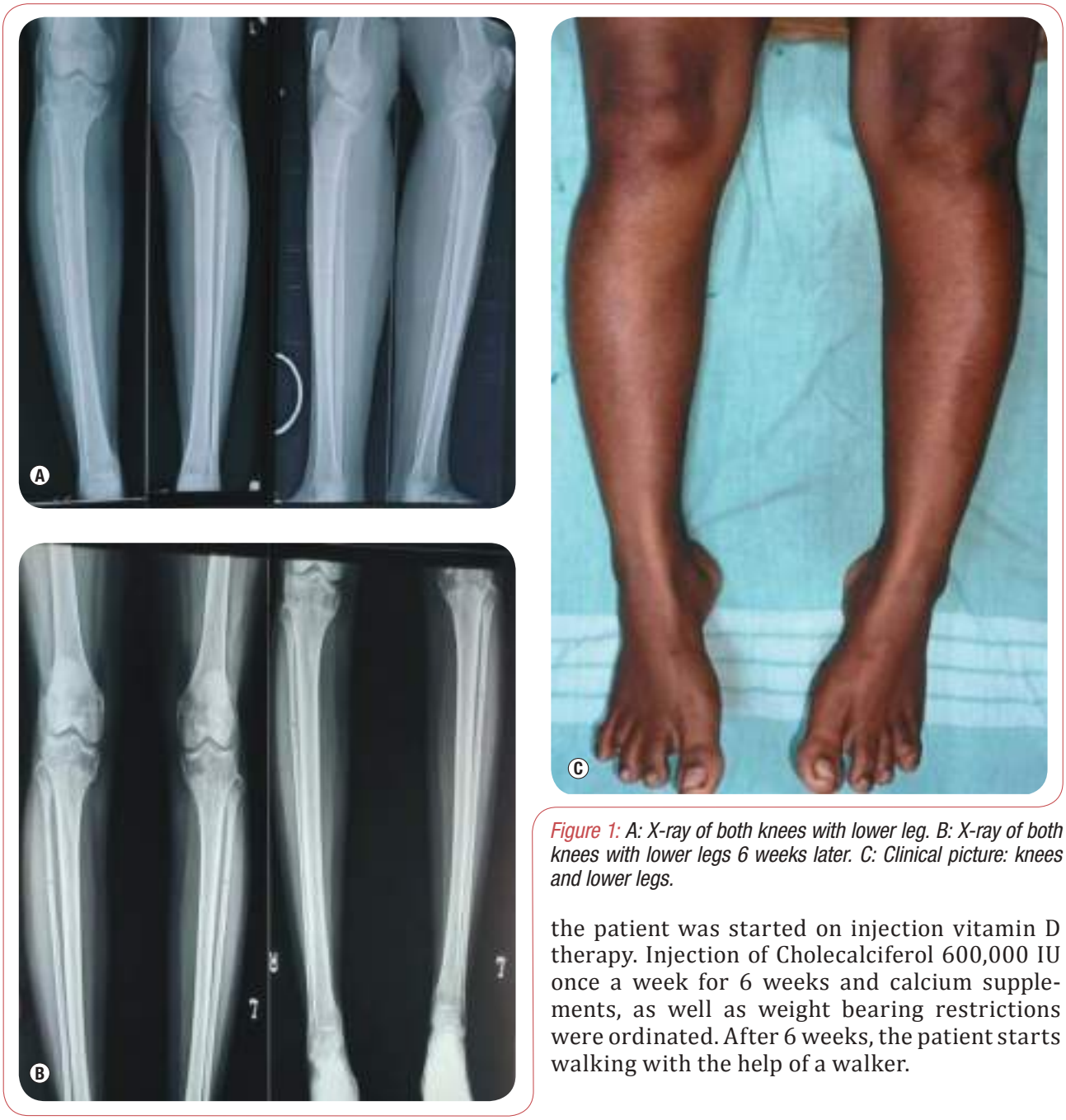

Figure 1: A: X-ray of both knees with lower leg. B: X-ray of both knees with lower legs 6 weeks later. C: Clinical picture: knees and lower legs.

tant insignificant trauma, but she was unable to remember.

Given that vitamin D deficiency is widespread in India, she was tested for complete blood count, c-reactive protein (CRP) level, parathyroid hormone (PTH) level, thyroid profile vitamin D and calcium level and found to have hypovitaminosis-D (24.1 ng/mL) (normal range: 30-50 ng/mL). Her serum calcium level was borderline at 9 mg %. Other parameters were within reference values.

Since no other cause for the fracture was found, the patient was started on injection vitamin D therapy. Injection of Cholecalciferol 600,000 IU once a week for 6 weeks and calcium supplements, as well as weight bearing restrictions were ordinated. After 6 weeks, the patient starts walking with the help of a walker.

Discussion

Normal or physiological muscle action exerts stress on bone that lacks mineral or elastic resistance, resulting in fragility fractures.[4] Fragility fractures become more common as people become older. They are more common in women and occur at trabecular bone-rich locations. Young people with underlying bone-weakening illnesses including osteoporosis, chronic inflammatory diseases like rheumatoid arthritis, vitamin D insufficiency, hyperparathyroidism, endocrine disorders and chronic renal failure can also suffer fragility

fractures.[2,6,7] Only 6 to 16 % of the stress delivered from the lower extremity is received by the fibula. X-rays in the early stages are usually normal. During the healing process, new-bone and fracture lines become visible. Healing bone's profuse callus production mimics peri-osteal new bone tumour and infection. A fibula stress fracture is indicated by a triad of bowed fibula peri-osteal thickening, a transverse region of radio-lucency, and/or sclerosis on X-ray. Each of the X-rays is normal. The radionuclide bone scan invariably shows localised enhanced radio-tracer uptake at the locations of these fractures in the early stages of stress fracture.[8] The most sensitive and precise method for determining the occurrence of peri-osteal and bone marrow oedema in stress fractures at an early stage is magnetic resonance imaging (MRI).[9] The treatment of choices for fragility fracture is conservative and consists mainly in vitamin-D intramuscular injection 600,000 IU once a week for 6 weeks, supplementation of calcium, analgesia and immobilisation. Management of the underlying cause is essential to preventing recurrence.

Conclusion

It is quite unusual to have a spontaneous atraumatic isolated bilateral fibular fracture. X-rays demonstrate peri-articular osteopenia, a narrowed lateral joint space and a tibial metaphyseal varus deformity. In presented case, an atraumatic isolated fibular fracture with a stress fracture were due to vitamin D insufficiency.

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None.

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