2020

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SCRIPTA MEDICA

ISSN 2490-3329 (Print)
ISSN 2303-7954 (Online)

ASSOCIATION OF MEDICAL DOCTORS OF THE REPUBLIC OF SRPSKA,
FACULTY OF MEDICINE, UNIVERSITY OF BANJA LUKA

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SCRIPTA MEDICA

ASSOCIATION OF MEDICAL DOCTORS OF THE REPUBLIC OF SRPSKA,
FACULTY OF MEDICINE, UNIVERSITY OF BANJA LUKA

CONTENT

BANJA LUKA, September 2020

ISSN 2490-3329 (Print) ISSN 2303-7954 (Online)

SCRIPTA MEDICA 2020 | 51 | Q3

Publishers
Association of Medical Doctors of the Republic of Srpska,
Faculty of Medicine, University of Banja Luka

Indexed/Abstracted by: EBSCO, Google Scholar, SCIndeks, Crossref, DOAJ, KoBSON.

CONTENT

Editorial

Original Articles

Review Articles

History of Medicine

Professional Articles

Case Reports

CONTENT

Janković. Scr Med 2020;51(3):131-3. DOI:10.5937/scriptamed51-28336
EDITORIAL

Antiviral Therapy of COVID-19

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Abstract

The COVID-19 pandemic required rapid response to the needs of critically ill patients, and one of the solutions was re-purposing of drugs with wide spectrum of antiviral action for treatment of the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection. The re-purposing characteristically started with out-of-label use in single or series of cases, to continue after the first promising results with randomised clinical trials. There are several drugs that are currently tested in ongoing clinical trials: antimalarials hydroxychloroquine and chloroquine, HIV protease inhibitors lopinavir/ritonavir, broad spectrum antivirals umifenovir (anti-influenza drug) and favipiravir, antiparasitary drug ivermectin and nucleotide analogue remdesivir. However, up to date only a few trials are completed and published, precluding definitive conclusions about efficacy and safety of these drugs. Until major clinical trials are completed, physicians who decide to use these drugs out-of-label should properly inform their patients of all potential risks and benefits and seek for their consent before administration of the drugs.

Key words: COVID-19; Antiviral agents; Drug repurposing; Out-of-label use; Adverse effects.

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ARTICLE INFO
Received: 10 September 2020
Accepted: 11 September 2020

Introduction

Pandemic character of coronavirus disease 19 (COVID-19) and anti-epidemic measures imposed by states that affected almost any person in the world raised unprecedented interest in finding drugs that will block replication of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) causing COVID-19. Several dozens of clinical studies of different design are already published in medical journals, so numerous research groups all over the world prepared and published systematic reviews or meta-analyses in an attempt to summarise the findings and give definite recommendations about particular drugs. Most of the drugs tested for antiviral activity are repurposed, ie, they were initially developed and/or approved for other indications, and then tested in patients with COVID-19: antimalarials hydroxychloroquine (HCQ) and chloroquine (CQ), HIV protease inhibitors lopinavir/ritonavir, broad spectrum antivirals umifenovir (anti-influenza drug) and favipiravir,[1] antiparasitic drug ivermectin and nucleotide analogue remdesivir.[2] Repurposing was a deliberately chosen strategy since there was no time to develop a completely new drug (usually it requires 7 or more years). Unfortunately, it is still far from having sufficiently efficient and safe drug to inhibit replication of SARS-CoV-2 and get it approved for antiviral treatment of COVID-19.

Clinical trials

Hydroxychloroquine and Chloroquine

Hydroxychloroquine and chloroquine are anti-

This article should be cited as follows: Janković SM. Antiviral therapy of COVID-19. Scr Med 2020;51(3):131-3.

malarials that block entry of SARS-CoV-2 in human cells through inhibition of terminal phosphorylation of angiotensin-converting enzyme 2 (ACE2 – serving as receptor for the virus on human cells), and elevation of the pH in endosomes. These drugs were tested in four randomised controlled trials, 10 cohort studies and 9 case series. The studies failed to prove significant effect of antimalarials on all-cause mortality, the disease progression, clinical picture and virologic clearance from upper respiratory tract. On the other hand, prolongation of QTc interval over 500 ms was reported in several studies, although with different rate and unknown clinical significance.[3]

Umifenovir

Umifenovir (arbidol) was used previously in China and Russia for treatment of influenza; it prevents entry of the influenza virus in human cells through binding for viral haemagglutinin. Precise mechanism of action of umifenovir on SARS-CoV-2 virus is not known, but in vitro experiments showed that it inhibits early phase of viral replication.[4] Recent systematic review has found only one randomised clinical trial and one observational study with umifenovir and COVID-19 that could pass minimum requirements for design quality. The randomised trial included only 23 patients and found decreased progression to severe disease forms and more rapid viral clearance with umifenovir, while the observational trial showed decreased mortality. However, potential for bias in these two studies was high, so definitive conclusion about efficacy of umifenovir in COVID-19 will have to wait for results of clinical studies with more appropriate design.[5]

Favipiravir

Favipiravir is an RNA polymerase inhibitor, designed and developed for treatment of influenza and tested in clinical trials with patients with Ebola.[6] Currently, there are 17 ongoing randomised clinical trials and two completed investigating efficacy of favipiravir in patients with COVID-19. The completed trials showed that favipiravir decreased chances of the disease progression, mitigated cough and improved viral clearance, but this could be regarded just as an interim result until the rest of the studies are published.[7] Although review of 29 clinical studies concluded that favipiravir has favourable safety profile, it may cause significant QTc prolongation and hyperuricaemia; its teratogenicity is also a concern, but additional studies are necessary to clarify this issue.[6]

Lopinavir/ Ritonavir

Combination of protease inhibitors lopinavir/ritonavir (being strong inhibitor of cytochromes, ritonavir just serves to increase plasma concentrations of lopinavir) used for the treatment of the Acquired Immune Deficiency Syndrome (AIDS) was repurposed for treatment of COVID-19 early in the course of current pandemic. Currently, there are results of two completed randomised clinical trials and 10 observational studies, which speak in favour of lopinavir/ritonavir efficacy: it reduced mortality inconsistently from study to study and reduced somewhat need for invasive mechanical ventilation and rate of respiratory complications. On the other hand, hospitalisation was not shortened with lopinavir/ritonavir and the rate of adverse events was increased.[8] Main problem with these clinical studies are deficiencies in design that created high potential for introducing various types of bias. The latest systematic reviews concluded that existing results of clinical studies are not sufficient to decide whether lopinavir/ritonavir has beneficial ratio of efficacy and safety in patients with COVID-19.[9]

Ivermectin

Ivermectin is a drug with broad spectrum of action against parasites, mycobacteria, nematodes and a number of viruses, especially flaviviruses. It is highly lipid-soluble and remarkably well tolerated. High activity of ivermectin against SARS-CoV-2 was first noted in vitro and inhibition of importin α/β receptor was proposed as its mechanism of action, resulting in decreased entrance of viral proteins to the cell nucleus.[10] However, although some clinical trials with ivermection in COVID-19 patients are ongoing,[11] there are no published results yet, precluding any conclusion about its efficacy and safety in this particular indication.

Remdesivir

Remdesivir is a nucleotide analogue that inhibits viral RNA polymerase, primarily designed for treatment of ebola. However, high activity against SARS-Cov-2 *in vitro* was noted in several studies and at the beginning of current pandemic it was used in some critically ill patients with COVID-19, mostly without appropriate controls.[12] Published case reports and case series from these early experiences found some clinical improvement in the treated patients, but it was not clear whether this was effect of the drug used or not. Only one small randomised clinical trial (n = 237) conducted in China with remdesivir in COVID-19 patients was

CONTENT

published until now; it did not show benefits of remdesivir. However, there are 8 ongoing large randomised clinical trials with remdesivir in COVID-19 patients, enrolling several thousands of subjects (the largest trial has enrolled as many as 6,000 patients); the results from the ongoing trials will give definite answer about the efficacy and safety ratio of remdesivir.[12]

Conclusion

This short review of the best evidence of antiviral drugs efficacy and safety against COVID-19 gives us little grounds to either recommend or reject any of them as potential therapeutic agent. Almost all of these drugs are currently tested in ongoing clinical trials and the results are awaited. In the meantime, physicians should avoid use of these drugs unless as part of clinical trials, because they may make more harm than benefit to their patients. If such use is still insisted on, the patients should be properly informed about all potential benefits and adverse effects, and the drugs should not be used until the patient signs consent to receive an out-of-label therapy.[13]

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Đukanović et al. Scr Med 2020;51(3):134-40. DOI:10.5937/scriptamed51-28131
ORIGINAL ARTICLE

Time-Dependent and Force-Dependent Vasoreactivity of Isolated Human Umbilical Arteries

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Abstract

Background/Aim: There have been different experimental conditions for *in vitro* studies on human umbilical arteries (HUA) in tissue bath system. This diversity was mainly reflected in variables such as stretching tension, incubation period and initial constriction challenging with potassium (KCl). The aim of the study was to establish optimal experimental conditions which will provide better responsiveness of HUA preparations, as well as to examine the impact of 24 h cold storage on viability and responsiveness of HUA to KCl and serotonin.
Methods: The KCl-induced constrictions at different stretching tensions (0.5 g, 1.0 g, 2.0 g, 4.0 g), incubation times (30 min, 60 min, 120 min), and after multiple initial constriction challenging were compared. Dose response curves for serotonin were obtained under different conditions (1.0 g and 60 min vs. 2.0 g and 120 min). The influence of 24 h cold storage on KCl- and serotonin- induced vasoconstriction of HUA preparations was examined as well.
Results: The strongest constrictions induced by serotonin or KCl were obtained when preparations were adjusted at 2.0 g and incubated for 120 min. The KCl-induced constrictions observed after 120 min were statistically higher ($p < 0.05$) when preparations were challenged three times (30 min, 60 min, 120 min), compared to those challenged only once. The preparations that were stored at 4 ^{0}C for 24 h showed significantly stronger serotonin-induced constrictions ($p < 0.01$). The cold storage had no influence on KCl-induced constriction.
Conclusion: For performing *in vitro* studies on HUA preparations in tissue bath, we propose stretching tension of 2.0 g, incubation period of 120 min and multiple initial constriction challenging with KCl as optimal experimental condition. We also showed that HUA preparations retained functional viability even after 24 h of cold storage.

Key words: Human umbilical artery; Stretching tension; Incubation time; Constriction; Serotonin.

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ARTICLE INFO
Received: 25 August 2020
Accepted: 11 September 2020

Introduction

In addition to numerous animal models of pregnancy and sporadic human clinical trials, studies on isolated umbilical blood vessels have a special place in research related to pregnancy complications. The *in vitro* studies on umbilical arteries and veins allow direct assessment of vascular reactivity of utero-placental and foeto-placental circulation.[1] However, studies on animal blood vessel preparations cannot completely replace human preparations and their pathophysiological models.[2] Therefore, human umbilical blood vessels are of particular importance, since they

This article should be cited as follows: Đukanović Đ, Gajić M, Škrbić R. Time-dependent and force-dependent vasoreactivity of isolated human umbilical arteries. Scr Med 2020;51(3):134-40.

can provide the closest possible image of the *in vivo* system. The importance of the HUA has long been recognised, and its use in research dates back to the 1950s.[3] Umbilical cord is one of the few human tissues that is widely available and provides a high yield of HUA preparations.

Majority of the research studies on HUA are focused on elucidating the vascular dysfunction that occurs in preeclampsia, which is associated with an increase in utero-placental vascular resistance.[4] It is well known that HUA are not innervated blood vessels,[5] and that their vasoactivity is regulated exclusively by autacoids from blood circulation, such as serotonin, histamine, bradykinin, angiotensin, oxytocin, as well as by different eicosanoids.[6] Due to the described specificity of umbilical circulation, it is assumed that these vasoactive agents may play an important role in the pathogenesis of preeclampsia.

Since it was found that mechanical properties and reactivity of umbilical blood vessels are changed in preeclampsia,[6, 7] it is important to establish optimal experimental conditions for physiological models that will allow for the interpretation of these changes. The length and stretch of HUA preparations, the duration of incubation time, the composition of the solution to maintain the viability of the preparations and the initial constriction challenging procedures are some of the key determinants in defining the conditions that must be achieved before performing experiments in the tissue bath system. The majority of authors apply the same conditions regarding the form and length of the HUA preparation (rings, 3-4 cm long), aeration of the HUA preparation (mixture of 95 % O_2 and 5 % CO_2) and composition of the nutrient solution (Krebs-Ringer bicarbonate solution),[6, 8-10] whereas the differences are reflected mainly in passive tension, incubation period and initial constriction challenging. Given the existence of great diversity in the application of physical properties for testing HUA vasoactivity, it is of particular importance to establish an optimisation of the tissue bath methodology that will allow better responsiveness of HUA preparations.

The aim of the study was to analyse the effects of different physical variables such as stretching tension, incubation time, initial constriction challenging and cold storage period to determine the optimal conditions for measuring the vasoactivity of isolated HUA.

Methods

Ethical principles

The present study was conducted with the approval of the Ethics Committee for Research on Humans and Biological Materials, at the Faculty of Medicine, University of Banja Luka, the Republic of Srpska, Bosnia & Herzegovina (B&H).

Tissue preparation

Umbilical cords were obtained from healthy pregnant women after full-term vaginal delivery or Caesarean section at the Clinics for Gynaecology and Obstetrics, University Clinical Centre of the Republic of Srpska (Banja Luka, B&H). After delivery, the segments of umbilical cords (5-10 cm in length) were immediately placed into the modified Krebs-Ringer bicarbonate solution and transported on the ice to the Laboratory of Pharmacology and Toxicology, Centre for Biomedical Research, Faculty of Medicine, University of Banja Luka. For this purpose, the modified Krebs-Ringer solution with less calcium ions than standard solution ($CaCl_2$ 0.16 mmol/L) was used.

The umbilical artery was cleaned of Wharton jell and the connective tissue was carefully removed. The cleaning was performed in Petri dishes filled with modified Krebs-Ringer solution on the flat ice pack. The preparation was cut into rings of 3–4 mm in length and each ring was suspended between two stainless steel wires in a jacketed tissue bath containing 20 mL Krebs-Ringer bicarbonate solution (37 °C, pH 7.4), aerated with a mixture of 95 % O_2 and 5 % CO_2.

One of the wire hooks was attached to a transducer (CH1-SN: IT) connected to the amplifier (SN:BS 007) with recording system (Fast Acquisition, Elunit Group, Serbia) that recorded changes in isometric tension, using an e-Lab software. The second wire hook was attached to a displacement unit allowing fine adjustments of passive tension (g). During equilibration time, the organ bath solution was changed every 10 minutes and tension was adjusted when necessary.

Experimental protocols

Resting tension and incubation time

In order to evaluate the optimal resting tension and incubation period for HUA preparations, the different stretching conditions and incubation times were performed to obtain maximal con-

CONTENT

striction induced by potassium chloride (KCl). At the beginning of experiment the rings were equilibrated unstretched in organ bath at 37°C for 30 minutes. After that, the preparations were divided in four groups and each preparation was stretched to a different resting tension: 0.5 g, 1.0 g, 2.0 g and 4.0 g. When rings reached the determined tension, KCl (40 mM) was applied in precisely defined time points: 30 min, 60 min and 120 min. Between each KCl addition, rings were washed for several times and then adjusted to a determined resting tension.

KCl-induced constriction challenge

The intention was to assess the influence of the repeated initial constriction with KCl when preparations were set on optimal resting tension of 2.0 g. After 30 min of incubation time, the preparations were stretched to a passive force of 2.0 g. One group of preparations were allowed to equilibrate for 120 min and then were exposed to KCl initial challenge (40 mM), while on the other group of preparations three initial KCl challenges (40 mM) were performed, at 30 min, 60 min and 120 min, as previously described.

Serotonin dose-response curve

The aim of this protocol was to compare the dose-response curves for serotonin in HUA preparations under different passive tension and incubation time variables. When the first incubation period of 30 min ended, the preparations were stretched and equilibrated at different conditions. One group of preparations was stretched at 1.0 g and incubated for 60 min, while the other group was stretched at 2.0 g and incubated for 120 min. All preparations were exposed to KCl (40 mM) in order to obtain a reference constriction (100 %). After several wash-out periods, when preparations achieved the stable resting tension, a cumulative concentration-response curve for serotonin (10^{-8}–3 x 10^{-5} M) was obtained. At the end of each experiment, the viability of preparation was tested by challenging it with KCl-induced constriction.

Cold storage

Within this protocol the intention was to examine the effect of cold storage on HUA vasoreactivity. Optimal conditions (incubation period of 2 h and passive tension of 2.0 g) established in previous protocols were applied at HUA preparations after 24 h of cold storage at 4 °C. At the beginning of experiment, KCl-induced constrictions (40 mM) were established and after several washing-out periods, when basal line was stable, a cumulative concentration response curves for serotonin (10^{-8} – 3 x 10^{-5} M) were obtained.

Drugs and solutions

A Krebs-Ringer bicarbonate solution of the following composition was used (mmol/L): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 5.6. All stock solutions and serial dilutions were made in distilled water and were prepared shortly before the start of each of the experiments. Serotonin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and potassium chloride (KCl) was obtained from Lach: Ner (Zagreb, Croatia).

Statistical analysis

Results were presented as mean ± standard error (mean ± SEM) for number of preparations studied. Figures and statistical analysis were performed using SigmaPlot 14.0 (Systat Software Inc.). Two-way ANOVA with repeated measures was used to compare concentration-response curves for serotonin. Statistical significance between two groups was determined by Student`s t-test and the p values less than 0.05 were considered to be significant.

Results

Resting tension and incubation time

Preparations of HUA showed different responses to KCl at various stretching tensions and incubation periods. Preparations that were stretched at 0.5 g and incubated for 30 min did not show any response to KCl, while other preparations that were stretched at tensions of 1.0, 2.0, 4.0 g showed very weak constrictions. By prolonging the incubation time to 60 or 120 min, all preparations showed stronger constrictions than those obtained at 30 min. When preparations were stretched at tension of 2.0 and 4.0 g the constrictions were stronger than those stretched at 0.5 g, but the significance was confirmed only for 2.0 g. Further prolongation of incubation time to 120 min resulted in significant increase in constrictions for preparations stretched at tension of 2.0 and 4.0 g. However, additional increase in stretching tension of 4.0 g was not followed by stronger constriction response, as it would be expected (Figure 1).

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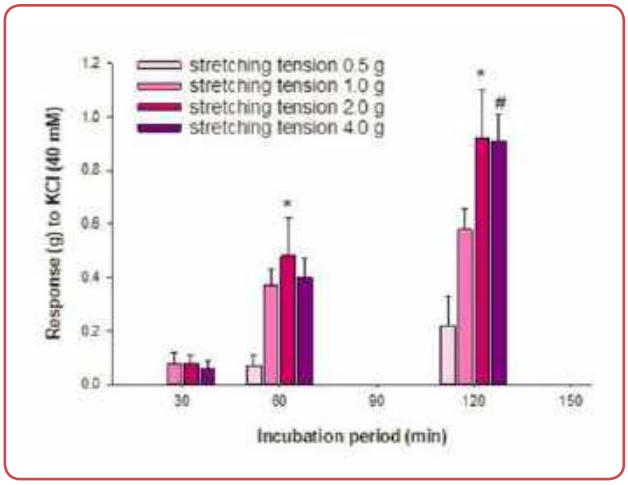

Figure 1: Comparison of KCl-induced constrictions, at a different stretching tensions and incubation periods, in human umbilical arteries in vitro. Preparations were adjusted at: 0.5 g (n = 6), 1.0 g (n = 8), 2.0 g (n = 10) or 4.0 g (n = 6) and each group was incubated for 30 min, 60 min and 120 min. Results (mean ± SEM) are expressed in grams of KCl-induced constriction.

** p<0.05; # p < 0.05 (2.0 g vs. 0.5 g; 4.0 g vs. 0.5 g, respectively).*

Potassium-induced constriction challenge

KCl-induced vasoconstriction (40 mM) was significantly stronger in HUA preparations that were exposed to three initial challenges (separate, non-cumulative additions of KCl at 30 min, 60 min and 120 min), compared to the preparations that were exposed to only one initial challenge (at 120 min) (Figure 2).

Serotonin dose-response curve

Concentration-response curve for serotonin, with the stretching tension of 2.0 g and incubation period of 120 min, showed a significantly stronger ($p < 0.001$) constrictions compared to the concentration-response curve at stretching tension of 1.0 g and incubation period of 60 min (Figure 3).

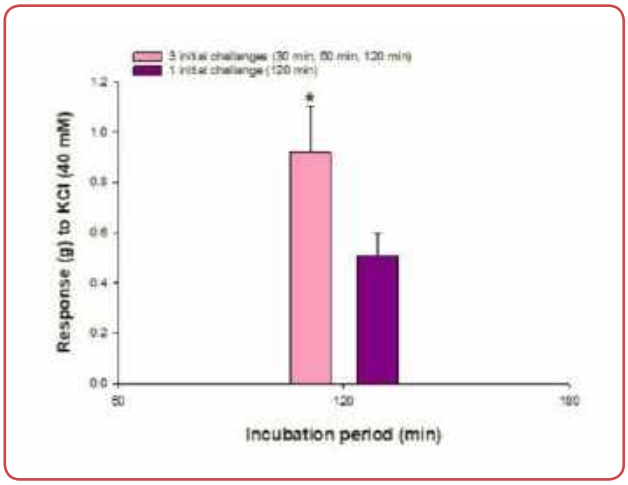

Figure 2: Comparison of KCl-induced constrictions of human umbilical arteries in vitro, in the absence or presence of first two initial challenges with KCl, at the optimal resting tension of 2 g. One group of preparations (n = 10) was exposed to three initial challenges: 30 min, 60 min and 120 min (pink bar), while the other group of preparations (n = 6) was exposed to one initial challenge: 120 min (purple bar). Results (mean ± SEM) are expressed in grams of KCl-induced constriction.

** p < 0.05 (significantly different from the respective control).*

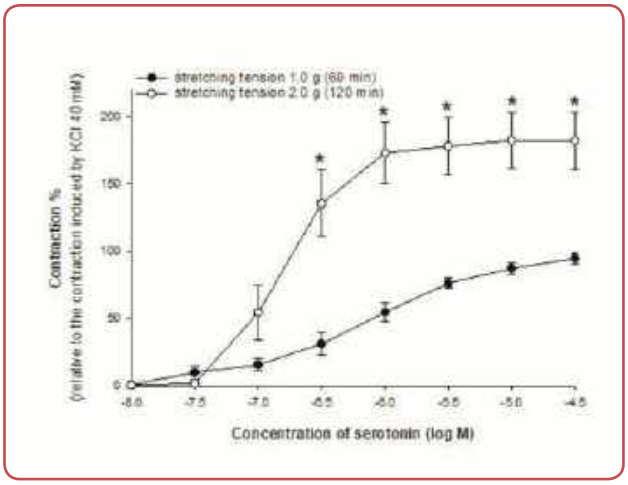

Figure 3: Comparison of concentration-response curves for serotonin, at different stretching tension and incubation time of human umbilical arteries preparations in vitro. Results (mean ± SEM) are expressed in percentage of reference KCl-induced constriction.

**p < 0.001 (significantly different from the respective control).*

Cold storage

Preparations that were stored at the temperature of 4 °C for 24 h did not show any significant difference in the magnitude of the KCl-induced constriction compared to the preparations of HUA that were used as fresh preparations, without cold storage (Figure 4).

Serotonin-induced constrictions were significantly stronger ($p < 0.01$) in cold-stored HUA preparations. Concentration-response curves for serotonin indicated higher efficacy of serotonin

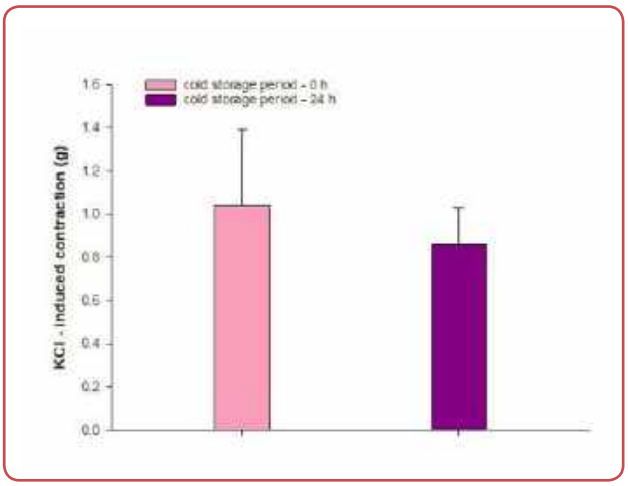

Figure 4: Comparison of KCl-induced constrictions of human umbilical arteries in vitro, at different cold storage times of human umbilical arteries preparations. Results (mean ± SEM) are expressed in grams of KCl-induced constriction

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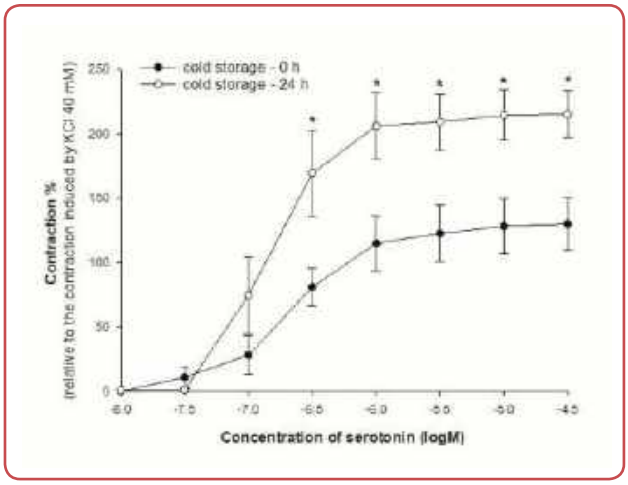

Figure 5: Comparison of concentration-response curves for serotonin, at different cold storage times of human umbilical arteries preparations in vitro. Results (mean ± SEM) are expressed in percentage of reference KCl-induced constriction.

** $p < 0.01$ (significantly different from the respective control).*

in cold-stored preparations, since significantly higher magnitude of responses were found at higher concentrations of serotonin (3×10^{-7}-10^{-5} M). However, at lower concentrations (10^{-8} - 10^{-7} M) there were no significance in the magnitude of the response, indicating that cold storage of the HUA preparation did not affect serotonin sensitivity (Figure 5).

Discussion

The main purpose of this study was to determine the optimal stretching tension and incubation period for researches that use HUA in tissue bath, as well as to examine the effect of multiple initial challenging and cold storage on HUA vasoactivity.

Bertrand et al reported that for reliable measurement of pharmacological properties it is necessary to stretch the HUA preparations to 5.0 or 6.0 g,[7] while other investigators in previous studies established the resting tension of 2.0 g as an optimal tension for HUA preparations.[8, 9] In addition, there are reports that applied a stretching tension of 4.0 g is optimal during 4 h of incubation,[10, 11] although in most studies the incubation time was 2 h.[8, 9, 12]

Results of these experiments have shown that the response of HUA to KCl was stronger after increasing stretching tension and incubating period. The maximal response was obtained when preparations were stretched at 2.0 g and incubated for 120 min. Despite the fact that after 120 min there was no significant difference between responses of arteries stretched at 2.0 g and 4.0 g, the stretching tension of 4.0 g was harder to achieve, thus, 2.0 g and 120 min are suggested as optimal experimental conditions. The sensitivity of HUA to KCl was not affected by stretching tension since no significant difference in responses was found between the HUA preparations stretched at 1.0, 2.0 or 4.0 g, although the differences in constrictions were significant when the mentioned tensions were compared with the lowest one applied, 0.5 g. This is in accordance with the intact sensitivity of chorionic vessels to KCl.1 However, in HUA preparations stretched at minimal passive tension (0.5 g), the incubation time might have an effect on the sensitivity to KCl, since constrictions were absent after 30 min of incubation, while prolongation of incubation time enhanced responsiveness.

In order to assess the functional integrity of HUA preparations, most authors use KCl as a reference contraction activator, applying different protocols to achieve initial challenge. The concentration of KCl that caused the maximal constriction for HUA preparations was found to be 80 mM, while the submaximal concentrations ranged from 20 mM to 60 mM.[9] However, Bertrand et al applied initial challenge with 100 mM of KCl, usually with repetitions of 3 to 4 times,[6] while Tufan et al used 40 mM KCl for contractile response and repeated it twice, the second constriction of which was taken as a reference one.[10] Estañ et al asserted that 40 mM KCl was the optimal concentration that would reach the submaximal constriction in foeto-placental blood vessels.[1] Considering the all above mentioned findings, it was decided to use KCl as a reference contractile agent at a concentration of 40 mM. Our results have shown that greater constrictions were obtained after multiple challenging with 40 mM KCl. The assumption for presence of greater constrictions and thereby increase of intracellular calcium ions (Ca^{2+}) could be that repeated KCl-induced membrane depolarisation lead to prolonged activation of Ca^{2+} voltage channels and release from intracellular depots.[13] It is also possible that during multiple challenging a higher amount of Ca^{2+} remains in the cell which is caused by inappropriate Ca^{2+} displacement after every wash-out period.[14]

It has long been known that serotonin is one of the most potent vasoconstrictors of HUA.[8] Finding that concentration of serotonin in umbilical

CONTENT

vessels at birth was 10^{-7} M[15] determined the range of serotonin concentrations most commonly used for *in vitro* studies.[6, 11, 16] In the present study the dose-response curves for serotonin (10^{-8} - 3 x 10^{-5} M), obtained after different stretching tensions and incubation periods, showed force- and time-dependent constrictions. It was found that the efficacy of serotonin was significantly higher under the optimal experimental conditions established in this study (2.0 g and 120 min). However, sensitivity to serotonin was not affected when preparations were stretched either at 1.0 g for 60 min or at 2.0 g for 120 min. It has been previously reported by Tufan et al that maximal serotonin constriction for HUA, expressed as percentage of KCl-induced constriction, was approximately 130 %.[10] Dayigolu et al applied the same experimental conditions (4.0 g and 4 h) and achieved a maximal constriction of approximately 145 %.[11] In the present experiment, higher maximal constriction for serotonin (approximately 180 %) were reached, using lower stretching tension (2.0 g) and shorter incubation time (120 min). Therefore, the advantages of this approach are reflected on savings of time and efforts in settings of HUA preparations for experiments.

In HUA preparations that were exposed to 24 h cold storage at 4 °C, the KCl produced qualitatively less constrictions compared to those in non-cold storage HUA preparations, but this difference was not statistically significant. Nevertheless, Sinanović and Chiba reported that KCl-induced constrictions were significantly decreased in dog and monkey skeletal muscle arteries after 3-5 days storage at 4 °C.[17, 18] They suggested that hypothermic conditions might have led to the change in sensitivity of Ca^{2+} voltage channels and thus Ca^{2+} influx. Moreover, it has been shown that cold storage (24-72 h at 4 °C) of rat thoracic aorta also diminished KCl-induced constrictions and that reactive oxygen species may be responsible for storage-induced changes in vascular reactivity.[19]

On the other hand, serotonin showed significantly higher efficacy, but not sensitivity in HUA preparations that were exposed to 24 h cold storage, compared to the control HUA preparations. Although the percentage of achieved serotonin constriction was almost twice as high in the preparations exposed to cooling, it still does not represent a realistic image of the increase in serotonin efficacy, taking into account that the reference KCl constrictions were lower in these preparations. The effects of cold storage on serotonin vasoactivity have been investigated in various blood vessel preparations, but the results were not consistent. There are reports that show that cold storage did not modify vascular response of serotonin on dog and monkey muscle arteries,[17, 18] whereas other reported that serotonin-induced constriction of rat aorta was almost completely diminished after 72 h of cooling at 4 °C.[19] Interestingly, Kevelaitis et al. found that endothelial dysfunction during cold preservation enhanced the sensitivity of serotonin on rat coronary arteries.[20] Since the results of the present experiments showed that cold storage increased serotonin constrictions, further studies are needed in order to examine the role of endothelial dysfunction on this phenomenon.

Like in the optimisations already performed for isolated human pulmonary arteries,[2] as well as for isolated human chorionic arteries,[1, 21] the results presented here represent an optimisation of the methodology for isolated HUA.

Conclusion

Results from the present study indicate that both KCl- and serotonin-induced constrictions were higher when HUA preparations were adjusted at 2.0 g stretching force and 120 min incubation period and that multiple initial challenging enhance vascular response to KCl. It was also shown that HUA preparations preserved functional sensitivity during 24 h cold storage for both KCl- and serotonin-induced constrictions. Considering all the above, mentioned experimental conditions are proposed as optimal to preform studies on HUA in isolated tissue bath, even 24 h after sampling.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Prtina et al. Scr Med 2020;51(3):141-6. DOI:10.5937/scriptamed51-28287
ORIGINAL ARTICLE

The Role of High-Dose Vitamin D Supplementation on Disease Severity and Lipid Profile in Psoriatic Patients - a Pilot Study

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Abstract

Background/Aim: Psoriasis is a chronic inflammatory skin disease that is associated with a higher prevalence of cardiovascular (CV) risk factors. The effect of vitamin D on bone health has been long known, but its extraskeletal role especially in cardiovascular disease and skin disease, is the subject of recent research. This study aimed to assess the influence of high-dose vitamin D supplementation on the Psoriasis Area and Severity Index (PASI) score and lipid profile in patients with psoriasis.
Methods: The study included 20 adult patients with chronic plaque psoriasis. They received vitamin D capsules in a daily dose of 5,000 IU over 12 weeks. Measured serum concentrations of lipid metabolism parameters were triglycerides (TG), total cholesterol (TC), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). PASI was used to determine the severity of the disease.
Results: High-doses vitamin D supplementation had a significant influence on reduction in PASI score in all patients (17.99 ± 12.42 vs 10.27 ± 8.53; $p < 0.001$). The supplementation of high dose vitamin D induced statistically significant lowering of the TC, LDL-C and TG in the psoriatic patients ($p < 0.05$). Furthermore, significant increase in serum HDL-C level was observed. The change of PASI score showed week positive correlation with the changes in serum TC and LDL-C ($r = 0.303$, $p = 0.03$ and $r = 0.357$ $p = 0.013$).
Conclusion: High-dose vitamin D supplementation had a positive impact on clinical status of the chronic plaque psoriasis patients, measured by PASI score. It also improved the serum lipid profile of these patients. Double-blinded prospective studies are needed in order to get more comprehensive data related to vitamin D, lipid metabolism and severity of psoriasis.

Key words: Psoriasis; Disease severity; Lipid profile; High-dose; Vitamin D.

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ARTICLE INFO
Received: 6 September 2020
Revision received: 21 September 2020
Accepted: 22 September 2020

Introduction

Psoriasis is a very common chronic inflammatory skin and systemic disease with various clinical manifestation that affects 2-3 % of the general population.[1,2] Aetiopathogenesis of the disease has not been fully understood yet and includes complex interaction of immune system, genetic background, autoantigens and environmental factors.[3]

A connection between psoriasis and higher cardiovascular (CV) morbidity and mortality is well recognised.[4] The CV risk factors such as lipid disturbance, hypertension, oxidative stress, diabetes mellitus and metabolic syndrome are more prevalent in patients with psoriasis.[4, 5] Dyslipidaemia is one of the most common CV risk factors and many authors have shown a direct link be-

This article should be cited as follows: Prtina A, Grabež M, Vujnić M, Rašeta Simović N. The role of high-dose vitamin D supplementation on disease severity and lipid profile in psoriatic patients. Scr Med 2020;51(3):141-6.

tween the serum levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and the risk of cardiovascular disease (CVD).[6]

The features of dyslipidaemia in psoriatic patients are very often associated with elevated triglyceride (TG) level, predominance of LDL-C and low level of HDL-C, all of which contribute to the increased CV risk. Therefore, more attention should be paid to routine screening for lipid disturbances in the psoriatic patients, which can contribute to an early determination of the risk for CVD.[7]

Vitamin D deficiency reached an epidemic proportions in the general population and has recently been associated with many CV risk factors like obesity, hypertension, insulin resistance, dyslipidaemia and chronic low-grade inflammation.[8] Besides that, there is a lot of data from available literature that have shown low vitamin D status in many non-skeletal diseases, including psoriasis. Some authors found that hypovitaminosis D in psoriatic patients correlated with clinical severity of disease calculated by the Psoriasis Area Severity Index (PASI) score.[9] It is unclear whether this association is causal or due to the underlying disease and the importance of hypovitaminosis D in psoriasis is still not entirely clear.[10]

There are limited data from available literature about the influence of high-dose vitamin D supplementation on disease severity and lipid profile in the psoriatic patients. The study aimed to assess influence of high-dose vitamin D supplementation on PASI score and lipid metabolism in patients with psoriasis.

Methods

Study design

This was a clinical study in chronic plaque psoriasis patients performed between June and December 2018. The patients were recruited at the Skin and Venereal Disease Clinic of the University Clinical Centre of the Republic of Srpska, Banja Luka, Bosnia & Herzegovina. A total of 20 adult psoriatic patients completed the study. All participants (> 40 years), received an oral dose of vitamin D (5,000 IU per day) for three months. The participants were given a precise number of capsules for three months of treatment and their compliance was checked on a monthly basis.

The participants who received some form of antipsoriatic systemic or local therapy, phototherapy and topical or oral vitamin D preparations not less than three months prior to being enrolled in the study were not included in the study. Participants who were treated with lipid metabolism affecting medications were not included in the study, either. Besides, the participants who had hypercalcaemia were not included. During the study, calcium values were measured on a monthly basis for all patients.

Ethical consideration

The study was approved by the Ethics Committee of the University Clinical Centre of the Republic of Srpska. All of the participants were informed of the study purpose and protocol, risks/benefits in the treatment course and the study schedule and signed informed permission prior to enrolment to the study.

Clinical and anthropometric measurements

Disease severity of skin lesions in chronic plaque psoriasis patients was scored by PASI values. Chronic skin lesions were monitored during this study. Based on PASI scores, the patients were divided into three groups: mild, moderate, and severe (PASI values < 10, PASI values from 10-20, PASI values > 20), respectively.[11]

Based on anamnestic data, psoriatic patients were split into two groups: early and late form of psoriasis (early form had onset of the disease before 30 years of age, and late form of psoriasis had onset of the disease after 30 years of age).

At the beginning of the intervention period, anthropometric measurements were taken in the morning, by qualified staff. Height was determined with an accuracy of 1 mm. Weight measurement was conducted using a standard scale to the nearest 0.1 kg. Body mass index (BMI) was derived using the standard formula.

Biochemical analysis

Lipid status profile and serum calcium levels were analysed. Blood samples were taken at the beginning and at the end of the treatment period, after 12-14 hours overnight fasting. Standard biochemical methods at Cobas 6000 analyser

CONTENT

(Roche Diagnostics, Mannheim, Germany) were used to analysed lipid profile parameters and serum calcium levels.

Statistical analyses

The Shapiro-Wilk's test was used for assessing data distribution normality. After the distribution check, an appropriate parametric or non-parametric test was used. The paired sample t-test or Wilcoxon Signed Rang test was performed for analysis of differences in the outcome variables. Correlation coefficients were assessed using the Pearson's correlation. Correlation coefficients are considered negligible if r = 0.0 < 0.30, week if r = 0.30 < 0.50, moderate if r = 0.50 < 0.70, and strong if r > 0.70.

The SPSS Statistic for Windows, version 20, was used to analyse data. Statistical significance was considered by *p-value* < 0.05. Numerical/categorical data were represented as mean ± standard deviation or number (%) where is appropriate.

Results

There were 20 patients of both genders that completed the study (10 males, 50.00 %). Demographic date concerning gender, age, BMI, disease duration, current smoking status, family history of psoriasis, and beginning of disease were presented in Table 1. The mean value of BMI was 27.67 kg/m^2, and higher BMI was noticed in the more severe form of disease group (28.38 kg/m^2) compared to the patients with mild or moderate forms of diseases. Nevertheless, this difference was found not to be statistically significant.

Table 1: Demographic characteristic of psoriatic patients

Variable	Total n = 20
Gender	
Male	18 (46.2)
Female	21 (53.8)
Age (year)	46.82 ± 15.05
Body Mass Index (kg/ m^2)	26.43 ± 2.60
Disease Duration (year)	13.69 ± 11.90
Smoking status	
Smoker	27 (69.2)
Non-smoker	12 (30.8)
Family history of psoriasis	
positive	21 (53.8)
negative	18 (46.2)
Beginning of disease	
early	19 (48.70)
late	20 (51.30)

Values are presented as number (%) or Mean ± Standard deviation

Twelve-week supplementation of vitamin D had a significant effect on the reduction in PASI score in all patients. The PASI score decreased from 15.54 ± 10.77 to 8.87 ± 7.38 (< 0.001) in all psoriatic patients. A significant reduction of PASI score after twelve-week supplementation of vitamin D in female and male patients (13.81 ± 10.58 vs 8.22 ± 7.41 and 17.58 ± 11.09 vs 9.67 ± 7.48) was noticed, respectively.

Table 2: The effect of vitamin D supplementation 5,000 IU/day over 12 weeks on PASI score in psoriatic patients (n = 20)

PASI score	Before vitamin D supplementation	After vitamin D supplementation	*p*
Female	16.17 ± 13.14	9.62 ± 9.09	0.002
Male	19.81 ± 12.01	10.92 ± 8.38	< 0.001
All patients	17.99 ± 12.42	10.27 ± 8.53	< 0.001

Data are expressed as Mean ± Standard deviation; PASI, Psoriasis Area and Severity Index

Serum lipid profile was analysed after 12 weeks of the intervention period. The supplementation of high dose vitamin D (5,000 IU) had a significant influence on the lipid parameters in psoriatic patients. As shown in Figure 1, the levels of TC, LDL-C, and TG were significantly decreased at the end of the study when compared with baseline values (5.61 ± 1.30 vs 5.37 ± 1.08; 3.25 ± 0.91 vs 2.98 ± 0.82 and 2.31 ± 1.53 vs 2.13 ± 1.37), respectively. Furthermore, a significant increase in serum HDL-C level was observed 1.22 ± 0.28 vs 1.47 ± 0.31, (p < 0.001).

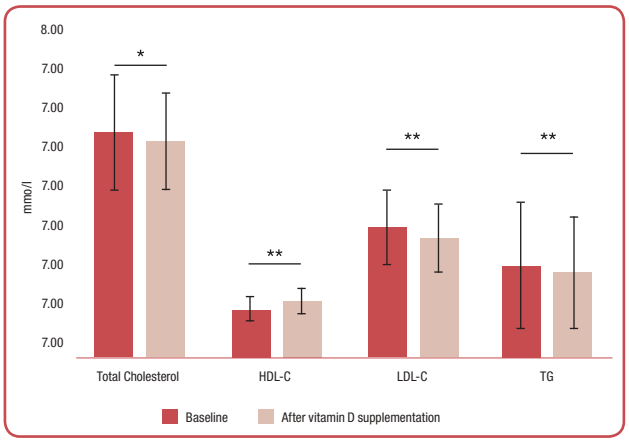

Figure 1: Effects of twelve weeks' supplementation with high dose of Vitamin D on serum lipid profile in psoriatic patients

*Data are expressed as the mean ± SD. HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; TG, triglycerides; *p < 0.01 **p < 0.001*

Table 3: Correlation between changes of PASI score with change of serum lipid profile after twelve-week of supplementation with high dose of Vitamin D (5,000 IU/day)

Lipid profile changes	r	p
Δ Total Cholesterol mmol/l	0.303	0.030
Δ HDL - C mmol/ l	0.099	0.274
Δ LDL - C mmol/l	0.357	0.013
Δ TG mmol/ l	0.043	0.398

Pearson's correlation was applied for verification of the relationship between the lipid profiles level changes in patients with psoriasis in relevance to PASI score after 12 weeks of vitamin D supplementation. Table 3 shows a weak, but statistically significant positive correlations between the change of PASI score with serum total cholesterol and LDL-C changes (Pearson's r = 0.303, p = 0.03 and Pearson's r = 0.357 p = 0.013, respectively), but there were no significant correlations between PASI score change with serum HDL-C and triglycerides changes.

Discussion

Psoriasis is a chronic skin and systemic disease interrelated with an increased risk of metabolic syndrome and CVD.[4] The chronic inflammation, usually common in psoriasis patients, can be a trigger for the structural protein changes, including the creation of neo-epitopes, which stimulate the HDL-C alterations and production of autoantibodies (anti–aHDL, anti-aApo-Al). Batuca et al found that elevated levels of autoantibodies were related with an increased cardiovascular risk and they could be involved in the development of atherosclerotic plaques.[12] These antibodies were also detected in patients with other autoimmune disease and their presence correlated with the severity of disease.[13] The previous studies reported that the vitamin D deficiency may be associated with dyslipidaemia in apparently healthy people and in diabetic patients.[14–16] Besides, vitamin D deficiency was related to an increased risk of CVD. A meta-analysis of 24 observational studies indicated an inverse relationship between the vitamin D serum level and risk of CVD.[17] The outcomes obtained by Bashir et al indicated that the vitamin D insufficiency or deficiency had significant effects on the lipid profiles, decreasing HDL-C level by almost 30 % and increasing the levels of TC, LDL-C and TG more than double.[18]

Lipid disorders in chronic psoriatic patients and their correlation with clinical progression have been described in the literature. The most common lipid abnormalities in psoriatic patients were reduced concentrations of HDL-C, apolipoprotein A (ApoA) and apolipoprotein B (ApoB) and increased concentrations of TC, LDL-C, and TG.[6,19–21] In another study, the inverse correlations between vitamin D serum level and atherosclerotic lipid profile in psoriatic patients was described.[22]

The current study results revealed a remarkable relationship between the twelve-week vitamin D supplementation and an improvement of lipid profile. A significant blood lipid reduction (TC, LDL-C, and TG), and an increase in HDL-C level after the intervention period were observed. These results are partially in agreement with the previous study conducted in diabetic patients.[23, 24]

However, the other trials have shown that vitamin D supplementation does not appear to improve the lipid profile.[25, 26] Additionally, discrepancies in the results of these studies could be possibly explained by variation in study protocols, different pathophysiologic conditions and the vitamin D interaction with certain drugs.

The decreased lipid absorption and endogenous synthesis are suggested mechanisms by which vitamin D treatment influences the lipid profile (total cholesterol and LDL-C).[27] Besides, the previous data have suggested that an increase in intestinal calcium absorption reduces the synthesis and secretion of TG. Another possible lipid-lowering pathway might be via the parathyroid hormone (PTH) regulation and consequent lowering of TG level.[16]

Several recent studies have indicated the role of vitamin D in the synthesis of glycosylceramides in the stratum corneum, in differentiation and proliferation of keratinocytes and migration of dendritic cells.[13,28] Additionally, vitamin D plays an important role in the formation of antimicrobial peptides in epithelial cells of the skin that maintain normal skin integrity.[29] Previously, observational studies demonstrated a negative correlation between the reduced level of vitamin D in psoriatic patients and the disease severity according to PASI score.[9, 30] There are limited data in the literature about the influence of high-dose vitamin D supplementation on the disease severity in patients with psoriasis.

The results of the present study demonstrated that a high dose of vitamin D supplementation significantly decreased the disease severity, determined by the PASI score. A significant reduction of PASI scores after twelve-week supplementation with a high dose of vitamin D in female and male patients was noticed.

In the three-quarters of patients, a significant improvement of the lesions was observed after oral treatment with 1 alpha-hydroxyvitamin D3.[31] A Brazilian study in 25 psoriasis and vitiligo patients who received a very high daily dose of vitamin D over six months, showed clinical improvement in all psoriatic patients and in 25–75 % of patients with vitiligo had repigmentation.[32] Similar results were obtained in the randomised, double-blind, placebo-controlled study conducted by Disphanura et al, however, the treatment period was the same, but the vitamin D supplementation was intermittent every two weeks.[33] In contrast to the results presented in this paper, Jarrett et al found that oral vitamin D administration had no significant difference in the PASI score in patients with mild form of the disease.[34]

The current study indicated the existence of a significant, but weak correlation between clinical improvements determined by change in PASI score and changes in TC and LDL-C serum level. In another placebo-controlled clinical study, the correlation between the severity of disease and anti-oxLDL and oxLDL concentrations was shown and a higher anti-ox-LDL concentration, along with HDLs antibodies or apolipoprotein was found, which indicated higher cardiovascular risk. It showed that the anti-ox-LDL/ox-LDL ratio correlated significantly with disease severity, its levels increasing linearly with increasing severity.[35]

Nakhwa et al did not find a statistically significant correlation between PASI score determined severity of disease and lipid profile abnormalities. At the same time, they found a reduced HDL-C level in patients with severe form of psoriasis.[7]

Conclusion

Although there are conflicting data in the literature about the vitamin D role in the pathogenesis of psoriasis, the results of this study found that high-dose vitamin D supplementation had a positive impact on the clinical status of the chronic plaque psoriasis patients, determined by PASI score. Besides, significant improvement in the lipid profile of those patients by lowering TC, LDL-C, and TG and increasing HDL-C, was observed. Nevertheless, prospective, double-blinded clinical studies are needed to get more data related to the role of vitamin D supplementation in psoriatic patients.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Grgić et al. Scr Med 2020;51(3):147-51. DOI:10.5937/scriptamed51-27558
ORIGINAL ARTICLE

Prognostic Significance of Intrathecal Oligoclonal Immunoglobulin G in Multiple Sclerosis

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Abstract

Introduction/Aim: Detection of intrathecal oligoclonal bands of immunoglobulin G (OB IgG), in addition to diagnostic, has a predictive significance in multiple sclerosis (MS). The aim of the study was to determine the prognostic significance of OB IgG and to correlate the presence of OB IgG with the progression of disability in MS patients.
Methods: A retrospective-prospective cohort study included 177 MS patients examined at the Centre for MS, Clinic of Neurology, University Clinical Centre of the Republic of Srpska. In all patients, demographic data, clinical parameters, Expanded Disability Status Scale (EDSS) score, isoelectric focusing (IEF) of cerebrospinal fluid (CSF), cyto-biochemical analysis of CSF, evoked potentials (EP) and magnetic resonance (MR) of the head were analysed. MS patients were divided in two groups: with and without intrathecal synthesis of oligoclonal IgG. According to the EDSS determined in both groups, the relation between the degree of functional disability and the presence of OB in the CSF and also with characteristics of the cyto-biochemical profile were analysed. Methods of descriptive and analytical statistics, analysis of variance, chi-square test, Bonferroni's post hoc test, correlation and regression analysis were used in the analysis of the results.
Results: In the examined cohort of MS patients, the sensitivity of IEF was 96.6 %. There was a statistically significant association between the detectability of intrathecally synthesised IgG and EDSS score ($p = 0.004$) so that individuals who do not have intrathecally synthesised IgG had lower EDSS scores. MS patients with a CSF protein concentration > 0.40 g/L were 2.45 times more likely to enter secondary progression and 2.51 times more likely to achieve EDSS 4.0.
Conclusion: IEF is a very sensitive diagnostic and prognostic method for MS patients, which indicates a more benign course of MS in patients without oligoclonal bands in the CSF.

Key words: Oligoclonal bands; Multiple sclerosis; Prognosis.

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ARTICLE INFO
Received: 17 July 2020
Revision received: 18 August 2020
Accepted: 19 August 2020

Introduction

By analysing the quantitative and qualitative disorders of cerebrospinal fluid (CSF) IgG in patients with multiple sclerosis (MS), it is possible to obtain very significant diagnostic and prognostic data.[1] Recommended criteria for CSF analysis indicate that isoelectric focusing (IEF) on agarose gel with immunofixation and the use of specific antiserum for human IgG are "gold standard" for oligoclonal bands (OB) detection.[2]

This article should be cited as follows: Grgić S, Dominović Kovačević A, Đajić V, Vukojević Z, Tadić D,Račić D, Vujković Z. Prognostic significance of intrathecal oligoclonal immunoglobulin G in multiple sclerosis. Scr Med 2020;51(3):147-51.

MS experts consider magnetic resonance (MR) alone to be insufficient for the diagnosis and prognosis of MS.[3] CSF testing helps predict conversion to MS in patients with negative MR or with lesions that do not meet diagnostic criteria.[4] In patients with negative MR, the presence of OB increases the risk of MS by 4-23 % .[5] Along with OB, numerous markers in the CSF are specific to the disease process, such as inflammation and immune dysfunction. Some of these markers have predictive significance in the conversion of the Clinically Isolated Syndrome (CIS) to MS.[6, 7] Increased conversion was observed in the presence of IgG antibodies to neurotropic viruses, rubella, varicella-zoster, which are responsible for nonspecific polyclonal activation of B cells within the central nervous system.[8] CSF markers of an axonal lesion may be more specific than MR to predict CIS conversion to MS.[9, 10]

Frederikson et al pointed out that, in addition to diagnostic significance, the detection of OB of immunoglobulin G has the highest predictive sensitivity of conversion of CIS to MS and that there is a correlation between OB and disability progression.[11] In some patients (5-10 %) with clinically confirmed MS, OBs in the CSF are not detected regardless of the sensitivity of the method. Among the patients without OB in the CSF, the majority are those with a benign course of the disease.[12, 13]

The aim of the study is to determine the prognostic significance of OB IgG and to correlate the presence of OB IgG with the progression of disability in MS patients.

Methods

The study is a cohort, partly retrospective, and partly prospective. It included all MS patients whose CSF was analysed by the IEF method with immunofixation at the Neuroimmunological Laboratory of the Clinic of Neurology, University Clinical Centre of the Republic of Srpska for three and a half years. All patients signed informed consent before entering the study. The study was approved by the Ethics Committee of University Clinical Centre of the Republic of Srpska, Banja Luka, Republic of Srpska, Bosnia and Herzegovina.

All CSF and serum samples were analysed by the standardised IEF method.[2, 3, 5] The study included 177 MS patients, of which 55 were men and 122 women (ratio 1: 2.2 in favour of women). The mean age ± standard deviation (SD) of MS patients at the time of the study was 38.8 ± 10.7 years and the mean age of patients at the disease onset was 34.4 ± 8.4 years. The average duration of the disease was 4.4 ± 6.0 years. According to the course of the disease, the most common is the relapsing-remitting (RR) form of MS (79.7 %), followed by the secondary-progressive (SP) (13.6 %) and primary-progressive (PP) (6.8 %) form of MS. The mean EDSS score in patients with MS was 2.7 ± 1.7. Progression index (PI = EDSS/duration of the disease)[14] was calculated for all MS patients and a mean value was 2.70 ± 4.78. The disease was more active in the first compared to the second year of the disease, because in the first year of the disease the average number of relapses was 1.5 ± 0.6 and in the second year 1.3 ± 0.7.

All material was analysed in the mentioned period and the IEF was interpreted with the data on the temporary diagnostic assumption. All MS patients were diagnosed according to McDonald criteria (2005 revision).[10, 14] The degree of functional disability was determined by using the EDSS score,[15] at least 30 days after relapse. Beside EDSS, for all patients, the PI was calculated.

All MS patients were examined for demographic and clinical parameters and the following diagnostic procedures were performed: cyto-biochemical analysis of CSF, IEF of CSF and serum, evoked potentials battery - visual evoked potentials (VEP), auditory evoked potentials (AEP) and somatosensory evoked potentials (SSEP) by stimulating median nerve and MR of the head.

Based on the IEF findings, the group of MS patients was divided into two groups:

(1) with intrathecal synthesis of oligoclonal IgG and

(2) without intrathecal synthesis of oligoclonal IgG.

Qualitative testing of IgG in CSF and serum was performed by the IEF method of CSF and serum on agarose with protein transfer to the nitrocellulose membrane, immunofixation and staining with immunoperoxidase. According to the EDSS determined in both groups of MS patients, the relation between the degree of functional disability

CONTENT

and the presence of OB in the CSF was analysed. The association of the cyto-biochemical profile with EDSS and the predictive significance of such association for MS were investigated.

Descriptive and analytical statistical methods were used in the analysis of the results. Analysis of variance (ANOVA) was used to estimate differences among continuous variables, and the χ^2 test was used for categorical variables. Bonferroni's post hoc test was used for multiple intergroup differences. Correlation analyses included the calculation of Spearman's correlation rank coefficient for nonparametric data. Statistical significance was determined at the level of 0.05.

Results

IEF of CSF and serum was done in all patients, and out of 177 MS patients, intrathecal synthesis was detected in 171 patients (96.6 %), which indicates a very high sensitivity of the method. Comparing the group of MS patients with OB and those without intrathecal synthesis, it was observed that all patients with regular IEF findings had the RR form of the disease, the disease lasted up to a year, average EDSS = 1.0 and the average number of relapses in the first year was one. From the above mentioned clinical parameters of the MS patients with OB, it can be seen that in the group without local synthesis there are no patients in SP and PP form of MS, that the disease lasted shorter, the mean EDSS score was lower and that the disease activity was lower in the first year. In this study, the presence of a statistically significant difference in the positivity of IEF

Table 1: Significance of the difference between IEF and EDSS positivity and progression index in MS patients

		N	Mean	Standard deviation
EDSS	Negative IEF	6	0.7500	0.61237
	Positive IEF	171	2.8099	1.71939
	Total	177	2.7401	1.73376
		p = 0.004		
Index of progression	Negative IEF	6	4.8333	6.21021
	Positive IEF	171	2.6301	4.73783
	Total	177	2.7047	4.78929
		p = 0.269		

IEF - isoelectric focusing of the cerebrospinal fluid
EDSS - Expanded Disability Status Scale
MS – multiple sclerosis

(intrathecally synthesised IgG) according to clinical parameters as variables was examined. The results indicated that there was no statistically significant association between IEF positivity and gender, age, level of education, age at onset, and duration of MS disease. There was a statistically significant association between the detectability of intrathecally IgG and EDSS score (p = 0.004) so that individuals who do not have intrathecally synthesised IgG have lower EDSS scores

Table 2: MS Outcome: secondary progression as a function of CSF variables

Variable	HR	95% CI	p
Proteins (> 0.40)	2.45	1.01 - 5.99	0.049
Cell number (> 5)	1.45	0.49 - 4.32	0.504

MS – multiple sclerosis
CSF – cerebrospinal fluid
HR - hazard ratio
CI - confidence interval

(Table 1). There was no statistically significant association between the positivity of the IEF and the progression index, protein concentration and cell number in the CSF of MS patients.

Cyto-biochemical analysis of CSF in MS patients found a mean protein value of 0.39 ± 0.1 g/L (range 0.1-1.0 g/L). The mean number of cells in the CSF of MS patients was 4.8 ± 6.8.

Table 3: MS Outcome: EDSS 4.0 scores as a function of CSF variables

Variable	HR	95% CI	p
Proteins (> 0.40)	2.51	1.23 - 5.12	0.049
Cell number (> 5)	1.24	0.54 - 2- 85	0.504

MS – multiple sclerosis
EDSS - Expanded Disability Status Scale
CSF – cerebrospinal fluid
HR - hazard ratio
CI - confidence interval

A statistically significant predictor of progression in this series of patients was the protein concentration > 0.40 g/L. MS patients with a CSF protein concentration > 0.40 g/L were 2.45 times more likely to enter secondary progression (Table 2). A statistically significant predictor of achieving EDSS 4.0 in this series of patients was a protein concentration > 0.40 g/L. This means that MS patients with a CSF protein concentration > 0.40 g/L are 2.51 times more likely to achieve EDSS 4.0 (Table 3). In this series of MS patients, no variable showed a statistically significant predictive value for achieving EDSS 6.0. IEF was not included in predictive models because in this series of MS patients there were only six IEF findings without intrathecal IgG synthesis, but a statistically sig-

nificant association between IEF and EDSS positivity indicates a more benign disease course in patients without local synthesis.

Discussion

Numerous studies have shown that IEF is the most sensitive method of detecting intrathecal IgG synthesis, which gives it a great diagnostic value.[16-19] For the method to have full clinical potential for OB IgG analysis in CSF, standardisation of laboratory methods and diagnostic criteria is necessary.[20, 21] Results of the present study showed high sensitivity of the IEF method (96.6 %) in the examined group of MS patients in this laboratory. Recent studies have shown that the detection of OB in the CSF has a predictive significance for the course of the disease and the progression of disability expressed by EDSS score.[22-25] This study indicated that there was a statistically significant association between the detectability of intrathecally synthesised IgG and EDSS score ($p = 0.004$) so that individuals who do not have intrathecally synthesised IgG have lower EDSS scores.

When the groups of examined MS patients with and without OB were compared, it was noticed that all patients without OB in the CSF had the RR form of the disease, that the disease lasted up to a year, the average EDSS was 1.0, and the average number of relapses in the first year was one. Therefore, in the group of MS patients without local synthesis there were no patients with the progressive forms (SP and PP) of MS, the disease lasted shorter, the average EDSS score was lower and the disease activity was lower in the first year. Significant prospective studies with similar results have indicated that patients with negative OB have a better prognosis in terms of neurological disability, due to a more benign course of the disease.[26-30] In 2013, Mero et al pointed out that MS patients without OB were immunogenetically different from typical MS patients with OB.[31] Imrell and co-authors showed that patients with OB had a more aggressive course of the disease compared to those without OB. This imposes the importance of IEF in a predictive sense, but also in an attempt to understand the pathogenic mechanisms in MS patients with and without OB.[32]

In 2012, Lourenco et al conducted a large retrospective study on a sample of 6,935 MS patients.[33] The results of the study presented in the paper here follow the results of the mentioned study and both studies have highlighted a significant difference in the positivity of the IEF according to the degree of the functional disability expressed by the EDSS score, without a significant difference regarding the progression index. This showed that MS patients without local synthesis had a more benign course and a lower EDSS score. Along with OB, numerous biological markers in the CSF are specific to the disease process, such as high protein levels as a sign of inflammation and immunodysfunction.[34, 35] Studies indicate that proteins at high concentrations in the CSF have a predictive significance for the progressive course of MS.[36-38] In the study presented here, a statistically significant predictor of secondary progression and reaching EDSS 4.0 in the examined MS patients was a protein concentration of more than 0.40 g/L. MS patients who have a CSF protein concentration of more than 0.40 g/L are 2.45 times more likely to enter secondary progression and 2.51 times more likely to reach EDSS4.0.

Conclusion

Numerous studies in this area have indicated that the presence of OB in the CSF of MS patients in addition to diagnostic has an exceptional predictive significance. The results of the present study indicate that MS patients with intrathecal IgG synthesis have a higher degree of functional disability expressed by the EDSS score. Also, an elevated CSF protein concentration was confirmed as a predictor of MS progression. Everything mentioned indicates that the study of numerous markers in the CSF enables a better understanding of the aetiology, pathophysiology, course and prognosis of MS, and allows us to make timely and adequate therapy choices for MS patients.

Acknowledgements

None.

Conflict of interest

None.

CONTENT

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CONTENT

Svolacchia and Svolacchia. Scr Med 2020;51(3):152-7. DOI:10.5937/scriptamed51-24968
ORIGINAL ARTICLE

Use of Microfiltered vs Only Disaggregated Mesenchymal Stem Cells from Adipose Tissue in Regenerative Medicine

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Abstract

Background: Clinical use of adult mesenchymal stem cells (MSCa) in medicine and regenerative surgery is constantly evolving. Adipose tissue-derived stem cells (ADSc) are capable of inducing the production of new extracellular matrix (ECM), deposition of new collagen and early revascularisation.
Methods: Flow cytometry was performed for 2 mL of cell colonies harvested from adipose tissue (AT). Comparation has been made of AT disaggregated only and the same AT disaggregated and microfiltered at 50 µm, 100 µm and 200 µm. Signs of inflammation after dermo-epidermal regeneration session through the mesotherapy method were observed and compared.
Results: Even after filtration, significant number of ADSc was collected. An increase in the size of the filter did not always translate into an increase in the number of cells that were found in the microfiltrate. In the non-filtered AT disaggregated in both cases, highest number of cells was found, as expected, but at the expense of more pronounced inflammation. Sampling with the 16 Gauge needle produces superior results compared to the cannula in all cases.
Conclusion: With this method in medicine and regenerative surgery it will be easier to exploit the growth factors, mRNA, MicroRNA, lipids and bioactive peptides emitted in the MSCa signalling micro-vesicles as they are isolated from the inflammatory component.

Key words: Mesenchymal stem cells; Adipose tissue-derived stem cells, Side-population cells; Filtration; Flow cytometry.

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ARTICLE INFO
Received: 26 January 2020
Revision received: 23 August 2020
Accepted: 24 August 2020

Introduction

Tissue progenitors with adult stem markers present in the mesenchyme and used in medicine as regenerative therapy induce their clinical response by means of a complex and dynamic interaction between the cells, growth factors and blood vessels present in the extracellular matrix (ECM).[1] Adult progenitors are those that can be isolated in an autologous tissue sample of mesenchymal origin and that best embed cells with characteristics of adult stem cell on cytometric examination. They are contained in the so-called side population (SP) and are also referred to as MSCa. The MSCa derived from adipose tissue (AT) or adipose tissue-derived stem cells (ADSc) and their secretion micro-vesicles are capable of inducing the production of new ECM, deposi-

This article should be cited as follows: Svolacchia F, Svolacchia L. Microfiltered vs only disaggregated mesenchymal stem cells from adipose tissue in regenerative medicine. Scr Med 2020;51(3):152-7.

tion of new collagen and early revascularisation not influenced by hypoxia.[2] Clinical success of a therapy carried out through a microfiltered AT disaggregated at predetermined measures of 50 and 100 micrometres (µm) in order to safeguard the tissue progenitors has been demonstrated both in chrono-ageing and in photo-ageing.[3, 4] After publishing the clinical success of a technique that included the exclusion of fibrous branches and cellular debris because they are potentially inflammatory.[5-7] By preserving the SP, the prore-generative properties related to the transmission of extracellular vesicles are preserved, which contain the different types of molecules including RNA, ranging in size from 30 to 100 nm, nucleic acids (DNA, mRNA and microRNA), signalling proteins and lipids for target cells.[8, 9] If excluding the inflammatory phase, it is possible to epigenetically reprogramme the receiving cells by altering their phenotype and activating an inversion of fibrosis.[10] This information is transferred through an intercellular communication of the signalling micro-vesicles that will induce the resolution of fibrosis, the inhibition of the activation of macrophages, the inhibition of the secretion of inflammatory cytokines and the remodelling of the ECM with reduction of scar tissue.[10] Through the micro-vesicles emitted by ADSc deprived of the inflammatory component, the functional phenotype of the mother cell as a therapeutic will be maintained.[11]

Methods

The quantities of viable cells contained in 2 mL of AT according to Tonnard et al[12] were microfiltered at various sizes and without any microfiltration by cytometric examination.[13] The samples were taken from the same patient, in the same operating session and in the same anatomical site of the abdomen both with a 16 Gauge (16G) needle and with a small hole multiport cannula, after carrying out a tumescent anaesthesia in order to preserve the vitality of the MSCa contained in the adult tissue compared to a simple local anaesthesia.[14] The patient signed an informed consent for the use of lipoaspirate for experimental procedures. The study was performed following the standards of the local ethics committee and in accordance with the Helsinki Declaration (2000).

After collection and disaggregation, all AT samples were cultured (1 flask T75 / sample) in the presence of 10 mL of ALFA-MEM medium with 10 % foetal bovine serum and antibiotic. After two days, cells were present in all the flasks. In the following two days the flasks of samples A, B and D, those containing the 16G needle collection, were confluent and the cells were then detached and the flow cytometric analysis of specific mesenchymal (CD29, CD44, CD73, CD105) and hematopoietic (CD45) markers was performed. After two more days the flasks containing the samples C, E, F and G, those extracted with the small hole multiport cannula, had also reached confluence and flow cytometric analysis of specific mesenchymal markers was carried out. All the samples were analysed for mesenchymal markers.

Signs of inflammation after dermo-epidermal regeneration session through the mesotherapy method with 1 mL syringe and Luer attachment and 30G 6 mm needle with the non-microfiltered disaggregated ADSc were observed and compared to the same treatment with disaggregated microfiltered ADSc at 50 µm.

Results

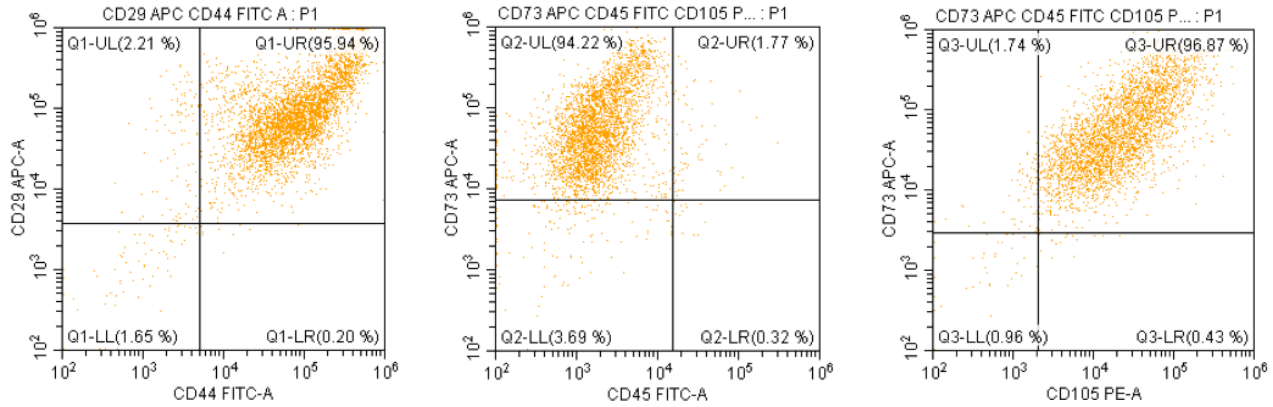
Two days after collection, disaggregation and cultivation of cells were present in all the flasks. The richest cell sample was D, the poorest C, while samples A and B were equivalent. Sample G was richer in E and F.

In samples collected with 16G needle (A, B and D) the following were recovered: in sample A (filtration at 50 µm): 1,350,000 cells; in sample B (filtration at 100 µm): 1,170,000 cells; in sample D (no filtration): 1,870,000 cells (Figure 1).

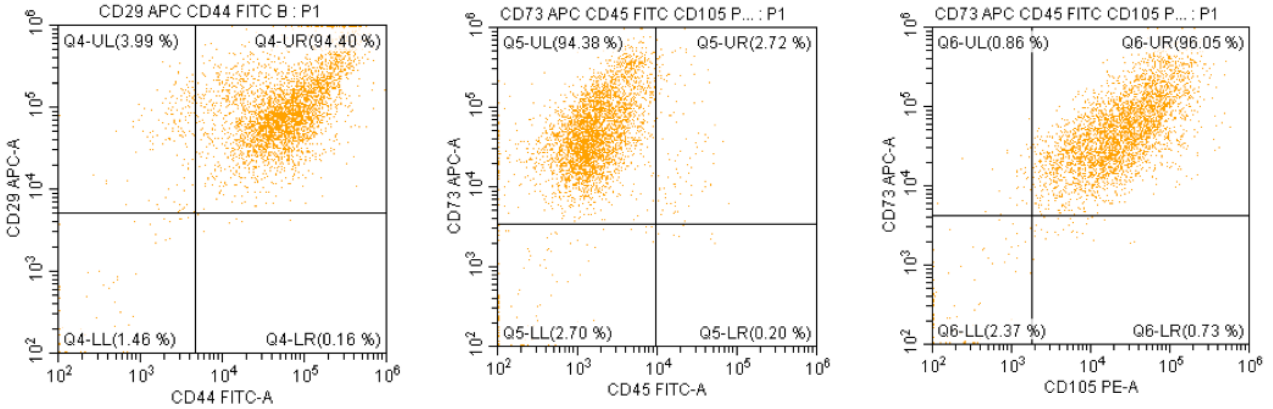
In samples extracted with small hole multiport cannula (C, E, F, G) the following were recovered: 980,000 cells were recovered in sample C (filtration at 200 µm); in sample E (filtration at 50 µm): 1,125,000 cells; in sample F (filtration at 100 µm): 1,364,000 cells; in sample G (no filtration): 1,560,000 cells (Figure 2).

All the samples analysed were positive for all

Sample A



Sample B



Sample D

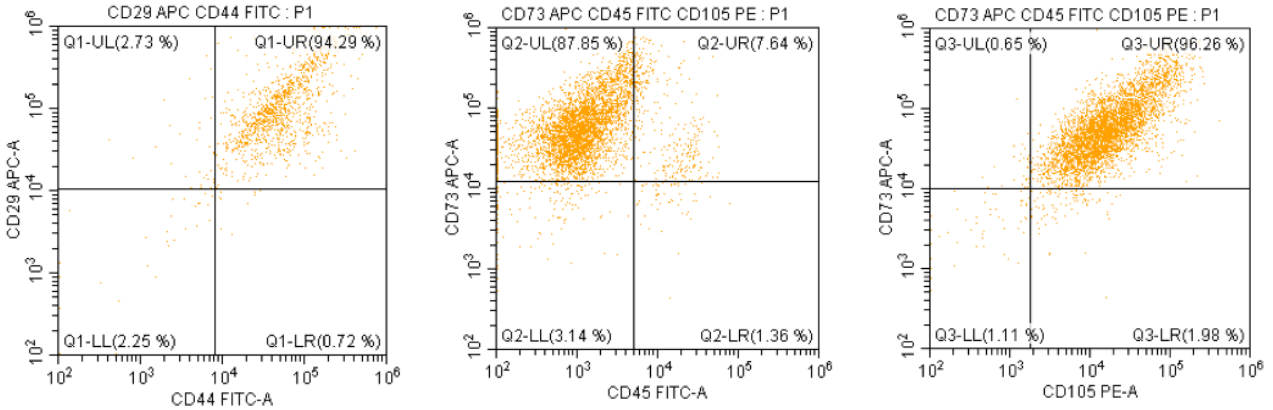


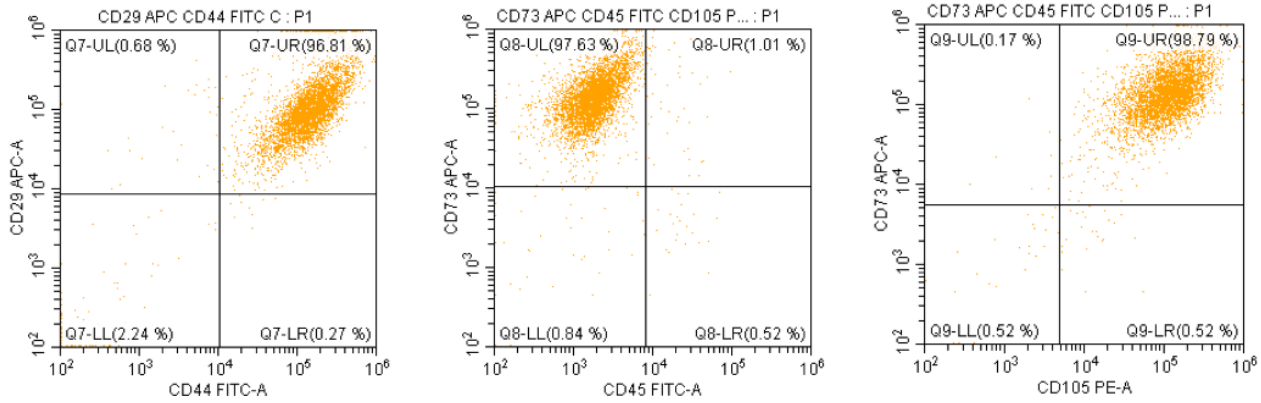
Figure 1: Flow cytometry of adipose tissue derived stem cells (ADSc) samples collected with 16G needle.
Sample A) with filtration at 50 µm (1,350,000 cells); Sample B) with filtration at 100 µm (1,170,000 cells); Sample D) no filtration (1,870,000 cells)

mesenchymal markers. The population of CD45 + cells (hematopoietic cell contamination) was minimal.

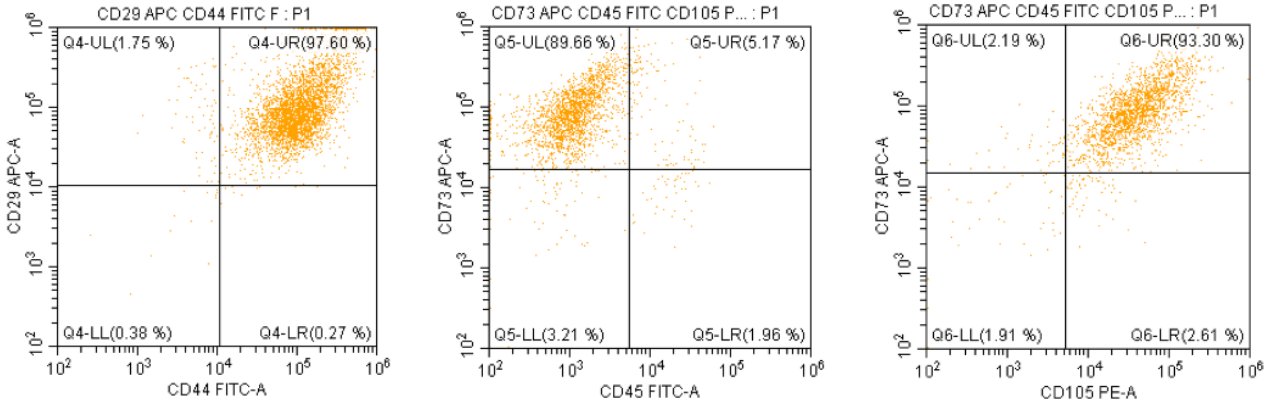
When comparing signs of inflammation (rubor, dolor, calor), temporary increase of the signs was noticed about 30 minutes after treatment. Clinically observed, signs of inflammation were more expressed with treatment with 1 mL syringe and Luer attachment and 30G 6 mm needle with the non-microfiltered disaggregated (Figure 3) compared to the same treatment with disaggregated microfiltered ADSc at 50 µm (Figure 4).

CONTENT

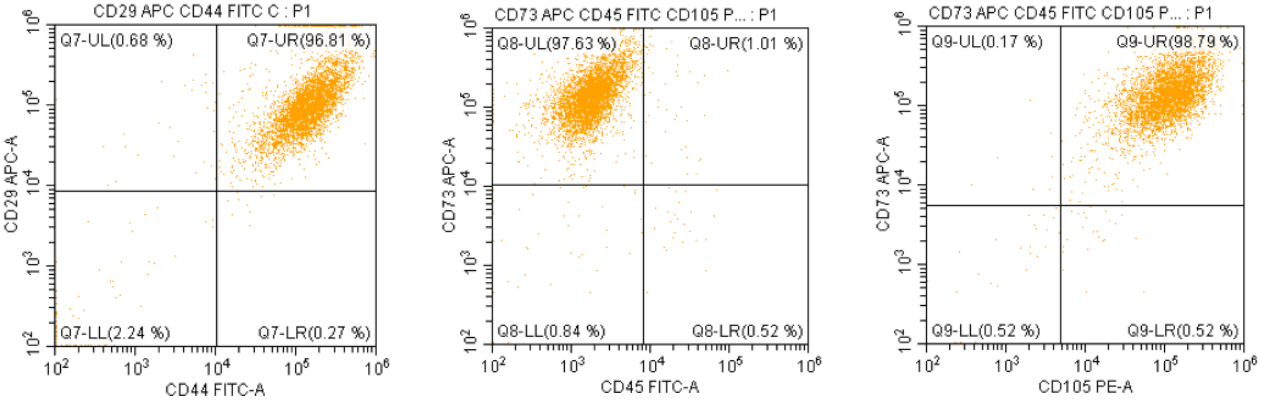
Sample E



Sample F



Sample C



Sample G

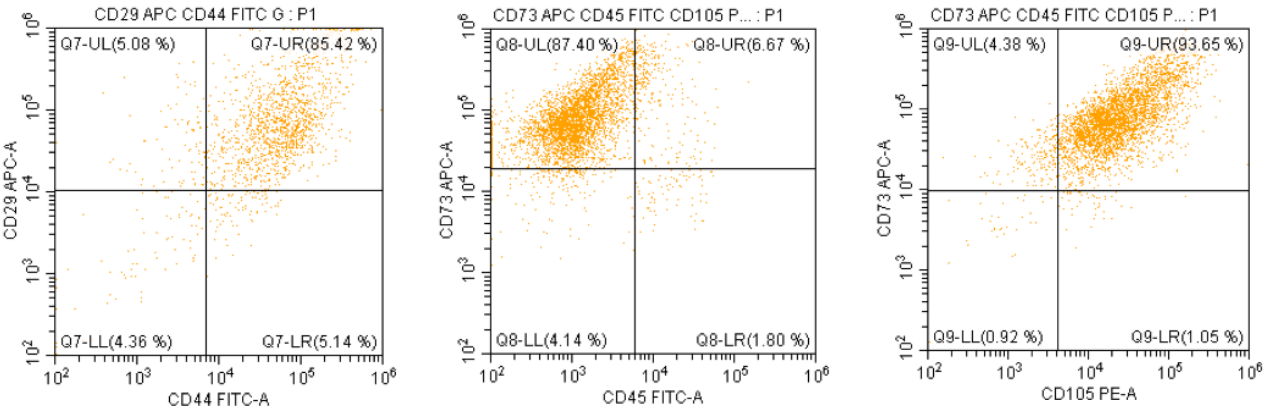


Figure 2: Flow cytometry of adipose tissue derived stem cells (ADSc) samples collected with lipoaspiration cannula.

Sample E) with filtration at 50 µm (1,125 cells); Sample F) with filtration at 100 µm (1,364,000 cells); Sample C) with filtration at 200 µm (980,000 cells); Sample G) no filtration (1,560,000 cells);

CONTENT

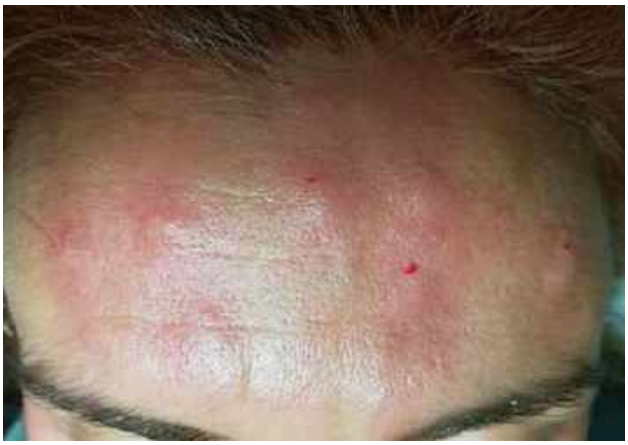


Figure 3: Signs of inflammation after treatment with 1 mL syringe and Luer attachment and 30G 6 mm needle with the non-microfiltered disaggregated adipose tissue derived stem cells (ADSc).

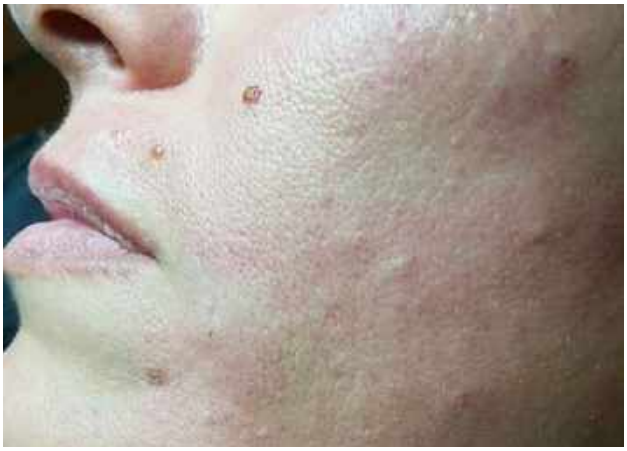


Figure 4: Signs of inflammation after treatment with 1 mL syringe and Luer attachment and 30G 6 mm needle with the microfiltered disaggregated adipose tissue derived stem cells (ADSc) at 50 µm.

Discussion

In this study it is compared some of the possible methods that allow us to use a small sample of AT for tissue regeneration. The comparison was made between sampling with a small hole multiport cannula versus a 16G needle and between a fat disaggregated according to Tonnard et al[12] and its filtrations at 50, 100 and 200 µm.

Collecting MSCa from AT is convenient, as it is easily available, repeatable and AT is non-essential. Also, ASCs have been shown to have higher proliferation capacities than bone marrow MSC.[15, 16] Differences in the proliferation time of ASCs originating from different regions of the body have been reported.[17-19]

Sampling with a 16G needle was richer in progenitors with adult stem cell outlines, those that best embody MSCa, compared to drawing with the cannula. Filtration maintained an excellent quantity of cells with stem markers allowing the elimination of fibrous branches and cellular debris potentially inflammatory in both extraction methods. The filtration of the sampling with a needle at 50 µm contrary to what could be expected gave a higher result than the filtration at 100 µm. The exact opposite occurred with the disaggregated and microfiltered AT from the collection with the cannula. Of this latter sampling, the filtration at 200 µm produced a numerically lower presence in cells but still very high, due to the small amount of AT used (2 mL).

In the non-filtered AT disaggregated in both cases, the greatest presence of cells was found, as expected, but at the expense of more pronounced inflammation. There were changes at the injection point on the dermis during the mesotherapy treatment between a microfiltrate and the AT disaggregated according to Tonnard et al.[12] During the procedure without filtration some of the clinical signs of inflammation that spontaneously regressed after about 30 minutes were observed. Those were practically absent in the mesotherapy treatment with the microfiltrate. Not only that, although there is a loss of cells during the disaggregation carried out according to Tonnard et al[12] and subsequent filtration, their vital and replication potential after the selection of the AT by filtration turns out to be higher than the simple disaggregation.[20, 21] Through the cytometry studies reported in this study, it was clear that the microfiltration technique is suitable for preserving the SP and with it the MSCa for each regeneration treatment by mesotherapy therapy supplementation of tissue progenitors, resulting clinically preferable to the unfiltered unbundled. In this study it is shown that during filtration there is a loss in the number of cells with adult stem cell markers that are stuck in the mesh-

CONTENT

es of fibrous branches and cellular debris. It is also shown that an increase in the size of the filter does not always translate into an increase in the number of cells that are found in the microfiltrate. Very high number of viable cells can be found even in a microfiltrate from only 2 mL of AT. That translate clinically into a better success since it lacks the inflammatory component. Data has shown that sampling with the 16G needle produces superior results compared to the cannula in all cases.

Conclusion

The filtering technique is simple, fast, reproducible and economic, it allows for to have a very high suspension of cells, is more efficient in terms of vital potential and replication for all medical and surgical regenerative techniques compared to the simple disaggregated according to Tonnard et al.[12] Its use can also be defined as safe and preferable based on the authors' clinical experience.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Maksimović et al. Scr Med 2020;51(3):158-65. DOI:10.5937/scriptamed51-26726
ORIGINAL ARTICLE

Significance of Dyspnoea as a Symptom in the Emergency Department of the Primary Healthcare Centre

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Abstract

Background/Aim: Dyspnoea represents a subjective feeling of laboured breathing of different intensity. The aim of this study was to determine how often patients come with dyspnoea to a local Emergency Department of the Primary Healthcare Centre (EDPHC) and to analyse the assumed aetiology, diagnostics and therapy of the dyspnoeic patients.
Methods: The records of the EDPHC of the City of Banja Luka for the period between 1 October 2019 to 31 December 2019 of all patients older than 15 years of age that have reported laboured breathing were identified and analysed. Following parameters were recorded: age, gender, diagnostics performed, administered therapy, whether the patient was examined in the field or in the ambulance, as well as whether the patient was referred to a hospital (Cardiology, Pulmonology or another department) or not.
Results: Laboured breathing was reported by 665 patients. Out of this number, 108 patients were examined by their family doctor, 407 patients in EDPHC ambulance and 150 patients in the field. The average age of the patient was 61.03 ± 19.17, with an equal distribution in males and females. The aetiology of dyspnoea was cardiac and pulmonary in one-third of patients each, whereas the diagnosis in the other patients was versatile, from anxiety disorders, musculoskeletal diseases and active malignancy to unspecified chest pain that could not receive a definitive diagnosis. One half of the patients (N = 261 or 46.86 %) was completely taken care of in the ambulance or in the field, 199 (35.73 %) patients were sent to a cardiologist, 87 (15.62 %) to a pulmonologist and 10 (1.80 %) of patients to another specialist. Referral to hospital was registered more often in men ($\chi^2 = 9.195$, $p = 0.027$), elderly ($\chi^2 = 53.29$, $p < 0.001$), people with lower peripheral oxygen saturation (SpO_2) ($\chi^2 = 120.61$, $p < 0.001$) and people with significant deviation of normal blood pressure values ($\chi^2 = 120.61$, $p < 0.001$).
Conclusion: Dyspnoea can be caused by an array of different diseases and more than one diagnostic method is necessary to confirm/exclude any of the most common causes of dyspnoea. A broader diagnostical palette in ED would be preferred for purposes of ascertaining a timely diagnosis.

Key words: Dyspnoea; Emergency Department; Primary Healthcare Centre; Differential diagnosis.

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ARTICLE INFO
Received: 24 May 2020
Revision received: 7 June 2020
Accepted: 7 June 2020

Introduction

Dyspnoea represents a subjective feeling of laboured breathing of different intensity.[1] Patients describe it differently, but the symptoms can be most often divided in three groups: effort (breath-

This article should be cited as follows: Maksimović ŽM, Banjac N, Čović M. Significance of dyspnoea as a symptom in the emergency department of the primary healthcare centre. Scr Med 2020;51(3):158-65.

ing requires labour), tightness ("thorax seems too small for comfortable breathing") and air hunger (the patient's feeling that he/she has not inhaled enough air, need for more air).[2, 3] It is considered that the air hunger originates from stimulating the breathing centre in the brain with hypercapnia and/or hypoxia and labour from muscle afferents and "afferents of breathing".[4]

Dyspnoea is a disabling symptom, trailing by importance only pain and it is estimated that a quarter of the total population in the ambulance complains of breathing discomfort.[5, 6] It is also an important predictive factor in many diseases, mortality as well as quality of life. Dyspnoea is a better prognostic factor of mortality in patients with chronic obstructive pulmonary disease (COPD) than forced expiratory volume in first second (FEV_1), and in patients with heart diseases it is better than angina during the stress test.[7, 8]

Causes of dyspnoea are many. They are most commonly of pulmonary (pneumothorax, pulmonary embolism, asthma, foreign body in respiratory tract, inhalation of toxic substances, pneumonia, exacerbated COPD, restrictive lung disease, interstitial lung disease, pleural effusion) and of cardiac (myocardial infarction, heart insufficiency, papillary muscle rupture, angina, pericardial effusion, cardiac tamponade) aetiology, but it can be also caused by anxiety disorders, anaemia, bad physical condition, diaphragm paralysis.[9] Dyspnoea is often the first symptom of some relatively rare diseases.[10-15]

Based on the notion that dyspnoea, as well as pain, are subjective feelings, questionnaires were created as an attempt to define the intensity and type of difficulty that the patients feels, with questionnaires such as Dyspnoea-12, Multidimensional Dyspnoea Profile (MDP), modified Medical Research Council scale (mMRC), Baseline Dyspnoea Index (BDI), Total Dyspnoea Scale for Cancer Patients (TDSC), however each one of them deals with a different disease, so it is impossible to implement any of them as an universal questionnaire of dyspnoea that would help the doctors in the ED.[16-19] Evaluation of the potential cause of dyspnoea begins initially with anamnesis and physical examination. Alongside, pulse oximetry is very important, then blood pressure values, electrocardiogram (ECG), chest X-ray and eventually, depending on doubt regarding potential causes, also blood gas analysis, complete blood count (CBC) and plasma D-dimer concentrations.[20, 21] With chronic dyspnoea additional examinations might be necessary, such as computerised tomography scan, lung function tests, echocardiography and bronchoscopy.

The goal of this research was to determine how often patients had reported dyspnoea in EDPHC of Banja Luka and to analyse the assumed aetiology, diagnostics and therapy of a dyspnoeic patient. It was also investigated how often a dyspnoeic patient had to be referred to a hospital and based on which parameters.

Methods

After obtaining the permission of the local Ethics Committee, a retrospective cross-sectional study was conducted. Analysing the protocol of the EDPHC of the City of Banja Luka, between 1 October 2019 and 31 December 2019 data were found for all patients that reported laboured breathing. Only patients older than 15 years of age were recorded, as younger ones were examined by a paediatrician. These patients were further included in analysis, so their age, sex, diagnostics (whether blood pressure was taken and their values, oxygen saturation, ECG, blood tests: CBC, glucose level, D-dimer, troponin), prescribed therapy, whether the examination of the patient was made in the field or in an ambulance, as well as whether the patient was referred to a higher referenced level or not (to cardiology, pulmonology or any other department) were all recorded.

The anonymity of patients was preserved because only the patient's age and sex were recorded, without name or surname, initials or date of birth. Blood pressure values were recorded as: a) normal blood pressure values: 100-140/60-90 mmHg, b) hypotension: <100/60 mmHg, c) stage 1 hypertension: 140-160/90-100 mmHg, d) stage 2 hypertension: 160-180/100-110 mmHg, e) stage 3 hypertension: >180/110 mmHg. If the values of systolic and diastolic blood pressure were found in different categories, the values were classified in the higher category.

Oxygen saturation was analysed by a pulse oximeter. It was analysed whether the abovementioned examination had been performed, as well as whether the values were: a) ≥ 94 %, b) ≥ 88 - 93 %, c) ≥ 80 - 87 %, d) ≤ 79 %.

CONTENT

D-dimer and troponin were analysed using quantitative turbidimetric immunoassay using a Cobas h-232 device. It was analysed whether the above-mentioned examination was performed, as well as whether the values were within the reference values.

The data were processed using IBM SPSS 21.0 software. Categorical data were compared by Chi-square test. After distribution inequality had been determined by Kolmogorov-Smirnov test, continual data were analysed by Mann-Whitney U-test or the Kruskal-Wallis test.

Results

In the EDPHC of the City of Banja Luka, in the period from 1 October 2019 to 31 December 2019, a total of 8,112 patients were treated. Laboured breathing was found to be a complain in 665 of them, regardless of whether it was the only or just one of the symptoms. Of the given number, 108 patients were examined by their family doctor, while the EDPHC doctors performed supervision and, if necessary, administered therapy while transferring the patients to a hospital. The remaining 557 patients were examined either in the ambulance of the EDPHC (407 patients, or 73.07 %), or in the field (150 patients, or 26.93 %).

The distribution of patients is shown in Table 1. It shows equal distribution according to gender. Dyspnoea was more often reported by older patients; half of them were aged over 65. The average age of patients was to 61.03 ± 19.17 years, (95% CI 59.43-61.62), with median of 65 years and interquartile range of 30.

Table 1: Gender and age distribution of dyspnoeic patients admitted to the Emergency Department of the Primary Healthcare Centre

		Referred to hospital - N (%)			
Variable	N (%)	No	Cardiology	Pulmonology	Other
Gender					
Male	281 (50.45)	115 (40.93)*	114 (40.57)*	48 (17.08)*	4 (1.42)
Female	276 (49.55)	146 (52.90)*	85 (30.80)*	39 (14.13)*	6 (2.17)
Age					
16-30	45 (8.08)	38 (84.44)*	4 (8.89)	3 (6.67)	0 (0.00)
31-64	232 (41.65)	126 (54.31)*	77 (33.19)*	25 (10.78)*	4 (1.78)
Over 65	280 (50.27)	97 (34.64)*	118 (42.12)*	59 (21.07)*	6 (2.14)
Total	**557 (100.00)**	**261 (46.86)**	**119 (35.73)**	**87 (15.62)**	**10 (1.80)**

N (%) = number and percentage of patients
** $p < 0.05$. Male patients were statistically more often reffered to hospital treatment (Chi-square test: $\chi^2 = 9.195$, df = 3, $p = 0.027$). Older patients were statistically more often reffered to hospital treatment (Chi-square test: $\chi^2 = 50.07$, df = 6, $p < 0.001$).*

The cause of dyspnoea was of cardiac and pulmonary aetiology in one-third of the patients each, whereas the diagnosis of the other patients was versatile, from anxiety disorders, musculoskeletal disorders and active malignancy to unspecified chest pain that has not received a definitive diagnosis (Table 2).

Table 2: Assumed causes of dyspnoea in patients admitted to the Emergency Department of the Primary Healthcare Centre

Diagnosis	N	%
Pulmonary: N (%) = 193 (34.65)		
Pulmonary embolism	6	1.08
Acute respiratory infections	55	9.87
Chronic respiratory infections	11	1.97
Exacerbation of COPD	62	11.13
Exacerbation of asthma	41	7.36
Pleural effusion	5	0.90
Respiratory failure	13	2.33
Cardiac: N (%) = 206 (36.98)		
Hypertension	31	5.57
Angina pectoris	86	15.44
Acute myocardial infarction	20	3.59
Heart failure	43	7.72
Atrial fibrillation	3	0.54
Pulmonary oedema	17	3.05
Hypotension	6	1.08
Other: N (%) = 158 (28.37)		
Anxiety disorders	39	7.00
Myalgia	15	2.69
Unclear aetiology	17	3.05
Active malignancy	3	0.54
Nonspecific chest sensations	84	15.08
Total	**557**	**100**

N - number of patients; % - percent of patients

Among the objective parameters, most patients had their peripheral SpO_2 measured, blood pressure value, heart rate and ECG taken and, when considered necessary by the doctor, blood analyses - glucose level, complete blood count (CBC), troponin and D-dimer level - determined. Results are shown in Table 3.

Half of the patients had normal values of blood pressure, 7 % of patients were hypotensive and the rest were to some extent hypertensive. Normal SpO_2 was present in a bit less than half of the patients, an additional one-third had slightly lower values, where approximately 15 % of the patients had significant hypoxia.

Two-thirds of patients had normal heart rate, 4 % were bradycardic, 10 % had slightly increased heart rate, and 12.57 % of patients had significant tachycardia with or without other heart rhythm disorders.

CONTENT

Table 3: Values of measured parameters in dyspneic patients admitted to the Emergency Department of the Primary Healthcare Centre

Parameter	N	%
TA: N (%) = 541 (97.13)		
Normotensive	291	52.24
Hypertension, stage 1	126	22.62
Hypertension, stage 2	38	6.82
Hypertension, stage 3	45	8.08
Hypotensive	41	7.36
TOTAL	541	97.12
SpO_2: N (%) = 500 (89.77)		
94 % and more	246	44.17
88-93 %	169	30.34
80-87 %	55	9.87
Less than 80%	30	5.39
TOTAL	500	89.77
Heart rate: N (%) = 516 (92.64)		
Bradycardia	21	3.77
60-100/min	370	66.43
101-110/min	55	9.87
>110/min	70	12.57
TOTAL	516	92.64

N: number of patients; %: percentage of total number of patients; SpO_2: peripheral oxygen saturation;

ECG was performed in 77.74 % of patients, which was either normal or led to the establishment of one of the before mentioned working diagnosis. CBC was analysed in 41 patient and blood glucoses level in one-third of the patients. Out of 182 patients, 132 had normal, 2 lower and 48 increased blood glucose values. Troponin was analysed in 64 patients, its values were increased in 10 patients and D-dimer was determined in 23 patients, where almost half of the patients (10 patients or 43.48 %) 10 patients or 43.48 % had D-dimer values above the upper reference limit.
Administered therapy in dyspnoeic patients is shown in Table 4. Patients received one or more drugs and two patients (0.36 %) were in such condition that they required cardiopulmonary resuscitation.

Table 4: Therapy administered to dyspnoeic patients admitted to the Emergency Department of the Primary Healthcare Centre

Therapy	N	%
Oxygen	162	29.08
Corticosteroids	178	31.96
Bronchodilatators	172	30.88
Analgesics	151	27.11
Diuretics and/or antihipertensives	142	25.49
Diazepam	81	14.54
Acetylsalicylic acid	72	12.93
Nitrates	40	7.18
Infusion solutions	31	5.57
Beta blockers	13	2.33
Antibiotics	11	1.97
Proton-pump inhibitor	10	1.79
Digoxin	9	1.61
Amiodarone	9	1.61
Cardiopulmonary resuscitation	2	0.36

N = number of patients; % = percentage of patients

One of the most important parameters observed is the percentage of patients referred to hospital treatment, as well as to which department. Half of the patients (N = 261; 46.86 %) were completely taken care of in the ambulance/in the field, without need for further diagnostics, 199 (35.73 %) patients were referred to a cardiologist, 87 (15.62 %) to a pulmonologist, and 10 patients (1.80 %) to a different specialist (neurologist, infectologist, internist, surgeon) (Figure 1).

It was also analysed whether there was a difference in referring the patients based on specific parameters.

Regarding the gender, men were significantly more often referred to hospital treatment, both to a cardiologist and a pulmonologist (Chi-square test: $\chi^2 = 9.195$, df = 3, p = 0.027).
There is also a statistically significant difference

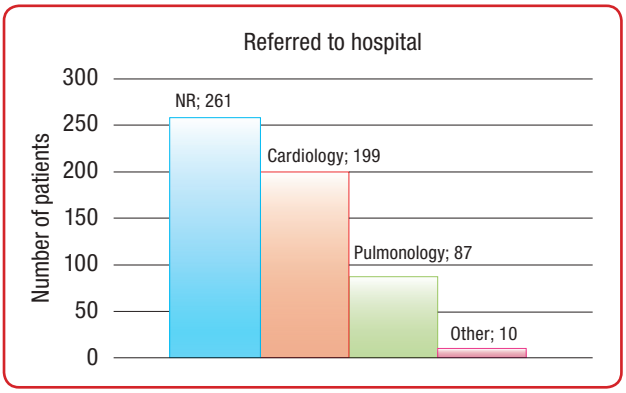

Figure 1. Number of patients treated by the Emergency Department of the Primary Healthcare Centre and referred to a specific department (Cardiology, Pulmonology, Other) or not referred at all (NR)

in the age of the referred patients (Kruskal-Wallis test: $\chi^2 = 53.29$, df = 3, p < 0.001). After a general statistical significance had been shown, differences among the groups were analysed by Mann-Whitney U-test, which showed that older people were significantly more often referred to both a cardiologist (U-test: 16991.00, p < 0.001) and a pulmonologist (U-test: 7085.00, p < 0.001), but also that there was no statistically significant difference to which department the patient was referred to based on age (U = 8347.00, p = 0.63).

SpO_2 values greatly influenced the doctor's de-

CONTENT

cision whether to refer the patient to hospital treatment or not (Chi-square test: $\chi^2 = 120.61$, df = 9, $p < 0.001$). Patients with normal blood oxygen saturation were most often not referred for further treatment, whereas those with the lowest values were more often referred to a pulmonologist.

The measured blood pressure values greatly influenced the doctor's decision (Chi-square test: $\chi^2 = 52.81$, df = 12, $p < 0.001$). Patients with both hypertension and hypotension were most often referred to a cardiologist and normotensive patients either to a pulmonologist or they were not referred for further specialist treatment at all.

Heart rate also significantly influenced the doctor's decision whether to refer the patient to hospital treatment or not (Chi-square test: $\chi^2 = 56.66$, df = 9, $p < 0.001$), with the main difference being in bradycardic patients being exclusively referred to a cardiologist, whereas other patients were further referred or not based on other symptoms/indications.

Discussion

Dyspnoea represents a serious differential diagnostic challenge for a doctor. The challenge is even bigger knowing that the subjective feeling often does not match the objective parameters.[22, 23]

Patient distribution by gender was even, which matches the data found in literature.[24] The interesting finding is that more than half of women were not directed to further treatment (50.29 %), unlike only 40.93 % of men who were. The reason for that can be found in detailed data analysis, where it was found that most common causes of dyspnoea in women were the ones that can be taken care of by the treatment in the EDPHC, like hypertension, exacerbation of COPD and anxiety disorder.

Half of the patients were aged 65 and above and only 8 % of them were under the age of 30. Age distribution makes sense and it matches the data found in literature, considering that almost all potential dyspnoea causes are more common in the elderly.[22] The youngest among the examined population were more often hit by unspecific difficulties, myalgia, acute respiratory infections, so only seven of the patients required further examination by a specialist. In the range of 31-65, half of the patients were directed to see a specialist, cardiologist being the most frequent one, three times more often than the others. The reason for that can be found in the fact that at this age, most of the cardiac difficulties appear for the first time. Two-thirds of the patients above the age of 65 are being directed to a hospital treatment, mostly to a cardiologist, unlike middle-aged people. The reason for directing elderly to a pulmonologist is that the pulmonary diseases are usually much more often and much more severe in them than in younger people, often with a respiratory insufficiency.[25] COPD and cardiac disease comorbidity are also common in the elderly, so the doctor has to determine which disease causes the newly created dyspnoea.[26]

How to differentiate the cause of dyspnoea in the ED, with limited diagnostics, is now the question. The doctor's experience is significant, because an experienced doctor can assume the potential cause of dyspnoea and with great probability, just by taking anamnesis and performing physical examination. For that reason, the diagnostic procedures should help the doctor with confirming/excluding the assumed diagnosis. SpO_2, ECG and blood pressure measurement should be performed in every dyspnoeic patient. The reason why it is not being done in practice is the fact that a certain number of difficulties comes from obvious lighter infections of the upper respiratory tract, such as nasopharyngitis and tonsillitis, where the aforementioned diagnostics are not really necessary. Unfortunately, a certain number of patients simulate their difficulties, making them appear more urgent and serious than they really are.

Almost half of the patients were directed to further treatment and two times more often to a cardiologist than to a pulmonologist, even though the number of cardiologic and pulmonary diagnosis was equal. Reason for that is that most pulmonary diagnosis represent either exacerbation of COPD/asthma or acute respiratory infection, which can be taken care of and treated in the ED, while the cardiologic diagnosis often require hospitalisation. Data indicate that one-third of dyspnoea causes were of pulmonary aetiology, half of which were exacerbation of COPD/asthma, similar to the literature data.[27, 28] Different studies reported different hospitalisation rates.[18, 28, 29] Reason for that can be a different organisation of the healthcare system, primary healthcare and ED availability.

On the other hand, hypertension, rhythm disorder, acute myocardial infarction and unstable angina pectoris are often first time discovered in the ED and therefore require a cardiologist examina-

CONTENT

tion.[30-32] Cardiac decompensation and lung oedema and directing them or not often depends on whether the patients appear in the earlier or the later stages of the disease.[24, 33]

Certain number of patients has dyspnoeic difficulties because of an earlier diagnosed or undiagnosed psychiatric disorder.[34] Their differentiation is made even harder by the fact that because of hyperventilation they often have tachycardia and tachypnoea in their report, sometimes even auscultatory wheezing or even hypertension because of the anxiety. Doctors' experience plays a huge part here. On the other hand, patients suffering from COPD and asthma, often remain undiagnosed for the anxiety disorder, which are common cause of disease exacerbations, so the patients show even worse clinical picture and are being treated inadequately.[35] Depression is also common in patients with cardiac decompensation and is directly related to the dyspnoea frequency.[36]

Values of blood oxygen saturation, blood pressure, frequency, blood glucose, ECG recording, should primarily help the doctor to differentiate the potential cause of dyspnoea, but also their values indicate the severity of the clinical picture. As previously mentioned, it is often the case that the subjective feeling does not match the objective state of the patient, so the objective parameters help the doctor significantly with the diagnostics and the therapy as well. Systematic review article that analysed the dyspnoea in ED showed that there was no single symptom/sign that could exclude heart failure, COPD or pulmonary embolism.[37]

SpO_2 values significantly affected the doctors' decision, especially the fact whether the oxygen therapy improved the saturation, so those patients whose saturation stayed low were most often directed to hospital treatment. Those ones with lowest values were usually sent to a pulmonologist.

Heart rate was observed within the whole clinical picture when considering therapy and directing the patient further. Only bradycardia as an individual symptom would affect the decision that such patients were, as a rule, directed to a cardiologist. Tachycardia was obviously regarded as a part of the clinical picture, with increased body temperature, hypertension, arrhythmia, pulmonary embolism, COPD. ECG is also a significant tool that helps the doctor, especially in case of pathological finding and sometimes it gives a reliable diagnosis in cases such as acute myocardial infarction and heart rhythm disorder.

Blood pressure value helped the doctor both with the diagnostics and choice of the therapy, so those patients with higher values were more often referred to a cardiologist. Hypotensive cardiac patients often had a severe clinical picture and their prehospital treatment was limited.

Considering the fact that D-dimer has a negative predictive value, it serves primarily for excluding pulmonary embolism and it is recommended not to be determined as a routine.[38] On the other hand, troponin values can help the doctor with unspecific changes in the ECG. CBC was determined, weather it was for suspected anaemia or acute respiratory infection.

It is the belief of the authors that the doctors in EDPHC would benefit from additional diagnostics when contemplating the differential diagnosis of dyspnoea, such as X-ray or ultrasonography, wider lab pallet, which at the moment, is not at the doctors' disposal. N-terminal ProBrain Natriuretic Peptide (NT-proBNP) as a reliable parameter of heart failure would be very useful for differentiating dyspnoea in the elderly. Besides, doctors should record the number of respirations per minute in every dyspnoeic patient, considering it was not the case so far. On top of that, it would be preferable to record the body mass index (BMI), considering the correlation found between obesity and dyspnoea.[34, 39, 40]

Conclusion

Dyspnoea can be caused by an array of different diseases and more than one diagnostic method is necessary to confirm/exclude any of the most common causes of dyspnoea in the EDPHC.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Paleksić. Scr Med 2020;51(3):166-73. DOI:10.5937/scriptamed51-24550
ORIGINAL ARTICLE

Professional Stressors in Prison Officers: a Cross-Sectional Study

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Abstract

Background/Aim: The aim of this study was to analyse the stressors on prison officers' workplace in facilities of closed and semi-open type and their differences and the interconnection between specific sociodemographic variables (sex, age, marital status, exposed working experience, education level) and stressors on workplaces.
Methods: The cross-sectional study included 330 prison officers, between 19 and 65 years of age, who have been divided in two groups depending on the security level of the facility (semi-open and close facility type). The research was conducted during 2015, using the following questionnaires: sociodemographic questionnaire, the organisational police stress questionnaire (PSQ.org) and the operational police stress questionnaire (PSQ.op).
Results: The results have shown significantly higher load with organisational and operative stress in facilities of closed type ($p < 0.001$) and that in both groups operative stress sources were slightly more represented than the organisational. The intensity of stress ranged from low to medium. Higher intensity of organisational stress was perceived regarding stressors related to work appreciation, than in regard to sources related to logistic support, while the lowest intensity of stress was in regard to interpersonal relations in the organisation. In closed facilities, divorced prison officers and those who were separated from their families for a longer time have experienced higher stress intensity. Total work experience and age of prison officers had a moderate and mild effect, respecitively, on organisational stressors in higher security facilities. "Fatigue", "traumatic event" and "favouritism" were the most important stressors.
Conclusion: The prison officers are exposed to stress of low to medium intensity, the operational stress sources being more represented then organisational. In higher security facilities total work experience and age had an influence on organisational stressors.

Key words: Prisons; Stress, psychological; Workplace; Occupational stress; Cross-sectional studies.

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ARTICLE INFO
Received: 23 December 2019
Revision received: 28 July 2020
Accepted: 29 July 2020

Introduction

There are five groups of factors that influence stress on the workplace: (1) the personality of the employee, (2) the conditions on the workplace, (3) the demands of the workplace, (4) the organisation of the workplace and (5) social environment and life conditions (family problems, living and material conditions, social and society changes, sickness, sudden life events).[1]

A prison is a complex institution with clearly defined rules and its own functioning and oversight system, in which people are serving sentences of imprisonment due to committed criminal offences. Prison facilities can be closed, semi-open, open and specialised facilities. Closed facilities are the most common type of facilities, known by high walls and barb wire, in modern times also by

This article should be cited as follows: Paleksić VM. Professional stressors in prison officers: a cross-sectional study. Scr Med 2020;51(3):166-73

security with highly sophisticated video-surveillance and similar equipment. Beside the technical equipment and technical security, these types of facilities also have a high level of physical security and departments for special surveillance. Semi-open facilities usually have some elements of external security and inner structure as closed ones (often seen are high walls and wire), but the internal structure is normally less strict. Aggression inside the facility is less frequently demonstrated, the interpersonal problems between the staff and the prisoners are not very expressed. Open prison facilities are defined as facilities in which there are no obstacles for the escape of prisoners, there are no walls, bars nor armed security staff. Specialised facilities can be juvenile prisons, specialised hospitals for medical treatment of prisoners, psychiatric facilities for mentally disturbed prisoners and similar. In penal-correctional facilities there are by rule organisational units for security issues, re-education, health protection, employment of prisoners, as well as units for general and common matters.[2] The security staff is made of prison officers (prison guards), who are armed, uniformed and of same sex as prisoners. In the Republic of Srpska there are six prison facilities of closed or semi-open types.

One of the first empirical stress evaluation studies on staff in prison facilities determined that, on average, prison staff had higher blood pressure than the inmates.[3] Several studies confirmed that stressors among prison staff can be divided into two broader categories: problems with prisoners and problems with management (operative and organizational, respectively).[4-6] In the research done by Rutter and Fielding it was concluded that stress connected with the inmates had biggest influence on the prison officers' health[7], while other research indicated management as the most important stress source.[5, 8] In a different research it was determined that prison staff, especially in prisons with a stricter regime, more often suffered from mental illnesses connected to stress.[9] In the literature there are consistent findings that the organisation, oversight, support between the colleagues, problems regarding working roles, ambiguous, unclear or conflicting roles (organisational stressors) are the most important sources of stress.[8,10-12] However, the results in the literature are not consistent in regard to the influence of the sociodemographic factors (sex, age, work experience, marital status, education) on the stress of prison officers.[13,14]

The goal of this study was to analyse the stressors in prison officers' workplace in facilities of closed and semi-open type and their differences and the interconnection between specific sociodemographic variables (sex, age, marital status, exposed working experience, education level) and stressors in the workplaces.

Methods

The research was designed as a cross-sectional study among 477 police officers aged 19 to 65 from 6 prison facilities in the Republic of Srpska with at least 12 months of working experience. It was performed during 2015, according to the Helsinki declaration and approved by the Ethics Committee of the Institute for Occupational Health and Sports of the Republic of Srpska. The author of the article conducted the interviews personally. Subjects were informed about the aim of study and questionnaires itself. Study was voluntary and anonymous.

Stratification according to security level in the penal-correctional facility was performed. Two groups were compared to each other: (1) prison officers from the facility of the closed type (in Banja Luka, Foča and Bijeljina) and (2) prison officers from facilities of the semi-open type (in Doboj, East Sarajevo and Trebinje). The main instrument of the study were questionnaires and participation that were voluntary and anonymous. A total of 330 questionnaires were completely filled with the response rate of 69.2%. Sociodemographic questionnaire contains the main data on the respondents: sex, age, marital status, living conditions, workplace, total work experience, experience on the current workplace, education, working time, night work, habits of alcohol or tobacco consumption, use of sedatives and other drugs.

For the assessment of stress sources on the work place questionnaires of the authors McCreary and Thompson were used: The Organisational Police Stress Questionnaire (PSQ.org) and The Operational Police Stress Questionnaire (PSQ.op).[15] PSQ.org contains 20 statements and is structured into three groups: (a) organisational stress sources in narrower sense (claims number: 1, 2, 3, 4, 5, 6, 7, 8, 11, 14, 18 and 19), (b) stress sources regarding work appreciation (claims number:

CONTENT

10, 12, 15, 16 and 17) and (c) logistic support (claims number: 9, 13 and 20). PSQ.op contains 20 claims with which stress sources related to direct prison officer's work and problems in social environment such as: night work, injury risk, health issues, social problems, social stigma and other, are assessed. Organisational and operative stress sources are rated with answers on numeric scales of Likert's type from 1 (not stressful) to 7 (very stressful).[15]

Statistical tests used are Kolmogorov-Smirnov test of normality of the distribution and χ^2 or Fisher's test for comparison. The significance of the difference was analysed with the Mann-Whitney-U-Test and Student's t-test. For the comparison of three groups of respondents, parameter ANOVA with post hoc analysis and non-parameter ANOVA (Kruskal-Wallis-Test) were used. As the correlation measure Spearman's coefficient was used. The importance level for all statistical analyses was 0.05 for statistically significant difference and 0.01 for highly significant statistical difference. Statistical package IBM SPSS 17.0 was used.

Results

Out of 477 penal-correctional police officers, 330 (69.2 %) were included in the study: Banja Luka 97/131 = 74.0 %, Foča 76/129 = 58.9 %, Bijeljina 47/51 = 92.2 %, Doboj 27/66 = 40.9 %, East Sarajevo 51/65 = 78.5 %, Trebinje 32/35 = 91.4 %. The study included 312 (94.5 %) men and 18 (5.5 %) women, with the average age of 38.3 ± 7.7 years (minimum 22 and maximum 62 years of age). (Table 1).

In prison facilities of the closed type there were 220 (67 %) employees and 110 (33 %) in facilities of the semi-open type. Average age of prison officers in facilities of closed type was 38.6 ± 7.8 and in semi-open it was 37.7 ± 7.3 years. Women more often worked in facilities of semi-open type ($p < 0.01$), while employees in closed facilities more often lived apart from their families ($p < 0.05$). Groups did not show any difference regarding other assessed parameters.

There are no significant differences between these two groups regarding the habits of alcohol, tobacco and sedative consumption, but there was a tendency for employees in facilities of closed type to use other drugs more often.

Prison officers in facilities of closed type longer commuted, their work experience on the current

Table 1: Main sociodemographic characteristics of respondents according to the type of penal-correctional facility

Assessed Characteristics	Closed Facilities	Semi-Open Facilities	p-value
Gender (% men)	97.3	89.1	p<0.01
Age (years: mean ± SD)	38.6 ± 7.8	37.7 ± 7.3	p=0.29
Marital status (%)			
single	19.1	15.5	
married	73.2	80	
living with partner	2.7	0.9	
divorced	4.1	1.8	
widower/widow	0.9	0.9	
living apart	0.0	0.9	p=0.38
Lifestyle (%)			
single	5.9	2.7	
family with children	60.5	71.8	
family without children	7.3	4.5	
single with children	0.9	0.9	
with parents	25.5	20	p=0.31
Separation (%)			
no	93.6	100	
during the week	3.6	0.0	
longer time	2.7	0.0	p=0.03
Education (%)			
apprenticeship	13.2	11.8	
high school	60.5	63.6	
college	4.1	5.5	
university	22.3	18.2	
post graduate degree	0.0	0.9	p=0.54
Total (N)	**220**	**110**	

Legend: Test: χ^2 – Chi-square test, except for age: Student's t-test;

Table 2: Main characteristics of respondents' jobs in two different types of penal-correctional facilities

Assessed Characteristics	Closed Facilities	Semi-Open Facilities	p-value
Working Time (%)			
full	10.5	9.1	
shift	11.9	15.5	
shift without weekends	0.5	0.9	
shift without weekends and night work	1.4	0.0	
shift of 12 h	75.8	74.5	p=0.62
Weekly overtime work (h)	3.8 ± 4.1	3.1 ± 3.6	p=0.08
Duration of commuting (h)	0.8 ± 1.3	0.5 ± 0.4	p=0.00
Total work experience (years)	16.1 ± 9.0	16.4 ± 8.4	p=0.13
Work experience on the current workplace (years)	8.0 ± 6.6	11.0 ± 5.9	p<0.01
Total shift work experience	12.3 ± 8.0	12.3 ± 7.1	p=0.91
Total (N)	**220**	**110**	

Legend: Test: Mann-Whitney U-test, except for Working time: Chi-square test (χ^2); h: hours

CONTENT

Table 3: Organisational stress sources on workplace depending on the type of penal- correctional facility

Organisational stressors	Facility Type	Mean value	SD	p (Mann-Whitney-U- test)
1 Dealing with co-workers	Closed Semi-open	3.1 2.7	1.8 1.6	0.03
2 The feeling that different rules apply to different people (eg, favouritism)	Closed Semi-open	4.7 3.9	5.2 1.7	0.02
3 Feeling like you always have to prove yourself to the organisation	Closed Semi-open	4.0 3.1	1.7 1.7	0.00 *
4 Excessive administrative duties	Closed Semi-open	3.4 2.8	1.7 1.6	0.00 *
5 Constant changes in policy/legislation	Closed Semi-open	2.8 3.6	1.6 2.0	0.00 *
6 Staff shortages	Closed Semi-open	3.5 3.1	1.9 1.8	0.08
7 Bureaucratic red tape	Closed Semi-open	3.4 2.6	1.8 1.5	0.00 *
8 Too much computer work	Closed Semi-open	2.2 1.8	1.5 1.2	0.03
9 Lack of training on new equipment	Closed Semi-open	2.9 2.5	1.8 1.7	0.07
10 Perceived pressure to volunteer free time	Closed Semi-open	4.3 3.4	1.9 1.9	0.00 *
11 Dealing with supervisors	Closed Semi-open	2.7 2.3	1.7 1.7	0.03
12 Inconsistent leadership style	Closed Semi-open	3.5 3.1	1.9 1.8	0.03
13 Lack of resources	Closed Semi-open	3.6 3.1	1.8 1.6	0.04
14 Unequal sharing of work responsibilities	Closed Semi-open	3.8 3.3	1.9 1.9	0.02
15 If you are sick or injured your co-workers seem to look down on you	Closed Semi-open	2.7 2.1	1.9 1.4	0.01
16 Leaders over-emphasise the negatives	Closed Semi-open	3.3 2.9	1.8 1.8	0.02
17 Internal investigations	Closed Semi-open	3.3 3.0	1.8 1.9	0.11
18 Dealing the court system	Closed Semi-open	2.6 2.5	1.7 1.7	0.39
19 The need to be accountable for doing your job	Closed Semi-open	3.3 3.1	1.8 1.9	0.31
20 Dealing the court system	Closed Semi-open	3.7 2.9	1.8 1.7	0.00*

*Legend: In order to avoid the first-level-error, the correction of the statistical importance level for multiple comparisons was used: p = 0.05/40=0.001, therefore *p < 0.001*

Table 4: Operative stress sources on workplace depending on the type of penal-correctional facility

Operative stressors	Facility Type	Mean value	SD	p (Mann-Whitney-U- test)
1 Shift work	Closed Semi-open	3.4 3.0	1.9 1.8	0.07
2 Working alone at night	Closed Semi-open	3.7 3.5	1.9 2.0	0.23
3 Over-time demands	Closed Semi-open	3.8 3.3	2.0 1.9	0.05
4 Risk of being injured on the job	Closed Semi-open	3.5 3.3	1.9 2.0	0.44
5 Work related activities on days off (e.g. court, community events)	Closed Semi-open	3.7 3.3	1.9 1.8	0.14
6 Traumatic events (e.g. death, injury)	Closed Semi-open	4.0 3.8	2.1 2.0	0.59
7 Managing your social life outside of work	Closed Semi-open	3.0 2.8	1.7 1.8	0.28
8 Not enough time available to spend with friends and family	Closed Semi-open	4.0 3.6	1.9 1.8	0.07
9 Paperwork	Closed Semi-open	3.7 2.8	1.9 1.6	0.00*
10 Eating healthy at work	Closed Semi-open	3.8 3.5	2.1 2.1	0.22
11 Finding time to stay in good physical condition	Closed Semi-open	3.5 2.8	1.7 1.8	0.00*
12 Fatigue (e.g. shift work, over-time)	Closed Semi-open	4.4 3.6	1.9 1.8	0.01
13 Occupation-related health issues (e.g. back pain)	Closed Semi-open	3.6 3.0	2.0 1.8	0.01
14 Lack of understanding from family and friends about your work	Closed Semi-open	3.2 2.6	1.8 1.6	0.01
15 Making friends outside the job	Closed Semi-open	2.7 2.1	1.8 1.7	0.00*
16 Upholding a "higher image" in public	Closed Semi-open	2.7 2.2	1.6 1.5	0.01
17 Negative comments from the public	Closed Semi-open	3.0 2.7	1.7 1.6	0.06
18 Limitations to your social life	Closed Semi-open	3.1 2.6	1.5 1.6	0.01
19 Feeling like you are always on the job	Closed Semi-open	3.8 2.9	2.0 1.8	0.00*
20 Friends / family feel the effects of the stigma associated with your job	Closed Semi-open	3.3 2.5	1.8 1.6	0.00*

*Legend: In order to avoid the first-level-error, the correction of the statistical importance level for multiple comparisons was used: p = 0.05/40=0.001, therefore *p < 0.001)*

workplace was on average by three years longer than that of employees in institutions of the semi-open type; the two groups did not show any other differences in regard to other job characteristics (Table 2).

In the facilities of closed type, both organisational and operative stressors were statistically significant ($p < 0.01$). Mean value for organisational stressors were 67.6 vs 57.3 (closed vs semi-open type), and for operative 69.8 vs 59.8 (Data not shown in the table).

Organisational stressors in narrower sense in facilities of closed type are in the domain moderate stressful (mean value 3.35) and in semi-open facilities in the domain not stressful (mean value 2.8). (Table 3). Highest mean value in the organisational stressors group was found for the stressor: "Feeling that different rules are applied for different persons, favouritism" (mean value = 4.7 and 3.9). A total of 19.5 % of prison officers in closed facilities and 9.1 % in the semi-open facilities have marked the question 10 ("Perceived pressure to volunteer free time") as the most stressful, that is with the grade 7 on Likert's scale. Operative stressors in facilities of closed type are in the domain moderate stressful (mean value 3.49) and in semi-open facilities in the domain not stressful (mean value 2.95) (Table 4). The highest mean value among respondents from closed facilities was found for the stressor: "fatigue, shift work, overtime work" (mean value = 4.4). Among prison officers from facilities of semi-open type, the highest mean value in the group of operative stressors was found for the stressor "traumatic event" (mean value = 3.8). A total of 19.5 % of the respondents in closed facilities and 16.4 % in semi-open facilities have marked the question 6 ("traumatic event") as the most stressful, that is with the grade 7 on Likert's scale.

Table 5. shows the correlation between the operative and organisational stress as dependent

CONTENT

Job Characteristics and sociodemographic variable	Stressor	Closed Facilities		Semi-Open Facilities	
		Spearmann's coefficient p	p	Spearmann's coefficient p	p
Total Work Experience	Organisational stressors	0.51	0.00	0.17	0.08
	Operational stressors	0.13	0.6	0.17	0.08
Work experience on the current workplace	Organisational stressors	0.13	0.06	0.03	0.73
	Operational stressors	0.12	0.03*	0.12	0.22
Working hours	Organisational stressors	-0.03	0.70	0.13	0.19
	Operational stressors	0.01	0.26	0.13	0.17
Age	Organisational stressors	0.15	0.02	0.12	0.20
	Operational stressors	0.14	0.04	0.11	0.25

Table 5: Correlation between age and job characteristics and perceived stress in closed and semi-open facilities

variables and the sociodemographic characteristics of respondents as independent variables (univariant analysis).

Sex, age, work experience, work experience in the current position did not have influence in facilities of semi-open type on operative stressors. Total work experience and age had a moderate positive influence on organisational stressors and age had a very low positive influence on operative stressors in facilities of closed type. Other variables that were examined did not corelate with stressors in both types of facilities.

Variant analysis has determined that there is a statistically significant difference between divorced and single respondents in closed facilities in regard to organisational stressors (p = 0.009) and in regard to operational stressrs (p = 0.045), as well as between the divorced and married respondents in regard to experience of organisational stressors (p = 0.02), the divorced experiencing higher stress.

Discussion

The results of this study have shown that prison officers in the Republic of Srpska are exposed to a larger number of professional stressors whose intensity was ranging from „moderate stressful" to „not stressful" and that sociodemographic characteristics did not significantly impact the perception of stress in the workplace. Moderate positive correlation in higher security facilities was found between the total work experience and organisational stressors and mild correlation between the age and the organisational stressors.

The results of this study have shown that in closed facilities divorced prison officers experience higher intensity of organisational and operative stress when compared to singles and higher intensity of organisational stress when compared to married participants. In semi-open facilities marital status did not have any influence on stress experience. Also, the research found that respondents who were separated from their families for a longer time experience higher stress intensity when compared to those not separated. In semi-open facilities it was found that prison officers with high alcohol consumption experienced higher stress intensity. Alcohol can be an attempt to "cure stress", but also the awardeness of excessive alcohol usage can also be a source of stress itself.[13, 14] No difference in stress experience was found in regard to education level, in both types of facilities. Most other studies, although not consistent, also show that there is no significant interconnection between the stress experience and sex, age, marital status, work experience and work satisfaction of employees in prison facilities.[4, 6, 13, 14] It should be noted though, that some researchers have found that female and older employees in prisons experience stress of higher intensity.[13, 14]

According to Morgan et al[16] prison staff in prisons of higher security experience stress of higher intensity, while Sudipto and Avdi[17] did not find that difference. This problem occupied many researchers, but the results are not consistent. In this research it was found that stress intensity was somewhat higher in closed facilities (higher

CONTENT

security), in the domain of "moderately stressful" and in semi-open facilities in the domain of "not stressful". In facilities of closed type there was a statistically much higher load both with organisational and operational stressors when compared to the semi-open facilities ($p < 0.01$, statistically highly important difference) was found. Besides that, operative stress sources are in both groups somewhat more represented than organisational, but this is of no statistical significance. The result of this study is in contrast to the studies from Europe and USA, which emphasise organisational stressors, that is stressors related to interpersonal relationships in the organisation.[7, 8] There are consistent findings in the literature that support from the management, from the organisation and from colleagues, role problems, ambiguous, unclear and conflict loaded roles, the conflict work-family and dangers are the most important sources of stress.[8, 10-12] In the study on stress with prison employees in Ireland, Regan stated that prison employees experienced significant stress in the workplace and predictors of stress were problems with the management, concern for own safety, overload, overtime work, work-family conflicts.[4]

In our country and in countries of Eastern Europe which have gone through economic, political and social reforms, the value system is somewhat different then in developed western countries, so that the perception of important work stressors is also different. Besides that, a pre-selection of candidates based on health demands of the workplace is done in the Republic of Srpska before employment and for police officers this is regulated in separate regulations, so this can also be a factor for the lesser experience of stress intensity in work. In this way some of the differences in the results and specifics regarding our prison facilities can be explained.

In the research which was conducted in 2009, in the prison facility East Sarajevo (facility of semi-open type), 55 % of prison officers have stated that they suffer from stress in their workplace and 41 % have stated that shift and overtime work are the most important stress sources in work.[18] These results are almost totally consistent with this research.

According to this research "fatigue due to overload, shift work, overtime work without compensation" is the most important stress source in facilities of closed type in the operative stressors group. In earlier research this stressor was marked as one of the most important stress sources, and its role remains important even today.[4, 19] In one English study overtime work, lack of support and appreciation were marked as significant stress sources in work.[20] According to the results of an Israeli study, the most important stressors of prison officers are overtime work without compensation, low wages and a difficult job.[21]

"Traumatic event" is the most important stress source in the operative stressors group in facilities of semi-open type. Traumatic events can be a physical attack on the prison officer, injury or death of other persons, suicide of inmates, prison riots, conflicts between prisoners, fear of contagious illness while contacting secretion of sick or infected inmates and similar. In USA there were 113 deaths at work among prison officers from 1999 to 2008, the death rate being 2.7/100,000 a year.22 According to the report of US Justice Department from 2000, non-fatal incidents on 1,000 prison officers are higher than in any other profession apart from police officers.[32] Lambert and Paoline have determined that perception of danger was the highest predictor of stress on work,[24] and fear of illness the second most powerful predictor.[25] The prison officers as well as the police officers confront the most violent, anti-social and problematic elements of the society and sometimes find themselves in life- threatening situations. This can lead to stress manifestations such as posttraumatic stress reactions, behavioural disorders, acute, chronic and permanent stress manifestations and health consequences. Prison staff working in prisons all over Texas showed that the perception of danger had the highest correlation with stress in work, while fear of sickness was in the second place.[26]

In this research the highest mean value in the organisational stressors group in prisons of closed and semi-open type was found for the stressor "favouritism". This finding is consistent with findings of other researchers.[11, 27] If in the same workplace the rules are not the same for all employees, the principle of justice is disturbed, and it comes to favouritism and not deserved privileges. This factor mirrors in situations when there is inequality in workload or in pay, then through the

CONTENT

experience of injustice or betrayal, when there is no reward or promotion to match the achievements. Over this dimension self-respect is shown and self-value is confirmed. Due to impairment of the possibility to show own's abilities and to get appreciation, promotion or reward for those, there comes to frustration and insatisfaction.[28] It is generally known that our society is suffering from corruption, nepotism and disturbed value system, which is a fertile ground for "injustice" and privileges in the workplace.

The advantage of this study is the representativity of the sample, which realistically describes the studied population. The study has a methodological limitation because it is designed as a cross-sectional study that shows the current state without changes in time and does not allow for conclusions on the direction of cause-and-effect connection. Apart from that, for the assessment of variables the method of self-report was used, which is subjective, and the possibility of wrong answers cannot be ruled out. According to the results of this research it is necessary to perform interventions for the prevention of professional stress with the population of prison officers.

Conclusion

According to the results, it can be concluded that in facilities of closed type both organisational and operative stressors were much more represented, while the operative stress sources were generally more represented in both groups. The results show their orientation towards lower values, from not stressful to moderately stressful. Sources regarding work appreciation are perceived with a higher intensity of organisational stress, then sources in regard to logistic support and stress sources related to interpersonal relationships in the organisation with the lowest intensity. In higher security facilities total work experience had a moderate positive influence on organisational stressors, and the age of respondents a mild positive effect. Divorced respondents experienced higher intensity of stress. Other sociodemographic factors did not influence stress. "Fatigue", "traumatic event" and "favouritism" were the most important stressors.

Acknowledgements

I thank to my daughter Ana Suljkanović for the help with the translation of this article into English.

Conflict of interest

None.

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CONTENT

Gajić et al. Scr Med 2020;51(3):174-80. DOI:10.5937/scriptamed51-27423
ORIGINAL ARTICLE

Efficiency of the Halliwick Concept in the Rehabilitation of Children With Cerebral Palsy

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Abstract

Background/Aim: Since 2010, the Halliwick Concept has been applied in the rehabilitation of children with neurological disorders at the Department of Habilitation and Rehabilitation of Children and Youth at the Institute for Physical Medicine and Rehabilitation (IPMR) "Dr Miroslav Zotović" in Banja Luka, the Republic of Srpska, Bosna and Herzegovina. Aim of this study was to analyse results of the implementation of the full Halliwick Concept programme over the period from 1 January 2016 to 1 January 2017 and points to the effectiveness of this type of hydrokinesitherapy.
Methods: After analysing 40 patients with different diagnoses of neurological disorders: cerebral palsy (CP), arthrogryposis (AG), Down syndrome (DS), and central nervous system (CNS) injury, only patients with CP were represented due to the homogeneity of the diagnosis and were included in analysis (N = 30). Rehabilitation treatment of patients according to the Halliwick concept was performed, over a period of one year, for 60 minutes once a week. Patients were tested by Swimming With Independent Measurement (SWIM) test, Gross Motor Function Measure (GMFM) and Barthel Index before and after treatment.
Results: Considering all patients, before and after the application of the concept of individual Halliwick swimming skills/movement in water, a statistically significant difference was detected by assessing the SWIM test and a highly significant difference was detected by evaluating the Gross motor function measure test of GMFM-66, as well as in evaluating the Barthel Index test. Assessment of swimming abilities through the SWIM test showed the least progression was in the ability in Getting out of the water, with the greatest progression in the ability in Breathing control.
Conslusion: The Halliwick Concept programme at the Department for the Habilitation and Rehabilitation of Children and Youth, IPMR "Dr Miroslav Zotović" in Banja Luka is effective and the results indicate the need for its application in the rehabilitation of children with CP.

Key words: Hydrokinesitherapy; Halliwick concept; Swimming; SWIM test; Cerebral palsy.

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ARTICLE INFO
Received: 7 July 2020
Revision received: 29 July 2020
Accepted: 31 July 2020

Introduction

In children with neurological disorders, kinesitherapy is the most important procedure in their rehabilitation. Early diagnosis and early initiation of treatment are very important due to better results. For the rehabilitation of movement disorders, it is important to determine the functional capacity of the musculoskeletal apparatus. Although there are various kinesitherapy methods, and they are the basis for the treatment of movement, coordination and movement disor-

This article should be cited as follows: Gajić D, Jokić S, Mraković B. Efficiency of the halliwick concept in the rehabilitation of children with cerebral palsy. Scr Med 2020;51(3):174-80.

ders, the most popular method is Bobath. More recent research indicates that the Bobath treatment is not overly better than other treatments in patients after stroke.[1]

In the literature related to medical rehabilitation of patients, the Halliwick concept is not present as a kinesitherapy method in general. Of the many approaches to the rehabilitation of children with special needs, the most important connection is between the Halliwick and Bobath concepts. The Halliwick concept is unofficially referred to as "Bobath in the Water". Both concepts began to develop in England in the middle of the last century, out of need for a different approach when working with children with neurodevelopmental and motor impairments.[2]

The Halliwick concept focuses on the biophysical principles of movement in water, especially the developmental sense of equilibrioception and fundamental stability. The Halliwick concept approaches teach all people in water activities, but it is especially focused on people with physical and/or mental disabilities, and their ability to move independently in water and, depending on ability, even to swim.[3] Kinesitherapy in combination with hydrokinesitherapy is the basic and most important form of treatment for children with CP, because the exercises are safe, simple and fun for them, indicating that the Halliwick concept is essential in their rehabilitation.[4]

In order to ensure long-term gross motor function improvement in children with CP, Ballington and Naidoo indicated that water-based programmes should be integrated and considered as an essential, ongoing mode of treatment for these children together with conventional therapies.[5] Researchers Christodoulaki et al claimed that water-based programmes in therapy of children with CP produced positive effects on children's respiratory system.[6]

Iranian researchers Khalaji et al published a comprehensive review of 33 research papers published from 2000 to 2016 on the results of hydrotherapy on patients with CP and concluded that this therapy, when applied in conjunction with other conventional therapies, has positive effects in all areas of the International classification of functioning, disability and health.[7]

The Halliwick concept improved motor function in children with CP and autism in swimming and gross motor skills depending on the individual condition of the patient.[8] Meyer et al concluded that for children with cerebral palsy, Halliwick therapy provided significant results in a global reduction in spasticity.[9]

Researcher Sršen, showed that the Swimming with Independent Measurement (SWIM) test was sensitive to changes in swimming abilities in patients.[10] Progress was noted in all patients tested. The SWIM test provides a detailed analysis of the swimmer's ability and allows accurate preparation of further activities for the purpose of the swimmer's progress. In preparation planning, it is essential to choose the tasks and activities that follow in order of difficulty.[10]

After the swimming program there is statistically significant effect on walk improvement, running and jumping as well as on the overall gross motor function of children with CP. Also, the statistically significant impact is on the increase of mental adaptation to the water environment, ability to move independently in water and ability of these children to swim.[11]

Researcher Tripp shown first clinical trial of aqueous physiotherapy in post-acute stroke patients in a hospital setting. They showed greater improvements in postural stability and functional walking ability after two weeks of intervention compared to conventional treatment. Further studies indicate that larger sample sizes and longer duration of therapy are necessary.[12]

In a case study from the University of Central England, the assessment was performed by SWIM and the Gross motor function measure (GMFM) tests and the therapy led to improvements in balance and dynamic systems in theoretical frameworks. Additional benefits of swimming included improved ability to maintain an appropriate level of fitness, integration with peer group and increased confidence.[13]

Since 2010, the Halliwick concept has been applied within the rehabilitation of children with neurological disorders at the Department of Habilitation and Rehabilitation of Children and Youth at the IPMR "Dr Miroslav Zotović" in Ban-

CONTENT

ja Luka. The aim of this research was to present a retrospective analysis of the efficiency of the complete Halliwick concept program within the rehabilitation treatment of children with CP treated at the Department of Habilitation and Rehabilitation of Children and Youth at the IPMR "Dr Miroslav Zotović" in Banja Luka, during the period from 1 January 2016 to 1 January 2017.

Methods

Rehabilitation with the Halliwick concept was carried out for years on the same patients (children with neurological disorders) and five years after the start of the concept, in 2016, was chosen as a reference at the Department of Habilitation and Rehabilitation of Children and Youth at the Institute for Physical Medicine and Rehabilitation (IPMR) "Dr Miroslav Zotović" in Banja Luka, the Republic of Srpska, Bosna and Herzegovina. The efficiency of this concept on children with CP, who were treated in the mentioned institution was analysed. After analysing 40 patients with different diagnoses of neurological disorders: CP, arthrogryposis (AG), Down syndrome (DS), and central nervous system (CNS) injury, only patients with CP were represented due to the homogeneity of the diagnosis, and were included in analysis. The approval of the Ethics Committee of the IPMR "Dr Miroslav Zotović" Banja Luka for rehabilitation of patients as well as for analysis of data was obtained.

Rehabilitation treatment of patients according to the Halliwick concept was performed, over a period of one year, for 60 minutes once a week.

During this period, 30 patients with CP were examined (10 patients in 3 groups each, groups formed according to the characteristics of the patients who were at that moment registered for the mentioned rehabilitation and the maximum number of therapists - instructors working with the patients in the pool). The concept is performed by “one-on-one and all together”. Age and gender of patients was recorded.

Upon admission to the aforementioned ward, patients underwent testing to determine their initial condition, prior to enrolment in rehabilitation treatment. All patients who were presented in this study were enrolled in kinesitherapy before the Halliwick concept of rehabilitation.

GMFM and Barthel index tests were performed in all patients prior to the application of the Halliwick concept and considered relevant for the initial condition of the patient, while for the SWIM test therapist / instructor derived input parameters at the beginning of the application. In this research the greatest attention was paid to the SWIM test as the only instrument for evaluating progress in the water movement, which is also a major rehabilitation feature of the Halliwick concept.

The SWIM test is intended to be a tool for assessing swimming abilities and analysis of movement in water of children with special needs and includes 11 abilities and movements, which are an integral part of the Halliwick Concept Ten Points program. Each movement has well-defined criteria that assess the ability in the range of 0 to 7 points. The maximum score can be 77 points.14

The test includes the following abilities / movement in water:

A. Entering the water (with or without help)
B. Adaptation to water (movement with full support to independent movement in water)
C. Respiratory control (ability to exhale air over water to control breathing underwater)
D. Balance (from full support to self-sustaining floating position on the back in turbulent water)
E. Transversal rotation back (occupying a back-float position from full support to self-rotation)
F. Transversal rotation forward (from full support to self-rotation)
G. Sagittal rotation (from full support to self-rotation)
H. Longitudinal rotation (from full support to independent rotation)
I. Combined rotation (from full support to independent rotation)
J. Swimming style development (from skating with support to solo swimming)
K. Getting out of the water (with or without help)

The Halliwick concept was introduced to promote joyful movements in water and swimming. To assess the swimming skills and advancement of an individual swimmer, in the Halliwick concept, SWIM was used as a valid and reliable mea-

CONTENT

sure.[15] SWIM is an assessment tool and it is used to monitor swimmer progress as well as for research purposes.[16]

GMFM is a standardised instrument for measure a gross motor function in children with CP. The GMFM-88 test evaluates 88 motor function items in five areas: a) lying and turning, b) crawling and kneeling, c) sitting, d) standing, e) walking, running and jumping and is applicable to children with DS and code lesion of the central nervous system, while GMFM-66 was modified from 88-items and used to assess the ability of children with CP. All items are usually achieved in children 5 years of age with normal motor skills.[17] Higher overall score, in GMFM-66 is indicating better motor function.

The Barthel Index monitors functional independence before and after treatment and the level of care required. It is intended for patients with long-term recovery but is also used as an evaluation measure. The Barthel Index can also be used for clinical assessment of function change as well as for research purposes. It is applicable to patients with neurological disorders.[18] The test contains 10 variables / items that have been assigned a certain number of points, depending on the level and rank of the variable, which describe the activities of daily living and patient mobility. Higher scores are associated with a greater likelihood that a patient will be able to live in a home with a degree of independence after discharge from hospital. In determining the allocation of points for each item, the amount of time and physical assistance required to complete each item is required.

Comparative analysis of the results of patients with CP before and after the application of the Halliwick concept for the individual SWIM test swimming ability and the total scores of the tests SWIM, GMFM-66 and Barthel index was performed. Results of tests were presented with total number of points (score) of the SWIM test for all patients, individually, and for group of all patients in statistical parameters of minimum, maximum, median, arithmetic mean, standard deviation and statistical significance, using the program IBM SPSS, version 21.0.

Results

Data for thirty patients was analysed. There were 20 male and 10 female patients, from 5 to 18 years of age.

Patients were rehabilitated from year to year, and data was analysed for 2016. Total number of points, scored on the SWIM test, before and after the application of the Haliwick concept for one year is shown in Figure 1.

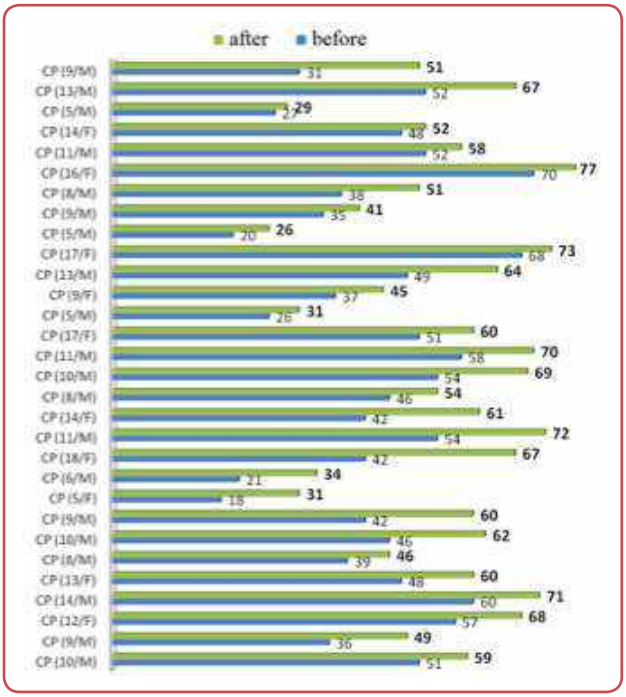

Figure 1. The total number of points (score) of the SWIM test before and after the application of the Halliwick concept program in all patients

Following the median value, which is used in the non-parametric tests, ability to Get out of the water (3.00) was lowest in individual swimming abilities in the SWIM test in patients with CP before applying the Halliwick concept. Also, same parameter remained at the same level after the application of the Halliwick concept (3.00). Highest ability of all individual abilities was Breathing control, before applying the Haliiwick concept amounted to 5.50, and after applying the Halliwick concept median was 7.00, which is shown in Table 1 and 2.

Comparison of the values of the medians and arithmetic means for the total score for the SWIM test before and after the Halliwick concept for CP patients (Table 1 and 2), showed the median in-

CONTENT

Group		Entering the water _SWIM	Adaptation to water _SWIM	Entering the water _SWIM	Breathing control _SWIM	Balance _SWIM	Transversal rotations back _SWIM	Transversal rotations forward _SWIM	Sagittal rotation _SWIM	Longitudinal rotation _SWIM	Swimming skills _SWIM	Getting out of the water _SWIM	SCORE_before_SWIM
	Minimum	2	2	2	2	2	2	2	2	2	2	2	2
CP	Maximum	7	7	7	7	7	7	7	7	7	7	7	7
N	Median	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
30	Mean	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
	Std. Deviation	1.339	1.339	1.339	1.339	1.339	1.339	1.339	1.339	1.339	1.339	1.339	1.339

Table 1: Values of each swim ability and the total score in the SWIM test before Halliwick swimming therapy

crease of 13.5 (22.69%), and mean increase of 11.34, or 20.52 %.

Comparison of the values of the medians and arithmetic means for the total score for the GMFM-66 test before and after the Halliwick concept, Table 3, shows that there is no change in the median for CP patients, whereas for the arithmetic mean an increase of only 0.50 or 0.87 %.

Comparison of the values of the medians and arithmetic means for the total score for the Barthel index test before and after applying the Halliwick concept, Table 3, showed that there is a slight change for patients with CP for a median of 0.50 points, or 0.68 for the arithmetic mean.

Using the Wilcoxon Rank Test, Table 4, a highly statistically significant difference was detected

Group		Entering the water _SWIM	Adaptation to water _SWIM	Breathing control _SWIM	Balance _SWIM	Transversal rotations back _SWIM	Transversal rotations forward _SWIM	Sagittal rotation _SWIM	Longitudinal rotation _SWIM	Combined rotation _SWIM	Swimming skills _SWIM	Getting out of the water _SWIM	SCORE_before_SWIM
CP	Minimum	2	2	2	2	2	2	2	21	2	0	1	18
N	Maximum	7	7	7	7	7	7	7	6	6	5	4	70
30	Median	4.00	5.00	5.50	4.00	4.00	4.00	4.00	4.00	4.00	4.00	3.00	46.00
	Mean	4.00	4.73	5.20	4.50	4.37	4.13	4.00	3.57	3.63	3.30	2.50	43.93
	Std. Deviation	1.339	1.258	1.243	1.432	1.474	1.456	1.414	1.223	1.159	1.393	0.861	13.383

Table 1: Values of each swim ability and the total score in the SWIM test before Halliwick swimming therapy

Group		Entering the water _SWIM	Adaptation to water _SWIM	Breathing control _SWIM	Balance _SWIM	Transversal rotations back _SWIM	Transversal rotations forward _SWIM	Sagittal rotation _SWIM	Longitudinal rotation _SWIM	Combined rotation _SWIM	Swimming skills _SWIM	Getting out of the water _SWIM	SCORE_before_SWIM
CP	Minimum	2	3	4	2	2	2	2	3	3	1	1	26
N	Maximum	7	7	7	7	7	7	7	7	7	7	7	77
30	Median	5.00	6.00	7.00	5.00	5.00	5.00	5.00	6.00	6.00	4.00	3.00	59.50
	Mean	4.60	5.80	6.57	5.00	5.00	4.70	4.57	5.67	5.73	4.07	3.30	55.27
	Std. Deviation	1.522	1.215	0.898	1.554	1.554	1.535	1.547	1.348	1.337	1.701	1.317	14.455

Table 2: Values of each swim ability and the total score in the SWIM test after Halliwick swimming therapy

CONTENT

Group		before_GMFM_66	after_GMFM_66	before_Barthel_index	after_Barthel_index
CP	Minimum	21.25	22.35	25	25
N	Maximum	89.07	90.26	99	99
30	Median	59.9200	59.9200	73.00	73.50
	Mean	57.0107	57.5143	72.03	72.43
	Std. Deviation	14.70908	15.04824	20.853	21.066

Table 3: Values of the total score in the GMFM and Barthel index test before and after Halliwick swimming therapy

Group	Entering the water	Adaptation to water	Breathing control	Balance	Transversal rotations back	Transversal rotations forward	Sagittal rotation	Longitudinal rotation	Combined rotation	Swimming skills	Getting out of the water	SCORE SWIM	GMFM-66	Barthel index**
CP N 30	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.001**	0.000**	0.000**	0.000**	0.000**	0.000**	0.003**	0.006**

Table 4: The significance of changes in swimming skills and the total score according to the SWIM test, GMFM-66 and Barthel index test /Wilcoxon's Rank Test/

before and after applying the Halliwick concept of all individual swimming abilities / movement in water by evaluating the SWIM test in patients with CP ($p < 0.001$** for all individual swimming abilities, and for sagittal rotation with exactly $p = 0.001$**).

Looking at all patients with CP there were statistically significant difference before and after the application of the concept of individual Halliwick swimming skills / movement in the water SWIM assessment test, as well as the total number of points / SCORE.

In patients with CP, a highly statistically significant difference was, before and after applying the Halliwick concept, when evaluating the gross physical (motor) ability test of GMFM-66 ($p = 0.003$**) and estimating by the Barthel index test ($p = 0.006$**), Table 4.

Discussion

Assessment of swimming abilities through the SWIM test showed, in the entire sample of 30 patients before and after applying the Halliwick concept, the least progression was in the ability to Getting out of the water, with the greatest progression in the ability to Breathing control, as it was also confirmed by other research.[4, 6]

The success of the application of the Halliwick concept is shown by the overall results. The average total score (following the median value for the non-parametric tests) for swimming ability assessment/movement analysis in water - SWIM before applying the Halliwick concept for patients with CP was 46.00, and after applying the concept it was 59.50. In the whole sample of 30 patients the median increased after applying the Halliwick concept program by of 13.50 points (22.68 %) for the median, while 11.34 (20.51 %) for the arithmetic mean.
Results showed that in CP, but also in other neurological disorders there was progression, but that it is not uniform, due to the different sub-classification within their primary diagnosis, and it is also manifested differently on their swimming skills after applying Halliwick concept, which was confirmed mostly by other research as well.[5, 7, 8, 11]

Using Wilcoxon Rank Test, with the SPSS, considering all patients, statistically significant difference ($p < 0.001$**) was detected before and after the application for the concept of individual Halliwick swimming skills/movement in water in total score on SWIM Test. Looking at all patients, a highly statistically significant difference was detected before and after applying the Halliwick concept by evaluating the Gross motor function measure test of GMFM-66 ($p = 0.003$**), which were confirmed by other studies.[5, 11] Highly significant difference was detected before and after applying the Halliwick concept by assessing the Barthel index test ($p = 0.006$**) which were not analysed by other studies.

CONTENT

Conclusion

In children with CP, the Halliwick concept adapts to the physical and mental abilities of the person and the results of the improvement are individual. According to the data from this study, the assessment of swimming abilities after application of concept is individual. Patients regardless of gender and age, within the same diagnosis, have different results.

The research confirmed most of the conclusions of other studies, that the application of the concept of the Halliwick program leads to the improvement of the patient's condition in certain swimming abilities. The overall results show that it is necessary to use this presented hydrokinesitherapy in the rehabilitation of patients with CP, because even a small improvement in any ability in such patients is of great importance.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Skrepnik. Scr Med 2020;51(3):181-9. DOI:10.5937/scriptamed51-28263
REVIEW ARTICLE

Therapies for Osteoarthritis Today and Tomorrow – Review

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ARTICLE INFO
Received: 3 September 2020
Revision received: 15 September 2020
Accepted: 16 September 2020

Abstract

Osteoarthritis is a common human disease with well understood pathophysiology, signs and symptoms, prevalence, risk factors, pain, and suffering with great understanding of personal, economic and social effects around the world. There are no drugs or treatments considered "disease modifying", with symptomatic control aiming to stave off the final solution of total joint replacement. Regenerative medicine and use of mesenchymal stem cells (MSC) promised hope to change that but have so far fallen short. This review focuses on current knowledge and use of MSC in clinic, completed research, and future directions for development of this once so promising biological treatment. Powerful treatment for pain in form of monoclonal antibodies against Nerve Growth Factor (NGF) are getting close to FDA approval in the US. Wnt signalling pathway modulators that decrease inflammation, increase function and potential to regenerate cartilage should be presented to the FDA early next year.

Key words: Osteoarthritis; Mesenchymal stem cells; Wnt pathway; NGF antibodies.

Introduction

Osteoarthritis (OA) is the most common form of joint disease worldwide. It effects 1 in 3 US adults according to Arthritis Foundation (www.arthritis.org) with radiographic evidence in more than 50 % of people over 65 years old and more than 80% at 75 years old. Symptomatic OA of the knee occurs in about 11% of people over 64 years old. By 2030, it is estimated about 20 % of the US population will be aged over 65 years.

For years, OA was labelled as "degenerative "disease; progressive loss of articular cartilage, remodelling and hypertrophy of bone, bone cysts formation and osteophyte development. Today, we have a much better understanding OA pathophysiology, explained as a "downward spiral" where cartilage degeneration is caused by injury, inflammation or metabolic defect, depletion of proteoglycans with compromised collagen ultrastructure and attempted repair by chondrocytes with increased proteoglycan (PGs) and collagen production. At the end, an increase in matrix metalloproteinases (MMPs), cartilage degrading enzymes and inflammatory cytokines all contribute to cartilage breakdown.[1] Chondrocyte apoptosis leads to further cartilage degradation with decreased concentration and viscosity of synovial fluid. Risk factors for OA are well described and classified in major categories as: (1) demographic (age, genetics, systemic factors like obesity), (2) biomechanical (trauma/injury, overload, instability) and (3) biochemical (cytokines, MMP's, PG's). All play a role in OA severity.[2] Even today, it is difficult to explain origins of pain associated with OA, as the mechanism is unclear and its presence does not consistently correlate with imaging studies. It appears many factors contribute to pain sensation including soft tissue damage, joint capsule (stretch), synovial membrane (synovitis), periarticular bursitis, tendinitis, muscle spasm,

This article should be cited as follows: Skrepnik N. Therapies for osteoarthritis today and tomorrow – review. Scr Med 2020;51(3):181-9.

ligament involvement, periosteum stretching, subchondral bone, osteophytes formation, microfracture presence, increased intra-osseous pressure, etc.[3]

Many studies confirm that OA has profound personal, economic, and social impacts in around the world. According to the US Center for Disease Control (CDC) in 2013 the total national arthritis attributable medical care cost and earning losses among adults with OA was $303.5 billion or 1 % of the 2013 US Gross Domestic Product (GDP).[4] Effecting more than 32.5 million adults in US,[5] it is also among most expensive conditions to treat when surgery is required. In fact, OA was the second most costly health condition treated at US hospitals in 2013.6 In that year, it accounted for $16.5 billion or 4.3 % of the combined cost for all hospitalisations.[6]

OA management guidelines have recently been revised and adopted by the American College of Rheumatology and Clinical Consensus Group of Orthopaedic Surgeons to reflect this "New Paradigm." After diagnosis of OA, a patient could be treated with non-pharmacologic therapy, simple analgesics or over the counter NSAID's, Rx NSAID's, and/or intra-articular (IA) corticosteroids all prior to surgical intervention. Even more interesting is a comparison between AAOS, ACR, OARSI[7] for US patient and what treatments are recommended vs. inconclusive evidence vs. not recommended. American Academy of Orthopaedic surgeons (AAOS) recommends only topical NSAID's, oral NSAIDs and Tramadol. Inconclusive evidence for acetaminophen, non-tramadol opioids and IA injections of corticosteroids (CS) and platelets rich plasma (PRP). Even IA injection of hyaluronic acid (HA) is not recommended together with Chondroitin and Glucosamine supplements.[7] Also, there are calls for use of IA corticosteroids to be reconsidered.[7]

The Cochrane report by Juni at all.[8] generates some controversy about the use of this most common treatment for OA even with limited evidence of efficacy. Cheap to purchase and administer, and approved by all insurance companies, the risk of corticosteroids may out-weight the benefits. However, until alternate treatments are approved it will remain a first-line intra-articular therapy used by physicians. Prospective randomised clinical trials did not find evidence[8] that IA HA is any better than IA CS despite fewer side effects. There was no evidence for AAOS to recommend IA HA for standard treatment. So, it is fair to say that there is no adequate therapy to offer once patient goes through topical and Rx NSAIDS, injections with limited evidence of efficacy and questionable safety for the patients not ready for total joint replacement. That opened the door for more research with regenerative medicine options in last decade. Prolotherapy, PRP, APS (Autologous Protein Solution) and especially stem cell injections took centre stage in ortho research and use. Also, the search is expanding for better pain control (Anti NGF MoAb) and injectable molecules with the potential to decrease inflammation and move MSC from bone metabolism to the cartilage area and repair defect (Wnt). All of them will be presented briefly in this paper with special focus on MSC potential, research done so far, use and presented evidence to clarify what the current standpoint is and what following research should be done in order to solve the "holy grail" of orthopaedics and human locomotor system - a "disease modifying agent," able to slow down the process of OA and potentially reverse damage of cartilage degradation, restoring new cartilage, decreasing inflammation, eliminating pain and increasing function.

Autologous Mesenchymal Stem Cell

Since a landmark MSC publication[10] in 2008 highlighting their potential to facilitate musculoskeletal repair by binding to the injury site and secreting large amounts of bioactive immunomodulating and trophic factors rather than differentiating into target tissue, many physicians implemented them in clinics and data began accumulating. What is known today and what is the standpoint with the research already done and recommendations for clinical use? In order to answer that question, manuscripts of randomised clinical trials (RCT) and recent review papers were reviewed.

One of the most frequently cited works from 2014[11] is a proof of concept clinical trial from S. Korea (Jo at al) that enrolled 18 patients; study was dose ranging with different number of cells injected IA (low dose, mid dose and high dose) and no placebo or any control with clear conclusion that more research needs to be done with randomized clinical trials (RTC), more consistency with cell isolation (techniques, sites, preparation etc.) and use of controls.

Quickly after deploying stem cells for OA treatment, some technical issues became apparent. Different techniques of preparation and manipulation damaged the cells and caused dissemination to non-target tissues. To minimise those issues, injectors sought different injectable vehi-

CONTENT

cles as containment systems to provide a better microenvironment for injected cells. Roffi et al[12] reviewed 40 studies (19 preclinical and 21 clinical trials) with platelet-rich plasma (PRP), hyaluronic acid (HA) and hydrogels to help delivery and function of MSC. Even though the authors reported negligible adverse events and promising clinical outcomes, the prevalence of low-quality studies prevented demonstration of benefit, calling for studies designed to more clearly demonstrate possible improved outcomes.

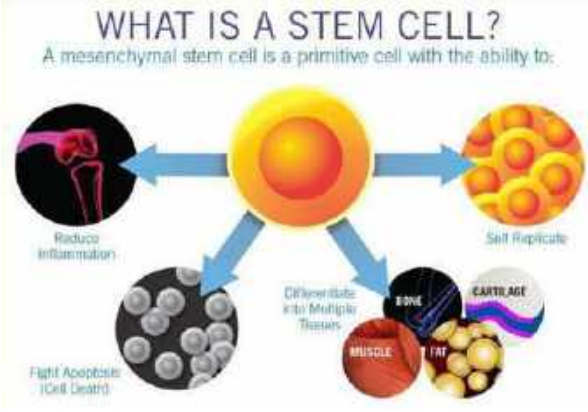
In 2019, Kim[13] published a review article covering five RCTs with 220 total patients. This meta-analysis demonstrated that IA MSC have limited evidence of pain relief and functional improvement in knee OA. It does not support the use of intra-articular MCSs for improving cartilage repair in knee OA.

In order to establish "standardised" injections, an effort was made to use "minimal manipulation" methods to increase the use of MSC in orthopaedic practice. Di Matteo et al[14] published a review article in 2019 to assess clinical applications of "minimally manipulated" MSC from either bone marrow (BMAC) or as stromal vascular fraction (SVF). Twenty-three papers were included in final analysis; only 4 were randomised clinical trials (RCT). They reported overall poor quality of the studies reviewed. Despite evidence of clinical safety in minimally manipulated MSC and the short term positive clinical outcomes, clinicians reported varying collection, preparation, and administration methods of MSC preventing any recommendation on the use of either product in clinical practice.

Contradictions surrounding the term "Mesenchymal Stem Cells "(MSC) are nothing new. It is fair to say since the early 2000s, various populations of these cells in the human body have been subject of controversy; origins, developmental potential, biological function, possible therapeutic uses, and even the name MSC itself[16] have the subject of debate (Figure 1).

Cursory literature search reveals over 3,000 research articles in just the last 5 years with MSC derived from bone marrow, adipose and umbilical tissue with capacity for self-renewal and differentiation in the chondrocyte lineage (Figure 2).

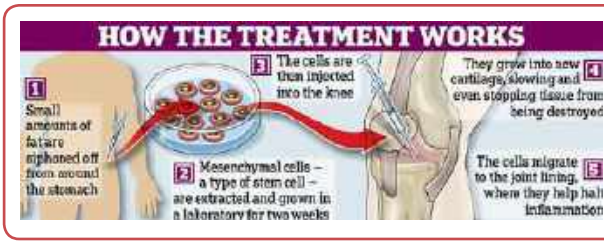
Sipp et al. 2018 explicitly asked to "clear up this stem-cell mess", claiming that wildly varying reports have helped MSCs to acquire a near-magical "all-things- to- all- people" quality in the media



http://www.regenorthopedics.com/wp-content/uploads/2014/09/Regen-Stem-Cell-Chart1-1200.jpg
Web based picture, published online, downloaded for this publication.

Figure 1: What is a stem cell?

and in the public mind. He elaborated that hype was easy to exploit, anointing it a go-to cell type for many unproven stem-cell interventions. The same authors stated "In most cases, rigorous preclinical studies of these cells are limited or non-existing."[16] In another editorial publication



https://nsistemcell.com/wp-content/uploads/2017/06/osteoarthritis.jpg
Web based picture, published online, downloaded for this publication.
Proposed mechanism of action for stem cells injected in osteoarthritic joint.

Figure 2: Explanation how the treatment with MSC works for OA of the knee

in 2018 "Emerging stem cell ethics,"[17] a different array of bioethical debates and issues triggered by stem cells are reviewed. Rushing new commercial MSC products into market, off label use and direct -to-consumer marketing of unproven therapeutic benefits of MSCs are all touched upon. Other big issues include safety and efficacy of fast-tracked product and financial support for post-market efficacy data collection and testing.

How big is the stem cell market? Who is paying for these injections, and how much? In 2018 Piuzzi et al.[18] published an interesting article about clinics in the US offering MSCs treatment for knee OA. All centres reported "good results" and "symptomatic improvement" in 82.2% of patients. Average cost was $5,156 ($1,150 - $12,000) based on a review of 273 US based treatment centres. It is difficult to explain and understand these numbers knowing direct cost for the centre, time, people and exper-

CONTENT

tise needed to perform injection vary widely. For comparison, sometimes a total knee replacement surgery (involving surgery, prosthesis, anaesthesia, hospital stay etc.) is less costly than MSC based interventions. The same group of authors reviewed 420 reports, with only 6 studies offering evidence level III or lower, suggesting some positive results and modest clinical improvement that could not rule out placebo effect.

Most published articles share a commonality; IA injections of MSC resuspended in saline (2.5 ml up to 8 ml of saline and different number of cells). Reviewing literature regarding saline injections for knee OA finds 2 reviews published by Altman et al[23] in 2016 and Saltzman et al[24] reporting all investigators have long suspected saline is not really "placebo," but rather a "comparator" due to respectable efficacy as a stand-alone intervention. Altman reported that a review of 38 eligible RCTs, IA saline was able to provide significant improvement in short-term knee pain (3 months) in 32 studies totalling 1,705 patients. Even long-term (6-12 months) knee pain was significantly reduced following IA injection of saline in 19 studies (1,445 patients) with no SAE's (serious adverse events) related to saline. Similar results are published by Saltzman et al.[24] in September 2017. 14 cohorts in 13 studies totalling 1,076 patients with KL grade 1 - 4, VAS and WOMAC met all inclusion criteria for enrolment. At 3 months there was significant improvement in VAS score, WOMAC approached that but did not reach statistical significance. At 6 months, both VAS and WOMAC total scores were significantly improved (statistically and clinically significant) in comparison to pre-injection values. The hypothesis that these "placebo" injections have therapeutic effect has been quantified in RCTs with active treatment group like HA (hyaluronic acid). Since almost all stem cells are resuspended in saline, it is not a surprise to see decreases in VAS and WOMAC at short and long duration follow-up.

Presented articles and reviews are from 2000 to 2019. Efforts were made to research 2020 publications to find more better designed studies for knee OA. Unfortunately, Vasiliadis et al[22] reported a review of 8 articles with varying OA grades and different scales of assessment (KL grading, IKDC). In KL grading studies, all grades were involved from KL 1 to KL 4, two studies have control without treatment, but received analgesia, weight management, exercise and injection of saline. Two studies used SVFT (stromal vascular fraction, 19 patients total) and 6 used cultured AD-MSC involving 96 patients. AD-MSC studies required culturing the cells before IA injection and reports are very different about the length of cultures (1 week, 3 weeks up to 6 weeks). The number of cells injected IA differed too, ranging from 5 million to 100 million per injection. Half of the studies used ultrasound for injection, half did not. Four studies reported using adipose tissue from the abdomen, while 2 studies used tissue from the thigh, flanks and abdomen and 2 studies did not even report where they obtain adipose tissue.

Arshi et al[15] published an interesting review article including a brief scientific stem cell overview, preclinical data and animal research, use of implantable scaffolds to enhance chondrogenesis and incorporation in cartilage defect of MSCs. They ultimately concluded that "extreme diversity in methodologies and therapeutics used in these studies obviates the need to higher quality study design to have reliable external validity into clinical application." Future directions are clean and clear: calling for RCT with control group, well powered, with long follow up, specific primary and secondary endpoints and adequate imaging (x-rays and MRIs). Also of note, regulatory efforts in this field are not easy to establish and enforce.[20,21] The American Academy of Orthopaedic Surgeons (AAOS) and National Institute of Health (NIH) had issued a statement of minimal standards for product development and clinical research for valid safety and efficacy data collection and ethical responsibility to patients.[19] Their great concern was that "misrepresentation of uncharacterised and unproven minimally manipulated products as stem cells may erode public trust and compromise development of legitimate cell therapies." Many professional organizations like the National Academy for Science, International Society for Cellular Therapy, the American Association for the Advancement of Science together with AAOS and NIH joined the consensus statement recognising the potential value of the cell therapies and the risk that current environment may erode public trust and investment needed to bring legitimate cellular and biological therapies to the patients. Recommendations include: define terminology to clearly distinguish uncharacterised minimally manipulated autologous cell products from rigorously characterised and culture-expanded and purified stem cell and progenitor cell population, standardise reporting requirements, establish registries for post-market monitoring and quality assessments of biologic therapies, and four additional tasks.[19] When implemented, these recommendations can create difference in designing and reporting results from RCT.

CONTENT

The Arthroscopy Association of Canada also issued a position statement on intra-articular injections for knee osteoarthritis in 2019, as follows.[25]

Corticosteroids (CS)

After a detailed literature review, recognising that AAOS found inconclusive evidence to recommend for or against IA steroid for knee OA, this Canadian association recommends their use based on short term moderate pain relief and restoration of function with good cost efficacy in patients with early OA. Grade A.

Hyaluronic acid (HA)

Numerous RCTs were reviewed with significant heterogenicity in trial designs, preparations, data collection and analysis of outcomes measured. However, a recommendation was given stating improved pain relief after IA HMW HA (high molecular weight HA) and restoration of function compared with placebo and can be helpful in patients with mild to moderate OA of the knee. Grade A.

Platelet Rich Plasma (PRP)

PRP received grade C, meaning conflicting or poor-quality evidence (level 4 or 5) not allowing a clean recommendation for or against an intervention. Studies did show the potential to relieve pain and improved physical function up to 1 year after injection in the knee with mild to moderate OA. There is no evidence of efficacy in more advanced OA like KL grade 4. Until further high-quality studies become available it is not possible to recommend for or against.

Cellular based BMAC

Grade I – insufficient evidence to support use of MSCs or BMAC in the treatment of OA of the knee and recommended that they should be limited to registered controlled clinical trials and did not recommend their use in routine medical practice until further evidence becomes available.

Search for new therapies

It is obvious that well evidenced non-surgical interventions are not possible currently to offer to patients suffering from osteoarthritis. Only topical and oral NSAIDs and Tramadol are recommended by AAOS. No recommendation for or against Acetaminophen, non-tramadol opioids and IA-corticosteroids or IA PRP. IA HA is not recommended, neither are glucosamine or chondroitin. Once a patient stops responding to above mentioned treatments, surgery is the final solution. Since many patients cannot take NSAIDs (bleeding issues and other side effects), acetaminophen has potential liver toxicity with high dose and long-term use, opioids are not recommended for long-term use in chronic diseases like OA, surgery is often the only options. However, some help may appear relatively soon for pain control and via the first "disease modifying "agent.

Anti NGF monoclonal antibodies (MoAb)

Nerve Growth Factor (NGF), a member of the neurothrophin family was discovered in the 1950's [26] and plays a critical role in normal development of sympathetic neurons as well as sensory neurons responsible for nociception and temperature sensation. There are many studies with additional evidence that NGF receptors play a role in pain propagation.[27-30] The mechanism by which NGF may impact pain remains under investigation (Figures 3 and 4).[29-32]

One of the first publications about fasinumab Tiseo et al was published in 2014 with a double-blind, placebo-controlled exploratory study in the OA of the knee.[33] All 3 doses of fasinumab significantly decreased pain in the study knee and WOMAC total and subscale scores.

In 2015, Schnitzer et al[34] published a systematic review of the efficacy and safety of antibodies to NGF in the treatment of OA of the hip and knee.

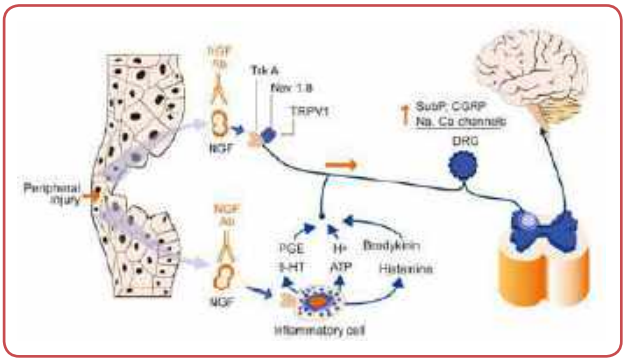


Figure 3: NGF mechanism of action after tissue injury, inflammation and chronic pain conditions

Neurotransmitters, receptors and ion channels get modulated and upregulated by nerve growth factor (NGF) binding to Tropomyosin-related kinase A receptor (TrkA) primary afferent sensory nerve fibers with their cell body in the dorsal root ganglia (DRG), transmitting sensory information from the periphery to the spinal cord and brain. During inflammation or injury, inflammatory cells (eosinophils, lymphocytes, macrophages, mast cells, schwann cells) release NGF that binds to TrkA directly activating nocioreceptors and triggering synthesis of neuropeptides (Substance P, ion channels like Na and Ca, calcitonine gene-related peptide (CGRP) etc.). Also, inflammatory cells release inflammatory mediators like histamine, serotonin (5HT), prostaglandins (PGE) and protons (H+). Binding of NGF to TrkA activates intracellular signaling pathways which results in increased expression or modulation at the membrane surface of number of receptors including bradykinin, transient receptor potential vanilloid 1 (TRPV1), voltage-gated sodium (Na), calcium (Ca) etc. These rapid changes in the afferent terminal modify the sensory fiber's response to sensory stimuli and propagation of sensory impulses to the dorsal horn.

Schematic and explanation adapted from Bélanger et al. J Toxicol Sci. 2018;43(1):1-10 and Mantyh PW et all.[30]

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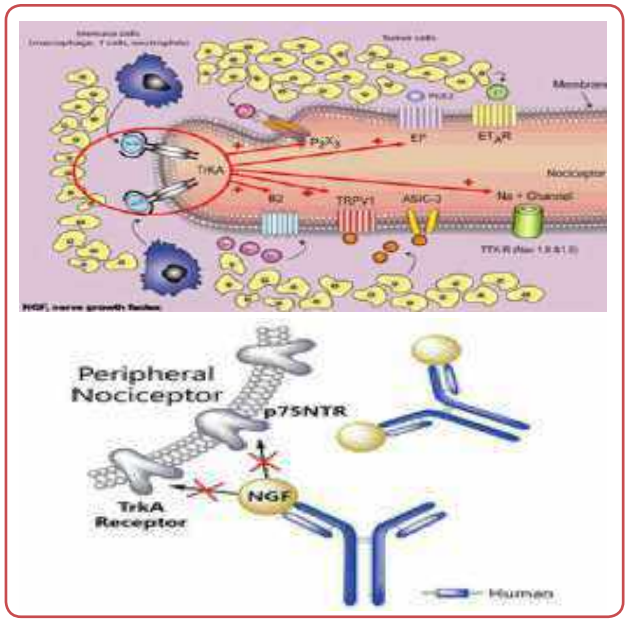

Adopted from Hefti at all. Web based pictures, published online, downloaded for this publication.

Figure 4: NGF receptors are highly involved in pain propagation. Blocking receptors with MoAb developed against NGF receptors , pain propagation is slowed down and diminished

Nerve growth factor (NGF) regulates multiple receptors /ion channels expressed by sensory nerve fibers that innervate bone and skin. There are differences in the percentage of tropomyosin-related kinase A receptors (TrkA) that innervate bone vs skin. The skin is mostly innervated by thickly myelinated A-beta fibers (TrkA -). The bone appears to be predominantly innervated with thinly myelinated A-delta fibers (mostly TrkA+) and peptide rich C-fibers (mostly TrkA+). According to published data, more than 80% of all sensory nerve fibers that innervate the bone are TrkA+ and only 30% that innervate the skin are TrkA+. This data may help explain why blocking NGF or TrkA is highly efficacious in diminishing skeletal pain. Modified from Mantyh et all. [30]

Tanezumab (Pfizer) and fasinumab (Regeneron) demonstrated superiority in efficacy compared to placebo and general safety was established in all RCT Phase II. Those results initiated clinical trials Phase III, some of the largest ever with 10,000 enrolled across the world. Regeneron announced in May 2016 positive topline results from Phase 2/3 fasinumab study in patients with OA pain. Pfizer and Lilly received FDA fast track designation for Tanezumab, was announced June 2017, and complete results from ongoing Phase 3 program for Tanezumab were published in Press Releases in October 2018 demonstrating significant improvement in pain and function in OA patients. In January 2019 Pfizer and Lilly announced top-line results from second Phase 3 study that this humanised monoclonal antibody acting as nerve growth factor (NGF) inhibitor demonstrated statistically significant improvement in pain and physical function over placebo. Regeneron just announced in a new press release in early August 2020 that all three Phase III RCT's for OA (knee and hip) met primary and secondary endpoints with clinical and statistical significance.

As of today, there is no FDA approval for tanezumab or fasinumab, but with overwhelming evidence of pain reduction, improved function and good safety profile, there will likely soon be possible to have "once a month, sub-cutaneous injection" of anti-NGF monoclonal antibodies for OA of the knee and hip. This Mo-Ab has a potential to replace use of other pain medication with multiple side effects. It is a pain killer, far superior to current options.

Wnt signaling pathway

As noted previously, current OA treatments are limited. They provide temporary symptomatic relief. No "disease modifying osteoarthritis drugs" (DMOADs) exist. Looking closely at the cellular level, many mesenchymal stem cells (MSC) are present in the synovial space and subchondral bone, fully capable of differentiation into cartilage forming chondrocytes, bone forming osteoblasts and adipose tissue.[35, 36] Joints effected with OA are rich with stem cells, especially synovium,[37, 38] clearly pointing that failure to regenerate articular cartilage is not a lack of supply but rather a failure to differentiate. The next logical question is why? Understanding the Wnt signalling pathway and the central role it plays in cell differentiation and tissue remodeling,[39] especially in the joint, provides some insight. It helps control tissue homeostasis through regulation of MSC differentiation in chondrocytes and osteoblasts[40, 41] (Figure 5).

Many publications indicate increased Wnt activity in OA joint drives MSC to osteogenic differentiation and stimulates metalloproteinase production, resulting in cartilage destruction.[42, 43] Downregulating Wnt activity locally could promote restoration of articular cartilage.[44] Preclinical studies demonstrated that Wnt inhibition results in reduced cartilage degradation and improved cartilage health (Figures 6 and 7). Figure 7

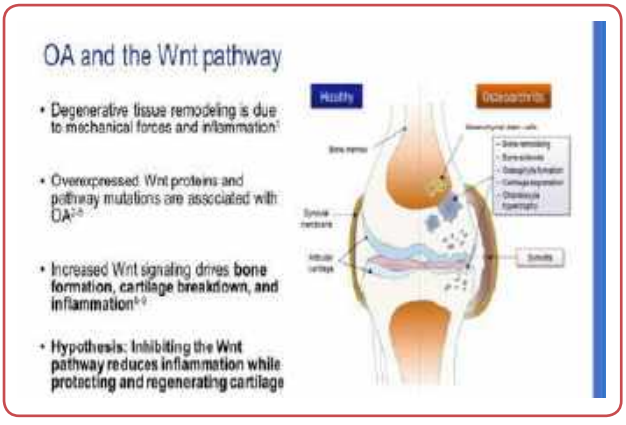

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Figure 5: Osteoarthritis and role of Wnt pathway

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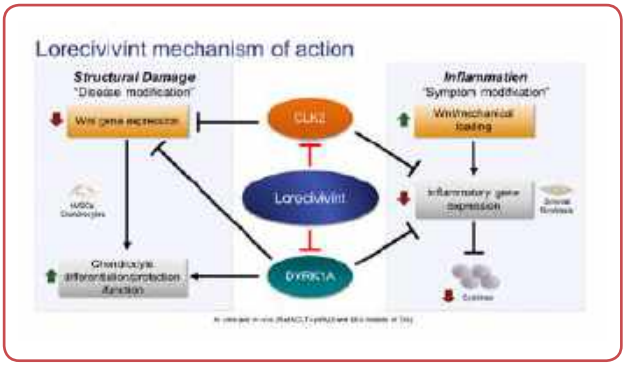
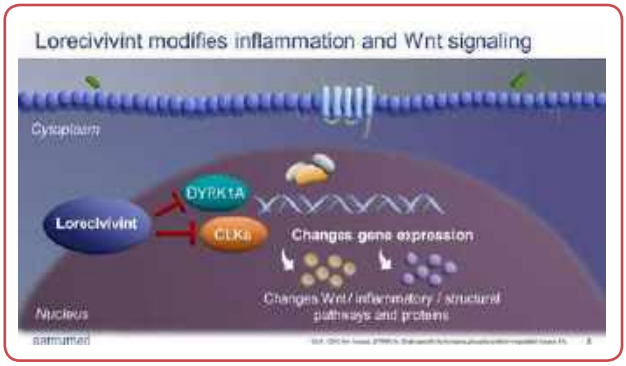
The first human Phase I RCT to assess the safety, tolerability, PK, dose limiting toxicities and exploratory efficacy of a single IA knee injection in patients with moderate to severe OA of the knee. Yazici et al[45] reported that molecule SM04690 appeared safe and well tolerated with no evidence of systemic exposure and exploratory efficacy analyses suggested positive trends for pain control and function, opening the door for additional phases to further assess the disease modifying properties.[46] Results of Phase III are already presented at a few international meetings and a manuscript with data is submitted for publication. FDA submission of this potential treatment for OA of the knee is anticipated in 2021.



Approved by Summed for publication. Presented and published before. Yazici Y. et all. Artritis & Rheumatology, Vol.0, No 0, Month 2020, pp1-13.

Figure 6: Lorecivivint is the first Wnt inhibitor for treatment of OA of the knee

Lorecivivint affects Wnt pathway activity via inhibition of two intranuclear targets, CDC-like kinase 2 (CLK2) and dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), through which it acts both independently and in combination to improve chondrocyte health and function while inhibiting inflammation.



Approved by Summed for publication. Presented and published before. Yazici Y. et all. Artritis & Rheumatology, Vol.0, No 0, Month 2020, pp1-13.

Figure 7: Lorecivivint mechanism of action in modifying inflammation in knee joint

The Wnt is integral pathway for tissue homeostasis and regeneration and is a key regulator of progenitor cell differentiation in the knee joint. Cartilage homeostasis requires a balance of Wnt activity. While necessary for chondrocyte differentiation and function, aberrant Wnt activity in OA directs progenitor cell differentiation in the joint toward development of osteoblasts instead of chondrocytes. Increased activation of Wnt pathway is known to increase OA development in humans whereas excessive inhibition of the Wnt pathway can cause cartilage and bone destruction. Potential DMOAD (disease modifying OA drug) approach would need to maintain signaling within an optimal range. CLKs = CDC-like kinase 2 (CLK2) and dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A).

Conclusion

All aspects of osteoarthritis: prevalence, pathophysiology, symptoms, oeconomical impact and current treatments are summarised in this paper. Pain control (paracetamol, tramadol, opioids) and NSAIDs as anti-inflammatory tablets or creams/gels, physical therapy, weight loss, IA articular injections of corticosteroids and hyaluronic acid have been the mainstream treatments for decades. Short acting, temporary relief and numerous side effects are limiting their use and pushing many patients to consider total knee replacement. Regenerative medicine was extremely promising in describing potential of mesenchymal stem cells to the point that after decades of use with aggressive marketing many patients are willing to pay a high price to prolong the use of their own joint. After reviewing literature in last 20 years with a focus on more recent robust articles, Level 1 evidence to justify hype and enthusiasm sold to patients does not exist. The most common conclusion in each review is similar; more good research needs to be done. Comparing modest results MSC produced from published data with role of saline, commonly used as "placebo" in ortho trials, most recently as comparator group since saline efficacy was established during last 10-15 years for short (3 months) and even longer (6 months) period of time. Since MSC are, after being harvested, from different places of the body, resuspended in saline (2.5 to 8 mL) there is definitely that "effect' of saline, explained as "therapeutic lavage" of the joint, simple dilution of what is left from synovial fluid in inflamed and arthritic joint and appears that saline "resets" synovium for a while and patient are reporting decreased pain and increased mobility. In the near future better options will be present, more objective parameters to follow patient mobility in real time, using already available trackers to count steps, calories spent, general activity[47, 48] etc. These should be complementary or better than metrics used today such as very subjective like VAS pain score, WOMAC and KOOS scores.

In the near future we hope to have at least two new, exciting drugs added to therapeutic arsenal. Based on my personal experience with both molecules (NGF antibodies since 2008 and Wnt blocking agent since 2012) and many studies from Phase I up to late Phase III with few hundred patients enrolled just at our research centre, I am very optimistic to see both therapies approaching date for FDA submission for approval.

CONTENT

Acknowledgements

None.

Conflict of interest

Dr Skrepnik is currently consultant for Sanofi, Regeneron, Samumed, Orthofix and was consultant for Zimmer, DePuy Johnson@Johnson, Chiltern International and 17 other companies and CRO's.

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CONTENT

Gazdić. Scr Med 2020;51(3):190-7. DOI:10.5937/scriptamed51-25992
HISTORY OF MEDICINE

A Brief History of Anaesthesia

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Abstract

According to the definition of the International Association for the Study of Pain (IASP), pain is defined as: "Unpleasant subjective feeling and emotional experience associated with current or potential tissue damage of a particular localisation", which, as such, poses a challenge for epidemiological research to determine its frequency and prevalence.

We have all heard the motto that surgery has experienced its unprecedented development on the wings of anaesthesia. This is most certainly the case, since it is precisely the pain that prevents any invasive procedure on the human body, hence the very elimination of pain has opened up the way for the application and development of surgery. For this reason, the skill and now the science of anaesthesia are epochal civilizational achievements, which is why it is worth remembering the attempts and successes of its application. The very beginning of mankind cannot be imagined without the humans facing some sort of pain. As long ago as about 460 to 370 BC, the renowned Greek physician Hippocrates (in Greek: *Ἱπποκράτης*), who is nowadays considered the founder of modern medicine, stated: "To reduce pain is a divine deed" or, in Latin: *Sedare dolorem, opus divinum est*!

The article presents Morton's discovery of inhalation anesthesia, now as far back as in 1846, its development, introduction of other modes of anaesthesia, local, infiltration and regional, use of neuromuscular blockers and auxiliary procedures, such as endotracheal intubation and fiberoptic bronchoscopy, without which modern anaesthesia is inconceivable today.

Key words: Pain; Inhalation anaesthesia; Local anaesthesia; Infiltration anaesthesia; Regional anaesthesia.

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ARTICLE INFO

Received: 4 April 2020
Revision received: 31 May 2020
Accepted: 2 June 2020

Introduction

Over the centuries, people have resorted to various methods and means to get rid of the uncomfortable feeling of pain. Back in about 3000 BC in Mesopotamia, patients were anaesthetised by pressing the carotid arteries to make them unconscious and thus feel no pain. The Egyptians were the first to apply snow to cause analgesia by cooling, ie, by body hypothermia. The eastern Chinese tradition had developed acupuncture as a special form of analgesia. In the ancient times, surgical operations were rarely performed, while the use of cruel physical measures, such as suffocation, knocking-down and such, was noted for pain relief, all with the aim of causing a deep sleep in which one would cease to go through this unpleasant experience.

The use of the first means of analgesia was mentioned in the ancient Greek and Roman texts of Hippocrates, Theophrastus, Aulus Cornelius Celsius, Pedanius, Dioscorides and Pliny the Elder, whereas the period of great scientific advances began in the late eighteenth and early nineteenth centuries. A rapid development of natural sciences led to the rapid development of medicine. Anaesthesia, the development of microbiology, the

This article should be cited as follows: Gazdić VS. A brief history of anaesthesia. Scr Med 2020;51(3):190-7.

discovery of X-rays, the discovery of blood groups and development of safe methods of blood transfusion and the discovery of penicillin and other antibiotics thereafter were the five major scientific advances that have enabled the development of modern surgery. Surgery was to experience its full take off on the wings of anaesthesia.

No real anaesthesia existed until the 19th century and the first research in this field began owing to the development of chemistry and physiology. In his book *The Century of the Surgeon*, Jürgen Thorwald wrote: "The century of modern surgery began in 1846 in the operating room of Massachusetts General Hospital in Boston. October 16 of that year marked the birth of the art of producing insensibility to pain by the inhalation of chemical gases. I believe that it is impossible for anyone in our times to grasp the vast significance of the revolution that began that day. Even to me it sometimes seems as if the cruel and frightful surgery, which I witnessed so often in my youth, never existed at all. Only a short time before that October 16 I had again watched another surgeon performing surgery of a cancerous tongue. I watched an operated woman fallen dead in shock when the broken iron began to squeak on the wound of the stump of her tongue. It was as if her last cry was still echoing the room, when the woman had already fallen dumb forever. And then, only a short while afterwards, a young man lay quietly under Warren's knife, uttering not a sound, unstirring, in a state of merciful insensibility, feeling none of the inconceivable pain that countless human beings had endured before him. Our whole world was transformed by an operation that lasted only a few minutes. A light burst forth from the darkness of those days, so bright that its first flash blinded us."[1]

The epoch-making discovery of ethereal anaesthesia was preceded by nearly fifty years of mental play and a series of attempts to relieve pain that had escalated in the consciousness of the humanity on that day. Subsequently, this new adjunct surgical device began to be successfully tested worldwide. The skill and science of anaesthesia have progressed steadily, contributing to the further development of surgery. The most important thing for anaesthesia is that it has become a separate specialisation. This has created a category of physicians who are both physiologists and high-level pharmacologists.

A brief historical overview

The end of the 18th and beginning of the 19th century brought numerous researches in the field of gases and their discoveries. Thus, Joseph Black discovered carbon dioxide, Henry Cavendish hydrogen, Antoine-Laurent de Lavoisier nitrogen and oxygen. Joseph Priestley discovered nitrous oxide (N_2O) and its analgesic effects were quickly recognised. It was the first gas used to reduce pain. Initial experiments on animals with nitrous oxide to induce sleep were performed by Henry Hickman in 1824, a young English surgeon who was working in France at the time. He did not receive a license to work on humans from the French medical authorities and, discouraged, he returned to England, where he soon died. In the 19th century, the so-called laughing parties were popular among the upper classes in Britain, to which people came and inhaled large quantities of nitrous oxide gas, which was better known as "paradise gas" or "laughing gas". In the year of 1800, the British chemists Humphrey Davy and Thomas Beddoes described the analgesic properties of nitrous oxide, but the era of its use in surgery had not begun at the time. It was in 1818 when Michael Faraday discovered that ether had similar properties to nitrous oxide, whereas, in 1824 Hickman discovered that carbon dioxide could also be used in anaesthesia; however, the era of surgical anaesthesia had not begun at the time either.[2]

It was another physician, Crawford Williamson Long, from Danielsville, Georgia, USA, who, while working with nitrous oxide and ether, observed their similar properties and it was him who noted that ether had stronger anaesthetic effect. Long was the first to successfully apply ether anaesthesia, on 30 March 1842, during surgery, giving his friend James M. Venable an excision, cutting out one of two tumours from his neck. For unknown reasons, he made this discovery public only in 1849, three years after William Morton celebrated the demonstration of ethereal anaesthesia in Massachusetts.[3]

Horace Wells, an American dentist, was the first who extracted a diseased tooth to a patient under anaesthesia using nitrous oxide in 1844. Wells came to this idea by watching a public performance of Gardner Colton, a travelling chemist, who made money entertaining people by inhaling nitrous oxide. Wells noted that people did not feel pain when they were injured stumbling around

CONTENT

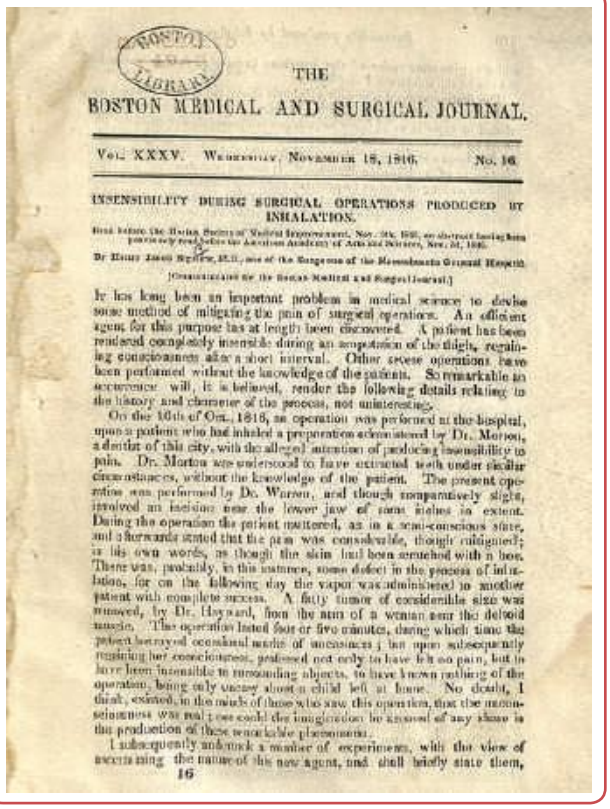
Figure 1: Dr William Thomas Green Morton (1819-1868), pioneer of inhalation anaesthesia

stunned with nitrous oxide. Unfortunately, he was unsuccessful in trying to publicise his method at the Massachusetts hospital and quickly lost the support of medical circles due to multiple professional errors, which caused Wells' disappointment and severe psychological depression, leaving his student, associate and colleague, William Morton (Figure 1), take over the primacy in anaesthesia.[4]

On 16 October 1846, William Thomas Green Morton (1819-1868), an American dentist, after unsuccessful attempt to administer nitrous oxide by his colleague Horace Wells, whom he successfully collaborated with, performed his famous demonstration of anaesthesia in surgery of the submandibular gland and tongue. He used ether as a stronger anaesthetic, as advised by his former mentor, Professor Jackson. The operation, performed by the Boston surgeon John Collins Warren, was to remove a large tumour from a man's face. Morton, at the same clinic where Wells failed, experienced the full success and recognition of his colleagues, after which the use of anaesthesia in surgery spread throughout the world. Therefore, 16 October 1846 is considered a date of discovery of anaesthesia (Figure 2). Morton's discovery was published next month and the news spread throughout the world (Figure 3).

Figure 2: "First Operation Under Ether" by Robert C. Hinckley, painting, oil on canvas

In 1847, James Young Simpson (1811-1870), a British obstetrician, proposed the use of chloroform, another significant drug in anaesthesia, discovered in 1831. The efficacy of chloroform has been proven by Simpson by using it in pregnant women to relieve childbirth pain. Thus, at the beginning of its development, in addition to nitrous oxide, anaesthesia received two more anaesthetic agents, ether and chloroform.[5]



THE

BOSTON MEDICAL AND SURGICAL JOURNAL.

Vol. XXXV. Wednesday, November 18, 1846. No. 16.

INSENSIBILITY DURING SURGICAL OPERATIONS PRODUCED BY INHALATION.

Dr Henry Jacob Bigelow, M.D., one of the Surgeons of the Massachusetts General Hospital.

[Communicated for the Boston Medical and Surgical Journal.]

It has long been an important problem in medical science to devise some method of mitigating the pain of surgical operations. An efficient agent for this purpose has at length been discovered. A patient has been rendered completely insensible during an amputation of the thigh, regaining consciousness after a short interval. Other severe operations have been performed without the knowledge of the patient. So remarkable an occurrence will, it is believed, render the following details relating to the history and character of the process, not uninteresting.

On the 16th of Oct., 1846, an operation was performed at the hospital, upon a patient who had inhaled a preparation administered by Dr. Morton, a dentist of this city, with the alleged intention of producing insensibility to pain. Dr. Morton was understood to have extracted teeth under similar circumstances, without the knowledge of the patient. The present operation was performed by Dr. Warren, and though comparatively slight, involved an incision near the lower jaw of some inches in extent. During the operation the patient muttered, as in a semi-conscious state, and afterwards stated that the pain was considerable, though mitigated; in his own words, as though the skin had been scratched with a hoe. There was, probably, in this instance, some defect in the process of inhalation, for on the following day the vapor was administered to another patient with complete success. A fatty tumor of considerable size was removed, by Dr. Hayward, from the arm of a woman near the deltoid muscle. The operation lasted four or five minutes, during which time the patient betrayed occasional marks of uneasiness; but upon subsequently regaining her consciousness, professed not only to have felt no pain, but to have been insensible to surrounding objects, to have known nothing of the operation, being only uneasy about a child left at home. No doubt, I think, existed, in the minds of those who saw this operation, that the unconsciousness was real; nor could the imagination be accused of any share in the production of those remarkable phenomena.

I subsequently undertook a number of experiments, with the view of ascertaining the nature of this new agent, and shall briefly state them,

16

Figure 3: Boston Medical and Surgical Journal, 18 November 1846, describing the 16 October 1846 successful operation under ethereal inhalation anaesthesia

CONTENT

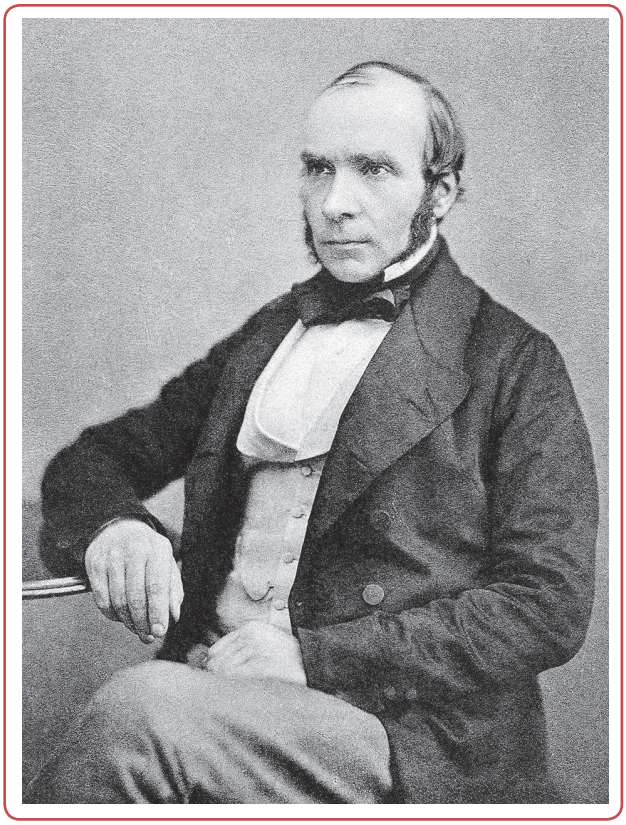


Figure 4: Dr John Snow (1813-1858), first qualified anaesthesiologist and father of modern epidemiology

In 1847, a Russian surgeon Nikolay Ivanovich Pirogov, performed the first successful operation under ether anaesthesia on the battlefield, which significantly reduced the mortality of the wounded on battlefields during the war as well as in the postoperative period.[6]

The first qualified anaesthesiologist was John Snow (1813-1858). This was a doctor who decided early on to deal exclusively with anaesthesia. Dr Snow was also involved in the practice of teaching, particularly of passing the knowledge to younger doctors. In 1841, he developed the first fan, called a pulmotor, for children in asphyxia. He also developed a type of inhaler through which the amount of ether given to a patient could be dosed. He discovered the importance of lack of oxygen as well as of problems occurring in the body due to the insufficient removal of carbon dioxide. Snow was the first to describe the stages of anaesthesia. He used chloroform in his work and became famous when he used the anaesthetic in 1853 during the childbirth of Queen Victoria with her eighth child, Prince Leopold. Snow gained fame beyond anaesthesia by alerting the public that cholera, with its London outbreak in 1854, was transmitted by water. As a result, the epidemic was stopped by the issuance of a regulation that all water had to be boiled before human use.[2,7] Dr John Snow thus remained remember not only as the first anaesthesiologist, but also as father of modern epidemiology (Figure 4). The first intravenous anaesthesia was used in 1872, which was performed with chloral hydrate by Pierre-Cyprien Ore (1828-1891). Due to delayed and slow recovery and high mortality of patients, this type of anaesthesia has long been unacceptable.[8]

The first anaesthesia using a cannula inserted through tracheostomy was performed by Friedrich Trendelenburg (1844-1924) in 1869 for successful performing of operations in the oral cavity,[9] while in 1878 the Scottish surgeon William McEwen (1848 - 1924) performed the first endotracheal intubation through the mouth as an alternative to tracheotomy, allowing the patient to breathe in oedema of the glottis, during general anaesthesia with chloroform. This anaesthesia technique is still in use today.[10]

The first local anaesthesia was performed in 1884 by Carl Koller (1857–1944), an Austrian ophthalmologist, anaesthetising the cornea of the eye by applying a solution of cocaine onto its surface, which was of great importance for further refinement of ophthalmic surgery. Due to reflex, involuntary eye movements that occur in response to a slightest touch, eye surgery without prior anaesthesia was difficult and limited. After numerous studies, Carl Koller discovered that a few drops of cocaine solution would solve this problem. Cocaine also causes mydriasis, an enlargement of the pupil of the eye, which is another trait for which it has also been used in ophthalmology.[11] It is interesting that Sigmund Freud, another prominent scientist of the time, mentioned in his autobiography his cooperation with Koller and commended him for his work.[12]

Already in the year of 1885, William Stewart Halsted (1852 - 1922), an American surgeon, performed the first blockade of the nerve with cocaine, only to become a victim of his own research. He became addicted to cocaine and morphine later in life, which was why he had to be sent to a sanatorium for treatment. Namely, during his stay in Europe, Halsted, along with his fellow students and doctors, experimented with cocaine. They injected cocaine into each other's nerves, which caused safe and effective local anaesthesia, but during the course of the study they became addicted to it.[13] A little later, in 1892, Carl Ludwig Schleich (1859–1922) in Germany demonstrated

CONTENT

his method of local analgesia by infiltration of cocaine into the tissue.[14] The field of use of cocaine as then only known local anaesthetic was in 1898 extended to anaesthetise nerve roots of the spinal cord when August Bier (1861–1949), a German surgeon, performed the first successful spinal anaesthesia by puncturing the dura mater.[15]

The development of local anaesthesia required discovery of anaesthetics that would work locally. In 1904 in France, Ernest Fourneau commercialised a new local anaesthetic amylocaine (Stovaine). Using the complex structure of cocaine, he invented the original molecule, with similar activities, but poorer toxic properties. This paved the way to developing of other local anaesthetics.[16]

Alfred Einhorn (1856 – 1917), a German chemist, patented novocaine the same year, which marked the beginning of the modern era of local anaesthesia. Until then, cocaine was used as a local anaesthetic, which due to side effects, including toxicity and addiction, forced scientists to search for new topical anaesthetics. Novocaine has been found to be relatively safe and effective, although its anaesthetic effects are slightly weaker than cocaine and some patients were allergic to it. However, no anaesthetic proved to be more effective than novocaine, so it quickly became the most commonly used topical anaesthetic. Although its use has been largely replaced by lidocaine today, novocaine is still in use, most commonly in dentistry.[17]

Using a mixture of ether and oil administered rectally to relieve birth pains, James Tayloe Gwathmey (1862-1944) introduced rectal anaesthesia to medical practice in 1913. This method of anaesthesia avoided inhalation of anaesthetics and their negative effect on the mother and foetus.[18, 19]

The introduction of neuromuscular blockers and the ability to control muscle tone is considered a turning point in the development of anaesthesia and, consequently, surgical work. In 1942, Harold Randall Griffith (1894-1985), a Canadian anaesthesiologist, introduced curare into anaesthesia (Figure 5). The introduction of curare, as a means of muscle relaxation, significantly reduced the amount of anaesthetic required, increased the volume of surgery, improved working conditions for the surgeon and reduced morbidity and mortality. The contribution of this Griffith's discovery to the further development of medicine and anaesthesia is best illustrated by medical historians, who often divide the development of anaesthesia into the periods before and after Griffith.[20, 21]

Figure 5: Harold Randall Griffith (1894-1985), pioneer of use of curare and other neuromuscular relaxants during anaesthesia

Another novelty introduced into anaesthesia practice, which modern anaesthesia is unthinkable without, was the use of a fibreoptic bronchoscope. Specifically, in 1967, Peter Murphy, an English anaesthesiologist, introduced it for tracheal intubation. By the mid-1980s, a flexible fibreoptic bronchoscope has become an indispensable instrument in pulmonology and anaesthesiology worldwide.[22]

History of the central nerve blocks

With the development of general anaesthesia, experience and knowledge about its harmful effects on human body were accumulating, taking into account the generality of its action. This was the reason for seeking to anaesthetise only the part of the body that would undergo surgery. For this purpose, the techniques of local and regional anaesthesia have found their place of application. The two most accepted regional anaesthesia techniques are spinal and epidural anaesthesia, which block nerve conduction at the level of nerve roots upon their exit from the spinal cord, in all its modalities: autonomic, sensory and motor. The development of regional anaesthesia required the accumulation of knowledge in the fields anatomy and physiology of the nervous system and pharmacology of local anaesthetic agents.

CONTENT

Figure 6: August Bier (1861-1949), pioneer of spinal anaesthesia

It was Hippocrates (470-400 BC) who discovered cerebrospinal liquor, while treating hydrocephalus with ventricular puncture and called it "brain water". The Hippocratic school described in detail the brain with two halves, brain sheaths, crossed brain syndromes, inflammation and brain tumours, etc. The first serious experiments on the nervous system were made by Aurelius Galen or Claudius Galen (129 - 200 AD), better known as Galen from Pergamum (in Greek: Γαληνός, Galēnos). Galen was a prominent Roman physician and philosopher of Greek descent and probably the greatest medical scholar of the Roman era. He was the first to carry out more serious tests of the nervous system, such as experiments on the ligation of nerves, to substantiate the theory, which still stands today, that the brain controls all muscle movements through the central and peripheral nervous systems.[23] In 1692, Valsalva was the first who claimed the existence of water fluid around the spinal cord of the dog, while in 1764 Domenico Cotugno was the first to explain this fluid.[24] In 1825, François Magendie, a French physiologist, was the first to describe the circulation of the cerebrospinal fluid and provide an explanation for it. He also called it cerebrospinal liquor.[25, 26]

Puncture of the spinal canal was also a challenge for nineteenth-century physicians. In 1855, James Leonard Corning, a neurologist from New York, accidentally punctured dura mater when investigating the effect of cocaine on the spinal nerves of dogs. Corning is considered the father of epidural anaesthesia. However, the origin of the first lumbar dural puncture was attributed to Heinrich Quincke of Cologne, Germany, in 1890.[27, 28]

A German surgeon August Carl Gustav Bier (1861-1949) was the first to use cocaine intrathecally in six patients for lower extremity surgery in 1898. He is considered the father of modern intrathecal anaesthesia (Figure 6). He hypothesised that cocaine administered around the spinal nerve roots may induce adequate surgical anaesthesia. In 1898, he performed experiments on his assistant August Hildebrandt after giving him spinal anaesthesia by hammering an iron hammer at his legs, twisting his testicles, all to test their pain after injecting cocaine intrathecally. However, it turned out that all these stimuli were painless for Hildebrandt. Hildebrandt subsequently suffered from headaches, nausea and bruises on his legs. Thus, Bier's lumbar puncture also provided the first description of a nine-day post-dural puncture headache.

Bier attributed it to the loss of cerebrospinal fluid and stated the importance of using thin needles for puncture for its prevention.[15]

There is a controversy in the medical world as to whether the first spinal block was performed by Dr August Bier or the American neurologist James Leonard Corning (1855-1923), of Acorn Hall, Morristown, NJ. Corning is officially considered the father of epidural anaesthesia because he was the first to perform neuro-axial blockade by injecting 111 mg of cocaine into the dog's epidural space and of ten healthy male volunteers. He published this experiment in 1885 in the *New York Medical Journal*.[29] On 16 August 1898 in Kiel, Germany, Dr August Bier performed the first surgery under spinal anaesthesia. The experiments of Dr Bier were published in 1899.[15] There was a controversy as to whether Dr Bier or Corning performed the first spinal anaesthesia. Undoubtedly, the experiment of Dr Corning chronologically preceded Dr Bier's experiments. It is unclear whether Corning's injection was a subarachnoid or epidural blockage. The dose of cocaine used by Corning was several times higher than that used by Bier. Dr Corning did not describe seeing the flow of cerebrospinal fluid in his report in the *New York Medical Journal*, while Bier made it clear. This suggests that the injections of Dr Corning were in the epidural space, not in the subarachnoid space. Indeed, Dr Bier deserved credit for introducing spinal anaesthesia into the clinical

CONTENT

practice of medicine for the first surgery done under spinal anaesthesia. Still, Dr Corning created the first experiment with neuraxial anesthesia.[30]

A year later, in 1899, the first spinal anaesthesia in the United States was performed by Rudolph Matas (1860-1957), a vascular surgeon from New Orleans, using cocaine and probably the first to inject morphine in the subarachnoid space. He was also the first who described a fatal outcome after lumbar puncture.[31] The first spinal anaesthesia for therapeutic purposes for the treatment of painful conditions in syphilis was performed by US medical doctors from San Francisco, Dudley Tait and Guido Cagliri, who injected mercury iodide into cerebrospinal fluid. Their studies include work on animal models, corpses and living patients, all in an effort to determine the benefit of lumbar puncture for the treatment of syphilis.[32]

The continuous intrathecal technique with a flexible needle was first used by William T Lemmon (1896-1974) at the Mayo Clinic in 1940.[33] The first sitting block was described by Adriani and Roman-Vega in the USA[34] in 1946, whereas the first intrathecal injection of morphine to a patient with malignancy was reported by Wang at the Mayo Clinic in 1979.[35]

In Europe, the first caudal approach to the epidural space was performed by Jean-Anthanase Sicard and Fernand Cathelin in Paris in 1901. Radiologist Jean-Anthanase Sicard described the injection of a dilute solution of cocaine through the sacral hiatus to a patient who suffered severe pain in the sacral and lumbar region. Working independently, Cathelin published a similar report three weeks later. He assumed that sacral injection of cocaine could also be used in surgery.[36, 37]

The lumbar approach to the epidural space would be applied only twenty years after the caudal approach, being first performed by the Spanish surgeon from Madrid, Fidel Pagés Miravé (1886-1923). Twenty years later, in 1921, he reported on his work on lumbar epidural anaesthesia. The text was as the birth of the modern epidural anaesthesia (EA). In *Anaesthesia Metamerica* he talks about 43 cases of epidural anaesthesia performed for operations inguinal hernias, adhesions and lower extremities.[38] Pagés died quickly after his work was published and his idea of lumbar epidural anaesthesia remained dormant until the year of 1933 when it was reconfirmed and popularised by an Italian surgeon from Turin named Achille Mario Dogliotti (1897-1966). Doglioti wrote a book entitled *Anasthesia, Narcosis, Local, Regional, Spinal* and it was the first foreign book of anaesthesia translated into English. Dogliotti devoted one chapter to epidural anaesthesia. He advocated the use of EA in a wide range of different procedures. He was the first to describe the technique of loss of resistance. Although a surgeon by profession, he had a strong influence on the development of anaesthesia.[39] Therefore, Pagés in Spain and Dogliotti in Italy applied, independently of each other, lumbar EA.

In 1932, Vincent Ruiz and Alberto Gutierrez from-Buenos Aires, Argentina began their practice of EA in the USA and published their work in 1939. They discussed the history of EA, Doglotti's first caudal anaesthesia, their drip stroke technique and their experiences with EA. During 1933, 81.5 % of all their cases were done in EA, 12.5 % in local, 4 % in general, 0.25 % in spinal and 1.75 % without anaesthesia. They used 2 % procaine in a volume of 25 to 50 mL and their failure rate was 1 % .[40]

Dr Angelo Luigi Soresi, an Italian surgeon, combined the spinal and epidural techniques into one combined technique "episubdural anaesthesia" in 1937, in an effort to achieve good analgesia with minimal motor blockage.[41] However, this technique was not initially accepted, to be reintroduced forty years later Ioan Curelaru, who is thus considered a pioneer of regional anaesthesia in obstetrics.[42]

Thus, 120 years after the establishment of regional anaesthesia techniques, these both anaesthetic techniques for surgery have become popular, because of their cost and relative ease of delivery and advantages in terms of patient safety. They have also found their place in the treatment of acute and chronic pain conditions.

Anaesthesiology, as a young medical discipline, has grown rapidly thanks to the rocketing development of science and technology and is now classified as a broad, if not the broadest, field of medical activity. Its field of work has long gone beyond just caring for sleeping patients and because of their knowledge of pharmacology, physiology, pathophysiology, surgery, acute and chronic pain therapy, anaesthesiologists today stand at the helm of operating theatres, intensive care, emergency departments, outpatient pain therapy

CONTENT

clinics. Due to their broad medical knowledge and mastery of many skills, they are expected to be trained in a wide range of subspecialties, cardiology, pulmonology, sleep electrophysiology, pain therapy, intensive care, pharmacology, all for the purpose of contributing to the development of medical science. In addition to understanding the medical community, this activity requires their personal engagement as well.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Sohipah et al. Scr Med 2020;51(3):198-202. DOI:10.5937/scriptamed51-26567
PROFESSIONAL ARTICLE

Development of Gel for *Aedes aegypti* Repellent With Combination of Cinnamon Oil (*Cinnamomum burmannii* Blume) and Fennel Oil (*Foeniculum vulgare* Mill)

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Abstract

Background/Aim: Aim of this study was to develop efficient mosquito repellent by combining fennel oil (*Foeniculum vulgare* Mill) and cinnamon oil (*Cinnamomum burmannii* Blume) in a gel preparation form.
Methods: Effectiveness of each oil and its combination as viral repellents was tested by the World Health Organization's Pesticide Evaluation Scheme (WHOPES) method. Repellent was prepared in gel form. Evaluation of gel preparation included organoleptic properties, homogeneity, pH, viscosity, flow properties, acute skin irritation test and effectiveness test as mosquito repellent.
Results: The mosquito protection test for cinnamon oil showed that cinnamon oil was most effective at concentration of 15 % (96.85 %), and fennel oil at 24 % concentration (79.26 %). Within 6 hours, gel made of 24 % fennel oil and 15 % cinnamon oil combination gave protection against 53.49 % mosquitoes.
Conclusion: The combination of cinnamon oil and fennel oil can be formulated into gel form with satisfying physical and chemical characteristics and effectiveness against *Aedes aegypti* mosquitoes for 6 hours.

Key words: Gel; *Aedes aegypti*; Cinnamon oil; Fennel oil; Repellent.

ARTICLE INFO
Received: 13 May 2020
Revision received: 26 June 2020
Accepted: 28 June 2020

Introduction

Aedes mosquitoes are a type of mosquito that is usually found in the tropics. Its name is derived from the Greek word aedes, which means "unpleasant". These mosquitoes are more than unpleasant, since they spread some dangerous diseases such as dengue fever. This species can often be found in Asia. Its specific characteristic is black and white legs. *Aedes aegypti* mosquitoes are also known as dengue propagators.[1-3]

Currently, the most available mosquito repellents on the market contain of anti-mosquito spray and anti-mosquito lotion are mostly made from diethyl-meta-toluamide (DEET) chemicals. Chemical insecticide has an impact on a residue and its active ingredients are difficult to decompose in nature. These negative effects need to be avoided by replacing chemical insecticide with natural insecticide.[4]

Based on the type form of mosquito repellent products on the market, mosquito repellents such as mosquito coil gel are classified as superior products. They can be used anytime and anywhere. In addition, gel formulations have many advantages when compared with other semi-solid prepara-

This article should be cited as follows: Sohipah E, Purba AV, Djamil R. Development of Gel for *Aedes aegypti* repellent with combination of cinnamon oil (*Cinnamomum burmannii* Blume) and fennel oil (*Foeniculum vulgare* Mill). Scr Med 2020;51(3):198-202.

tions such as creams or ointments. Gelled preparations have good active absorption capability on the skin, it is easy washed it away with water, they are highly adhesive when used on hair and skin without clogging the pores.[5, 6]

Plant ingredients such as fennel oil (*Foeniculum vulgare* Mill) contain anethole that can serve as a repellent. Anethole is a group of essential oil compounds that have a very distinctive aroma and flavour that mosquitoes do not like. Cinnamon oil (*Cinnamomum burmannii* Blume) has cinnamaldehyde compounds that are toxic to mosquitoes. This substance has a potential to be used to make mosquito gel preparations, because the smell and taste is not favoured by mosquitoes. Cinnamon oil exhibits antibacterial, antifungal, antioxidant and anti-thrombotic effects. The combination of both plants is expected to work synergistically as a mosquito repellent.[7, 8]

Methods

Material

The material consists of eggs from *Aedes aegypti* L mosquitoes, Carbopol U21 (dope lubrizol), propylene glycol (The Dow Chemical Company), triethanolamine (Merck), phenoxyethanol (Clariant), *C burmannii* blume oil, *F vulgare* oil and aqua.

Methods

The concentration of cinnamon oil and fennel oil was used in the formula that is determined based on preliminary test results, fennel oil was used in 2 %, 4 %, 6 %, 24 % concentration and cinnamon oil was used in 5 %, 10 %, 15 % concentration. In this research concentration of combination of 15 % cinnamon oil and 24 % fennel oil was used to assess the effective dose of cinnamon oil and fennel oil test effectiveness mosquito repellent performed following the procedure recommended by the current guidelines by the World Health Organization's Pesticide Evaluation Scheme (WHOPES). The effectiveness test of mosquito repellent using human subjects' method has been chosen according to actual conditions of use.[9]

Maintenance of *Aedes aegypti* eggs

Aedes aegypti eggs were inserted into plastic cups or bowls containing water for 2-3 days to drip down the larvae. During the development period the larvae were fed in pellets until the pupa phase was formed. The larval stage usually lasted 6-8 days to become the pupa, then the pupa was transferred into a bowl of water and left in the cage for 1-3 days to develop into an adult mosquito. Female mosquitoes were separated from male mosquitoes and fed in the form of sugar water.[9, 10]

Preparation of gel formulation

Carbopol U21 was dispersed in distilled water with 500 rpm stirrer speed for 24 hours. Phenoxyethanol was dissolved in propylene glycol and stirred with a stir bar until the homogeneous mixture was produced. The developed Carbopol U21 was neutralised with triethanolamine stirred with a stir bar until homogeneity. All bases were stirred at 200 rpm for 30 minutes, then the fennel oil and the cinnamon oil were added and stirred until homogeneity. Then, evaluation of anti-mosquito gel was performed.[11-13]

Evaluation of physical and chemical preparations gel

Physical and chemical properties such as organoleptic properties (colour and odour), homogeneity and pH of gel were determined. Determination of viscosity and flow properties were performed with a Brookfield viscometer at a rate of 0.5; 1; 2.5; 5; 10; and 20 rpm and turn 20; 10; 5; 2.5; 2; 1; and 0.5 rpm. The result obtained was plotted against shear stress (dyne/cm^2) and shear rate velocity (rpm). Viscosity checks were performed for 3 months at 40 °C temperature storage, at room temperature and at temperature of 400 °C.[14]

Effectiveness of gel protection

Before and after the experiment each test area of the forearm volunteers was washed with soap and rinsed with water, then dried. The left arm as control was inserted into the mosquito coil, observed and the number of mosquitoes perched within the brackets within 5 minutes were recorded. When mosquito was perched, the test had begun, and after 5 minutes the arm was carefully removed from the mosquito cage. Then the right leg was smeared with gel and placed into the mosquito coat to be observed for 5 minutes. Arms and legs should not move during the test. Number of mosquitoes perched on the volunteer arms and legs were recorded. Observation lasted for 6 hours, with continuous monitoring and recordings every hour (on start, 1st, 2nd, 3rd, 4th, 5th and 6th hour). The number of mosquitoes that alighted were recorded as well.

Stability test

The gel stability test was performed and it includ-

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ed organoleptic properties (colour and odour), homogeneity, pH, viscosity and flow properties. Evaluated at low temperature (4 ± 2 °C) and room temperature (25 ± 2 °C) for 3 months with evaluation every 2 weeks, and high temperature (40 ± 2 °C) for 4 weeks, with once a week evaluation.[15-16]

Irritation test

Toxicity test was performed by acute dermal irritation test on rabbit. Rabbits used in test were white male rabbits, 4 - 6 months of age, weight between 1.8 - 2 kg. Regulation of the head of BPOM No. 7 Year 2014 concerning the non-clinical *in vivo* toxicity test was considered and it was complied with the regulation.[17]

Results

The results of effectiveness test of *Aedes aegypti* mosquito cinnamon oil and fennel oil

The mosquito protection test for cinnamon oil is most effective at concentration of 15 % and its activity protection was 96.85 % (Table 1).

Table 1: Result of effectiveness test of protection power of cinnamon oil to Aedes aegypti mosquitoes

Days	Replication	Alcohol	Concentration		
			5 %	10 %	15 %
1	I	24	6	2	1
	II	24	4	3	2
	III	23	4	2	1
2	I	25	8	2	0
	II	23	8	2	1
	III	27	5	1	0
3	I	25	4	1	1
	II	27	6	2	1
	III	24	3	1	0
Average mosquitoes on hand		**24.67**	**5.33**	**1.78**	**0. 80**
Activity protection			**78.38 %**	**92.79 %**	**96.85 %**

Table 2: Test results of effectiveness of fennel oil protection against Aedes aegypti mosquitoes

Days	Replication	Alcohol	Concentration			
			2 %	4 %	6 %	24 %
1	I	21	14	11	12	5
	II	20	14	11	10	3
	III	19	12	10	13	4
2	I	24	15	13	14	5
	II	22	16	12	11	4
	III	20	13	12	11	5
3	I	19	16	14	13	5
	II	22	14	13	11	4
	III	21	15	14	10	4
Average mosquitoes on hand		**20. 89**	**14. 33**	**12. 22**	**11. 67**	**4. 33**
Activity protection			**31.38 %**	**41.49 %**	**44.15 %**	**79.26 %**

The mosquito protection test for fennel oil is most effective at 24 % concentration and its activity protection was 79.26 % (Table 2).

The test of mosquito protection from combination cinnamon oil and fennel oil for 6 hours compared with positive control (Figure 1).

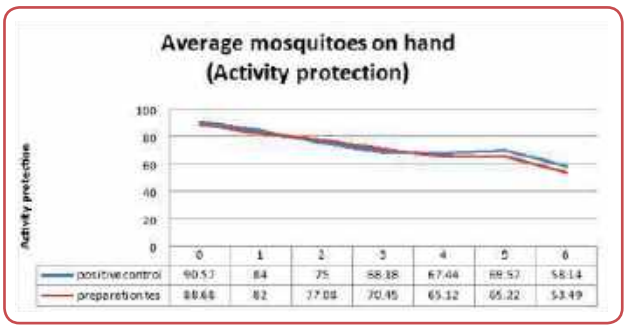

Figure 1. Result of activity protection combination of cinnamon oil and fennel oil mosquito repellent

Table 3: Formulation design of the mosquito repellent gel

Materials	Concentration (%) (b/b) Formula	Function
Fennel Oil	24	Active ingredients
Cinnamon Oil	15	Active ingredients
Carbopol U 21	3,01	Thickener
Propylene glycol	6,03	Humectan
Triethanolamin	0,5	pH adjuster
phenoxyethanol	0,2	Preservative
Aquadest	51,26	solven

Formulation design

Table 3 contains the chemical composition of the repellent gel based on fennel oil and cinnamon oil.

Evaluation of gel preparations

Organoleptic

The mosquito repellent gel was made to show a homogeneous physical appearance, clear white colour and pleasant aroma.

The results of viscosity testing and flow properties

The viscosity of gel on third temperature after storage for 12 weeks decreased. At 5 rpm speed using spindle No. 3 temperature 400 °C has viscosity of 7,050 centipoise (cP), temperature 250 °C has viscosity 3,600 cP, and temperature 40 °C has viscosity 2,550 cP (Figure 2).

CONTENT

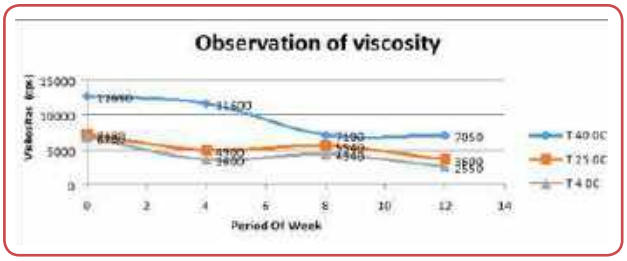

Figure 2. Graph of changes in viscosity values to three temperatures during 12 weeks.

Storage flow properties

The pseudo plastic flow properties begin at zero and there is no yield value. Viscosity decreases with increasing rate of shearing. Increased shearing stress causes the regularity of the polymer thereby reducing the resistance and further increasing the rate of share in subsequent shearing stress (Figure 3).

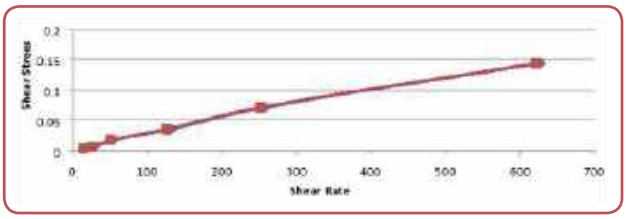

Figure 3. Rheology pseudo plastic

pH

pH of gel was 6.00 - 6.25. The addition of a combination of cinnamon oil and fennel oil can cause a decrease in the pH.

The result of mosquito repellent stability test

The mosquito repellent formulas are stable at temperature (25 ± 2 °C) and cold temperature (4 ± 2 °C) with homogeneous physical appearance, clear white colour, and aromatic viscosity of 7180 - 2550 cps, pseudoplastic flow properties pH 6.00 up to 6.25 (skin pH 4.5 to 6.5).

Table 4: Response of rabbits to irritation caused by mosquito repellent gel containing fennel oil and cinnamon oil administered over 72 h

Irritation response category in rabbits	Score
No Irritation	0
Very Low Irritation	1
Low Irritation	2
Middle Irritation	0
High irritation and exfoliation	0

Result of irritation test

The combination of mosquito repellent oil gel and fennel oil resulted in mild irritation of the skin of experimental animals after application for 3 consecutive days (72 h) with a mild irritation index of 2 (Table 4).

Discussion

With observation for 6 hours, combination of fennel oil in 24 % concentration and cinnamon oil in 15 % concentration, gave protection from 53.49 % *Aedes aegypti* mosquitoes. Chemical insecticides due to their longevity have a great impact on environment. If there is a possibility of finding a natural insecticide that is effective it is worth the effort.

This decrease in viscosity can be caused by storage packaging. It is assumed that it causes the gel to absorb water from the environment so that the volume of water in the formula increases. In addition, the arrangement of initially irregular molecules into long axes in the flow direction may result in some of the solvents binding to lose molecules, thereby causing a decrease in viscosity.

pH of gel was 6.00 - 6.25. The addition of a combination of cinnamon oil and fennel oil can cause a decrease in the pH which is welcomed since acid repels mosquitoes.

Conclusion

Based on the observation and test of protection power it can be concluded that the combination of fennel oil and cinnamon oil are effective repellents against *Aedes aegypti* mosquitoes for 6 h. The combination of cinnamon oil and fennel oil can be formulated into gel preparations and meets the physical and chemical requirements.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Jain et al. Scr Med 2020;51(3):203-8. DOI:10.5937/scriptamed51-28092
PROFESSIONAL ARTICLE

A Cross-Sectional Study of Prescribing Pattern of Antimicrobial Agents Among Inpatients of a Tertiary Healthcare Centre

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Abstract

Background: Antimicrobial agents are the mainstay of treatment in modern medicine. In view of emerging threat of Antimicrobial Resistance (AMR), there was a requirement aimed at identifying patterns of antimicrobial prescribing. The prescribing pattern of antimicrobial agents (AMAs) among the medicine inpatients of SMS Medical College & Hospital, Jaipur, India was studied. The objectives were to evaluate, monitor and suggest rational prescribing practices.
Methods: This was a cross-sectional study that was done for a period of one year from July 2016 to June 2017. The rationality of antimicrobial drug use was evaluated by analysing the prescriptions.
Results: A total of 400 patients were included in the study. Percentage of indoor patients with one or more AMAs prescribed was 48.75 %. The average number of AMAs prescribed was 1.67. Percentage of AMAs prescribed consistent with the National List of Essentials Medicines (NLEM) was 89.73 %. The average AMAs duration prescribed was found to be 4.24 days. The most commonly prescribed antibacterial agent in medicine in patients was a beta-lactam AMA with 57.29 % of all prescriptions.
Conclusion: AMR is strongly linked to improper antimicrobial use. This study suggests that certain interventions are required to minimise toxicity and lessen the chances of emergence of resistance. It is therefore proposed that there should be continuous education on rational use of drugs among healthcare professionals.

Keywords: Anti-Infective Agents; Drug Resistance, Microbial; Essential Medicines List; Prescription Drug Monitoring Programmes.

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ARTICLE INFO
Received: 22 August 2020
Revision received: 2 September 2020
Accepted: 3 September 2020

Introduction

Antimicrobial agents (AMAs) have continued to remain the basis of treatment in modern medicine, since their development.[1-3] AMA use has resulted in a worldwide decline in infection-associated mortality.[4, 5] Today, the most commonly prescribed medication in any hospital settings is an antimicrobial agent.[6] Judicious use of antimicrobials is rapidly becoming a major public health need.[7] Inappropriate/improper antimicrobial use can result in increased adverse drug reactions (ADRs), suboptimal therapy, treatment failures, polypharmacy and the most significant of all, the emergence of antimicrobial resistance (AMR).[8, 9]

The pattern of antimicrobial use varies in different geographical regions and from centre to centre, depending on the strains of pathogens, the pattern of nosocomial infections, cost and availability of AMA's. In view of the up-and-coming worldwide menace of AMR, there is a requirement to identify patterns of AMA prescribing. Therefore, the present study was designed to

This article should be cited as follows: Jain M, Kakkar S, Bansal A, Kapadia R. A cross-sectional study of prescribing pattern of antimicrobial agents among inpatients of a tertiary healthcare centre. Scr Med 2020;51(3):203-8.

study the prescribing pattern of AMAs (according to the World Health Organization's *How to investigate AMA use in hospitalised patients: 2012*)[10] among the medicine indoor patients of SMS Medical College & Hospital, Jaipur, India. This study helped in evaluating, monitoring and suggesting modifications, all in an attempt to rationalise the prescribing practices.

Methods

The objectives of this observational study were to describe and analyse the prescribing pattern of AMAs among medicine indoor patients of SMS Medical College and Hospital, Jaipur, India and it specifically related to the appropriateness of use of antimicrobials. The administrative permission to conduct the study was obtained from the Medicine Department & Institutional Research Review Board. The study was a cross-sectional in design. It was conducted for the duration of 12 months (in order to include seasonal variations in diseases). The inclusion and exclusion criteria are listed below.

Inclusion criteria:

1. Patients of any sex, aged 18 to 60 years.
2. Inpatients from the Medicine Department.
3. Patients who gave written informed consent.

Exclusion criteria:

1. ICU admitted patients.
2. Patients on antitubercular or antiretroviral therapy.
3. Post-surgical patients.

Informed consent was obtained in writing from all the patients after fully explaining the study procedure to their satisfaction. This study was approved by the Research committee at the SMS Medical College & Hospital, Jaipur, India.

Data were collected from the selected Medicine Department inpatients in a predesigned case report form (CRF). The CRF contained dates of admission and discharge, patient demographics, age, sex, outcome, history of presenting illness, general physical examination, diagnosis, investigations, drug details and other relevant information. The data obtained was assessed for the prescribing pattern, as per World Health Organization's *How to investigate AMA use in hospitalised patients, 2012*.[10]

Statistics

The sample size at 95 % confidence level expecting 50 % use of antimicrobials (maximum variance) in the Medicine Department of the SMS Hospital. At the precision (relative allowable error) of 10 %, minimum of 384 patients were required as a sample size which was further increased and rounded up to 400 patients as a minimum sample size required for the present study (Davey et al, 2005).[11]

Data collected was tabulated and analysed using descriptive statistical tools (mean, standard deviation and percentage wherever applicable). Probability $p < 0.05$ was considered as significant.

Results

A total of 400 patients from the Medicine Department were included in the study. Out of these patients, 281 (70.25 %) were males while 119 (29.75 %) were females. The age of the study patients was in categories; 59 patients (14.75 %) were aged 18-20, 66 patients (16.5 %) were aged 21-30, 87 patients (21.75 %) were aged 31-40 and 188 patients (47 %) were above 40 years. The most commonly prescribed AMAs were antibacterials with a frequency of 285 (85.84 %).

The most commonly prescribed antibacterial group in medicine in patients were beta-lactams with a frequency of 165 (57.29 %) (Table 1). Oxazolidinones and tetracyclines accounted for 37 (12.85 %) and 32 (11.11 %) respectively. The least prescribed antibacterial was aminoglycosides with a frequency of 1 (0.35 %).

Table 1: Frequencies of prescription of antibacterial agents in inpatients of a tertiary healthcare centre

No.	Name of Antibacterial Agent	Number of patients prescribed	Percentage of patients prescribed
1	Aminoglycosides	1	0.35 %
2	Beta-lactams	165	57.29 %
3	Lincosamides	25	8.68 %
4	Macrolides	9	3.13 %
5	Oxazolidinones	37	12.85 %
6	Quinolones	16	5.56 %
7	Tetracyclines	32	11.11 %

Conditions recorded on prescription and antimicrobials prescribed are listed in Table 2.

CONTENT

Table 2: Conditions of inpatient of a tertiary healthcare centre and prescribed antimicrobial agents

Condition	Antimicrobial agent prescribed
Acute pancreatitis	Meropenem (2), Clindamycin (1), Linezolid (1)
Alcoholic hepatitis	Ceftriaxone (3), Metronidazole (2)
Anaemia	Albendazole (3), Artesunate (1), Cefpirome (1), Ceftriaxone (1), Metronidazole (1)
Chronic liver disease	Linezolid (4), Meropenem (2), Piperacillin (2), Albendazole (1), Ceftriaxone (1), Clindamycin (1), Metronidazole (1)
Chronic obstructive pulmonary disease (COPD)	Amoxicillin Clavulanate (6), Cefoperazone (3), Ofloxacin (3), Cefpirome (1), Fluconazole (1)
Cerebrovascular incident (CVI), Stroke/ Paralysis	Ceftriaxone (17), Linezolid (2), Acyclovir (1), Cefpirome (1), Cefuroxime (1), Metronidazole (1), Moxifloxacin (1), Ticarcillin (1)
Dengue/ Pyrexia-Thrombocytopenia	Artesunate (2), Ceftriaxone (2), Doxycycline (2), Cefixime (1), Primaquine (1)
Diabetes/ Complications	Ceftriaxone (4), Moxifloxacin (3), Cefoperazone (1), Doxycycline (1)
Encephalopathy	Ceftriaxone (6), Cefoperazone (1)
Enteric Fever	Azithromycin (1), Cefixime (1), Ceftriaxone (1)
Guillian-Barre syndrome/ Muscle weakness	Ceftriaxone (4)
Generalised tonic - clonic seizures/ Seizure disorder	Ceftriaxone (6)
Hepatitis C	FDC (1)
Infectious diarrhoea	Ceftriaxone (4), Metronidazole (3), Ciprofloxacin (1)
Malaria	Artesunate (17), Doxycycline (14), Clindamycin (1), Primaquine (1)
Pneumonia	Ceftriaxone (25), Amoxicillin Clavulanate (9), Cefotaxime (9), Aztreonam (3), Clindamycin (3), Linezolid (3), Amoxicillin (1), Clarithromycin (1)
Pyrexia of unknown origin	Doxycycline (9), Ceftriaxone (8), Artesunate (6), Amoxicillin Clav (5), Azithromycin (4), Levofloxacin (1), Moxifloxacin (1)
Scrub typhus	Azithromycin (3), Doxycycline (3)
Sepsis	Linezolid (26), Clindamycin (19), Meropenem (16), Aztreonam (7), Artesunate (2), Piperacillin (2), Cefoperazone (1), Ceftriaxone (1), Moxifloxacin (1)
Snake Bite/ Unknown Bite	Amoxicillin Clav (2), Linezolid (1), Metronidazole (1), Piperacillin (1)
Uper respiratory tract infection	Cefoperazone (1), Doxycycline (1)
Urinary tract infection	Levofloxacin (4), Gentamicin (1), Meropenem (1), Norfloxacin (1)

Table 3 contains local data for the WHO Prescribing indicators of antimicrobial agents.

Table 3: WHO Prescribing indicators of antimicrobial agents in inpatients of a tertiary healthcare centre

Percentage of indoor patients with one or more AMAs prescribed	48.75 %
Average number of AMAs prescribed	1.67 %
Percentage of AMAs prescribed consistent with the National List of Essentials Medicines (NLEM)	89.73 %
Average duration for AMAs prescribed	4.24 days
Pneumonia patients who were prescribed AMAs in accordance with standard treatment guidelines (STG)	81.58 %
Percentage of AMAs prescribed by their generic names	100 %
Additional Indicator: Number of drug sensitivity tests (DST) reported per hospital admission with AMA prescription	8.21 %

However, the average number of medicines prescribed was found to be 7.12. Proton Pump Inhibitors (PPIs) contributed to being most routinely prescribed among patients.

Discussion

Antimicrobials used in hospitals for treating infections are often used unacceptably. The study revealed that the percentage of medicine indoor patients with AMAs prescribed was neither very

CONTENT

high nor extremely low. The percentage of AMAs prescribed consistent with the NLEM suitable. Similarly, the percentage of pneumonia patients who are prescribed AMAs in accordance with standard treatment guidelines (STG) was found to be appropriate. In the study hospital, it was found that the percentage of AMAs prescribed by their generic names is absolute which is most creditable.

The percentage of indoor patients with one or more AMAs prescribed was 48.75 %. Although, this is more than the typical values obtained from previous studies from developing countries like Bangladesh (25 %), Malawi (34 %), Tanzania (39 %) and Indonesia (43.1 %) (Janković et al, 2006 and Massele et al, 2001)[12, 13] but acceptable. It is comparable to the study by Odunsanya et al (2004)[14] and the study in Iran[15] that showed the value close to 50%. This study was done for a period of 1 year, so the seasonal variations in diseases were taken into account. Reports from other studies were 60 % (Bosu et al, 2000),[16] 60.9 % (Otoom et al, 2002),[17] 61.9 % (Moghadamnia et al, 2002)[18] and 72.8 % (Hazra et al, 2000).[19] Some of the reports from Oman,[20] Malaysia,[21] Saudi Arabia[22] and China[23] had values of the order of 64 %, 67 %, 72 % and 77.8% respectively, which are way higher than the one from this study. This indicator finds out the degree of AMA use in hospitals. When used over a period of time, it is able to observe the changing trends.

The average number of AMAs prescribed was 1.67. Indoor patients may receive more than one AMA during the course of their treatment. This prescribing may be warranted or unwarranted. Even duplication occurs in exceptional events. The study from Oman reported the average number to be 2.5, which is higher in comparison to the present study.[20] In contrast the study from Ghana reported the average number to be 1.1.[24] Since in the medicine indoor patients who were prescribed AMAs were prescribed an average of 1.67 AMA per hospitalisation, the rate is satisfactory in most situations.

National List of Essentials Medicines (NLEM) represents the medicines that fulfil the priority health care needs of the patients in a hospital, is used to rationalise the use of medicines. Non- adherence to this can have many causes including lack of awareness, not being in conformity and listed medicines (AMAs) not being available at the hospital. Percentage of AMAs prescribed consistent with the NLEM was 89.73 %. This indicator measures the degree of prescriber adherence to the NLEM. Since a total of 89.73 % of AMAs prescribed were on the NLEM,[25] this percentage is acceptable, and no further assessment was done to examine adherence. It can be said that the prescribers are aware and in agreement with the list. The routine training programme and workshops in SMS Medical College and Hospital can be attributed to this achievement. These academic activities in a tertiary care teaching hospital give physicians an up to date on treatment protocols. Another factor responsible for this is the fact that the NLEM is also updated regularly and revised accordingly. It can be hoped that in future this indicator would improve even further and there will be a suitable use of AMAs that are on the NLEM.

In SMS Medical College and Hospital the average AMAs duration prescribed was found to be 4.24 days. This indicator measures the strength of patient exposure to AMAs. The number of days on AMAs includes the number of days of all AMAs prescribed for treatment (not prophylaxis). It includes the duration of AMAs prescribed on discharge. Since the average duration of prescribed AMAs was 4.24 days, it conforms to other studies that suggest reducing the duration of antimicrobial use.[26] Also, this duration of therapy is at par with most international guidelines on antimicrobial use. But the discontinuing the antimicrobials post discharge may have been overlooked.

The adherence with hospital treatment standards is reflected in prescribing AMA of choice as per the STG. [27, 28] Pneumonia patients who were prescribed AMAs in accordance with STG was found to be 81.58 % This indicator measures whether the patient prescription adheres to the treatment guidelines or not. The therapeutic options to pneumonia were analysed according to 2016 guidelines.[28] In SMS Medical College and Hospital, 81.58 % of pneumonia cases were treated with the recommended AMAs according to the latest guidelines. It suggests that most of the prescribers are aware of these guidelines and are in wilful compliance. Also, all prescribers used the recommended doses as well. This is contrary to the study by Sharma and Kapoor in 2003 which showed that a large number of prescriptions did not match the STG.[29] However, in the study by Hariharan et al in 2009, 67% prescriptions adhered to hospital protocol.[30]

In the study hospital, it was found that the percentage of AMAs prescribed by their generic

names is 100 %. This indicator deals with the percentage of AMAs that are prescribed using their generic names, as per the WHO list of international non-proprietary names (INN) (WHO 2008).[31] The availability of generic drugs in the Government supply and the awareness and agreement of prescribers was looked for. In Rajasthan, after the implementation of free drug scheme, there is strict adherence to generic prescribing in all the Government-run hospitals. In addition, there are Medical Council of India (MCI) guidelines for ensuring the same.[32] The myth about free drugs being not effective, toxic and contributing to resistance is found to be false in the present study. Since it was ascertained that 100 percent of drugs were prescribed by their generic names, the role of State Government in implementation of MCI guidelines and ensuring compliance of the same is indeed commendable.

The use of AMAs depends on the knowledge of their spectrum. The rate of sensitivity tests done is a measure of the ability of the prescriber to provide rational treatment. The number of drug sensitivity tests reported per hospital admission with AMAs prescribed was found to be 8.21 %. This value is lesser in comparison to the Oman study which found that 25 % of drug sensitivity was done.[20] However, the Chinese study showed that a mere 39 specimens in the 1025 cases were sent for testing.[23] Whereas, Ider et al (2010) in Mongolia showed values similar to the present study.[33] This indicator measures the availability of drug sensitivity to establish best possible treatment to infective diseases. Here, since drug sensitivity test was performed for 8.21 % of hospital admissions treated with AMA, an intervention to improve the microbiology lab services may be required to improve this indicator to acceptable limits.

The average number of medicines prescribed was found to be 7.12. However, the study from Pakistan showed polypharmacy of 12 drugs/prescription.[34] Similarly, polypharmacy was found in GMC, Dhule by Deshmukh et al (2013).[35] The present study found that among AMAs, the antibacterial agents were prescribed for 85.84% of patients. Among antibacterials, as the beta-lactams account for 57.29 % of prescriptions; this value corresponds to the worldwide consumption of antimicrobials reported by Boeckel et al (2014) who showed that penicillins and cephalosporins accounted for 37 % and 24 % respectively.[36] Therefore, the present results correspond to the global trend in antimicrobial consumption.

Conclusion

Antimicrobial prescribing among medicine in patients at SMS Medical College and Hospital, Jaipur was found to be satisfactory with an area of concern. There are chances of antimicrobial resistance and toxicity of drugs. A practical way to address this urgent need is education and awareness about AMR among prescribers. Rational use of drugs should be promoted, as advocated by the WHO.[37] Besides, it is recommended to develop a restrictive antimicrobial use policy. This would ensure not only tailored intervention for empirical therapy but adjusted antimicrobial use once drug sensitivity test results are available.

Limitations

There are several limitations to this study. It is a single centre, observation study describing only the prescribing indicators. A preliminary comparison and evaluation of findings with other hospitals and countries is done. More studies would be required to further monitor, modify and rationalise drug use across the country.

Acknowledgements

The contributors of the article acknowledge the invaluable inputs and support provided by Departments of Medicine, Microbiology and Community Medicine, SMS Medical College & Hospital, Jaipur, India.

Conflict of interest

None.

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CONTENT

Pašagić et al. Scr Med 2020;51(3):209-14. DOI:10.5937/scriptamed51-28407
CASE REPORT

Combined Orthodontic and Surgical Treatment of Impacted Maxillary Canine in Young Patient with Class II Malocclusion: a Case Report

Ljubica Pašagić,[1] Ivana Ilić,[1] Vesna Kecman,[2] Marko Bulajić,[2] Nina Zubović,[3] Branislav Glišić[4]

Abstract

The impaction of maxillary canines is one of the biggest challenges in orthodontics practice. This case report describes successful surgical and orthodontic approach to the treatment of palatally impacted maxillary canine in a 14-year-old boy. Intraoral clinical examination revealed an absence of the upper right canine, an ectopic position of the upper left canine and crowding in the maxillary arch. The impaction of right maxillary canine and class II malocclusion were confirmed by lateral cephalogram, orthopantomogram and cone beam computed tomography. In the first phase, a transpalatal arch to the upper first molar teeth was applied, first premolars were extracted, and brackets were placed on all teeth and nickel-titanium arch wire was applied. The initial orthodontic phase was soon thereafter followed by the surgical exposure and orthodontic traction of the impacted canine using ligature wire attached from the button with chain to the open coil on the arch wire. The orthodontic treatment took two years with satisfactory aesthetic and functional results at the end. This clinical case has shown that adequate treatment of impacted maxillary canine can be achieved by using combined surgical technique and appropriate orthodontic approach.

Key words: Impacted canine; Malocclusion, Class II; Orthodontic-surgical approach; Canine traction.

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ARTICLE INFO
Received: 14 September 2020
Revision received: 20 September 2020
Accepted: 20 September 2020

Introduction

The presence of canines in dental arch are extremely important for facial harmony, smile aesthetic and stabile occlusion. Many factors can influence the functional and aesthetic balance, including the canine impaction. The impaction of the maxillary permanent canines is the second most common form of tooth impaction after the third molars with the rate that varies from 0.2 to 2.8 %.[1, 2] Girls are much more frequently affected than boys, with the prevalence ratio from 1.3 : 1 to 3.2 : 1, and with bilateral occlusion occurring in 8 % of cases.[3] The impacted canines occur 10-20 times more frequently in the upper jaw than in the lower jaw4 and the impaction occurs more frequently in the palatine region than in the buccal one (85 % vs 15 %).[5]

The causes of the maxillary canine impaction can be classified into several distinct groups: local tissue obstruction, local pathology, disturbance of normal development and hereditary or genetic factors.[4, 6] Canine impaction is very often accompanied by the persistence of deciduous teeth, cysts or dental ankyloses.[7]

This article should be cited as follows: Pašagić Lj, Ilić I, Kecman V, Bulajić M, Zubović N, Glišić B. Combined orthodontic and surgical treatment of impacted maxillary canine in young patient with class II malocclusion: a case report. Scr Med 2020;51(3):209-14.

There are several options for treatment of maxillary canine impaction. Some of them include extraction followed by an implant support or a prosthetic replacement procedure. However, if the position of impacted canine in the bone allows an orthodontic-surgical method, the orthodontic treatment should be started as soon as possible to avoid secondary problems.[8] The treatment procedure is based on surgical release and orthodontic traction of an impacted canine to the dental arch.

The aim of this report is to describe the orthodontic treatment in patient with impacted maxillary permanent canine using a combined surgical-orthodontic approach, and to validate the functional and aesthetic results.

Case history

A 14-year-old male patient was referred to the Department of Orthodontics, Dental Clinic of the Faculty of Medicine, University of Banja Luka, the Republic of Srpska, Bosnia & Herzegovina, for orthodontic consultation related to aesthetic reasons. The extraoral clinical examination revealed that the patient's face was symmetric with convex profile and unaesthetic smile. During intraoral clinical examination the absence of upper right canine, ectopic position of upper left canine and crowding in the maxillary arch were diagnosed. In the following steps the orthodontic records were taken including maxillary and mandibular impressions, extraoral and intraoral photographs, lateral cephalogram, panoramic radiograph and cone beam computed tomography (CBCT) of the maxilla (Figures 1 and 2). Gnatometric analyses of the study models revealed the Class II malocclusion. The panoramic radiograph showed the presence of all permanent teeth in-

Figure 1: Extraoral and intraoral pretreatment photographs.

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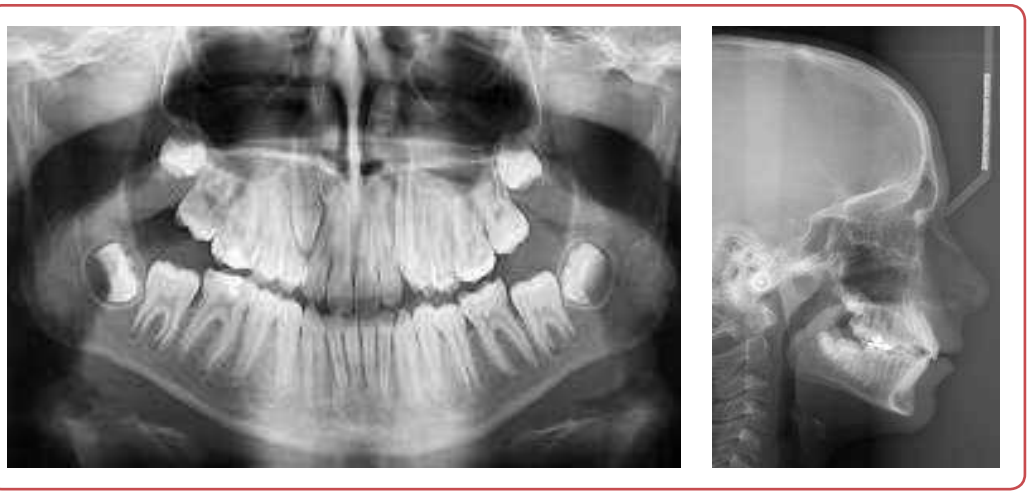

Figure 2: Initial radiographs; panoramic radiography (left) and lateral teleradiography (right).

cluding the developing third molar buds and intraosseal impaction of right permanent canine in the maxilla (Figure 2). The orthopantomogram analysis revealed an increased angle between the maxillary canine and lateral incisors indicating mesial inclination (Figure 2). The CBCT of maxilla demonstrated that the canine was impacted on palatal site. The cephalometric analysis on the lateral cephalogram confirmed a skeletal Class II malocclusion. After the diagnostic analyses was completed, the patient was scheduled for the combined surgical-orthodontic treatment.

Course of treatment

After a detailed analysis of the study model was done, the surgical and orthodontic treatment was applied. In the initial orthodontic phase the transpalatal arch to the upper first molar teeth was applied to maintain an anchorage (Figure 1). After that, the first premolars were extracted, brackets were placed on all teeth and a 0.012 nickel-titanium (NiTi) arch wire was applied. In this case the metal brackets of 0.018 slot were used. Open coil spring was positioned between 1.2 and 1.4 to increase the space for the permanent canine. Levelling and alignment phase was done by wire sequences of 0.012", 0.014", 0.016", 0.018", 0.016" x 0.022" NiTi, followed by 0.016" x 0.022" stainless steel arch wire. After orthodontic initial phase was completed the surgical exposure of 1.3 was performed using an open eruption technique. Surgical circular excision of the overlying palatal mucosa was performed and a small amount of bone covering the impacted canine was removed. The enamel surface of the impacted canine was washed and dried and then an orthodontic button with a chain for the guided traction was bonded at the crown level. Three weeks later the patient

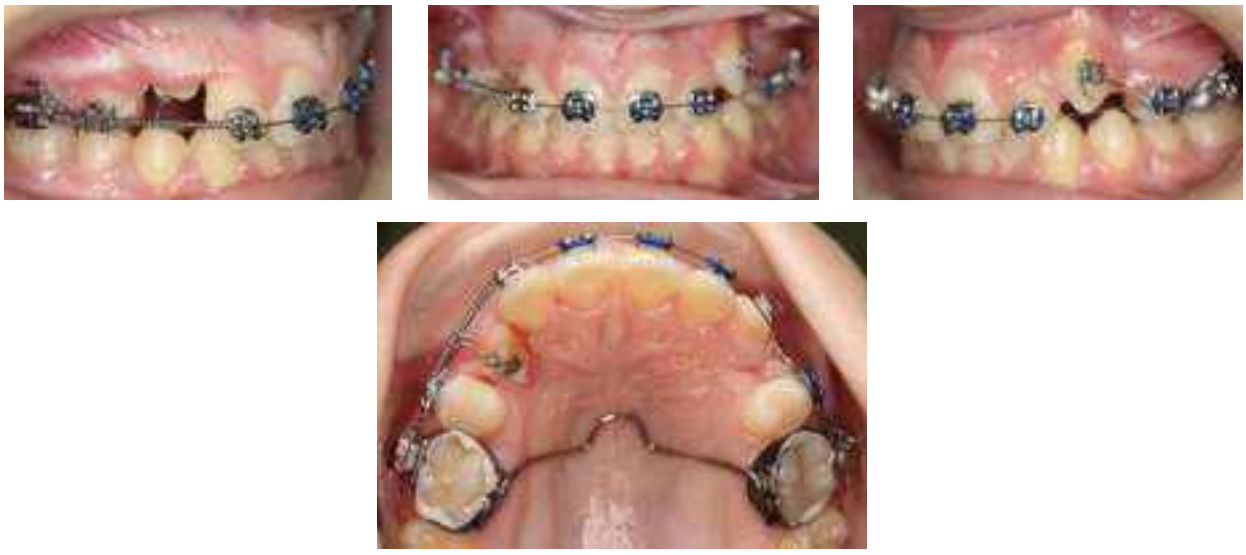

Figure 3: Orthodontic traction of the canine into the dental arch.

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Figure 4: Post-treatment extraoral and intraoral photographs.

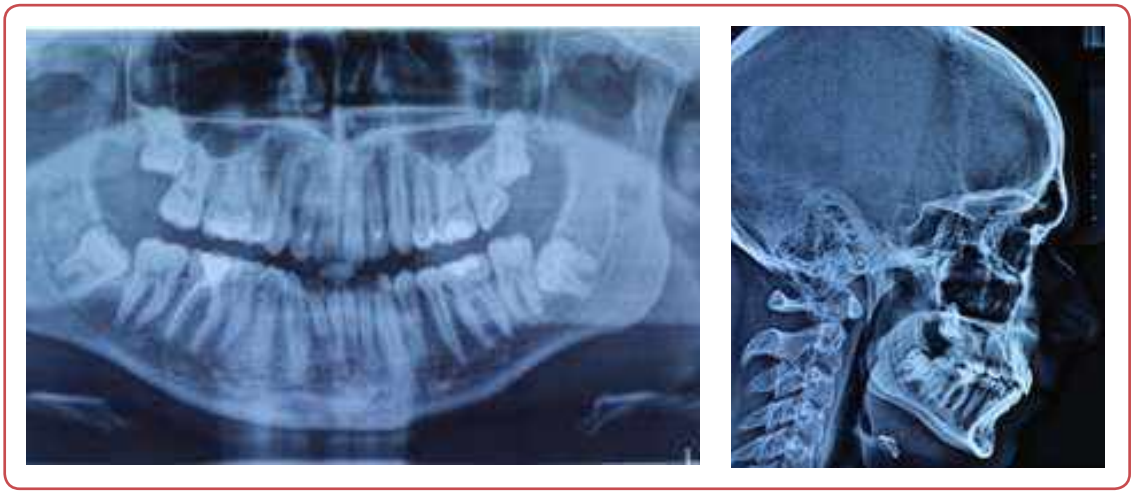

Figure 5: Post-treatment radiographs; panoramic radiography (upper) and lateral teleradiography (right).

returned to the orthodontic department after the soft tissues had healed. Traction of the impacted maxillary canine was made with ligature wire attached from the button with chain to the open coil on the arch wire. The patient came for check-ups every three weeks (Figure 3). Eight months later the left maxillary canine was near the arch and the bracket was bonded to the labial surface of its crown and tied to arch wire of 0.016" NiTi to begin the final orthodontic phase. Maxillary left canine alignment and levelling was done by following wire sequence of 0.018" and 0.016" x 0.022" NiTi. Finalising was achieved with stainless steel arch wire of 0.016" x 0.022". The active treatment took 24 months and retention was accomplished with a removable acrylic retainer. The final aesthetic result was satisfactory and the final radiographs indicated intact roots and proper root alignment (Figures 4 and 5).

CONTENT

Discussion

The treatment of impacted teeth is among the most complex procedures in orthodontics that requires multidisciplinary approach. In order to identify and locate the position of impacted teeth adequate tomographic images are required. It also helps to evaluate possible injuries to adjacent roots, to estimate the quality and quantity of bone around the teeth, to detect the existence of possible tooth ankylosis and occurrence of follicular cysts or infections.[7, 9] In this clinical case the post-treatment tomographic image evaluation did not show any signs of external root resorption.

Besides the position of the teeth, the patient's age can also determine the type of treatment.[10] For adult patients the tooth extraction is recommended method of treatment.[3] However, for younger patients with favourable position of impacted canine, combined surgical and orthodontic treatment is suggested. The best treatment for impacted canine is surgical exposure and orthodontic traction in order to bring the impacted tooth to the line of occlusion using light and slow orthodontic forces.[11] The movement of 1 mm per month has been considered as an optimum traction.[12] However, concerning the surgical exposure it is important to emphasise that good collaboration between the orthodontist and the surgeon is of crucial importance to define and select the most appropriate technique.[13] Similarly to the spontaneous tooth eruption, the combined surgical and orthodontic approach to the treatment of an impacted canine has to provide conditions to facilitate the physiological eruption.[14] It has been shown that combined orthodontic-surgical traction of impacted canines provides near- natural conditions for tooth eruption and reduce the need for prosthetic therapy.[15] This report has described a case of unilateral impaction of permanent maxillary canine in a young male patient. In this case the open eruption method was used in order to reduce the risk of the second surgical exposure.[11, 16]

Conclusion

This clinical case has shown that treatment of impacted maxillary canine can be achieved by using combined surgical technique and appropriate orthodontic approach in order to obtain good aesthetic and functional results. An adequate orthodontic force with optimum traction is a key component in orthodontic treatment needed for satisfactory occlusion and good aesthetic results.

Acknowledgements

None.

Conflict of interest

None.

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All measurements should be reported in the metric system of the International System of Units (SI), and the standard internationally accepted terms (except for mmHg and °C).

MS Word is recommended for word processing. Illustrations should be made using standard graphic formats (*pdf, *Ai, *cdr).

Photos should be delivered in *jpg format, 1:1, 300 Dpi as separate files.

Papers should be prepared in accordance with the Vancouver Convention.

Scripta Medica uses the British spelling of the English orthography. American spelling should be limited to the official titles of the US institutions and the references only.

Preparation of the manuscript
Parts of the manuscript are: Title page; Abstract with Key words; Text; Acknowledgements (optional), References.

1. Title page
a) The title should be concise but informative, while subheadings should be avoided. Words in the title should be capitalised according to the Rules of the AP Title Case.
b) Full names of the authors marked with ascending Arabic numerals in superscript (eg 1, 2, 3).
c) Exact names and places of department(s) and institution(s) of affiliation where the studies were performed, city and the state for any authors, clearly marked by standard footnote signs;
d) Contact data of the corresponding author.

2. Abstract and key words.
The second page should contain the title of the article and a structured abstract (250-300 words for original articles). In short, clear sentences the authors should write the Background/Aim, major procedures – Methods (choice of subjects or laboratory animals; procedures, methods for observation and analysis), the obtained findings – Results (concrete data and their statistical significance), and the Conclusion. It should emphasize new and important aspects of the study or observations. A non-structured abstract of maximum 150 words should be used for editorials, review articles, current topics, special articles, professional articles, history of medicine articles and case reports. Letters to the editor and obituaries do not have an abstract. Below the abstract 3-5 key words should be provided that describe the topic of the article. The key words should be selected from Medical Subject Headings (MeSH). Letters to the editor and obituaries do not need to have key words.

3. Text
The text of the articles includes the following chapters: Introduction, Methods, Results and Discussion. Longer articles may need subheadings within some sections to clarify their content. Case reports should have four sections: Introduction, Case history, Discussion and Conclusion.

Introduction. After the introductory notes, the aim of the article should be stated in brief (the reasons for the study or observation), only significant data from the literature, but not extensive, detailed consideration of the subject, nor data or conclusions from the work being reported.

CONTENT

Methods. Selection of study or experimental subjects (patients or experimental animals, including controls) should be clearly described. Methods, apparatus (manufacturer's name and address in parentheses) and procedures should be identified in sufficient detail to allow other workers to reproduce the results. References should be cited for established methods, including the statistical ones. Identify precisely all drugs and chemicals used, with generic name(s), dose(s), and route(s) of administration. Statements on the study approval by the Ethics Committee for human or animal studies should be included.

Results should be presented in logical sequence in the text, tables and illustrations. Emphasise or summarise only important observations.

Discussion is aimed to emphasise new and significant aspects of the study and conclusions that result from them. Relate the observations to other relevant studies.

Conclusion should be linked with the goals of the study, but avoid unqualified statements and conclusions not completely supported by your data.

Tables
Each table should be typed single-spaced on a separate sheet, numbered in the order of their first citation in the text in the upper right corner and supplied with a brief title each. Explanatory notes are printed under a table. Each table should be mentioned in the text. If data from another source are used, acknowledge them fully. Each table should be named in order to indicate the author's name the number of the table, (eg Johnson_Table 1). The place of the table in the text should be marked as in the following example: "(Table 1 near here)".

Illustrations
Any forms of graphic enclosures are considered to be figures and should be submitted as additional databases in the System of Aseestant. Letters, numbers, and symbols should be clear and uniform, of sufficient size that when reduced for publication, each item will still be legible. Each figure should be named in order to indicate the author's name and the number of the figure (eg Johnson_Figure 1). If a figure has been published, state the original source.

Captions for illustrations are typed on a separate page, with Arabic numbers corresponding to the illustrations. If used to identify parts of the illustrations, the symbols, arrows, numbers, or letters should be identified and explained clearly in the legend. The method of staining and magnification in photomicrographs should be explained. Captions should be detailed enough to allow for understanding of the content of the figure without previous reading of the text. The place of the figure in the text should be marked as in the following example: "(Figure 1 near here)".

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Authors are encouraged to use abbreviations and acronyms in the manuscript in the following manner: abbreviations and acronyms must be defined the first time they are used in the text and thereafter must be consistently used throughout the whole manuscript, abbreviations should be used only for terms that appear more than three times in text; abbreviations should be sparingly used. Use of abbreviations in the titles should be avoided. Even if used after its definition in the abstract, the same definition and repetition of the abbreviation should be performed the first time it is used in the text. In order to assure self-explanatory nature of the tables and figures, abbreviations and acronyms should be defined in the captions and then introduced, irrespective of whether it was done earlier in the text.

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Examples of references:

Gillespie NC, Lewis RJ, Pearn JH, Bourke ATC, Holmes MJ, Bourke JB, et al. Ciguatera in Australia: occurrence, clinical features, pathophysiology and management. Med J Aust 1986;145:584-90.

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Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

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CONTENT