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EDITORIAL

Multi-Omics Approaches in Research of Cardiovascular Diseases

Guo-Wei He[1]

Abstract

Cardiovascular diseases remain number 1 killer world-wide. New technologies, particularly the novel approaches at genetics, transcriptomics, proteomics and metabolomics levels have provided new insights into the disease aetiology, pathophysiology and diagnosis, as well as therapeutics from molecular level. These omics by integrating the nuclear, cellular components, proteins, enzymes generate large data sets gather the information with support of organised and systematic computational methods. The integrated analyses may give deeper view on the development, treatment and prevention of the disease. In recent years, multi-omics technologies have been used in research on cardiovascular diseases including coronary artery disease, heart valve disease and congenital heart disease. These studies help in understanding the aetiology and pathophysiology of the disease and facilitate the precision diagnosis, therapy and prognosis.

Key words: Omics; Cardiovascular; Coronary artery disease; Heart valve; Congenital heart.

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Introduction

Cardiovascular diseases remain number 1 killer world-wide. New technologies, particularly the novel approaches at genetics, transcriptomics, proteomics and metabolomics levels have provided new insights into the disease aetiology, pathophysiology and diagnosis, as well as therapeutics from molecular level. These omics by integrating the nuclear, cellular components, proteins, enzymes generate large data sets gather the information with support of organised and systematic computational methods. The integrated analyses may give deeper view on the development, treatment and prevention of the disease. In recent years, the multi-omics approach has become very useful tool for medical research. This review will briefly describe our multi-omics studies in cardiovascular research.

Coronary artery disease (CAD)

Proteomics of multi-vessel disease in CAD

World Health Organisation has indicated that the world's biggest killer is CAD (ischaemic heart disease), responsible for 16 % of the world's total deaths. Since 2000, the largest increase in deaths has been for CAD.[1]

We have focused the multi-omics studies on coronary artery bypass grafting (CABG) related issues. First, a study to identify the protein profiling in patients with triple vessel coronary artery disease (CAD) undergoing CABG, in order to detect CAD-related differential proteins (DPs) in these patients was designed. Plasma samples of 20 males and 20 females in each group were analysed with iTRAQ technique. ELISA test was used

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to test the chosen proteins from iTRAQ results in plasma samples from a new cohort of the CABG group (n = 120) and control (n = 120). iTRAQ detected 544 proteins with 35 up-regulated and 41 down-regulated. Three proteins including platelet factor 4 (PF4), coagulation factor XIII B chain (F13B), and secreted frizzled-related protein 1 (sFRP1) were selected for validation by using ELISA that demonstrated significant up-regulation of PF4 and sFRP1. There was a positive correlation between these proteins and CAD and myocardial infarction history. Thus, the thrombotic disease/inflammation progress-related protein PF4 and sFRP1, a member of the Wnt/fz signal-transduction pathway and related to myocardial repair, are significantly up-regulated in triple-vessel disease with/without left main stenosis. PF4 may be developed as a biomarker for the diagnosis of the severity of CAD requiring CABG procedure.[2]

Proteomics and metabolomics of postoperative atrial fibrillation (POAF)

POAF is a common complication in CABG. A prospective study aimed to investigate predisposition of proteins and metabolites correlated to POAF after CABG and related cellular pathways was performed. Preoperative plasma samples from CABG patients were prospectively collected. After CABG, the patients were grouped to POAF or sinus rhythm. The plasma samples were analysed using proteomics, metabolomics and bioinformatics to identify the differential proteins (DEPs) and metabolites (DEMs). The correlation between DEPs and POAF was also investigated by multivariable regression analysis and receiver operator characteristic (ROC) analysis. It was found that in POAF(+) group, 29 DEPs and 61 DEMs were identified compared to POAF(-) group. The analysis of integrated omics revealed that preoperative alteration of PPAR-alpha and glutathione metabolism pathways increased the susceptibility of POAF after CABG. There was a correlation between plasma levels of APO-CIII, PLTP, GPX3, CETP and POAF. It was then concluded that at multi-omics levels pre-existing DEPs and DEMs in the plasma of patients prone to POAF after CABG. Dysregulation of PPARα and glutathione metabolism pathways related to metabolic remodelling and redox imbalance-associated electrical remodelling may play a key role in the pathogenesis of POAF. Lower plasma PLTP, APO-CIII, higher CETP and GPX3 are linked with POAF. These proteins/metabolites may be developed as biomarkers to predict POAF.[3]

Transcriptomics, proteomics and metabolomics in heart valve disease (HVD)

Plasma proteomics in HVD

A proteomic study was conducted to exam the hypothesis that certain proteins may be associated with the pathological changes in the plasma of HVD patients. Differential protein analysis in the plasma identified 18 differentially expressed protein spots and 14 corresponding proteins or polypeptides by two-dimensional electrophoresis and mass spectrometry in 120 subjects. Two up-regulated (complement C4A and carbonic anhydrase 1) and three down-regulated proteins (serotransferrin, alpha-1-antichymotrypsin and vitronectin) were validated by ELISA in enlarging samples. The plasma levels (n = 40 for each) of complement C4A in RVD and carbonic anhydrase 1 in DVD patients were significantly higher and that of serotransferrin and alpha-1-antichymotrypsin in RVD patients were significantly lower than those in controls. The plasma vitronectin level in both RVD and DVD was significantly lower than those in normal controls. This study identified alterations of 14 DPs or polypeptides in the plasma of patients with various HVD. The elevation of plasma complement C4A in RVD and carbonic anhydrase 1 in DVD and the decrease of serotransferrin and alpha-1-antichymotrypsin in RVD patients may be useful biomarkers for these valvular diseases. The decreased plasma level of vitronectin – a protein related to the formation of valvular structure – in both RVD and DVD patients might indicate the possible genetic deficiency in these patients.[4]

Transcriptomics and proteomics in atrial tissue in atrial fibrillation (AF) associated with HVD

In this study, multi-omics approach with combined transcriptomic and proteomic technologies in the human atrial tissue was used to investigate the correlation between electrical and structural remodelling processes in HVD patients with AF. Human atrial tissues from the patients with rheumatic mitral valve disease in either sinus rhythm or persistent AF were analysed. An up-regulation in chloride intracellular channel (CLIC) 1, 4, 5 and a rise in type IV collagen were revealed. Combined with the results from immunohistochemistry and electron microscope analysis, the distribution of type IV collagen and effects of fibrosis

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on myocyte membrane indicated the possible interaction between CLIC and type IV collagen, confirmed by protein structure prediction and co-immunoprecipitation. These results indicate that CLICs play an important role in the development of AF and that CLICs and structural type IV collagen may interact on each other to promote the development of AF in rheumatic mitral valve disease.[5]

Transcriptomics and proteomics in human atrial tissue identified PPAR pathway in AF associated with HVD

In this study, human atrial tissues from patients with HVD in persistent AF preoperatively for at least 6 months or remaining sinus rhythm (as a control) undergoing heart valve replacement surgery were studied by combined transcriptomics and proteomics analyses. The total RNA of the

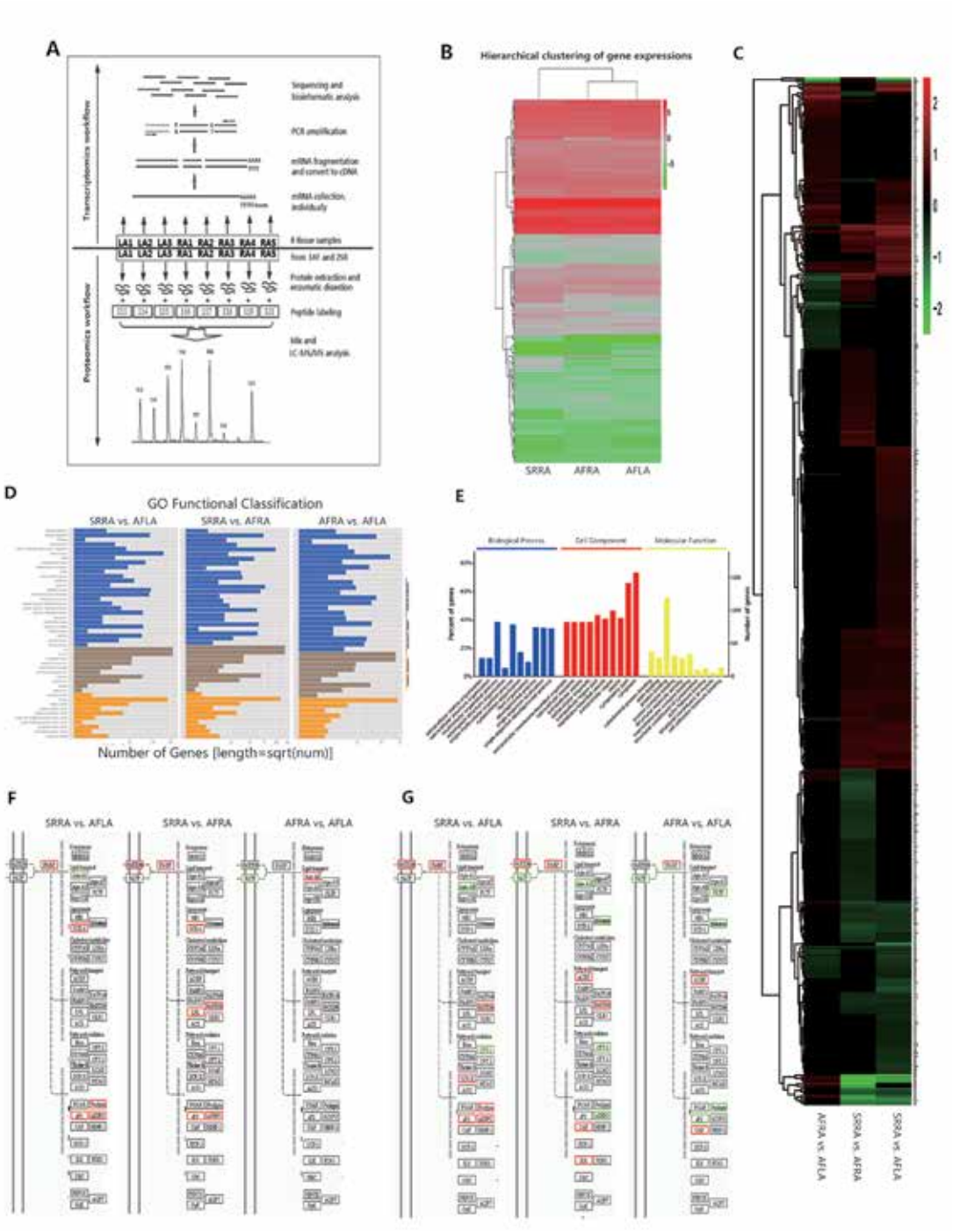

Figure 1: Integrated transcriptomics and proteomics in human atrial tissue identified PPAR pathway in atrial fibrillation (AF) associated with heart valve disease (HVD) (reproduced from reference 6 with permission)

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left and right atrial tissue samples (AFLA and AFRA) from the same AF patients and right atrial tissue from SR (SRRA) patients was extracted. cDNA library was sequenced in a single lane of the Illumina HiSeq™ 2000 system using paired end protocols according to the manufacturer's instructions. The total protein from each sample was extracted and labelled with a unique iTRAQ tag. Big data obtained from the omics studies were analysed by bioinformatics tools. This study provides an integrative analysis of transcriptomics and proteomics in the human atrial tissues of AF-associated HVD patients and reveals that the PPAR signalling pathway plays an important role in the pathogenesis of AF and that some proteins may serve as biomarkers for AF. Owing to the importance of the PPAR pathway in the pathology of AF found in HVD in this study, targeting differential proteins in the PPAR pathway may become new diagnostic and therapeutic methods in the future (Figure 1).[6]

Plasma biomarkers in AF associated with HVD identified by multi-omics methods

Further, the molecular mechanisms and possible biomarkers for chronic AF in HVD was investigated by using multi-omics methods with combined proteomics and metabolomics technologies. This prospective study enrolled patients with mitral valve disease (n = 100) undergoing mitral valve replacement surgery. The patients were allocated into chronic AF and sinus rhythm (SR) groups. Plasma samples were collected preoperatively. Proteomics was performed with iTRAQ to identify DEPs between the two groups. The selected DEPs were then validated in a new cohort of patients by ELISA. A gas chromatography-mass spectrometer was used in the metabolomics study to identify differential metabolites (DEMs). Bioinformatics analyses were performed to analyse the results. Among the 447 plasma proteins and 322 metabolites detected, 57 proteins and 55 metabolites, including apolipoprotein A-I (ApoA-I), apolipoprotein A-II (ApoA-II), LIM domain only protein 7 (LMO7), and vitronectin (VN) were differentially expressed between AF and SR patients. Bioinformatics analyses identified enriched pathways related to AF, including peroxisome proliferator-activated receptor alpha (PPARα), the renin angiotensin aldosterone system (RAAS), galactose, biosynthesis of unsaturated fatty acids and linoleic acid metabolism. Using integrated multi-omics technologies in HVD-associated AF patients, this study revealed important signalling pathways, such as PPARα, as well as possible roles of other signalling pathways, including the RAAS and galactose metabolism to understand the molecular mechanism of MVD-associated AF. It also identified a large number of DEPs and DEMs. Some identified proteins and metabolites, such as ApoA-I, ApoA-II, LMO7 and VN, may be further developed as biomarkers for MVD-associated AF.[7]

Congenital heart disease (CHD)

CHD is the most prevalent primary type of congenital anomalies.[8] Genetic studies may reveal the aetiology of the CHD. Under most circumstances, genomic and genetic studies are useful to identify the genetic variants of CHD. However, proteomics studies are also important in identifying the most important protein changes during disease progress. This was the aim in the present studies on CHD.

Genetic studies on 22q11.2 deletion syndrome

The 22q11.2 deletion syndrome is considered the most frequent chromosomal microdeletion syndrome in humans and the second leading chromosomal cause of CHD. The aim was to identify the prevalence and the detailed genetic characterisation of 22q11.2 region in children with CHD including simple defects and to explore the genotype-phenotype relationship between deletion/amplification type and clinical data. Patients with CHD were screened by multiplex ligation-dependent probe amplification and capillary electrophoresis methods. Universal Probe Library technology was applied for validation. In 354 CHD patients, 40 (11.3 %) carried different levels of deletions/amplifications at the 22q11.2 region with various phenotypes. The affected genes at this region include CDC45 (15 patients), TBX1 (8), USP18 (8), RTDR1 (7), SNAP29 (6), TOP3B (6), ZNF74 (4) and other genes with less frequency. Among those, two patients carried 3 Mb typically deleted region from CLTCL1 to LZTR1 (Low Copy Repeats A-D) or 1.5 Mb deletions from CLTCL1 to MED15 (Low Copy Repeats A-C). Clinical facial manifestations were found in 12 patients. This study revealed an unexpected high prevalence of chromosome 22q11.2 variations in CHD patients

CONTENT

even in simple defects. The genotype-phenotype relationship analysis suggests that genetic detection of 22q11.2 may become necessary in all CHD patients and that detect of unique deletions or amplifications may provide useful insight into personalised management in CHD patients.[9]

Complex CHD with polydactyly

CHD associated with polydactyly involves various genes. In this study, it was aimed to identify variations from genes related to complex CHD with polydactyly and to investigate the cellular functions related to the mutations. Blood was collected from a complex CHD case with polydactyly and whole exome sequencing (WES) was performed. The CRISPR/Cas9 system was used to generate human pluripotent stem cell with mutations (hPSCs-Mut) that were differentiated into cardiomyocytes (hPSC-CMs-Mut) and analysed by transcriptomics on day 0, 9 and 13. Two heterozygous mutations, LTBP2 (c.2206G>A, p.Asp736Asn, RefSeq NM_000428.2) and TCTN3 (c.1268G>A, p.Gly423Glu, RefSeq NM_015631.5) were identified via WES but no TBX5 mutations were found. The stable cell lines of hPSCs-LTBP-2^{mu}/TCTN3^{mu} were constructed and differentiated into hPSC-CMs-LTBP2^{mu}/TCTN3^{mu}. Compared to the wild type, LTBP2 mutation delayed the development of CMs. The TCTN3 mutation consistently presented lower rate and weaker force of the contraction of CMs. For gene expression pattern of persistent up-regulation, pathways in cardiac development and CHD were enriched in hPSCs-CM-LTBP2^{mu}, compared with hPSCs-CM-WT. Thus, the heterozygous mutations in TCTN3 and LTBP2 affect contractility (rate and force) of cardiac myocytes and may affect the development of the heart. These findings provide new insights into the pathogenesis of complex CHD with polydactyly.[10]

Genetic studies in CHD

The DNA on a series of CHD was sequenced and genetic variants of ventricular septal defect (VSD) were identified.[11-14]

Proteomics studies in CHD

Owing to the importance of protein profiling in CVD, a series of studies on proteomics in CHD was also performed.

Plasma proteomics in CHD

In this early study, the hypothesis that plasma proteins may be altered and related to the pathological changes of CHD was examined. Differential protein analysis was performed in the plasma of patients with tetralogy of Fallot (TOF), isolated VSD and normal controls by using two-dimensional electrophoresis and mass spectrometry. Candidate proteins that might be related to disease processes were further confirmed by ELISA in the new samples. Eighteen differentially expressed protein spots and 10 corresponding proteins or polypeptides were identified. Among those, two down-regulated proteins (gelsolin, ficolin-3) with significant clinical relevance were further analysed for validation. The plasma gelsolin and ficolin-3 levels in TOF patients were significantly lower than those in normal controls. This proteomic study demonstrated the plasma protein changes in CHD patients.[15, 16]

Plasma proteomics in CHD after repair surgery

The final goal for treatment of CHD is to resume not only the normal heart structure but also physiology. This study was designed to evaluate surgical results at molecular basis on the proteomic pattern in the pre- and post-operative period in TOF and VSD in order to find whether structure repair is associated with clinically important molecular changes in CHD. Differential protein analysis by using two-dimensional gel electrophoresis and mass spectrometry followed by ELISA validation was performed in the plasma samples of patients with TOF (n = 82) or VSD (n = 82) preoperatively, 6-month postoperatively and in normal controls (n = 82). A total of 473 protein spots in preoperative patients and 515 in postoperative patients were detected. Significantly downregulated or upregulated proteins were detected. Validation of proteins in the new cohort of patients demonstrated that in VSD patients, postoperative complement component C3c was partially and serum amyloid P-component was completely recovered. In TOF patients, postoperative gelsolin was partially recovered. In contrast, the elevated fibrinogen gamma chain level in preoperative patients became normal postoperatively. Thus, it was for the first time demonstrated by using proteomic methods that repair surgery for CHD not only corrects the structure malformation but also resumes the normality of certain altered proteins at molecular level. Identification of the recovered or unchanged proteins may facilitate the evaluation of the surgical results and the personalised management in postoperative period and long-term.[17]

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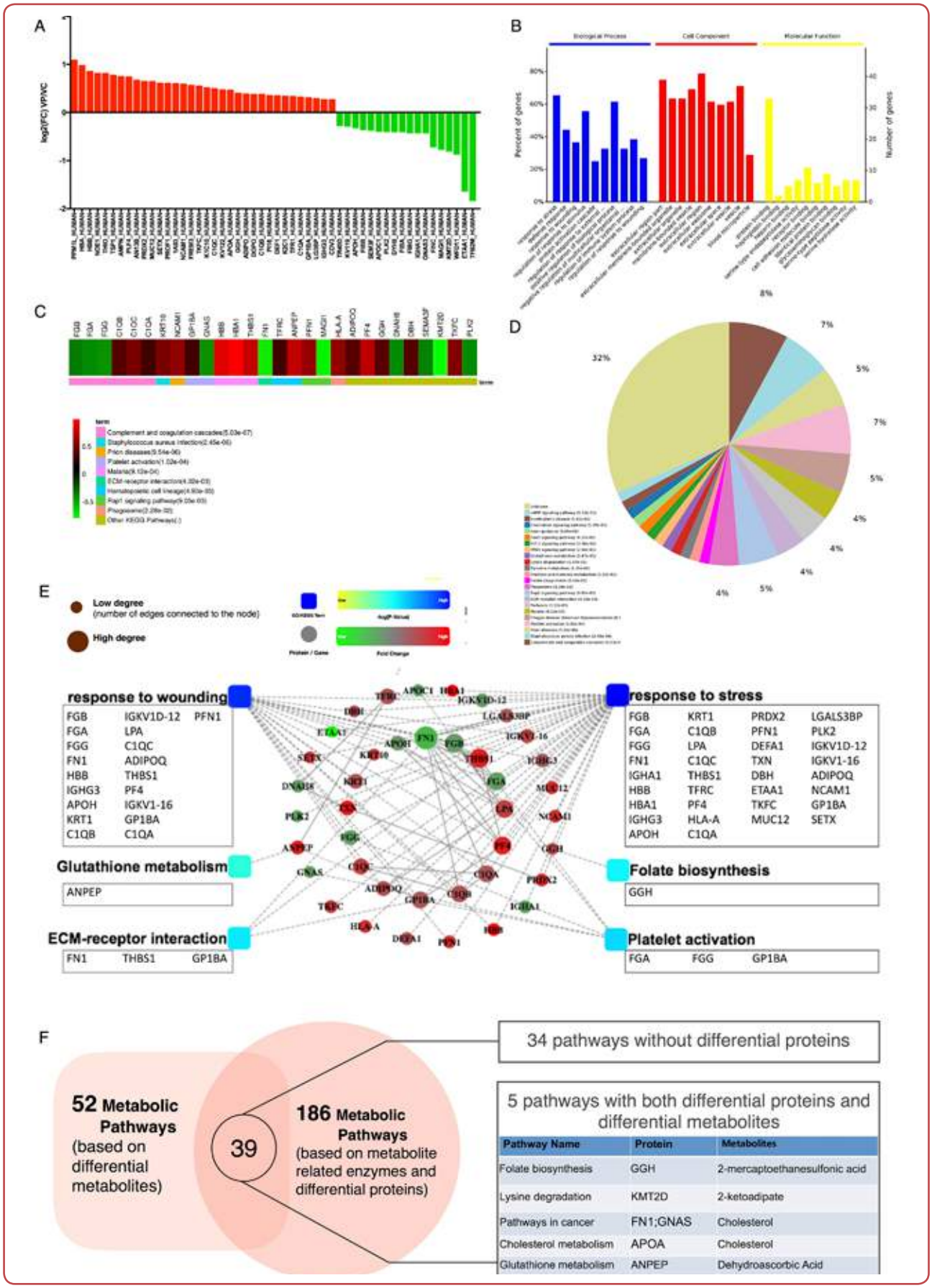

Figure 2: Multi-omics study on CHD-PAH to identify biomarkers (reproduced from reference 20 with permission)

Proteomics in patent ductus arteriosus (PDA) patients

PDA is the third most common congenital heart disease and resulted from the persistence of ductal patency after birth. Ductus arteriosus closure involves functional and structural remodelling, controlled by many factors. The changes in plasma protein levels associated with PDA closure are not known. Here, there were for the first time demonstrated six key differential plasma proteins in human PDA patients using proteomic technology and present a model to illustrate the constriction and closure of ductus arteriosus. Differentially expressed proteins were analysed by using isobaric tags for relative and absolute quantification and validated by enzyme linked immunosorbent assay in new samples. There were 74 up-regulated and 98 down-regulated proteins in the plasma of PDA patients. Five decreased proteins (platelet factor 4, fibrinogen, von Willebrand factor, collagen and mannose binding lectin-associated serine protease-2) and one increased protein (fibronectin) may increase the risk of PDA. Those proteins are closely related to platelet activation and coagulation cascades, complement mannan-binding-lectin and other systemic signalling pathways. These findings for the first time indicate that the differential proteins involved in different pathways may play key roles in the non-closure of the ductus arteriosus in humans and may be developed as biomarkers for diagnosis. All those findings may be served as the basis of understanding the aetiology and pathogenesis of PDA.[18]

Plasma proteomic study in pulmonary arterial hypertension (PAH) associated with CHD

Pulmonary arterial hypertension associated with congenital heart disease (CHD-PAH) has serious consequence and plasma protein profiles in CHD-PAH are unknown. It was aimed to reveal the differential plasma proteins in 272 CHD patients with or without PAH. Various types of CHD-PAH were studied. Differential plasma proteins were first detected by iTRAQ proteomic technology and those with significant clinical relevance were selected for further ELISA validation in new cohort of patients. Among the 190 differential plasma proteins detected by iTRAQ, carbamoyl-phosphate synthetase I (CPSI, related to urea cycle and endogenous nitric oxide production) and complement factor H-related protein 2 (CFHR2, related to complement system and coagulant mechanism) were selected for further ELISA validation in new cohort of 152 patients. Both CPSI and CFHR2 were down-regulated with decreased plasma levels. Thus, for the first time in CHD-PAH patients, a large number of differential plasma proteins was identified. The decreased CPSI expression in CHD-PAH patients may reveal a mechanism related to endogenous nitric oxide and the decrease of CFHR2 protein may demonstrate the deficiency of the immune system and coagulation mechanism. The findings may open a new direction for translational medicine in CHD-PAH with regard to the diagnosis and progress of the disease.[19]

Biomarkers in CHD-PAH

Further, a study was designed to investigate the signalling and metabolic pathways and risk scores of protein biomarkers in CHD-PAH by using integrated multi-omics studies. Plasma from 160 children, including 120 VSD patients with/without PAH and 40 healthy children was studied by integrated proteomics, metabolomics and bioinformatics analyses. Proteomics (iTRAQ) identified 107 DEPs between patients with/without PAH including significantly increased ADIPO, DBH, ANPEP, TRF1, GP1BA and decreased GNAS in VSD-PAH. Metabolomics discovered 191 differential metabolites between patients with/without PAH including elevation of 5-HT, taurine, creatine, sarcosine and 2-oxobutanoate and decrease of vanillylmandelic acid, 3,4-dihydroxymandelate, 15-Keto-$PGF_{2\alpha}$, fructose-6-phosphate, L-glutamine, dehydroascorbate, hydroxypyruvate, threonine, L-cystine and 1-aminocyclopropane-1-carboxylate. The DEPs were validated in a new cohort of patients (n = 80). Integrated analyses identified the key pathways including cAMP signalling pathway, ECM-receptor interaction, AMPK signalling pathway, HIF-1 signalling pathway, PI3K-Akt signalling pathway and amino acid metabolisms. Increased plasma protein levels of DBH, ADIPO and ANPEP were found to be independently associated with the occurrence of PAH with a new total risk score from these three proteins developed for clinical diagnosis. In this integrated multi-omics analysis in VSD-PAH patients, it was for the first time found that VSD-PAH patients present important DEPs, metabolites and key pathways. A total-risk-score (based on the plasma concentration of DBH, ANPEP and ADIPO) was developed as a predictor of development of PAH in CHD-VSD patients. Therefore, these proteins may be used as biomarkers and the new total-risk-score has significant clinical implications in the diagnosis of PAH (Figure 2).[20]

CONTENT

Conclusion

Multi-omics technologies have been used in research on cardiovascular diseases including CAD, HVD and CHD. These studies help in understanding the aetiology and pathophysiology of the disease and facilitate the precision diagnosis, therapy and prognosis. New technologies such as single cell sequencing may also be used in future studies to advance the understanding in the disease aetiology and development.

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Conflict of interest

None.

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ORIGINAL ARTICLE

In Vitro Assessments of Microencapsulated Viable Cells as a Result of Primary Bile Acid-Encapsulated Formulation for Inflammatory Disorders

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Abstract

Background / Aim: Metformin is widely used in type 2 diabetes and exhibits many positive biological effects on pancreatic β-cells and muscle cells, such as supporting insulin release by β-cells and glucose uptake by muscle cells and reducing oxidative stress, particularly due to diabetes-associated hyperglycaemia. Interestingly, for type 1 diabetes, transplantation of healthy β-cells has been proposed as a novel way to replace insulin therapy. Recently, bile acid-formulations containing transplantable β-cells showed best stability. Hence, this study aimed to explore the effects of metformin-bile acid formulations in β-cell encapsulation and on the biological activities of β-cells and muscle-cells.
Methods: Two sets of biological effects were examined, using metformin-bile acid formulations, on encapsulated β-cells and on muscle cells exposed to the formulations.
Results: Various encapsulated β-cell formulations' cell viability, insulin levels, cellular oxidative stress, cellular inflammatory profile and bioenergetics at the normo- and hyper-glycaemic states showed differing results based upon the metformin concentration and the inclusion or absence of bile acid. Similar effects were observed with muscle cells. Low ratios of metformin and bile acids showed best biological effects, suggesting a formulation dependent result. The formulations' positive effects were more profound at the hyperglycaemic state suggesting efficient cell protective effects.
Conclusion: Overall, metformin had positive impacts on the cells in a concentration-dependent manner, with the addition of chenodeoxycholic acid further improving results.

Key words: Bile acids; Chenodeoxycholic acid; Bioenergetics; Pancreatic beta-cells; Muscle cells.

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Introduction

Diabetes mellitus continues to be a significant global health problem, hence the importance in the development of an overarching treatment to assist in all aspects and subsequent complications of the condition.[1] Metformin is a drug which has anti-hyperglycaemic effects and is commonly prescribed in the treatment of type 2 diabetes mellitus. The widely accepted mechanism of action of metformin is its ability to decrease glucose production in hepatocytes and increase glucose uptake and utilisation in other peripheral tissues, including muscle tissue. There are also

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several theories about other mechanisms and manners in which metformin may act.[2, 3]

The role of inflammation in diabetes and subsequent complications has garnered interest, with the potential that anti-inflammatory treatments may be of assistance in diabetes management.[4] Drugs may also ameliorate hyperglycaemia which may in turn have anti-inflammatory effects, which can be manipulated for wider use.[5] This includes metformin, which has demonstrated anti-atherosclerotic properties in studies, which has been established to be likely due to anti-inflammatory effects of metformin.[6, 7] Furthermore, metformin has demonstrated anti-inflammatory effects in vascular smooth muscle cells and other tissues.[8, 9]

Stability profiling of metformin using HPLC conducted by Gedawy et al demonstrated that the drug metformin undergoes minimal oxidative degradation.[10] Piro et al showed metformin's ability to reduce oxidative stress caused by high free fatty acids in rat pancreatic islets.[11] As such, metformin may assist in improving antioxidant activity of cells, including encapsulated cells, which may in turn assist in inflammation control.

Antioxidant properties may also have protective effects, including on mitochondria. Mitochondria are important organelles, whose function are impacted by several factors, including cellular stress. The dysfunction of such mitochondria can also lead to insulin resistance, and mitochondrial changes in skeletal muscle of type 2 diabetic patients.[2] Hence, the importance of a drug which may protect mitochondria, such as metformin. Ahangarpour et al demonstrated the protective effects of metformin, likely due to its antioxidant properties.[12]

Metformin has also been shown to have protective effects on islet cells. Lupi et al demonstrated metformin to assist in protection of pancreatic islets from oxidation caused by free fatty acids, in addition to improving glucose utilisation.[13] Alternative studies by Lupi et al also demonstrated that metformin gave the ability for islet cells to maintain their insulin release, even when exposed to hyperglycaemic conditions. The authors showed metformin's potential to prevent the desensitisation of pancreatic islets even under high glucose concentration exposure.[14] Such properties would assist in the encapsulation of islet cells.

Microencapsulation offers the potential for an improved delivery of a variety of drugs, hence may be utilised with metformin via the addition of excipients including sodium alginate and poloxamer.[15-18] Microencapsulation of cells also has the potential to be translated into bioartificial organs, including an artificial pancreas to be used in diabetes treatments, replacing insulin therapy.[19-23]

A primary bile acid, such as chenodeoxycholic acid (CDCA) can also assist with stabilisation and has many promising properties which allow it to be utilised as an anti-inflammatory agent and permeation enhancer, making it ideal for use in microencapsulation.[24, 25] CDCA also has the potential to assist in cell survivability.[26] Previous research from this team has shown specific concentrations of CDCA with encapsulated with NIT-1 cells to offer optimised ani-inflammatory, pharmacological and cellular effects.[27, 28]

When encapsulated with pancreatic islets, metformin's beneficial properties may act on the islets offering improved protection via both the metformin and the encapsulation. Hence, the inclusion of such in this investigative study. Two formulations of equal metformin concentration, one with CDCA and one without will also be tested to see the impact of the addition of a bile acid such as CDCA, at six different metformin concentrations.
The properties of metformin, including anti-inflammatory and reduction in oxidative stress will be investigated within the context of this study, on both encapsulated MIN6 pancreatic β-cells and tested upon C2C12 muscle cells. C2C12 muscle cells will be investigated due to metformin's ability to act on them, including evidence of anti-inflammatory responses. Furthermore, previous research has utilised muscle cells for studies using bile acid ursodeoxycholic acid, due to its ability to be taken up by muscle cells.[29] Hence, both muscle and β-cells will be investigated due to their cellular uptake. Thus, providing a broader image of the impacts of such encapsulation and potential use in treatments of inflammatory disorders.

Methods

Metformin, sodium alginate, poloxamer and chenodeoxycholic acid were obtained from Sigma

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Chemical Co, USA. The MIN6 pancreatic β-cell line was kindly provided by Dr Jun-ichi Miyazaki, and the C2C12 cells were a generous gift from Professor Deidre Coombe. The formulations stock consisted of sodium alginate (1.6 %), poloxamer (4 %) and the drug metformin (0.4 % in F1 and F2, 1 % in F3 and F4, 2 % in F5 and F6, 3 % in F7 and F8, 4 % F9 and F10 and 6 % F11 and F12) and CDCA (0 % for control and 0.3 % for test formulations, being F2, F4, F6, F8, F10 and F12).

Microencapsulation

The above 12 formulations (F1-F12) were mixed with ultrapure water to form the stock mixtures for encapsulation. The BÜCHI-based microencapsulating system (BÜCHI Labortechnik, Switzerland) with a built-in concentric system and a Flow-Vibrational Nozzle was used to make the microcapsules.[30-32] This system makes use of a gelation bath below to capture the microcapsules.[17, 28, 31, 33, 34] In this experiment, a 2 % $CaCl_2$ gelation bath was used. $CaCl_2$ dihydrate was bought from Scharlab S.L (Sentrnenat, Spain), with the appropriate weight added to ultrapure water. In terms of cell encapsulation, the internal nozzle of the encapsulation system was utilised for the MIN6 pancreatic β-cells, whilst the prepared stock mixtures went through the external nozzle. The built-in concentric system ensures evenly formed and distributed microcapsules.[21-23]

Glucose-Induced Assessments of Microencapsulated Cells

Glucose-induced assessments of cell viability, antioxidant activity and insulin release were conducted on both the encapsulated MIN6 pancreatic β-cell and the additional C2C12 cells which were exposed to the microcapsules. Cells were incubated 48 h prior to assay. A validated method utilising MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co, USA) was used. Such a method allows assessment of cell viability microcapsules without having to rupture the capsules. Insulin release in regards to MIN6 cells was assessed using an Ultrasensitive Mouse Insulin ELISA kit (Mercodia Cooperation, Uppsala, Sweden).[35] Antioxidant activity measurements were conducted via fluorescent measurements from a plate reader (Enspire, PerkinElmer, USA). Cells were incubated with a mixture of dichloro-dihydro-fluorescein the oxidised radical species to provide oxidative stress measurements, with lower fluorescent readings corresponding to increased antioxidant activity.[29,36] All assessments were conducted post glucose exposure, with MIN6 cells exposed to either to 25 mmol/L or 35 mmol/L of glucose. C2C12 cells were exposed to conditions representative of normoglycemic and hyperglycaemic conditions, 5 mmol/L or 30 mmol/L of glucose.

Cytokine Assessments

Cytokine Bead Array technologies (BD Biosciences, San Jose, California, USA) was used for the assessment of pro and anti-inflammatory cytokines. Pro-inflammatory TNF-α, IFN-γ, IL-6 and IL-1β, and anti-inflammatory IL-10 cytokine biomarkers were utilised. Inflammatory profile assessments were conducted on both the encapsulated MIN6 pancreatic β-cells and the C2C12 cells exposed to the microcapsules after 48 h incubation.[37]

Bioenergetic Assessments

The Seahorse Flux Analyzer XF 96 (Seahorse Biosciences, USA) was used to conduct bioenergetic assessments on the encapsulated MIN6 pancreatic β-cells and the C2C12 cells exposed to the microcapsules. A fluorescent biosensor was used to measure markers including ATP production, respiratory and glucose-induced assessments. These analysis were conducted utilising established methods, with controls being unencapsulated MIN6 pancreatic β-cells after 48 h incubation period.[35, 37]

Statistical Analysis

Prism® version 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA), was utilised for statistical analysis. The assessments were done via one-way analysis of variance and Student's t-test. Statistical significance was indicated at $p < 0.05$.

Results

Glucose-Induced Assessments MIN6

Via MTT assay, the cellular viability of encapsulated MIN6 pancreatic β-cells was assessed at two concentrations of glucose treatment, 25 mmol/L and 35 mmol/L, as can be seen in Figure 1, with the control as unencapsulated MIN6 pancreatic β-cells. In terms of the 25 mmol/L study, F2 had the highest viability, closely followed by F6 and the control which were equal. All formu-

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lations with CDCA had a higher cellular viability than their counterparts with equal metformin concentrations without CDCA. These results indicate CDCA improves the cellular viability of encapsulated MIN6 pancreatic β-cells post treatment with 25 mmol/L of glucose via their stabilising and protection effects. In terms of the hyperglycaemic conditions of 35 mmol/L glucose, F6 had the highest viability, followed by the control then F2. Once again, all CDCA formulations had an increased cellular viability compared to their formulation counterparts without CDCA. Statistically, F1, F5, F9 and F11 had decreased viability to the control, ($p < 0.05$); as did F3 to the control,

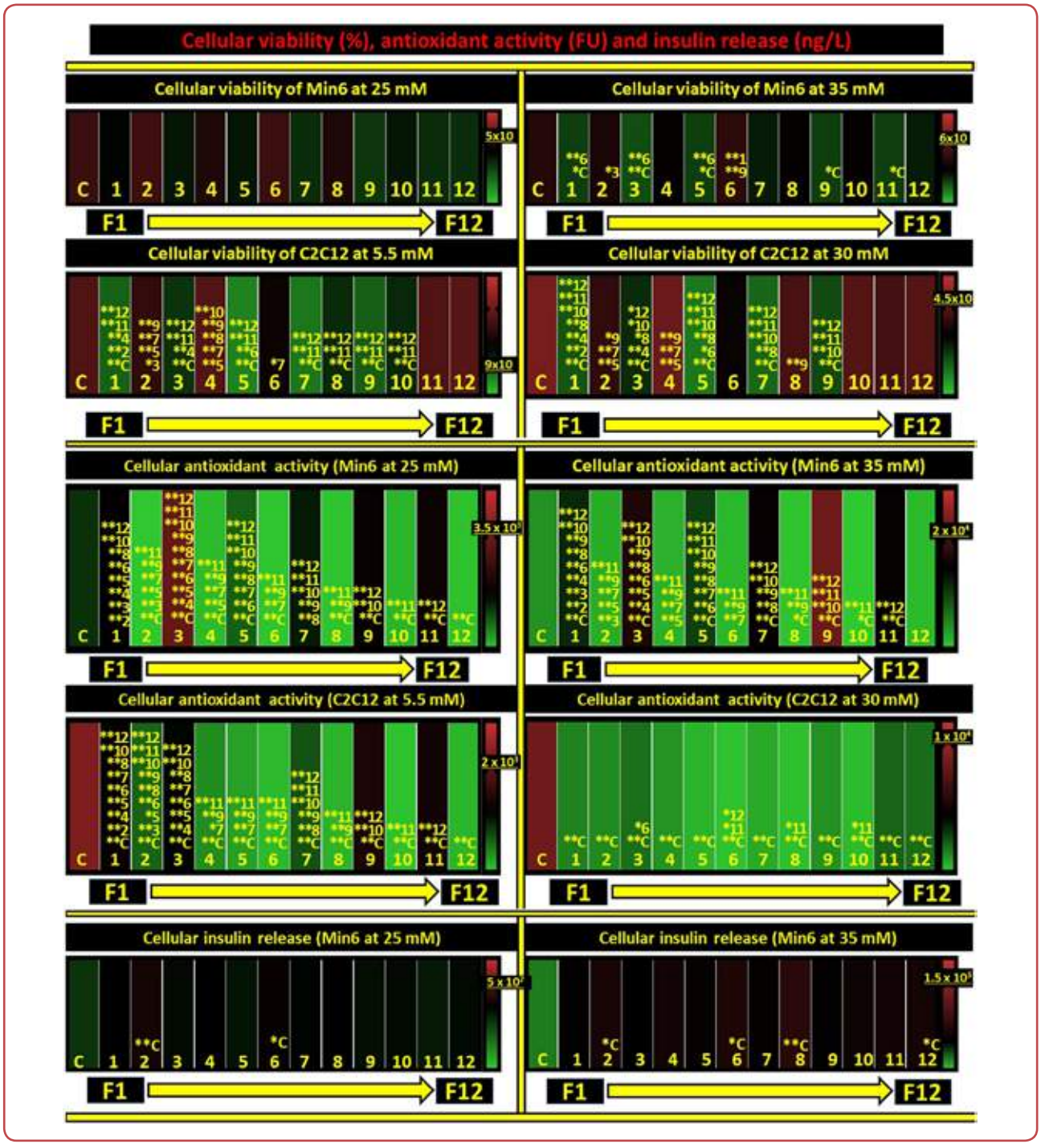

Figure 1: Glucose-induced assessments of cell viability, antioxidant activity and insulin release
*Heat maps showing the cellular viability of MIN6 cells (%) at 25 mM and 35 mM of glucose and viability (%) of C2C12 cells at 5 mM and 20 mM glucose. Cellular antioxidant activity (FU) of MIN6 cells at 25 mM and 35 mM of glucose and of C2C12 cells at 5.5 mM and 30 mM of glucose. Cellular Insulin Release (ng/L) from MIN6 cells after glucose treatment at 25 mM and 35 mM of glucose. Image demonstration of an ELISA assay used in the assessments. Measurements taken on all 12 formulations (F1 to F12). Unencapsulated MIN6 pancreatic β-cells were the control for MIN6 and C2C12 cells were the control for the C2C12 assessments. *p < 0.05, **p < 0.01.*

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($p < 0.01$). In terms of formulations with equal metformin concentrations, F5 had a statistically decreased cellular viability to F6 ($p < 0.01$). Overall, the results indicate that CDCA assists in the stabilisation and subsequent improved MIN6 pancreatic β-cellular viability in these studies.

Cellular antioxidant activity was assessed in terms of oxidative stress post-treatment with 25 mmol/L and 35 mmol/L of glucose. In the 25 mmol/L glucose study, all formulations without CDCA and the control performed poorly, with high levels of oxidative stress compared to the CDCA formulations. All CDCA formulations; F2, F4, F6, F8, F10 and F12 were significantly lower than the control, ($p < 0.01$). All formulations with CDCA were also statistically significantly lower than their formulatory metformin equivalent without CDCA, ($p < 0.01$). In terms of oxidative stress in the 35 mmol/L glucose study, results were similar, with high levels of oxidative stress detected in all formulations without CDCA and the control. F8 and F10 had a statistically significant decrease in oxidative stress to the control, ($p < 0.05$). All CDCA formulations were also significantly lower than the equal concentration of metformin formulation without CDCA, ($p < 0.01$).

Insulin release from the MIN6 pancreatic β-cells was also assessed via ELISA with glucose treatment concentrations of 25 mmol/L and 35 mmol/L, with MIN6 pancreatic β-cells as the control. For the 25 mmol/L study, all formulations had a higher insulin release than the control. Statistically, F2 and F6 had an increased insulin release to the control, ($p < 0.01$) and ($p < 0.05$), respectively. All formulations containing CDCA had higher insulin release than their equal concentrations of metformin formulation counterpart, indicating the assistance of CDCA in the improvement of insulin release. In terms of the 35 mmol/L analysis, once again all formulations had a higher release of insulin than the control, as did the CDCA formulations compared to their metformin counterparts. F2, F6 and F12 had statistically significant higher insulin release than the control, ($p < 0.05$); as did F8 ($p < 0.01$). Overall, results indicate that the metformin formulations all outperformed the control, with the CDCA formulations performing best.

Glucose-Induced Assessments C2C12

Cellular viability of C2C12 cells were also assessed via MTT following glucose treatment at 5.5 mmol/L and 30 mmol/L. The C2C12 cells were exposed to all 12 formulations, with the C2C12 cellular viability assessed. C212 cells served as the control, as can be seen in Figure 1. In terms of the 5.5 mmol/L results, F12 has the highest viability, followed by F4, F11 then the control. Statistically, F1, F3, F5, F7, F8, F9 and F10 had a lower C2C12 cellular viability than the control ($p < 0.01$). In terms of formulations with the same metformin concentration, F1's viability was lower than F2, F3 lower than F4 and F5 lower than F6 ($p < 0.01$). All formulations with CDCA had a higher cellular viability than their metformin counterparts. Studies were also conducted post exposure to 30 mmol/L glucose. In terms of C2C12 viability, the control performed best with the highest viability, followed by F10 and F12, F11, then F8. Formulations F1, F3, F5, F7 and F9 had a statistically lower viability than the control, ($p < 0.01$). In terms of formulations with equal metformin concentrations, F1 was statistically lower than F2, F3 lower than F4, F7 lower than F8 and F9 lower than F10, all ($p < 0.01$). F5 was also statistically lower than its counterpart F6 ($p < 0.05$). All CDCA formulations had a higher cellular viability than their counterparts. Overall, these results demonstrated the dosage effect of metformin, with the higher concentrations of metformin performing best in the C2C12 cellular viability studies.

Antioxidant activity studies were also conducted via the analysis of oxidative stress, with C2C12 cells as the control. In the study at 5.5 mmol/L glucose, the control exhibited the highest level of oxidative stress, with CDCA reducing levels of oxidative stress compared to their formulatory counterpart without CDCA. All 12 formulations examined had a statistically significant decrease in oxidative stress compared to the control ($p < 0.01$). This indicated metformin's antioxidant activity in C2C12 cells. In terms of oxidative stress between equal metformin concentrations, F1 was statistically higher than F2, F3 higher than F4, F7 higher than F8, F9 higher than F10 and F11 higher than F12, all ($p < 0.01$). The study conducted from 30 mmol/L glucose demonstrated the highest oxidative stress from the control, which was statistically significant to all 12 formulations ($p < 0.01$). CDCA did also reduce the oxidative stress in all formulations compared to the equivalent metformin formulation, although not significantly. Due to all formulations having a lower oxidative stress than the control, metformin's benefits were demonstrated at reducing the oxidative stress exhibited by C2C12 cells. The oxidative stress studies on C2C12 also highlighted the benefit of all formulations and microencapsulation,

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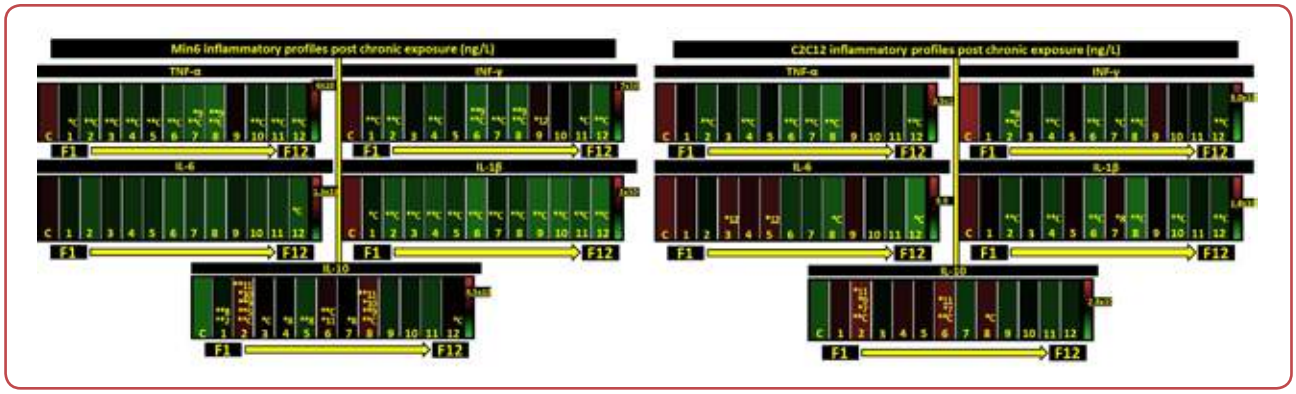

Figure 2: Inflammatory profiles post chronic exposure of MIN6 and C2C12 cells
*Heat maps showing the measurements (ng/L) of pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β as measured from MIN6 cells and C2C12 cells. Anti-inflammatory profile as measured by IL-10 (ng/L) from MIN6 and C2C12 cells. Inflammatory measurements were conducted on all 12 formulations (F1 to F12). Unencapsulated MIN6 pancreatic β-cells were the control for MIN6 and C2C12 cells were the control for the C2C12 assessments. *p < 0.05, **p < 0.01.*

and therefore, metformin on reducing oxidative stress levels, with the addition of CDCA further improving this.

Inflammatory Profiles - MIN6

Inflammatory profiles of MIN6 cells were established from each formulation. Pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β; and the anti-inflammatory IL-10 were examined to form an overview of the inflammatory profile, as demonstrated in Figure 2. Pro-inflammatory TNF-α had the highest detection in the control, with F9 the highest from the formulations. Statistically, F2, F3, F4, F5, F6, F7, F8, F10, F11 and F12 were lower than the control, ($p < 0.01$); as was F1 ($p < 0.05$). F7 and F8 were also statistically lower than F9 ($p < 0.01$). All formulations without CDCA had a higher TNF-α than their counterparts containing both metformin and CDCA. Pro-inflammatory IFN-γ followed a similar pattern, whereby the highest detection was in the control, then F9 and all formulations without CDCA had a higher release than their CDCA counterparts. F1, F2, F4, F6, F7, F8 and F12 were statistically lower in IFN-γ than the control ($p < 0.01$); as was F11 ($p < 0.05$). F6 and F8 were also statistically lower than F9 ($p < 0.01$). F9 was statistically higher than F12 ($p < 0.05$). In terms of pro-inflammatory IL-6, the control had the highest levels. All CDCA formulations had lower cytokine levels than their metformin CDCA counterparts. F12 was statistically lower than the control ($p < 0.05$). Pro-inflammatory IL-1β's control had the highest release and like all of the pro-inflammatory markers tested, the CDCA formulations had a lower cytokine level than their metformin CDCA counterparts. Statistically, all formulations had significance to the control, F1 with ($p < 0.05$) and F2 to F12 ($p < 0.01$).

In terms of anti-inflammatory IL-10, F8 had the highest release, followed by F2 and F6. The control of MIN6 cells had the lowest inflammatory biomarker detection. Similar to the pro-inflammatory markers, formulations with CDCA performed best. The CDCA formulations had higher levels of anti-inflammatory IL-10 than their counterparts without CDCA, with the exception of F3 and F4, with both formulations containing 1 % metformin. Statistically, F2, F6 and F8 had higher levels than the control ($p < 0.01$); as did F3 and F12 ($p < 0.05$). In terms of CDCA versus not with equal metformin concentrations, F1 was lower than F2 ($p < 0.01$) and F7 was lower than F8 ($p < 0.05$). Overall, microencapsulation reduced the levels of pro-inflammatory cytokines compared to the control, indicating that the coat of the capsule is protective and that metformin can exhibit anti-inflammatory properties. Furthermore, the formulations with CDCA had lower pro-inflammatory biomarker levels than their metformin counterparts without CDCA, indicating that CDCA assists in anti-inflammatory properties. Adding to this, the formulations had higher levels of anti-inflammatory cytokine than the control and all but one set showed increased levels when CDCA was added. This indicates that the formulation excipients and CDCA assist in the anti-inflammatory effects demonstrated by these biomarkers.[38]

Inflammatory Profiles - C2C12

Inflammatory profiles of C2C12 were determined post exposure to the microcapsules of each formulation, with pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β; and the anti-inflammatory IL-10, as seen in Figure 2. The control was C2C12 cells. In terms of pro-inflammatory TNF-α, the control had the highest profile, with

all formulations falling below this. All CDCA formulations had a lower TNF-α level than those of equal metformin concentrations without CDCA. Statistically, F2, F4, F6, F7, F8 and F12 had lower levels than the control, ($p < 0.01$). Pro-inflammatory IFN-γ, had a similar pattern to TNF-α, in which the control had the highest level of IFN-γ and the CDCA formulations had lower levels than those without CDCA. F2, F4, F6, F8 and F12 had statistically lower levels than the control ($p < 0.01$); as did F7 ($p < 0.05$). Pro-inflammatory IL-6 also had the highest levels from the control, with CDCA reducing levels compared to their formulatory equivalent without CDCA. F8 and F12 had statistically significant lower levels of IL-6 than the control ($p < 0.05$). In terms of pro-inflammatory IL-1β, a similar pattern to the other pro-inflammatory biomarkers was seen, with the control having the highest result and CDCA reducing levels compared with their metformin counterpart. Statistically, F2, F4, F6, F8, F10 and F12, all CDCA formulations, were lower than the control ($p < 0.01$). In addition, F8 was significantly lower than F7 ($p < 0.05$).

Anti-inflammatory IL-10 was also assessed in the C2C12 cells, with the control having the lowest detectable levels. From the tested formulations, F2 was highest, followed by F6 the F8. In all formulations, ones containing CDCA outperformed their metformin equivalent without CDCA. F2 and F6 had statistically higher levels of detection ($p < 0.01$); as did F8 ($p < 0.05$). Overall, all formulations improved the inflammatory profiles compared to the control in the C2C12 studies, decreasing pro-inflammatory biomarkers and increasing the anti-inflammatory biomarker. These results reflected those seen in the MIN6 studies. Furthermore, akin to the MIN6 cells, all CDCA in C2C12 studies improved the inflammatory profile when compared to the same concentration of metformin without the addition of CDCA. These results indicate the benefit of microencapsulation and metformin, in addition to supporting the inclusion of bile acids in such formulation design as assisting to further improve the overall inflammatory profile.

Bioenergetic Measurements – MIN6

Bioenergetic measurements were taken from the MIN6 cells, with the control being unencapsulated MIN6 cells, with results demonstrated in Figure 3. In terms of F1 and F2, with 0.4 % metformin and F2 containing CDCA, F2 outperformed F1 in all measurements, however, both F1 and F2 outperformed or, in the case of F1, were equal to the control. For non-mitochondrial oxygen consumption rates, rates of glycolysis and non-glucose-derived extracellular acidification rates, F1 had lower levels than F2 ($p < 0.01$). For F3 and F4, with 1 % metformin and F4 also containing CDCA, F4 outperformed F3 in all measurements, aside from the exceptions coupling efficiency and spare respiratory capacity, where they were equal and proton production rates in which F3 had higher levels than F4. F4 was significantly higher than F3 in non-mitochondrial oxygen consumption rates and rate of proton leak ($p < 0.01$). F3 and F4 had higher measurements than the control, aside from rates of glycolysis, in which F3 was equal to the control and proton production rates in which F4 was equal to the control. In terms of F5 and F6, with 2 % metformin and F6 also containing CDCA, F6 had higher levels in all measurements aside from rate of proton leak, in which F5 and F6 were equal. Both F5 and F6 had higher measurements in all areas than the control, aside from non-mitochondrial oxygen consumption rates, in which F5 was equal to the control.

Bioenergetic measurements from F7 and F8, both containing 3 % metformin, with F8 also having CDCA, demonstrated higher levels from F8 than F7 in all areas. F8 had higher levels than the control in all measurements, as did F7, with the exception of non-mitochondrial oxygen consumption rates and rate of proton leak, in which F7 was lower than the control. In terms of F9 and F10, with 4 % metformin and F10 containing CDCA, F10 had higher bioenergetic measurements than F9, with the exception of coupling efficiency in which F9 was higher. F10 was statistically higher than F9 in both rate of glycolysis and non-glucose-derived extracellular acidification rates ($p < 0.01$). F10 was higher than the control in all measurements, as was F9, aside from rates of glycolysis, which was equal to the control and rate of proton leak, in which the control was higher. For formulations with 6 % metformin, F11 and F12, with F12 also having CDCA; F12 had higher bioenergetic measurements than F11, aside from oxygen consumption rates and non-mitochondrial oxygen consumption rates, where F11 and F12 were equal. F12 was statistically significantly higher than F11 in spare respiratory capacity, rate of glycolysis and non-glucose-derived extracellular acidification rates ($p < 0.01$). F11 and F12 both outperformed the control of MIN6 cells in all bioenergetic measurements.

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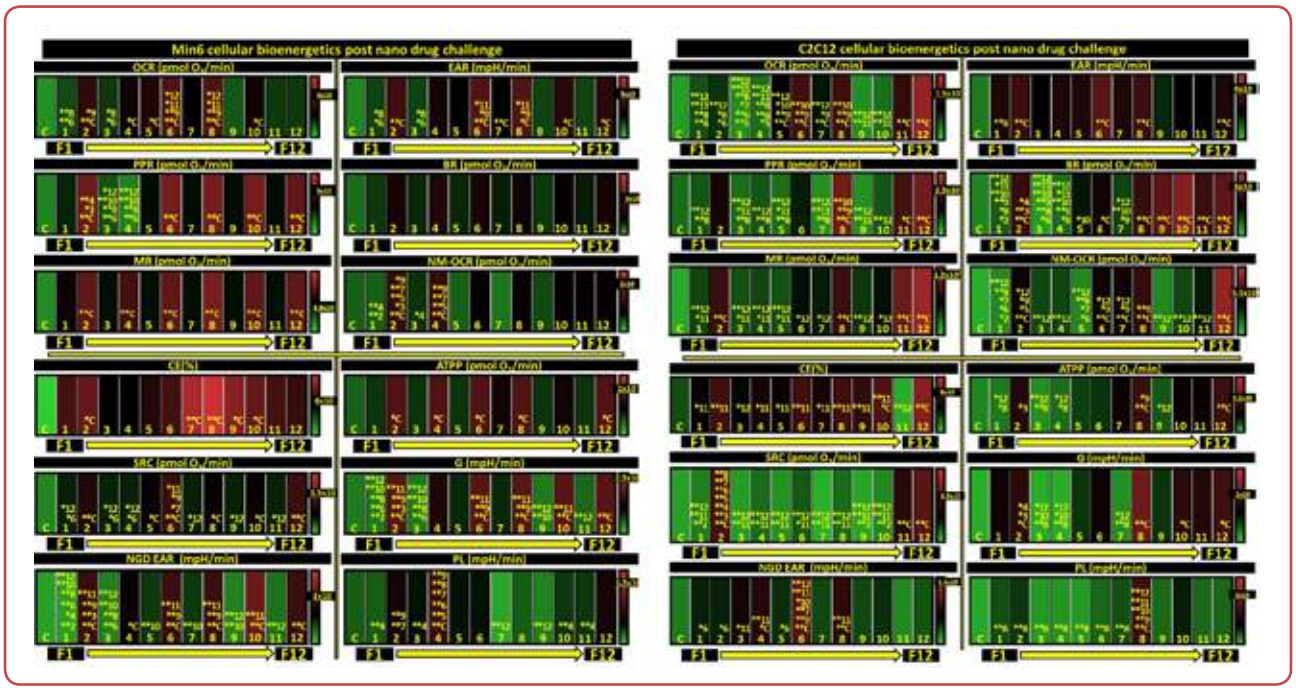

Figure 3: Cellular bioenergetics post-nano-challenge for MIN6 and C2C12 cells
*Heat maps showing the various bioenergetic measurements. Oxygen consumption rate (OCR) (pmol O2/min). Extracellular acidification rate (EAR) (mpH/min). Proton production rate (PPR) (O2/min). Basal rate (BR) (O2/min). Maximal respiration rate (MR) (pmol O2/min). Non-mitochondrial oxygen consumption rate (NM-OCR) (pmol O2/min). Coupling efficiency (CE) (%). ATP production rate (pmol O2/min). Spare respiratory capacity (SRC) (pmol O2/min). Glycolysis (G) (mpH/min). Non-glucose-derived extracellular acidification rates (NGD EAR) (mpH/min). Proton leak (PL) (mpH/min). Assessed for all formulations (F1 to F12) for MIN6 cells and C2C12 cells. Unencapsulated MIN6 pancreatic β-cells were the control for MIN6 and C2C12 cells were the control for the C2C12 assessments. *p < 0.05, **p < 0.01.*

Bioenergetic Measurements – C2C12

Bioenergetic measurements were also taken from C2C12 cells, following their exposure to the formulations, with untreated C2C12 cells serving as the control, as seen by Figure 3. In terms of F1 and F2, with 0.4 % metformin, and F2 also containing CDCA, F2 demonstrated higher measurements in all areas, aside from non-glucose-derived extracellular acidification rates, in which they were equal. F2 was statistically increased to F1 in basal oxygen rates and non-mitochondrial oxygen consumption rates ($p < 0.05$); as well as spare respiratory capacity ($p < 0.01$). Both F1 and F2 had higher measurements in all areas than the C2C12 control. Formulations F3 and F4, both containing 1 % metformin, with F4 also having CDCA, demonstrated F4 to have higher levels than F3 in all areas, aside from non-mitochondrial oxygen consumption rates and rate of glycolysis in which F3 and F4 were equal. The rate of glycolysis was also equal to the control. In all other measurements, F4 was higher than the control. F3 was equal to the control in proton production rates and lower in basal oxygen rates and ATP production rates. F3 was higher than the control in other measurements. In terms of F5 and F6, both containing metformin concentrations of 2 % metformin and F6 also containing CDCA, F6 outperformed F5 in all measurements. Statistically, F5 was lower than F6 in non-mitochondrial oxygen consumption rates and non-glucose-derived extracellular acidification rates ($p < 0.05$). Both F5 and F6 had higher bioenergetic measurements than the control, aside from proton production rates, in which F5 was equal to the control.

Bioenergetic measurements of F7 and F8, with 3 % metformin and F8 containing CDCA, showed F8 to be higher than F7, with significance in proton production rates, rate of glycolysis and rate of proton leak ($p < 0.01$). F8 had higher levels than the control in all measurements, as did F7 with the exception of proton production rates and spare respiratory capacity, in which the control was higher. In terms of F9 and F10, with 4 % metformin and F10 containing CDCA, F10 outperformed F9 and the control in all measurements. F9 outperformed the control in all measurements, with the exceptions of proton production rates, non-mitochondrial oxygen consumption rates and spare respiratory capacity. Formulations F11 and F12, with 6 % metformin, with F12 also containing CDCA, had bioenergetic results in which F12 had higher measurements than

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F11 in all tested, with significance in non-mitochondrial oxygen consumption rates and coupling efficiency ($p < 0.01$). F12 was higher than the control in all measurements, with statistical significance in ten of the twelve measurements. F11 was higher than the control in all measurements, with the exception of coupling efficiency and non-glucose-derived extracellular acidification rates. Different formulations performed best for the various measurements, however, the best performing formulation in each measurement always contained CDCA.

Disscusion

Glucose-Induced Assessments

The oxidative stress results showed a reduced level of oxidative stress observed in formulations with CDCA when compared to their equal concentration counterpart without CDCA, highlighting the importance of a bile acid such as CDCA in the formulation to decrease the oxidative stress experienced by the MIN6 cells, improving the antioxidant activity. Previous studies have also highlighted similar results, demonstrating in a formulation-dependent manner, that the addition of a variety of bile acids improve antioxidant effects.[36] Furthermore, CDCA is a natural agonist of Farnesoid X Receptor (FXR), with multiple studies indicating the activation of FXR to have antioxidant effects via a multitude of mechanisms.[39-41] Noh et al showed CDCA activation of FXR to result in the downstream effects of activating CCAAT/enhancer binding protein-β (C/EBPβ) via the adenosine 5′-monophosphate-activated protein kinase (AMPK) pathway which in turn activated extracellular signal-regulated kinase 1/2 (ERK1/2), resulting in the induction of antioxidant enzymes. Hence, via this pathway, CDCA can have antioxidant effects.[42]

Insulin studies showed metformin formulations to improve upon the control and the addition of CDCA to, overall, further improve this. NIT-1 encapsulated cells with CDCA in a previous study showed the microcapsules containing CDCA increased insulin production to a statistically significant level ($p < 0.01$), compared to microcapsules without CDCA. This study supports these results, with the indication that CDCA may have a biological effect on encapsulated β-cells.[43] Furthermore, Shihabudeen et al FXR activation by CDCA to reduce insulin resistance in high fat diet rats via the modulation of adipokines. The activation of FXR had anti-inflammatory effects, improving the sensitivity of insulin.[44] Hence, CDCA has the potential to not only increase insulin secretion as seen in the CDCA formulations, but also to improve insulin sensitivity *in vivo*.

Inflammatory Profiles

Pro-inflammatory marker assessments demonstrated the CDCA formulations to have lower cytokine levels than their metformin CDCA counterparts. In a recent study by this group, microcapsules of CDCA, sodium alginate, poly-l-ornithine and islets were created and investigated. In preclinical examinations using mice, plasma levels of pro-inflammatory biomarker IL-6 were measured. The mice with CDCA-islet microcapsules had levels of IL-6 60 % lower than mice which received microencapsulated islets without CDCA, alluding to immune-protective impacts from CDCA.[45] This experimentation helps to support the *in vitro* results of this study, in which IL-6 levels were reduced via the incorporation of CDCA. The reduction in pro-inflammatory biomarkers from the MIN6 pancreatic β-cells indicates a reduced cellular stress which leads to improved viability and functionality, and, importantly, biocompatibility.[22, 23, 37, 38]

The anti-inflammatory effects, in which there is an increased detection of the anti-inflammatory marker in all formulations, compared to the control, are supported by previously published works which have highlighted the fact that metformin can exhibit anti-inflammatory effects, including in non-diabetics. Metformin has been shown to reduce the presence of pro-inflammatory cytokines in various studies.[46-48] One of such investigated the potentials for use of metformin in the treatment of endotoxin-induced myocarditis. Results from endotoxin-challenged mice showed metformin to reduce cardiac expressions of TNF-α, IL-6, and IL-1β, three pro-inflammatory interleukins. The researchers determined their results to be from an anti-inflammatory mechanism dependent on AMPK, which was activated by metformin.[49]

As previously highlighted, the reduction in pro-inflammatory biomarkers via the addition of CDCA is key to β-cells survival and biocompatibility.[23] Inflammation, as indicated by pro-inflammatory biomarkers, is a key factor in the inability for bioartificial organs to be effective and result in the dysfunction of the transplanted cells. The reduc-

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tion of such pro-inflammatory cytokines is key to the production of non-immunogenic, functional microencapsulated cells, with considerations to be taken to reduce pro-inflammatory responses caused by microcapsules, in order to reduce inflammation post-transplantation.[50-52] Hence, the inflammatory profiles of the microcapsules are key, including both profiles, those of microencapsulated MIN6 cells and those of cells they may be in contact with, C2C12 cells; with both sets showing improved inflammatory profiles to the control, particularly via the addition of CDCA.

CDCA itself has also been demonstrated to show anti-inflammatory effects. As discussed, CDCA is a natural agonist of FXR. FXR, a ligand-dependent transcription factor's activation has previously demonstrated the ability to show anti-inflammatory effects. Hence, the addition of CDCA has the ability to activate FXR and reduce overall inflammation.[25, 53, 54] Therefore, both metformin and CDCA have anti-inflammatory properties, which are able to potentially induce the positive effects seen in this study.

Bioenergetic Measurements

As seen by the results of bioenergetic measurements for MIN6, overall, the formulations outperformed the controls and the addition of CDCA further improved the results, hence indicating that the biological function and activity of the MIN6 cells was enhanced via encapsulation. Overall, the C2C12 bioenergetic measurements showed the formulations and therefore, metformin, to improve such bioenergetic measurements, which were further improved by the addition of CDCA. This includes enhanced energy production and cellular respiration, indicating increased mitochondrial respiration, indicating an enhanced bioenergetic profile and metabolism, further enhanced via the presence of the bile acid CDCA in the capsules. Furthermore, in combination with a strengthened anti-inflammatory profile, the improved bioenergetics suggests a contribution from the reduced pro-inflammatory biomarkers on the capsules and their stability profiles via the inclusion of CDCA. The resultant positive impacts on the biological measurements of cells is supported by previous studies conducted by this research team.[55]

Conclusion

Overall, the encapsulation of MIN6 pancreatic β-cells with metformin improved the antioxidant activity of both the MIN6 and C2C12 cells. The insulin release was also improved with the metformin formulations. In both cell lines assessed, the inflammatory profile was improved to the control, as was the overall bioenergetic measurements. The results indicated the formulation-dependent effect of metformin on measurements taken. The addition of a bile acid, in this case CDCA, resulted in further improvements to results, indicating the potential benefit of including a bile acid in microencapsulated pancreatic β-cells.

Future Perspectives

The formulation-dependent results from this study and other research highlight the key requirement for a robust, functional formulation-base to be developed in order to continue this field of study. Furthermore, the technology and ongoing developments in the encapsulation of pancreatic cells indicate their strong potential *in vitro*, which must be further examined in an attempt to translate the research into a potentially functional bioartificial organ.

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Conflict of interest

None.

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ORIGINAL ARTICLE

New Approaches in Management and Treatment of *Hidradenitis Suppurativa*

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Abstract

Background / Aim: *Hidradenitis suppurativa* (HS) is a chronic inflammatory disease that most often affects apocrine gland-bearing areas of the skin. The treatment depends on the severity of the clinical presentation. The paper objective was to present new modalities in management and treatment of HS.
Methods: The subjects in this research included the patients suffering from the severe form of HS, who were treated in the University Clinical Centre of the Republic of Srpska for the past three years. The effect of treatment of HS were monitored. In four patients, biologic therapy with adalimumab or adalimumab biosimilars was administered, while four patients received radiotherapy and 17 of them, were treated surgically. Depending on the type of treatment, the effects of therapy were monitored after 6-12 weeks by using clinical examination and by assessing the disease stage according to the Hurley staging. Due to a small number of subjects, especially in patients treated with biologic and radiotherapy, it was not possible to perform any statistical analysis and the results were presented by description, in tables and photographs.
Results: Biologic therapy: Adalimumab was administered subcutaneously 80 mg, twice a month. After 12 weeks, in 4 patients was observed a regression of changes by 60-70 % when compared to previous skin changes. Radiotherapy: the total radiation dose was 5 Gy, distributed in 5 or 10 fractions. After 12 weeks an improvement by 60-70 % was observed. Surgical treatment: after 6-8 weeks, the patients were fully recovered.
Conclusions: Application of biologic and radiotherapy after 12 weeks had similar results, ie it brought to regression of changes by 60-70 %. The best results were achieved after surgical treatment of HS.

Key words: *Hidradenitis suppurativa*; Adalimumab; Radiotherapy; Skin autografting.

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Introduction

Hidradenitis suppurativa (HS) is a chronic, purulent disease with increased inflammatory component and tendency towards fibrosis in apocrine gland-bearing areas of the skin. However it should be pointed out that the HS also occurs in skin areas without apocrine glands. Namely, it was confirmed that the foundation of the disease development is basically folliculitis caused by occlusion of the follicular epithelium and that apocrine gland involvement was, both clinically and aethiopathogenically, secondary. A small number of patients, in addition to HS, can also have *acne conglobata*, *folliculitis et perifolliculitis capitis* - the so-called 'follicular occlusion triad' or additional pilonidal sinus in case of follicular occlusion tetrad.[1]

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HS occurs in both sexes, but in males it is mostly localised in the anogenital region. The correlation between obesity and the severity of the clinical presentation has been confirmed, as well as the effect of sex hormones to the disease occurrence and course.[2]

HS most often occurs in armpits, groin and perianal area. However, the disease can also be confirmed in the areas underneath the breasts, on the stomach, gluteus, scalp and eyelids. According to the clinical presentation, erythematous painful nodules appear first and they usually soften after a few days, after that are formed abscesses and thick purulent, seropurulent and bloody contents are oozing out of them. New nodules appear soon after in the same place or near the previous changes and the process is repeated. This creates sinus tracts between changes that never fully heal. The tendency towards fibrosis changes and scarring is particularly emphasised. Due to the chronic nature of the disease, there is a high risk of complications such as the spread of the disease to the rectum, bladder or urethra with the formation of fistulas. Sepsis is the most serious complication of this disease. Regarding the differential diagnosis of the disease, the following can be considered: carbuncles, lymphadenitis, inflamed cysts, granulomatous diseases and the possibility of skin tuberculosis should be particularly excluded.[1, 2]

HS significantly affects the patients' life quality. Due to the chronic nature of the disease, the appearance of painful nodules and malodorous discharge, patients withdraw from the community because they feel less valuable to other people. The presence of fistulas, scars in the anogenital region also reduces the sexual activity of patients. They sometimes isolate themselves even from their own family members, with the possibility of suffering from severe forms of depression. Due to physical and emotional pain, the patients are sometimes suicidal.[3]

Hurley staging describes three clinical stages of HS. Stage I is represented by solitary abscess formations, without scarring and sinus tracts. In stage II, recurrent abscesses and lesions with scarring and sinus tracts occur, and in stage III is affected the entire anatomical region with interconnected abscesses and sinus tracts.[4] Although Hurley staging system is simple, it is not quantitative, so it was modified for the purpose of accuracy of diagnosis and treatment protocol planning: the Modified Hidradenitis Suppurativa Score (HSS score, better known as the Sartorius score),[5] along with the Hidradenitis Physician Global Assessment Scale (HS-PGA) (Table 1).[6]

Table 1: Hidradenitis suppurativa staging according to the Physician Global Assessment Scale (HS-PGA)

Stage (form)	Description
Clear, minimal	No abscesses, fistulas, inflammatory nodules or non-inflammatory nodules
Mild	No abscesses, fistulas, 1-4 inflammatory nodules or 1 abscess and fistula, 0 inflammatory nodules
Moderate	No abscesses, fistulas, but more than 5 inflammatory nodules or 1 abscess and fistula and more than 1 inflammatory nodules or 2-5 abscesses or fistulas and less than 10 inflammatory nodules
Severe	2-5 abscesses or fistulas and more than 10 inflammatory nodules
Very severe	More than 5 abscesses and fistulas

The treatment protocol for HS is determined in accordance with the stage of the disease and the severity of the clinical presentation (Table 2). It is very important to point out the change in the patient's lifestyle: abstinence from smoking, weight loss, change in diet, wound treatment and care for the patient's mental health. Pain is one of the dominant symptoms and therefore it is necessary to use analgesics: lidocaine + diclofenac, topical nonsteroidal anti-inflammatory drugs (NSAIDs), gabapentin and pregabalin, tramadol and ultimately opiates for severe pain that does not stop with NSAIDs.[7-9]

Table 2: Guidelines in hidradenitis suppurativa treatment according to the clinical severity

Hurley I (PGA mild)	Hurley II (PGA moderate)	Hurley III (PGA severe -very severe)
Topical: 1 % clindamycin lotion for 12 weeks or 15 % resorcinol cream	Clindamycin tbl. 2 x 300 mg + Rifampicin tbl. 2 x 300 mg per day (10 weeks)	Biological: adalimumab 160 mg in the first week, 80 mg for two weeks, then 40 mg a week or 80 mg every two weeks
Tetracycline 2 x 500 mg per day or Doxycycline 2 x 100 mg per day (4 months)	Triamcinolone acetonide (TAC) intralesional injection	Laser therapy
TAC intralesional injection	Laser therapy	Surgical: local or wide excision
Surgical: local excision	Surgical: local or wide excision	

PGA: Physician global assessment scale

In the second line of biologic treatment, it is recommended to administer acitretin (0.25-0.88 mg/kg, 3–12 months), and in the third line it is possible to use dapsone (25-200 mg for 3 months), cyclosporine A (2-6 mg/kg for 6 weeks to 6 months), zinc gluconate (90 mg), isotretinoin (0.5-1.2 mg/kg/day for 4-12 months), hormone therapy and botulinum toxin. In case of hormone imbalance, after the recommendation of the endocrinologist, hormone therapy is included: metformin, cyproterone acetate + ethinyl oestradiol, finasteride, spironolactone.[8-10]

One of the latest trends in treating HS is the use of the monoclonal antibody adalimumab (Humira). Adalimumab has a selective immunosuppressive effect and it reduces the inflammatory process in diseases in which it is used for therapy. The active substance, adalimumab, is a human monoclonal antibody derived from cell culture. Monoclonal antibodies are proteins that recognise and bind to other specific proteins. Adalimumab binds to specific proteins (tumour necrosis factor or TNFα), which is present in increased concentrations in inflammatory diseases. According to the recommendations of the Agency for Medicinal Products and Medical Devices of Bosnia and Herzegovina, it is used for the treatment of rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, enthesitis-related arthritis, ankylosing spondylitis (AS), non-radiographic axial spondyloarthritis, psoriatic arthritis, psoriasis, HS, Crohn's disease, ulcerative colitis and non-infectious uveitis affecting the back of the eye. The recommended dosing regimen for adalimumab for adult patients with purulent inflammation of the sweat glands is the initial dose of 160 mg on the first day (given as four 40 mg injections in one day or two 40 mg injections per day for two consecutive days), followed by dose of 80 mg two weeks later, on day 15 (administered in the form of two injections of 40 mg in one day). Two weeks later (day 29), the treatment continues with a dose of 40 mg every week. If necessary, the use of antibiotics can be continued during treatment with adalimumab. It is recommended for the patient to use a local antiseptic solution daily to treat lesions caused by purulent hidradenitis during treatment with adalimumab. In case there is no improvement even after 12 weeks, it is necessary to carefully consider the continuation of the treatment of such a patient. If treatment needs to be temporarily discontinued, adalimumab may later be reintroduced at a dose of 40 mg every week.[11]

It is necessary to point out the positive effects of radiotherapy, ie of low-dose radiation of the affected region. Although the radiobiological effect of radiation on benign lesions has not been defined yet, there are several assumptions about achieving a favourable therapeutic effect of ionising radiation. It is believed that the effect of ionising radiation in the treatment of HS is based on the destruction of inflammatory cells and the release of mediators, cytokines and proteolytic enzymes. Before starting the treatment, a treatment protocol is established for each patient, which includes precise determination of the target volume, therapeutic dose, number of fractions and total duration of treatment. According to the protocol, it is necessary to determine the organs at risk (thyroid gland, eyes, gonads, breasts, bone marrow) that need to be protected during radiation.[12]

Surgical treatment may include incision and drainage of individual changes, superficial excision or wide excision, depending on the clinical severity. Incision and drainage are applied with minor changes but are avoided due to frequent recurrences. Superficial excision does not require general anaesthesia, the appearance of a scar is acceptable and there is a small number of recurrences. Wide excision is used in long-term and severe cases of Hurley III. It is recommended to apply the coverage with a skin autograft, and it is significant there is a possibility of local flaps in inaccessible regions.[13, 14]

The paper objective was to present new modalities in management and treatment of HS.

Methods

The subjects in this research included the patients who were treated in the University Clinical Centre of the Republic of Srpska (UCC) for the past three years. The subjects were treated with various topical, systemic antibiotics and retinoids.

Due to the duration of the disease and the failure of the therapy used, specialists in dermatovenereology suggested further treatment with biolog-

Table 3: The Modified Hidradenitis Suppurativa Score as modified by Sartorius, HSS score

Analysed factors	Manner of calculation
Anatomic region involved (armpit, pectoral, inguinal, perianal, perineal)	Number of regions involved x 3
Type of lesions	Number of fistulas x 4 Number of nodules x 2 Number of abscesses x 1 Number of scars x 1 Other changes (folliculitis, pustules) x 0.5
Total skin surface affected with lesions Distance between lesions or Size of the lesion, if only one lesion is present in the region	Number of regions with the distance of less than 5 cm between two lesions x 2 Number of regions with the distance from 5 to 10 cm between two lesions x 4 Number of regions with the distance of more than 10 cm between two lesions x 8
Are all lesions clearly separated by normal skin?	Yes - 0 points No - 6 points

Table 4: Severity Assessment of Hidradenitis Suppurativa, SAHS score

Score / Category	Number of regions involved	Number of inflamed and/or painful lesions besides fistulas	Number of fistulas	Number of new or extended existing abscesses during the last 4 weeks	Pain Rating (NRS 11)
0	0	0	0	0	0 - 1
1	1 - 2	1 - 4	1 - 2	1 - 2	2 - 4
2	3 - 4	5 - 9	3	3 - 4	5 - 6
3	≥ 5	≥ 10	≥ 4	≥ 5	≥ 7

NRS 11: The Numeric Rating Scale;

ic therapy, surgical therapy or, more recently, the use of radiotherapy in the treatment of HS.

The effects of treatment of HS were monitored:
- by applying biologic therapy with adalimumab or adalimumab biosimilars in 4 patients treated at the Clinic for Skin and Venereal Diseases UCC,
- using radiotherapy in 4 patients treated at the Affidea Radiotherapy Centre in Banja Luka, and
- using surgical therapies (skin autografting and reconstruction of the defect with local lobes) in 17 patients treated at the Clinic for Plastic and Reconstructive Surgery UCC.

All subjects had a severe form of HS (Hurley II/III).

To evaluate the effectiveness of biologic therapy, the Hidradenitis Suppurativa Clinical Response Score (HiSCR) was used, which implies a positive effect of treatment-regression of more than 50 % of changes (abscesses and nodules), without the occurrence of new abscesses and fistulas.

The Sartorius score is more dynamic and it provides a more detailed evaluation of HS. The score takes into account: the region of the body involved, the number and types of lesions, the longest distance between two lesions, whether all lesions are clearly separated by normal unaffected skin. Three points are given in each category to give a regional and overall score (Table 3). The Sartorius score is used primarily for research purposes, whereas the Hurley staging is more commonly used in clinical settings.[5]

The criteria for monitoring the clinical severity of HS should be simple to use in clinical practice, dynamic (able to measure the chronic recurrent nature of the disease), sensitive enough to measure treatment outcomes and time-efficient. Based on these, the new scoring system - Severity Assessment of Hidradenitis Suppurativa (SAHS) score has been designed (Table 4).[15]

Each item is scored accordingly and calculated for an overall SAHS score with an upper limit of 15. Mild form of the disease is considered for the SAHS score of 4 or less, moderate form of the disease is considered for the SAHS score of 5 to 8 and severe form of the disease is considered for the SAHS score of 9 or higher.

Due to a small number of subjects, especially in patients treated with biologic and radiotherapy, it was not possible to perform any statistical analysis and therefore the results were presented by description, in tables and photographs.

Results

Patients treated with biologic therapy

Biologic therapy with adalimumab or adalimumab biosimilars was administered to 4 patients suffering from HS at the Clinic for Skin and Venereal Diseases of the UCC. Adalimumab was administered subcutaneously in two ampules of 40 mg (the total of 80 mg) twice a month. According to the clinical severity, all four patients had Hurley III stage. All patients were male. The youngest patient was 21 and the disease appeared at the age of 14. The oldest patient was 53, and the disease appeared at the age of 34 and in two patients between the ages of 20 and 30. In terms of localisation, the changes in all patients were extensive and involved multiple regions: axillary, submammary, lumbosacral, scrotum and groin and gluteal (Figures 1a, 2a). All four patients had a negative family history of HS and they were all smokers. The most common comorbidities included obesity, arterial hypertension, ulcerative colitis, acne conglobata, fistula perianalis complexa, pilonidal sinus, anxiety and depression. Regression of changes by 60-70 % in all 4 patients was observed in the 12th week from the beginning of the therapy (Figure 1b, 2b).

Figure 1-a: Hidradenitis suppurativa of the inguinal region before the therapy with adalimumab

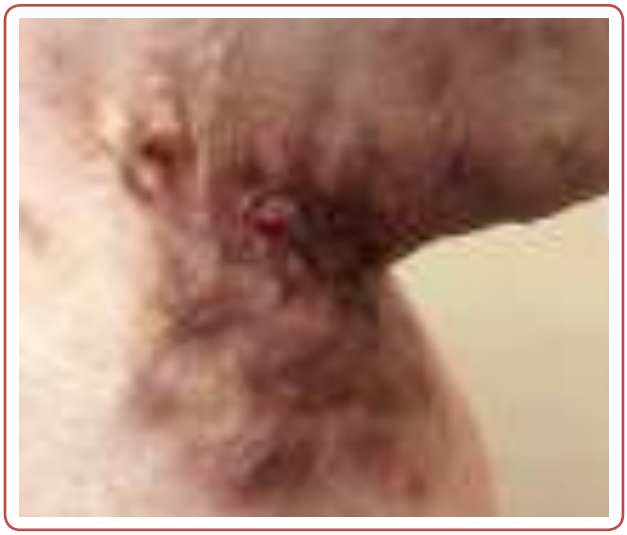

Figure 2-a: Hidradenitis suppurativa of the axillary region before the therapy with adalimumab

Figure 1-b: Hidradenitis suppurativa after 12 weeks of the therapy with adalimumab

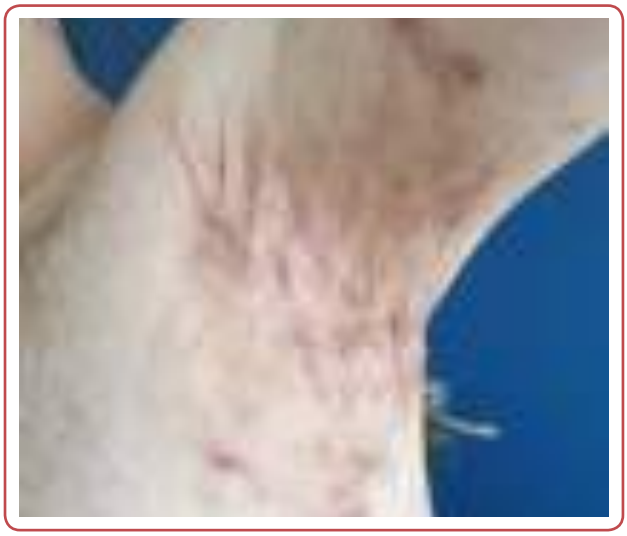

Figure 2-b: Hidradenitis suppurativa of the axillary region after 12 weeks of therapy with adalimumab

Patients treated with radiotherapy

For the past two years, four patients with HS were treated at the Affidea Radiotherapy Centre in Banja Luka. They were all men and their average age was 33. Table 5 shows the data on the localisation of changes, as well as the total dose and number of fractions in patients treated with radiotherapy.

The patients were examined by a dermatologist one month and three months after applying the last dose of radiation, noticing the improvement and regression of changes by 60-70 % compared to the examination before applying radiotherapy (Figure 3a, 3b, 3c).

Surgical treatment

For the past three years, 17 patients with HS have been treated at the Clinic for Plastic and Reconstructive Surgery of the UCC (Table 6).

Table 5: Overview of patients treated with radiotherapy regarding age, sex, localisation of changes and dose/number of fractions received by the patient

Age	Sex	Localisation	Dose (Gy) / number of fractions
59	Male	Gluteus	5 Gy / 5 fractions
21	Male	Gluteus	5 Gy / 10 fractions
28	Male	Abdominal wall	5 Gy / 5 fractions
25	Male	Axillae, on both sides	5 Gy / 10 fractions

**Gy (Gray)*

The average age of patients was 43 years. The oldest patient was 74 years old and the youngest was 17 years old. The patients were mostly male, 13 of them and 4 patients were female. According to the

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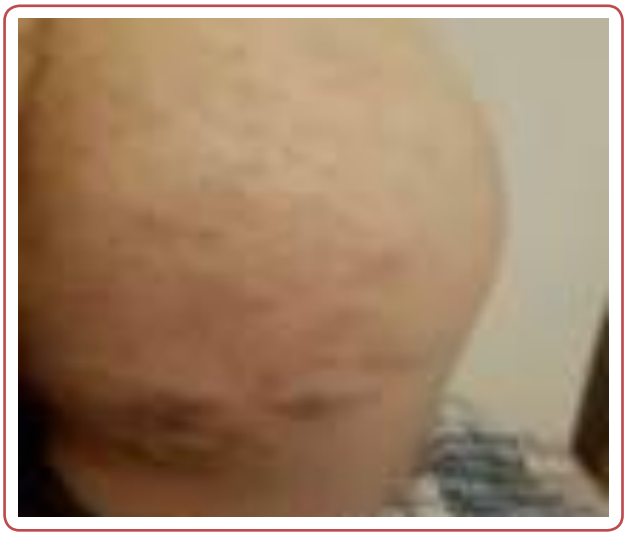

Figure 3-a: Hidradenitis suppurativa of the gluteal region before applying radiotherapy

Figure 3-b: Hidradenitis suppurativa one month after applying the last dose of ionising radiation

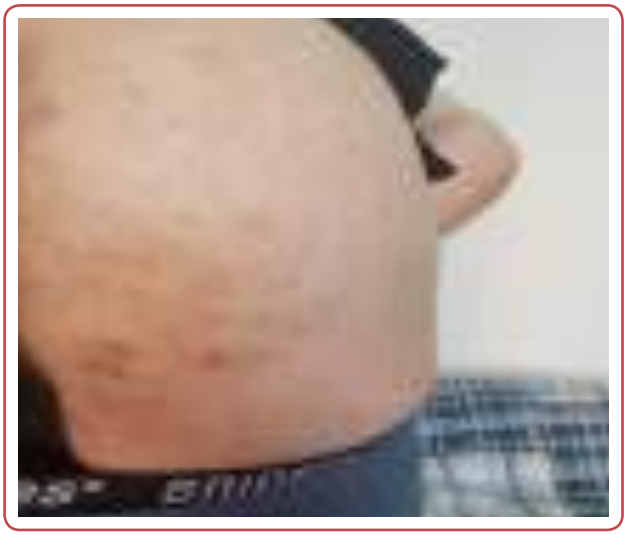

Figure 3-c: Hidradenitis suppurativa three months after applying the last dose of ionising radiation

Table 6: Overview of patients treated with surgical therapy with regard to age, sex, localisation of changes, type of surgery and duration of treatment

Age	Sex	Localisation	Type of surgery	Duration of treatment / day
44	Male	Axillae, on both sides	Skin autografting	12
52	Male	Scrotum and pubic area	Skin autografting	15
74	Male	Gluteal, on both sides	Skin autografting	13
27	Male	Axillae, on both sides	Skin autografting	14
42	Male	Axilla, on one side	Defect reconstruction with a local flap	4
43	Male	Scrotum	Skin autografting	17
32	Male	Axilla, on one side	Defect reconstruction with a local flap	4
39	Male	Axilla, on one side	Defect reconstruction with a local flap	5
50	Female	Axillae, on both sides	Defect reconstruction with a local flap	4
21	Female	Axilla, on one side	Skin autografting	9
50	Male	Axillae, on both sides	Skin autografting	10
31	Male	Axillae, on both sides	Skin autografting	12
43	Male	Axillae, on both sides	Skin autografting	12
17	Female	Axillae, on both sides	Skin autografting	12
45	Male	Axillae, on both sides	Skin autografting	17
43	Male	Scrotum	Skin autografting	13
73	Female	Axillae, on both sides	Skin autografting	12

localisation in 13 patients the changes involved the axillary region, in three patients hidradenitis affected the scrotum and in one patient it involved the gluteal region.

Regarding the type of surgery, 13 patients underwent skin autografting (Figure 4a) and in 4

Figure 4-a: Hidradenitis suppurativa after surgical therapy – skin autografting

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Figure 4-b: Hidradenitis suppurativa, completed recovery after the surgical procedure

patients defect reconstruction with a local flap. The duration of the hospitalisation was from 4 to 17 days, 11 days on average. Complete recovery of the patient did not occur immediately after the end of hospitalisation, because complete healing and adaptation of the autograft is necessary. The positive effects of surgery are usually observed 15 days after the end of hospitalisation in case there are no postoperative complications and there is a proper wound healing and removal of sutures (Figure 4b). After a period of 6-8 weeks, in most cases the patient can return to his normal work commitments.

It should be mentioned that during the last two years, the number of surgeries, including the surgical treatment of patients with HS, was significantly lower due to the COVID-19 pandemic. Namely, there were occasional suspensions of surgeries or only surgeries involving acute diseases and malignancies were performed.

Disscusion

HS is a chronic disease that most often affects apocrine gland-bearing areas of the skin. According to the information referred to in literature, it is more common in female, which is not in accordance with results in this study since the majority of subjects were male. The youngest subject was 17 and the oldest one was 74 years old. In most of presented subjects, the disease appeared immediately after puberty, which is in line with the previous research.[2, 16] Lifestyle, obesity and smoking are in direct correlation with the clinical severity of HS. The effect of sex hormones to occurrence and the course of the disease has been confirmed.[17]

Skin changes in the form of erythematous nodules, fistulas and scars are most often located in the armpits, groin, genital and gluteal region. Most of analysed subjects had axillary and gluteal changes and on the scrotum. In one subject, skin changes were located on the anterior abdominal wall and in the other below the breast (submammary on both sides). HS can also occur in both the occipital and retroauricular regions and on the eyelids.[18, 19]

The treatment of severe forms of hidradenitis (Hurley II/III) (Hurley II and Hurley III) starts with the application of the systemic antibiotic therapy. According to the previous research, the best effect is achieved by using the combination of rifampicin and clindamycin in the daily dose of 600 mg for four months. Acitretin, which is recommended in treatment of mild to moderate forms of HS, may be used as the continuation of therapy in treating the severe forms of HS after achieving a good therapy response by using systemic antibiotics, since it decreases the inflammatory component in the dermis by inhibiting the chemotaxis of polymorphonuclear with a consequential decrease in production of inflammatory cytokines, particularly interleukin 6 (IL-6). Biologic therapy with monoclonal antibody adalimumab, which is the only approved biologic medicine for HS treatment, is the first line of treatment in moderate and severe forms of HS that had no response or tolerance to antibiotic therapy.[20-23] The application of adalimumab in subjects resulted in regression of changes and improvement by 60-70 %.

Recently, Radiotherapy Centre Affidea started using ionising radiation for HS treatment. It is believed that the effect of ionising radiation in the HS treatment is based on the destruction of inflammatory cells and the release of mediators, cytokines and proteolytic enzymes. Radiation in small doses has the best effect in the early phase of inflammation when vasodilation, oedema and leukocyte accumulation are present. Endothelial cells have an important role in the further spread of inflammation.[12] After applying the dose of 5 Gy in 5 or 10 fractions, subjects had an improvement and regression of changes by 60-70 %. In addition to the treatment of HS, a positive effect was observed in the treatment of other benign diseases such as keloids, pterygium, endocrine orbitopathy, haemangiomas, gynaecomastia, endometrial

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hyperplasia and other benign tumours of the central nervous system, soft tissues, bones and locomotor system.[24-26]

If we compared the effects of applying the biologic therapy and radiotherapy in treating severe forms of HS, we could conclude that they both brought significant regression of changes and improvement. During the research, we had a small number of subjects treated with biologic and radiotherapy, so this conclusion should be taken with a reserve. Also, when selecting the therapy, it should be taken into consideration that the costs of biologic therapy are significantly higher compared to the cost of radiotherapy.

Subjects from this study achieved the best results after surgical therapy, ie after skin autografting and reconstruction of the defect with a local flap. They were able to return to their normal life activities after a period of 6-8 weeks. Data from the literature confirm that surgical therapy has the best results in treating severe forms of HS. Surgical therapy is applied in case all previous protocols did not give satisfactory results.[13, 14, 27]

Conclusion

The recommended therapies for treatment of severe forms of HS include the application of biological therapy, radiotherapy and surgical therapy. The application of biological and radiotherapy both provide similar results after the 12 weeks of treatment, bringing the regression of changes by 60-70 %. The best results in HS treatment were achieved using surgical therapy.

Acknowledgements

None.

Conflict of interest

None.

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ORIGINAL ARTICLE

Assessment of Prognostic Markers of Heart Failure Following Acute Myocardial Infarction in Patients Treated With Primary Percutaneous Coronary Intervention

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Abstract

Background / Aim: The concentration of N-terminal brain natrium peptides (NT-proBNP) is an important marker within the diagnostic and prognostic analysis of patients with chronic heart failure. In patients with ST-segment elevation myocardial infarction, natriuretic peptides are dominant predictors of death, heart failure and additional myocardial infarctions. The aim of this study was to correlate prognostic markers of heart failure following acute myocardial infarction.

Methods: 193 patients with myocardial infarction were divided into two groups: 69 patients with NT-proBNP ≤ 1000 pg/mL and 124 patients with NT-proBNP > 1000 pg/mL. During the hospitalisation, laboratory data, clinical data and information on previous medications were collected. Echocardiography was used to identify left ventricular ejection fraction (LVEF). All statistical analysis were done in SPSS, version 23.

Results: The group with elevated NT-proBNP (> 1000 pg/mL) was older ($p < 0.001$) and suffered more often of arterial hypertension ($p = 0.04$) and atrial fibrillation ($p = 0.003$). Heart rate was higher and LVEF was lower in patients with elevated NT-proBNP values ($p < 0.001$). Mean LVEF in the 193 patients was 46.86 %. In both linear and binary logistic regression analysis multiple predictors of elevated NT-proBNP have been identified.

Conclusion: Increased ranges of NT-proBNP in patients following acute myocardial infarction are in correlation with decreased LVEF, elevated high-sensitive troponin I, lactate dehydrogenase, urea, creatinine, C-reactive peptides. This may guide clinicians to assess and treat early stages of heart failure.

Key words: NT-proBNP; Acute myocardial infarction; Heart failure.

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Introduction

Defining the concentration of circulating natriuretic peptides is a crucial in the diagnostic and prognostic assessment of patients with persistent coronary heart disease.[1, 2] In patients who suffered from ST-segment elevation myocardial infarction, natriuretic peptides can be dominant predictors of left ventricular dysfunction and death.[1, 3]

The aim of this study was to correlate N-terminal brain natrium peptides (NT-proBNP) ranges with indicators of myocardial necrosis, left ventricular ejection fraction (LVEF), heart rhythm and other measures such as creatinine, cholesterol, sodium, potassium, C-reactive peptides (CRP) as well as with smoking habits and on-going medications ie, antiplatelet therapy, beta-blockers, ACE-inhibitors.

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Methods

Study population and data collection

In this study, 193 adult consecutive patients who were treated with primary percutaneous coronary intervention (PCI) between January 2021 and October 2021 at the University Clinical Centre of Republic of Srpska were included. All patients with measured NT-proBNP levels were included.

Clinical data

During the hospitalisation, clinical documentations on previous medications were collected. Study included data on hypertension, diabetes mellitus, family history of coronary artery disease, smoking status and previous interventions (PCI, coronary artery bypass grafting). Heart rhythm was analysed by electrocardiography for detecting normal sinus rhythm and abnormalities (atrial fibrillation, atrial flutter and patients with implanted pacemaker).

Laboratory data

Blood samples were collected in first 24 hours after admission. Parameters were NT-pro BNP levels, markers of myocardial necrosis (creatine kinase (CK), CK-MB, lactate dehydrogenase (LDH), troponin), total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, urea, creatinine, sodium, potassium, magnesium, calcium, CRP, D-dimer, prothrombotic time, activated partial thromboplastin time (aPTT), prothrombin time - international normalised ratio (INR).

Echocardiography

Echocardiography was used to determine LVEF.

Statistical analysis

Continuous variables were presented as mean with standard deviation or median and interquartile range, based on data distribution. For normal distribution was used a T-test and in cases with abnormal distribution Mann Whitney test was used. Categorical variables were presented as number with percentage and compared using a Chi-square test. In order to assess correlation between different parameters, linear regression analysis and Person´s coefficient were used. All variables were implemented into a univariate ie, multivariate binary logistic regression model. Independent predictors of elevated NT-proBNP were identified. All analysis was done in SPSS, version 23.

Results

Clinical data

Patients were divided into two groups according to the NT-proBNP levels. First group, 69 patients (NT-proBNP ≤ 1000 pg/mL) and second group 124 patients (NT-proBNP > 1000 pg/mL). The mean age of the 193 patients was 74.6 years. There was a predominance of male patients, 120 patients ie, 62.18 %. The vast majority had hypertension (65.8 %). There were 27.46 % smokers (53 patients) and 57 out of 193 had positive family history for cardiovascular diseases. Patients had mainly sinus rhythm (83.42 %), whereas in 29 patients (15.06 %) there was atrial fibrillation. Atrial flatter was found at 1 patient (0.52 %) and there were 2 patients with pacemaker (1.04 %). Mean LVEF in the 193 patients was 46.86 % (Table 1).

Table 1: Baseline characteristics in patients with heart failure following acute myocardial infarction related to NT-proBNP values

	Total n = 193	NT-proBNP (pg/mL) ≤ 1000 (n = 69)	NT-proBNP (pg/mL) > 1000 (n = 124)	p
Age		61 (IQR 19)	72 (IQR 19)	
Male	120	43 (62.3 %)	77 (62.1 %)	< 0.001
Hypertension	127	40 (57.9 %)	87 (70.2 %)	0.97
Diabetes mellitus	47	12 (17.4 %)	35 (29.0 %)	0.04
Smoking	53	23 (33.3 %)	30 (24.2 %)	0.28
Family history	54	27 (39.1 %)	27 (21.8 %)	0.37
Previous PCI	39	13 (18.8 %)	26 (20.9 %)	0.07
Previous CABG	7	2 (2.9 %)	5 (4.0 %)	0.72
ACE inhibitor	101	36 (52.2 %)	65 (52.4 %)	0.69
Beta blocker	138	43 (62.3 %)	95 (76.6 %)	0.97
Acetylsalicylic acid	104	34 (49.3 %)	70 (56.5 %)	0.04
Clopidogrel	73	23 (33.3 %)	50 (40.3 %)	0.34
Rhythm				0.34
Sinus rhythm	161	65 (94.1 %)	96 (77.4 %)	0.003
Atrial fibrilation	29	2 (2.9 %)	27 (21.8 %)	
Atrial flutter	1	1 (1.5 %)	0 (0 %)	
Pacemaker	2	1 (1.5 %)	1 (0.8 %)	
Heart rate		81.22 ± 19.127	94.69 ± 23.266	< 0.001
LVEF (%)		55 (IQR 15)	38 (IQR 15)	< 0.001

PCI: percutaneous coronary intervention; CABG: coronary artery bypass graft; ACE: angiotensin-converting enzyme; LVEF: left ventricular ejection fraction; NT-pro-BNP: N-terminal proBrain natriuretic peptide;

The group with elevated NT-proBNP (> 1000 pg/mL) were older (p < 0.001) and suffered more often of arterial hypertension (p = 0.04) and atrial fibrillation (p = 0.003). As expected (Table 1), heart rate was higher and LVEF was lower in patients with elevated NT-proBNP values (p < 0.001).

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Table 2: Laboratory findings in patients with heart failure following acute myocardial infarction related to NT-proBNP values

	NT-proBNP (pg/mL)		p
	≤ 1000 (n = 69)	> 1000 (n = 124)	
Hs troponin I (µg/L)	62.6 ± 4355.2	1345 ± 14057.2	0.001
LDH (U/L)	229 ± 237	298 ± 352.8	0.005
CK (U/L)	164 ± 400	121 ± 358.5	0.378
CK-MB (U/L)	24 ± 30	23.5 ± 59.8	0.334
Total cholesterol (mmol/L)	5.04 (IQR 1.46)	4.79 (IQR 1.46)	0.441
HDL (mmol/L)	1.1 (IQR 0.4)	1 (IQR 0.4)	0.318
LDL (mmol/L)	3.44 (IQR 1.29)	3.29 (IQR 1.17)	0.563
Triglycerides (mmol/L)	1.74 ± 0.98	1.656 ± 0.87	0.675
Urea (mmol/L)	5.9 ± 2.3	7.25 ± 6.7	0.002
Creatinin (µmol/L)	78 (IQR 31.8)	94 (IQR 47)	0.002
Sodium (mmol/L)	140 (IQR 3)	139 (IQR 6)	0.195
Potassium (mmol/L)	4.26 ± 0.42	4.34 ± 0.64	0.406
Magnesium (mmol/L)	0.83 ± 0.09	0.78 ± 0.09	0.018
Calcium (mmol/L)	2.27 ± 0.3	2.2 ± 0.2	0.034
CRP (mg/L)	2.8 ± 16.2	13.5 ± 59.3	< 0.001
D dimer (µg/L)	5.97 ± 11.4	2.42 ± 10.3	0.788
Prothrombotic time	1.62 ± 2.1	2.29 ± 3.4	0.765
aPTT	58.29 ± 32.5	57.18 ± 29.48	0.909

PCI: percutaneous coronary intervention; ACE: angiotensin-converting enzyme; LVEF: left ventricular ejection fraction; NT-pro-BNP: N-terminal proBrain natriuretic peptide; CK: creatine kinase; LDH: lactate dehydrogenase; CRP: C-reactive protein;

Regression analysis

Regression analysis data are given in Table 2. Patients in whom NT-proBNP was higher of 1000 pg/mL had values of high-sensitive troponin I, LDH, urea, creatinine and CRP. In the linear regression analysis, serum NT-proBNP levels correlated well with LVEF, serum creatinine (µmol/L) and CRP (Figure 1). When univariate binary logistic regression analysis were applied, multiple predictors of elevated NT-proBNP (> 1000 pg/mL) were identified (Table 3).

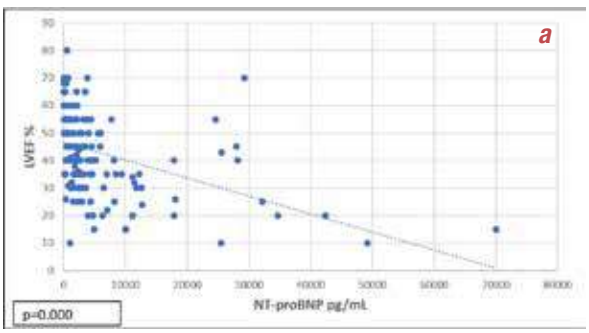
Table 3: Univariate binary logistic regression analysis in patients with heart failure following acute myocardial infarction related to NT-proBNP values

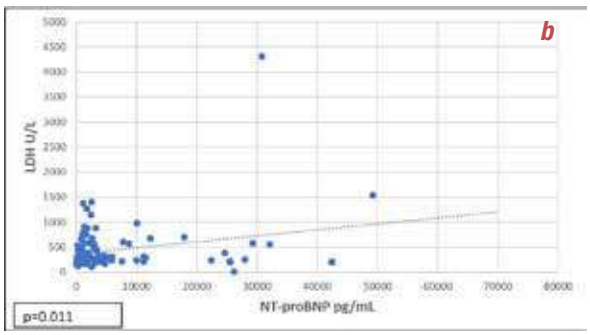
Variable	Univariate logistic regression		
	OR	95 % CI	p
Age	0.984	0.97-1.01	0.100
Male	1.009	0.55-1.85	0.976
Hypertension	1.705	0.92-3.15	**0.088**
Diabetes	1.868	0.90-3.90	**0.096**
Smoking	0.638	0.33-1.22	0.174
Prior PCI	1.143	0.54-2.40	0.724
Atrial fibrillation	2.747	1.12-6.73	0.027
LVEF (%)	0.914	0.89-0.94	**< 0.001**
ACEi	1.010	0.56-1.82	0.974
Beta-blockers	1.981	1.04-3.76	**0.036**
Acetylsalicylic acid	1.334	0.74-2.41	0.338
CK (U/L)	1.000	0.99-1.01	0.285
LDH (U/L)	1.003	1.00-1.05	**0.028**
CK-MB (U/L)	1.001	0.98-1.04	0.484
hsTroponin (µg/L)	1.005	1.00-1.10	**0.078**
Cholesterol (mmol/L)	1.002	0.99-1.01	0.715
LDL (mmol/L)	0.997	0.97-1.03	0.840
Triglycerides (mmol/L)	1.010	0.96-1.06	0.672
Urea (mmol/L)	1.013	1.01-1.02	**0.003**
Creatinine (µmol/L)	1.019	1.01-1.03	**0.002**
Sodium (mmol/L)	0.927	0.84-1.02	0.133
Potassium (mmol/L)	0.983	0.96-1.01	0.203
Magnesium (mmol/L)	0.994	0.98-1.01	0.563
CRP (mg/L)	1.001	1.00-1.01	**0.077**
D-Dimer (µg/L)	1.00	0.99-1.01	0.614

PCI: percutaneous coronary intervention; CABG: coronary artery bypass graft; ACE: angiotensin-converting enzyme; LVEF: left ventricular ejection fraction; NT-pro-BNP: N-terminal proBrain natriuretic peptide; OR: odds ratio; CI: confidence interval;

Discussion

This study was focused on patients with acute myocardial infarction treated with primary PCI. The major result of this study was the positive correlation between NT-proBNP levels with LVEF and other indicators (troponin I, LDH, urea, creatinine and CRP). LVEF was lower in patients with elevated NT-proBNP values ($p < 0.001$). Troponin I ($p = 0.078$) and LDH ($p = 0.028$) levels were considerably related with NT-proBNP levels. Previous study showed that kidney failure and aging are significant factors for elevated plasma NT-proBNP levels.[4] When serum creatinine levels are higher than 2.0 mg/dL, NT-proBNP levels elevate remarkably.[5-7] This study showed posi-





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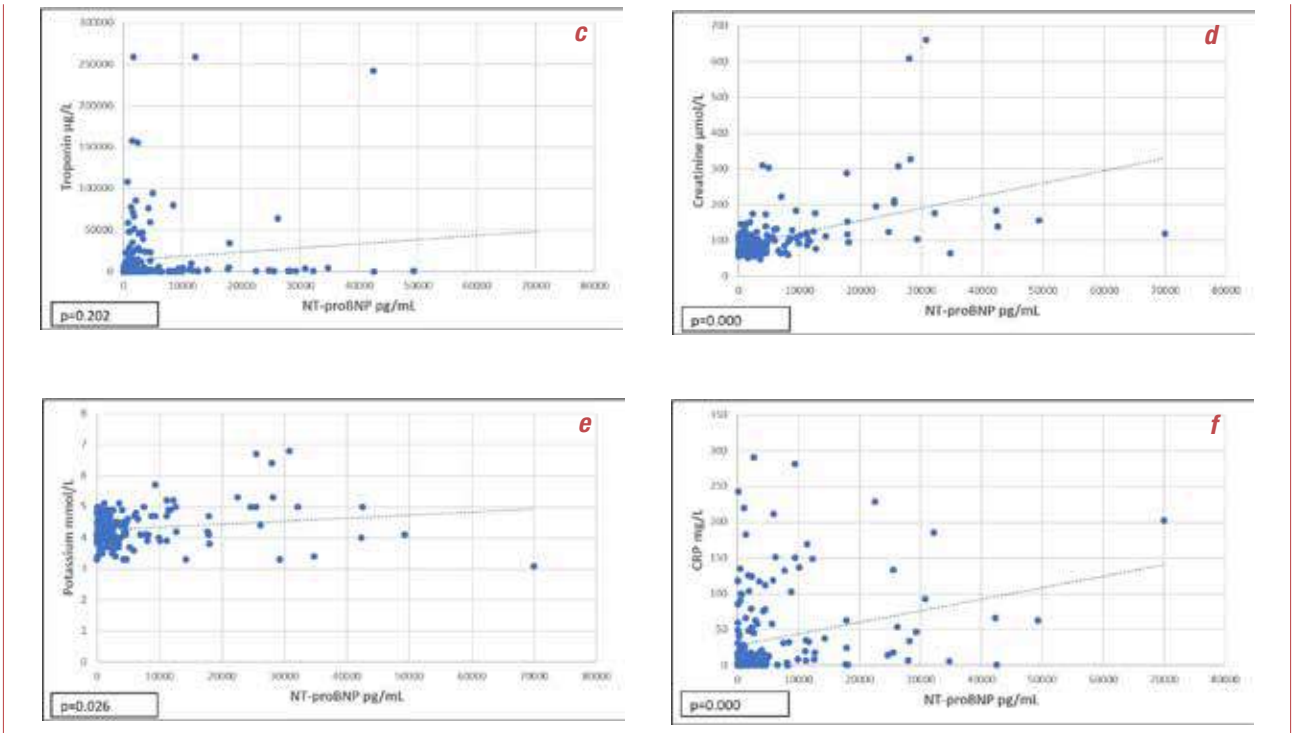

Figure 1 a-f: Linear regression analysis of parameters in patients with heart failure following acute myocardial infarction

tive correlation with urea (p = 0.003) and creatinine (p = 0.002) levels. The group with elevated NT-proBNP (> 1000 pg/mL) had higher CRP levels (p = 0.077) and suffered more often of arterial hypertension (p = 0.04) and atrial fibrillation (p = 0.003).

Study showed negative correlation with CK-MB (p = 0.484) and atherosclerosis markers (cholesterol and triglycerides). Cholesterol (p = 0.715) and triglycerides (p = 0.672) were not associated with higher NT-proBNP levels.

High NT-proBNP levels are in some measure related to the degree of ischaemic myocardial infarction, which may have prognostic value.[8] In previous study, older patients formed an enlarged amount of individuals with acute myocardial infarction and age was a powerful predictor of significant complications and death after acute myocardial infarction.[8] However, this study did not find significantly association with age and high NT-proBNP levels (p = 0.100).

Findings from this study indicate that high NT-proBNP levels can bring relevant information because of their possibility to outline the quantity of injured myocardium.

Limitations

The study has some limitations, which needs to be acknowledged. Retrospective nature of the study cannot exclude potential selection bias as well as confounding variables. Relatively small number of patients. Data on total ischaemic time were not available.

Conclusion

There are many important predictors for worse patient condition and poor outcomes. NT-proBNP levels correlated with LVEF, but also with troponin I, LDH, urea, creatinine and CRP levels. There was a relation of high NT-proBNP levels with hypertension and atrial fibrillation. This may guide clinicians to assess and treat early stages of heart failure.

CONTENT

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Conflict of interest

None.

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CONTENT

Dadhich et al. Scr Med 2022 Jun;53(2)130-4. DOI:10.5937/scriptamed53-35899
ORIGINAL ARTICLE

Prescribing Patterns in Type 2 Diabetes Mellitus Outpatients at a Tertiary Care Centre in Jaipur, India

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Abstract

Background: Over the last few years, an unexpected increase in the prevalence of diabetes in India have been witnessed. The present study was planned to analyse prescribing patterns of anti-hyperglycaemic drugs and assess the influence of Chief Minister's Free Drug Scheme in Rajasthan, India. It aimed to evaluate, monitor and if possible, suggest modifications in prescribing practices to make medical care rational and also to assist minimising adverse drug reactions (ADRs).
Methods: This was a cross-sectional, observational study carried out for a 12-month period. A total 400 known patients of type 2 diabetes mellitus (T2DM) from endocrinology outdoor of SMS Medical College Hospital (a tertiary care hospital in Jaipur, Rajasthan, India) were recruited and their prescriptions were analysed using the World Health Organization (WHO) prescribing indicators.
Results: Most commonly observed age group was of 40-50 years (mean age 53.76 ± 8.84), with a male preponderance (57.5 %). Among them, 67.5 % of patients were found to be obese (mean BMI 29.79 ± 3.26). All anti-hyperglycaemic were prescribed in their generic names only. Metformin was the most frequently prescribed anti-hyperglycaemic agent. Among the fixed dose combinations, the most common was that of glimepiride and metformin (40.75 %), while most prescribed add on anti-hyperglycaemic was teneligliptin (51.5 %), followed by pioglitazone (30.5 %). A total of 53.25 % of these patients received insulin along with oral anti-hyperglycaemic agents.
Conclusion: The anti-hyperglycaemic agent prescribing among endocrinology outpatients at tertiary care hospital in Jaipur was found to be satisfactory.

Key words: Glimepiride; Metformin; Teneligliptin; WHO prescribing indicators.

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Introduction

According to the World Health Organization (WHO), "Diabetes is a chronic, metabolic disease characterised by elevated levels of blood glucose (or blood sugar), which over time leads to serious damage to the heart, blood vessels, eyes, kidneys and nerves. The most common type is type 2 diabetes mellitus (T2DM), which affect mainly adults. This occurs when the body becomes resistant to insulin or doesn't make enough insulin."[1]

Glucose regulation is mainly done by two hormones insulin and glucagon secreted by pancreas and this regulation gets interrupted in diabetes mellitus.[2] According to global report of WHO, in October 2018, 72.9 (8.8 %) million people in India were living with diabetes in 2017.[3] And this has increased to 77 million in 2019 according to International Diabetes Federation report, 2019.[4]

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The initial treatment strategies are mainly based on the severity and type of diabetes. The subsequent addition of anti-hyperglycaemic and other agents would depend on the co-morbid conditions of the patient. For the management of T2DM, both the pharmacological approach, in form of anti-hyperglycaemic agents and non-pharmacological approach, in the form of lifestyle modifications (diet, exercise, reduced alcohol and smoking cessation) are applied.[5]

Rational use of medications in such chronic conditions can prevent the complications and suffering.[6] It is a complex issue with a goal that is difficult to achieve, it is defined as: "Patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements for an adequate period of time and at the lowest cost to them and their community".[7]

Multiple reasons have been observed behind the persistence of disease and suffering which include improperly prescribed, dispensed and sold medications, failure of patients to take the medications as advised and failure of access to the essential drug list (EDL). Thus, it is necessary to identify irrational prescribing patterns.[8] The WHO defines drug utilisation as "The marketing, distribution, prescription and use of drugs in a society, with special emphasis on the resulting medical, social and economic consequences." The WHO has also formulated a set of "core prescribing indicators" for improvement in the rational drug use. It includes the prescribing indicators, the patient care indicators and the facility indicators.[9, 10]

Therefore, this study was planned to analyse the prescribing patterns of anti-hyperglycaemic agents among the outpatients of endocrinology department of SMS Medical College Hospital, Jaipur. This study was aimed to evaluate, monitor and if possible, suggest modifications in prescribing practices to make medical care rational and also to assist in minimising adverse drug reactions (ADRs).

Methods

Study design and sample size determination

It was a cross-sectional, observational study which was carried out in the outpatients of endocrinology department of SMS Medical College Hospital (a tertiary care hospital in Jaipur) after taking permission from the research review board of the institute (protocol No 36600 dated 10/07/2018). The sample size for the study was calculated at 95 % Confidence Level expecting 50 % adherence (maximum variance) to the treatment of type 2 diabetes mellitus (T2DM) in endocrinology department. At the precision (relative allowable error) of 10 %, a minimum of 400 patients of T2DM were recruited as sample size.

Data collection and statistical analysis

A total 400 known cases of diabetes in age group of 40 to 70 years, irrespective of their co-morbid status were recruited and the data in the form of socio-demographic profile, personal history and WHO core indicators were collected in a predesigned study forms. Data collected was tabulated and analysed using descriptive statistical tools (mean ± standard deviation and percentage). Chi-square test was used for categorical data. The data were analysed using SPSS for Windows (version 16.0 Chicago, SPSS Inc.) with the statistical significance evaluated using two-sided P value at a 5 % level of significance.

WHO Core Indicators

The drug data obtained was assessed for "Prescribing Indicators" by the WHO.[10]

i. Average number of drugs per encounter = Total number of drugs prescribed / Number of medication charts.

ii. Percentage of drugs prescribed by generic name = Number of generic drugs prescribed / Total number of drugs × 100.

iii. Percentage of patient medications charts with antibiotics prescribed = Total number of charts with antibiotics prescribed / Total number of charts.

iv. Percentage of patient medications charts with injections prescribed = Total number of charts with injections / Total number of charts.

Percentage of drugs prescribed from Essential Drug List of Rajasthan[11] = Total number of drugs from EDL / Total number of all drugs prescribed × 100.

Results

Out of the 400 patients, 57.5 % were men and 42.5 % were women. The recruited patients in this study were between 40-70 years of age. Mean age was found to be 53.76 ± 8.84 years.

A total 39.56 %, out of the 230 men gave history of current alcohol intake while 17.3 % stated that they have quit alcohol. Rest denied any history of alcohol intake. Similarly, 35.21 % of men were currently smokers, while 28.69 % men said that they have quit smoking. Rest said they have never smoked. All the women denied any intake of alcohol and/or smoking, although, tobacco chewing history was present in 33.5 % of the women and 52 % of men. Smokers were asked about their number of cigarettes smoked per day, it was more than 5 cigarettes a day in 76.5 % of smokers and less in the rest. Out of the total patients, 67.5 % were found to be obese (BMI ≥ 30), 24.5 % patients were overweight and only 8 % had normal weight (Table 1).

Table 1: Distribution of patients with diabetes mellitus type 2 according to body mass index

BMI (in kg/m²)	Men	Women	p value*
18.5 - 24.9	24	8	0.085
25 - 29.9	58	40	
≥ 30	148	122	
Total	230	170	
Mean ± SD	29.79 ± 3.26		

** p value calculated using Chi square test*

WHO core indicators were assessed after seeing their prescriptions and they gave results as below (Table 2).

Table 2: Estimated WHO Core Prescribing Indicators

Number of prescription analysed	400
Average number of drugs per encounter (Mean ± SD)	4.58 ± 1.8
Percentage of encounter with antibiotics prescribed (%)	4
Percentage of encounter with an injection prescribed (%)	53.25
Percentage of drugs prescribed by generic name (%)	100
Percentage of drugs prescribed from Rajasthan Essential Drug List 2019 (%)	94

Individual drug distribution

Most commonly prescribed anti-hyperglycaemic agent was metformin, prescribed in 154 patients. Metformin was also prescribed in fixed dose combinations with glimepiride, gliclazide and teneligliptin in 163, 59 and 24 patients respectively (Figure 1).

In patients with poor glycaemic control other add-on anti-hyperglycaemic drugs were also prescribed. Teneligliptin was prescribed in a total of

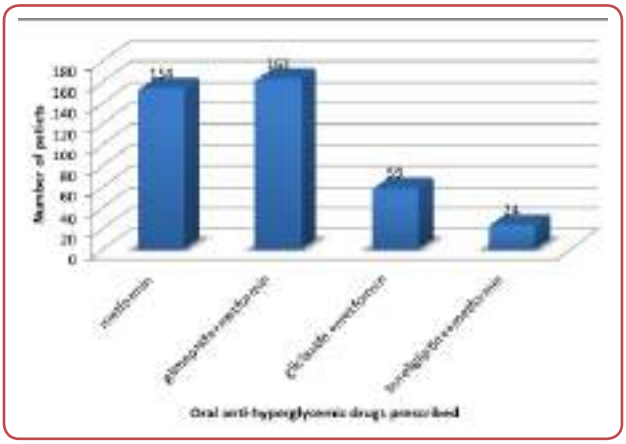

Figure 1: Distribution of patients according to oral anti-hyperglycaemic drug prescribed

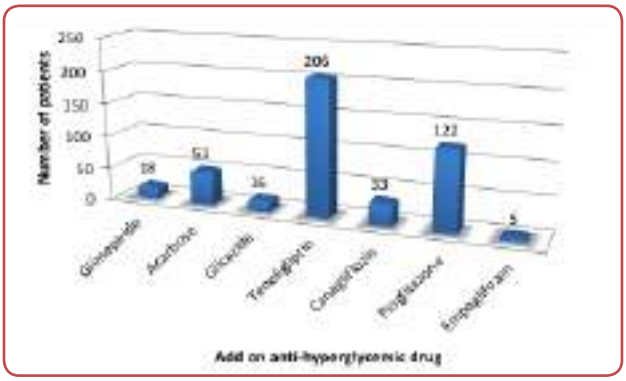

Figure 2: Distribution of patients according to add on anti-hyperglycaemic drugs prescribed

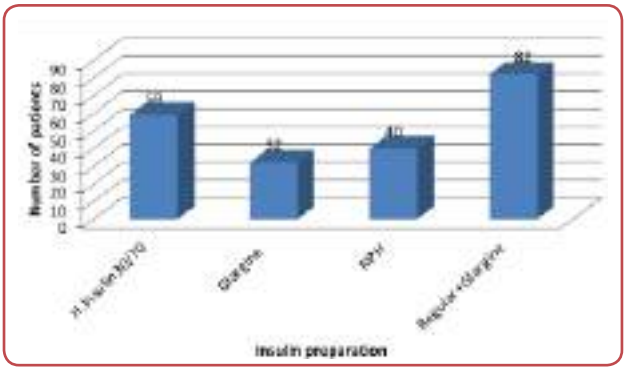

Figure 3: Distribution of patients according to insulin preparations prescribed

NPH: Neutral protamine Hagedorn insulin;

206 patients as an add-on drug with either fixed dose combination or with metformin. Second most common as an add-on was pioglitazone in 122 patients. Acarbose, glimepiride, gliclazide, canagliflozin and empagliflozin were prescribed in 51, 18, 13, 33, 5 patients respectively (Figure 2).

Along with oral anti-hyperglycaemic agents human insulin was prescribed in 59 patients (Figure 3).

Conclusion

The anti-hyperglycaemic agent prescribing among endocrinology outpatients at tertiary care hospital in Jaipur was found to be satisfactory. The average number of drugs per prescription was found to be 4.58. This seems to be justified as a chronic disease like diabetes mellitus is associated with comorbidities and deteriorates over time might require multiple drugs for its management. The most common add on drug was teneligliptin. Antibiotics were prescribed to only 4 % patients, which seemed judicious. Patients were also taught about the importance of lifestyle modifications and diet. This is also in accordance with the American Diabetes Association (ADA) recommendations. Adherence to lifestyle and dietary modifications will not only improve glycaemic control but will also help in reducing the long term complications of diabetes mellitus.

Limitations

The sample was not random and could have been a potential source of bias. The inherent limitations of a cross-sectional study cannot be ignored. Indeed, only prospective studies can demonstrate a causal association between the determinants and uncontrolled T2DM. Besides, the self-reporting of participants could not rule out the possibility of bias in the participant's responses. Glycosylated HbA1c was not recorded but recommended in the study.

Future perspectives

In future, similar studies can be planned in private tertiary care hospital settings. Studies for evaluating defined daily doses (DDD) for anti-hyperglycaemic agents can also be envisaged.

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Conflict of interest

None.

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ORIGINAL ARTICLE

The Awareness Survey of Clinical Trials Among Medical Students of South Rajasthan, India

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Abstract

Background / Aim: Clinical trials are becoming more popular in India, but its awareness among the medical professionals remains far from satisfactory. Clinical research/trial can help medical students in developing the critical thinking abilities necessary for medical practice. In this era of evidence-based medicine, the integration of medical education and clinical research is crucial to ensure that scientific findings are translated into clinical practice. The present study aimed to find out the awareness about clinical trial among undergraduates.

Methods: After obtaining approval from the Institutional Ethics Committee, this cross-sectional study was conducted on students from first to final year and interns after taking their consent. A sample of 390 respondents was analysed. A structured questionnaire was used to measure the objective of this study. The proportion of successfully answered questions in each group was computed and the results were sorted into pre-determined grades as follows: As excellent - 80-100 %, moderate - 50-80 % and terrible – less than 50 %.

Results: Out of 390 undergraduate students, for the statement regarding the concept of clinical trials, around 28.2 % fell in the good category, 57.7 % in the average category and 14.1 % in the poor category. Regarding the statement about role of the United States Food and Drug Administration (US FDA) in approving new drug, 34.1 % were poor responders, 52.6 % were average and 13.3 % were good. The statements regarding the participation in the clinical research study showed that majority were in the poor and average response category (45.6 % and 41.5 %, respectively) as compared to only 12.8 % in the good category.

Conclusions: The overall awareness of clinical trials was low among students, the medical undergraduates are future innovators, clinicians and scientific explorers. It would be better if they are trained at earlier days of learning about clinical trials/research and medical ethics. These can be made a part of medical curriculum so that they can build their concrete future.

Key words: Clinical trials; Knowledge; Undergraduate medical students; Awareness.

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Introduction

Clinical trials have a significant role in improving the quality of healthcare practice in a timely manner.[1] These trials are conducted at the majority of teaching medical institutes. The numerous steps of clinical trials, the process of putting together a study and giving the patient informed consent are included in the pharmacology curriculum for medical students at the undergraduate level.[2] Undergraduate medical students can be benefitted from basic research in order to strengthen their critical thinking skills, which are necessary for medical practice. The integration of medical education with clinical research is critical in this era of evidence-based medicine to see that scientific

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breakthroughs are translated into therapeutic practice. Teaching medical students about clinical research and trials may motivate some of them to seek a career in clinical trials and some of them may even participate in clinical trials.[3] The purpose of this study was to see how well undergraduate medical students knew about clinical trials in the Government Medical College, Kota, Rajasthan, India.

Methods

The research was done on undergraduate medical students in a cross-sectional study during the month of February 2020, after acquiring clearance from the Government Medical College's Institutional Ethics Committee in Kota, Rajasthan, India. After receiving their consent, a pre-tested questionnaire was used to interview the participants in this study. Medical students of first to final year, as well as interns, were given the questionnaire. Before the study began, the main investigator explained the study's purpose and obtained signed consent from the participants. The participants were also told that participation in the interview was completely optional and that they might leave at any time. All information acquired from participants was kept confidential. Participants who refused to participate in the study were excluded from the study.

A questionnaire was used in a similar study conducted by Vittalrao et al.[3] In the current study, the same questionnaire was employed and it was administered in English, which was the language of instruction. Part one of the questionnaire featured demographic profiles, part two was a general statement on the participant's status as a student, research scholar, or postgraduate and part three had 14 main statements with several sub statements. Certain statements were purposefully reframed as negative questions to gain a better understanding of the information and prevent prejudice. The following were the prizes for correctly answering the questions:

A. Correctly answered positive or negative questions +02 points;
B. Incorrectly answered positive or negative questions +00 point;
C. If the question had numerous positive or negative responses, each option that was properly ticked received a bonus of one point.

The proportion of successfully answered questions in each group was computed and the results were sorted into pre-determined grades as follows: As excellent - 80-100 %, moderate - 50-80 % and terrible – less than 50 %.[3] The data was imported into Ms Excel and analysed with the SPSS 22.0 programme.

Results

Ninety first-year medical students, 84 second-year medical students, 85 pre-final-year medical students, 71 final-year medical students and 60 interns were among the 390 respondents. Less than 5 % of medical students were currently involved in clinical trial study (Figure 1).

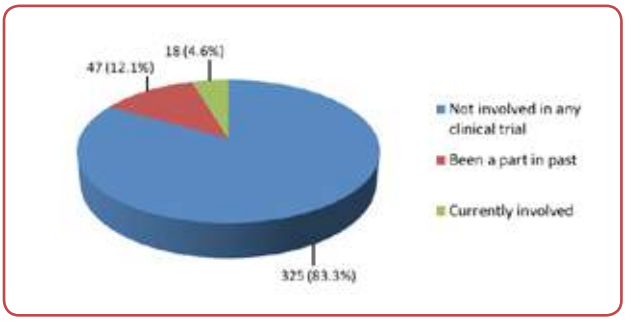

Figure 1: Status of students regarding participation in clinical trials

Clinical trials were familiar to the majority of them (82.1 %). There is a need for clinical trial research, according to 87.2 % of respondents. Clinical trials are a basic requirement for the advance-

Table 1: Questions to determine the level of interest in clinical trials

Questions	Yes N (%)	No N (%)	Don't know N (%)
Awareness about clinical trials	320 (82.1)	52 (13.3)	18 (4.6)
Any need for clinical trial research	340 (87.2)	12 (3.1)	38 (9.7)
Basic requirement for humanity's advancement and wellbeing	302 (77.4)	60 (15.4)	28 (7.2)
Would like to participate as researcher	280 (71.8)	48 (12.3)	62 (15.9)
Believe that clinical trial are unethical, incorrect and purely inhuman	32 (8.2)	308 (79.0)	50 (12.8)
Harmful events are more than the benefits	78 (20.0)	180 (46.2)	132 (33.8)
Clinical trials are a waste of time, money and resources	18 (4.6)	320 (82.1)	52 (13.3)

ment of mankind and health condition, according to 77.4 % of those polled. If any possibilities for clinical trials were available, 71.8 % of the students expressed an interest. Clinical studies are immoral, erroneous and totally inhuman, according to just 8.2 % of participants. Clinical trials are useful to the patient, according to nearly half of students (46.2 %). The vast majority agree that clinical trials are not a waste of time, energy or money.

Table 2: The awareness survey of clinical trials among medical students

Questions	Yes N (%)	No N (%)	Don't know N (%)
Concept of clinical trial	110 (28.2)	225 (57.69)	55 (14.10)
Need of clinical trial	96 (24.61)	220 (56.41)	74 (18.97)
Where can people find out about clinical trial	135 (34.61)	28 (7.17)	227 (58.20)
Participation in clinical trial	50 (12.82)	162 (41.53)	178 (45.64)
Pre-clinical and clinical testing	65 (16.67)	210 (53.84)	115 (29.48)
Institutional review board (IRB)	55 (14.10)	157 (40.25)	178 (45.64)
Role of US_FDA in approving new drug	52 (13.33)	205 (52.56)	133 (34.10)
DCGI role	124 (31.79)	102 (26.15)	164 (42.05)
Informed consent	132 (33.84)	172 (44.10)	86 (22.05)
Potential benefits	162 (41.53)	185 (47.43)	43 (11.02)
Potential risks	42 (10.76)	196 (50.25)	152 (38.97)
Leaving a research study	176 (45.12)	132 (33.84)	82 (21.02)
Knowing CRS results	74 (18.97)	140 (35.89)	176 (45.12)
Design of study/placebo/ control group	87 (22.30)	196 (50.25	107 (27.43)
Sponsors	84 (21.53)	198 (50.76)	108 (27.69)
Side effects	87 (22.30)	180 (46.15)	123 (31.53)

US FDA: the United States Food and Drug Administration; DCGI: Drugs Controller General of India;'

The average response for the assertions was given by more than half of the survey participants like concept of clinical trial (57.7 %), need of clinical trial (56.4 %), pre-clinical and clinical testing (53.8 %) and role of the United States Food and Drug Administration (US FDA) in approving new drug (52.6 %). Almost half students had average knowledge of potential risks of clinical trials, design of study/placebo/control group and sponsors. Out of total respondents, 58.2 % had poor knowledge of statement of what is the best way for people to learn about clinical trials. The knowledge of Drugs Controller General of India (DCGI) role was good in 31.8 %, average in 26.2 % and poor in 42.0 %.

Disscusion

The purpose of this study was to determine the level of awareness about clinical trials among undergraduate medical students and interns. The majority of the questions had an average response. In a studies done by Vittalrao et al[3] and Sharma and Jindal[4] similar outcomes were discovered. This could be due to a lack of organisation in medical education when it comes to student involvement in clinical trials and research. Early engagement in research by undergraduate students will benefit them in making evidence-based decisions for their future practice. Poor understanding and interest could be one of the explanations for students' lack of participation in clinical trials.[5] Similar outcomes have been recorded in various nations throughout the world. Doctors' participation in clinical research was found to be low in a study conducted in Pakistan.[6] The other factors include time restrictions and cumbersome paperwork.[3, 7] So far, research has not been made a requirement in India's undergraduate medical education. Undergraduate medical students were involved in 28 % of publications in one institution in Germany, where research is an integral part of the undergraduate medical curriculum.[8] According to a survey, 23 % of undergraduate students in Croatia, a European country, took part in research projects.[9] Sharma and Jindal[4] found that for the statement about the notion of clinical trials, roughly 20.2 % went into the good group, 61.4 % into the average category and 18.4 % into the bad category in a comparable research of 155 students. The bulk of the remarks on involvement in the clinical research study were in the bad response group (89.2 %), compared to 9.8 % in the excellent response category.[4] This difference could be because of the involvement of only final year students, while in the present study students from first to final year as well as interns were included. The knowledge of second to final year students could be better due to pharmacology background. According to Goel et al[10] a one-day training session on GCP standards can enable medical practitioners, dentists and nurses to have a better understanding of the concepts and practices of clinical research, hence increasing the credibility of clinical research in the nation.

Due to the low rates of medical professionals participating in clinical trials, greater emphasis should be placed on improving the research experience of medical students and interns in order

CONTENT

to boost their participation in clinical trials.[11] According to Chatterjee and Sarkar[12] just 10.9 % of students were aware of the existence of an institutional ethics committee and 42.8 % were unaware of the committee's specific job. Clinical research instruction should be included in the undergraduate pharmacology curriculum, according to Goel et al.[10] The findings in terms of clinical trial idea and understanding are comparable to those of Sharma and Jindal.[4] Complicated documentation and time restrictions are two of the challenges to starting clinical investigations.[7] Meenakumari et al[13] discovered that 90 % of doctors desired clinical trial training to be included in the undergraduate curriculum. As many as 5 % of medical students may go on to become researchers in the future. As a result, clinical research and medical ethics must be taught to them during their study time. To perform clinical trials, as Thatte and Bavdekar[14] suggests, one must have a solid understanding of basic concepts of clinical research, ethical and regulatory standards and appropriate clinical practices.

The limitations of current study were:

1. Pharmacology subject was not the part of first year medical student's curriculum, so they were less aware about clinical trials.
2. There is a need of multi-centric study for generalisation of the findings as only one medical college was involved in the present study.

Newer developments in medical education, in contrast to factual learning, emphasise the acquisition of skills, knowledge and attitudes. Clinical research is not only an appealing career opportunity, but it is also the cornerstone of evidence-based medicine. As a result, future doctors must be taught how to conduct scientific research in a well-structured, fruitful and ethical manner. Students can improve their clinical research ideas and perspectives by participating in monthly undergraduate clinical research projects, continuing education, workshops and symposiums, as well as organising excursions to contract research organisations (CRO).[15] Students should be encouraged to establish the habit of reading medical journals, since this will aid in the development of the skills required to work as a clinical trial investigator. With all of the aforementioned steps, developing medical community can surely position India at the forefront of the global pharmaceutical business in the hunt for high-quality and cost-effective services to assist drug research.[16]

Conclusion

It can be concluded from the findings of this questionnaire survey that undergraduate medical students have a good comprehension of clinical trial basics. Necessary efforts must be taken to increase student knowledge of clinical trials. Students should be encouraged to take part in clinical trials.

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None.

Conflict of interest

None.

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ORIGINAL ARTICLE

Procalcitonin is One of the Predictive Factors of Dehiscence of the Colorectal Anastomosis

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Abstract

Background/Aim: Dehiscence of the colorectal anastomosis is one of the most serious complications in digestive surgery that is still present in a large percentage today, which significantly increases the cost of treatment and can lead to death. Due to all the above, early detection of anastomotic dehiscence is very important, as well as the decision on surgical treatment. Procalcitonin (PCT) is thought to be an important marker of inflammation and sepsis. Aim of this paper was to confirm PCT as a marker of great sensitivity in early diagnosis of anastomotic leakage.

Methods: The study included patients who underwent surgery for colorectal cancer in the period from 2016 to 2020. Patients were operated according to an elective protocol and with an open surgical approach. In patients, PCT values were measured on the 2nd and 4th postoperative day (POD) to determine the association between elevated PCT values and the onset of dehiscence of the colorectal anastomosis.

Results: A study was conducted in 118 patients in whom a stapler colorectal anastomosis was created. Colorectal anastomosis dehiscence occurred in 10 patients. In 4 patients with dehiscence, no re-surgical intervention was required, but they were taken care of by conservative methods. Repeated surgery was performed in 6 patients. In all patients with dehiscence, there was a multiple increase in the value of PCT above normal.

Conclusion: PCT has high sensitivity and specificity (85 and 74 % respectively) as a marker in dehiscence of colorectal anastomosis. In this study it was found that PCT values were significantly correlated with the dehiscence of anastomosis 2nd POD and especially 4th POD.

Key words: Colorectal anastomosis; Anastomotic dehiscence; Procalcitonin.

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Introduction

Dehiscence of colorectal anastomosis is still one of the most serious complications in colorectal surgery, which significantly worsens the patient's oncological status, prolongs hospitalisation and increases treatment costs and can even lead to death. Even today, the frequency of dehiscence is up to 20 %.[1] The cause of dehiscence itself has not been fully elucidated, but it is believed that several factors may influence its occurrence. These are primarily male sex, high age, duration of surgery and preoperative radiotherapy. Also important are the blood supply to the anastomosis, the tension of the anastomosis, as well as the height of the created anastomoses and the type of surgical technique.[2-4] Diabetes mellitus type II may lead to numerous chronic complications that are mainly caused by pathological influence of hyperglycaemia on blood vessels. Diabetes is also related to high blood lipid levels, hypertension and changes of blood vessel walls.[5] Also, it causes changes

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of blood itself, like increased activation of platelets and blood coagulation disorders. Changes of blood vessels caused by diabetes mellitus may affect small blood vessels (microangiopathy) and large ones (macroangiopathy).[6] Also, body mass index (BMI), due to its metabolic and cardiovascular complications, significantly affects the perfusion in the area of the anastomosis and thus the more frequent occurrence of anastomotic leakage.

Clinical signs of infection are usually manifested in an advanced period, so it is important to have a reliable marker that would be very reliable for early detection of anastomotic leakage and thus to make the right decision on further therapeutic treatment.[7] There are several parameters of inflammation that can be used as indicators of infection, leukocyte count (WBC), C-reactive protein (CRP), Interleukins 6, procalcitonin (PCT) and fever. PCT is a protein made up of 116 amino acids and produced by thyroid C cells. Under normal conditions, PCT values are very low and less than 0.1 ng/mL. It is very significant that the PCT value is multiplied in infections and in septic conditions, so that the monitoring of PCT values can be effectively used as a marker of anastomotic leakage and for the assessment of necessity of surgical reintervention.[8, 9]

Methods

This prospective study included 118 patients who were operated at the Clinic of General and Abdominal Surgery of the University Clinical Centre of the Republic of Srpska in Banja Luka. All patients agreed to participate in the study. All study data were used from medical histories, operative protocols and clinical examinations of patients operated at the General Surgery Clinic. In all operated patients, rectal cancer was endoscopically verified and pathohistologically confirmed. All patients underwent preoperative value of tumour markers (C19-9, CEA) and computed tomography (CT) of the abdomen and pelvis. All patients were operated on in an elective protocol when an open laparotomy was performed and a stapler anastomosis was created. The study was conducted in the period from September 2016 to December 2020. The age of the patients included in the study ranged from 53 to 79 years of age. There were 71 male and 47 female patients. All patients who underwent surgery had risk factor I-III according to the American Society of Anaesthesiologist (ASA score). The operation was performed according to all standards of oncological surgery, which included high ligation of the lower mesenteric blood vessels and total or partial mesorectal excision depending on the height of the rectal resection. In all operated patients on the 2nd and 4th postoperative day, patients had blood drawn and measured values of the WBC and PCT. Alinity and the BRAHMS PTC chemiluminescent immunochemical assay processed on an Alinity analyser were used to determine plasma PCT values. In patients with anastomotic leakage, PCT levels were multiplied and dehiscence of the anastomosis was confirmed by the presence of intestinal contents on the drains or on laparotomy and a control finding of CT of the abdomen and pelvis. According to their severity, two types of anastomotic leakage were verified, those that could be solved by conservative methods and those that required surgical reintervention.

Results

Out of the total number of operated, it was determined that the largest number of operated was over 65 years of age. The number of male patients was 71 (60.2 %) and the Fishers Exact test method determined that there was no statistically significant influence of gender on the development of dehiscence of the anastomosis. Preoperative radiotherapy was performed in 33 (28 %) patients and based on the analysis, it was determined that there was no statistically significant effect of preoperative radiotherapy on the development of dehiscence of the anastomosis. Also, the Fishers Exact test analysis showed that there was no statistically significant effect on the height of the anastomosis and the onset of dehiscence of the anastomosis (Table 1). The total number of dehiscence of the colorectal anastomosis was recorded in 10 (8.47 %) patients. Six (60 %) patients with dehiscence of the anastomosis required re-surgery, while in 4 (40 %) patients with dehiscence conservative treatment was sufficient. Of all operated patients, 1 (0.8 %) was a patient with a fatal outcome, in the group with dehiscence of the anastomosis.

Dehiscence of the anastomosis with anastomotic leakage significantly prolonged hospitalisation

Table 1: Review of factors that may lead to dehiscence of colorectal anastomosis

Dehiscence		Yes	No	Total
Age				
from 55 to 65	N	2	33	35
	%	20.0	30.6	29.7
older then 65	N	8	75	83
	%	80.0	69.4	70.3
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**
Sex				
Male	N	7	64	71
	%	70.0	59.3	60.2
Female	N	3	44	47
	%	30.0	40.7	39.8
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**
Chemoradiotherapy				
Yes	N	5	28	33
	%	50.0	25.9	28.0
No	N	5	80	85
	%	50.0	74.1	72.0
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**
Anastomosis height				
Less than 10 cm	N	5	35	40
	%	50.0	32.4	33.9
More than 10 cm	N	5	73	78
	%	50.0	67.6	66.1
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**
Body mass index (BMI)				
Less than 19	N	0	8	8
	%	0.0	6.8	6.3
From 19 to 25	N	3	60	63
	%	30.0	50.8	49.2
More than 25	N	7	50	57
	%	70.0	42.4	44.5
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**
Diabetes mellitus				
Yes	N	4	34	38
	%	40.0	31.5	32.2
No	N	6	74	80
	%	60.0	68.5	67.8
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**
Mortality				
Yes	N	1	0	1
	%	10.0	0.0	0.8
No	N	9	108	118
	%	90.0	100.0	99.2
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**

of patients so that in patients without dehiscence the average hospitalisation lasted 8 days, while in patients with dehiscence hospitalization averaged 19.5 days.

Table 2: Procalcitonin 2nd postoperative day (POD) values in patients with dehiscence and no dehiscence anastomosis

Dehiscence		Yes	No	Total
Prokalcitonin ≥ 2 ng/mL				
Yes	N	9	32	41
	%	90.0	29.6	34.7
No	N	1	76	77
	%	10.0	70.4	65.3
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**

Table 3: Procalcitonin 4th postoperative day (POD) values in patients with dehiscence and no dehiscence anastomosis

Dehiscence		Yes	No	Total
Prokalcitonin ≥ 4 ng/mL				
Yes	N	10	21	31
	%	100.0	19.4	26.3
No	N	0	87	87
	%	0.0	80.6	73.7
Total	N	10	108	118
	%	**100.0**	**100.0**	**100.0**

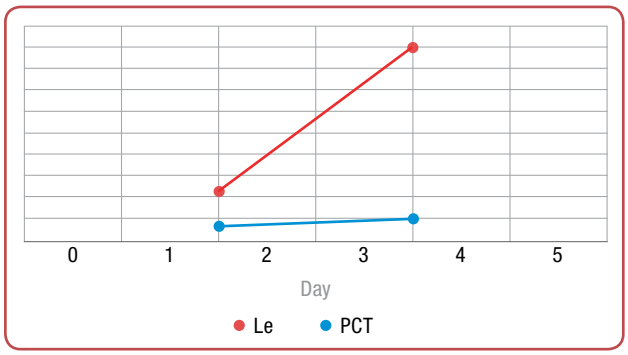

Figure 1: Correlation of procalcitonin (PTC) and total leucocyte count (Le) values 2nd and 4th postoperative day (POD)

The Fishers Exact test found that there was a statistically significant correlation between elevated PCT values above 2 ng/mL on the 2nd POD and 4 ng/mL on the 4th POD and dehiscence of the anastomosis. Thus, in patients who had a significantly higher value of PCT on the 2nd and 4th day, this was an important predictive factor in the occurrence of dehiscence of the anastomosis and anastomotic leakage (Tables 2, 3). Additionally, WBC level at the same time points was not markedly or statistically correlated with PCT vales showing different dynamic among the patients (Figure 1).

Discussion

In this study, patients operated for rectal cancer were followed and it was tried to verify the development of dehiscence of colorectal anastomosis at an early stage by measuring the value of PTC in order to conduct the best possible treatment. There are several parameters such as CRP, interleukin 6 and PCT that are considered important markers for early diagnosis of anastomotic leakage.[10] PCT is a protein that is significantly increased in generalised infections and sepsis when PCT is produced in large quantities by various types of body cells.[9, 11]

The increase in PCT concentrations reaches its maximum in 6-8 hours and in the case of sepsis values can reach up to 1000 ng/mL.[12] Dehiscence of the colorectal anastomosis is still one of the most difficult complications of colorectal surgery that occurs in a high percentage, which significantly increases the cost of treatment and can be fatal.[13, 14] Mortality in dehiscence of the colorectal anastomoses ranges up to 30 %.[15] Because of all this, early diagnosis of dehiscence of the anastomosis is very important to reduce treatment costs and mortality.[16] Some studies have shown that PCT is one of the best markers for distinguishing sepsis from non-infectious inflammatory reactions, even more so in intra-abdominal infections and especially anastomotic leakage, the increase in PCT is much higher than in extra abdominal infections.[17-19]

In their study, Meisner et al found that in the first days after colorectal surgery there was a physiological increase in PCT as a result of contamination when working with the intestines as well as due to the preparation with the intestines.[20, 21] Giaccaglia et al also confirmed in their experiences that measuring PCT values on the first postoperative day was not necessary, so they measured PCT values on the third and fifth POD in their study. PCT values were significantly higher on the third and fifth postoperative day in patients with anastomotic leakage than in patients without anastomotic leakage.[7]

In this study, PCT values were measured on the 2nd and 4th POD in all patients operated on for rectal cancer. PCT values on the 2nd day were significantly increased in all patients with anastomotic leakage. By nine patients with anastomotic dehiscence, PTC values were greater than 3 ng/mL, while in only one patient with anastomotic dehiscence, PTC values were less than 3 ng/mL. Fourth POD value of PTC by all ten patients with dehiscence anastomosis was higher than 7 ng/mL and in one patient the values reached 19 ng/mL.

In their study, Sarbinowski et al examined PCT, CRP, WBC and interleukin 6 in patients operated on for colorectal cancer. In the study, they determined the importance of these markers for the assessment of the occurrence of anastomotic leakage and for the development of systemic inflammatory syndrome. They found that only an increase in PCT concentration was statistically significant in patients with anastomotic leakage and systemic inflammatory syndrome.[22] In their study of 100 patients operated on for colorectal cancer on the first, second, third and fourth postoperative days, Lagoutte et al found that CRP was a more appropriate marker of anastomotic leakage than PCT, with a specificity of 87 % to 75 %.[23] Elevated PCT values were found to have much higher specificity than CRP values as a marker of anastomotic leakage. In their study of 154 patients, Tatsuoka et al found that PCT 4th POD has higher specificity and sensitivity than CRP values in patients with dehiscence of colorectal anastomosis.[24]

Besides, Takakura et al in their study confirmed PCT as an important prognostic marker of anastomotic leakage in patients operated on for colorectal cancer.[25] In this study, which included 118 patients operated on colorectal cancer, it was found that all patients with anastomotic leakage had a significant increase in PCT values on the second and fourth day compared to patients without anastomotic leakage. Biomarker for acute infection and inflammation such as WBC was insignificantly correlated with PCT level confirming their less sensitivity for dehiscence. Therefore, monitoring of WBC is not considered as a relevant biomarker for dehiscence although they are commonly measured in our centre. Also, a certain number of observed patients without anastomotic leakage showed an increase in PCT values, but these values were elevated in less than 30 % of patients, unlike patients with anastomotic leakage where PTC was elevated in 100 % of patients. Significantly, PCT 4th POD values in patients without anastomotic leakage were always significantly lower than in patients with anastomotic leakage. In a patient without anastomotic leakage, PCT was elevated in patients with pneumonia, urinary tract infection and local operative wound infection. In his study of 100 patients after colorectal surgery, Elyazed found that PCT was elevated in patients with pneumonia, which was a postoperative complication. PCT was elevated at 2nd, 3rd and 5th POD in nearly 84 % of patients with pneumonia.[26]

Conclusion

PCT value is an important predictor of anastomotic dehiscence and anastomotic leakage in colorectal surgery. An increase in PCT on the 2nd and especially on the 4th POD is one of the safest indicators of anastomotic leakage, which requires additional diagnosis and adequate therapy.

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Conflict of interest

None.

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CONTENT

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ORIGINAL ARTICLE

Therapeutic Effect of Two Fluoride Varnishes on Remineralisation of White Spot Lesions Evaluated by Laser Fluorescence

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Abstract

Background / Aim: The presence of white spot lesion (WSL) is considered the first stage of dental caries. The early detection and diagnosis of WSL is of crucial importance, since caries can be prevented at this stage, reversed and/or controlled by elimination of etiological factors and by use of fluorides. The aim of this study was to compare the efficacy between the two fluoride varnishes on WSL remineralisation evaluated by laser fluorescence.
Methods: A total of 30 children and 60 WSL cases (2 per each child) were included in this study. The selected WSL were randomly divided into two groups in each child: G1 applying Fluor Protector S®, Vivadent, Lichenstein (n = 30) and G2 applying MI varnish®, GC, Tokyo, Japan (n = 30). The fluoride varnishes were submitted to three applications: at baseline, four weeks and eight weeks following the baseline, according to the manufacturer's recommendations. Mineral density of the enamel was measured using laser fluorescence (DIAGNOdent® 2095, KaVo, Biberach, Germany) for each WSL. Laser fluorescence (LF) measurements were performed at baseline and at fourth, eighth and twelfth week after starting the treatment and LF scores were calculated.
Results: By comparing LF scores at each measurement after treatment initiation, it was found that the scores were significantly lower in all groups when compared to baseline.
Conclusion: The results of this study indicate that both fluoride varnishes used were capable of remineralising WSL as evaluated by LF measurements. No difference was noted in the remineralising efficacy of the varnishes despite their different compositions. The main limitations of this study are small sample size and short follow up period. Therefore, further studies with large sample size and a longer follow up are, however, necessary.

Key words: Remineralisation; Laser fluorescence; Enamel mineral density.

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Introduction

Dental caries is non-transmissible multifactorial disease, known to be related to the presence of the microorganisms from dental biofilm and to be modulated by diet.[1] It is one of the most widespread disease and significant health problem worldwide, also present in the Republic of Srpska, Bosnia and Herzegovina.[1] Obradović et al stated that 34 % of the children from municipality of Banja Luka at the age of two are having caries present.[2] Latest studies confirmed the presence of caries in 99 % of the thirteen years old children from municipality of Banja Luka, with mean number of 6 cavities per child.[3]

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CONTENT

Pathogenesis of tooth decay involves phases of demineralisation and remineralisation. The presence of white spot lesions (WSL) is considered the first stage of caries, that is characterised by demineralised enamel, with roughness and opacity.[4] The early detection and diagnosis of WSL is of crucial importance, since at this stage the caries can be prevented, reversed and/or controlled by elimination of an etiological factors such as diet and dental biofilm control, as well as by use of fluorides.[4, 5] The use of fluoride treatment is considered the golden standard for dental remineralisation.[6] Currently, there are several fluoride treatments available on the market, out of which 1.5 % ammonium fluoride (NH_4F) is considered one of the most commonly used (Fluor Protector S®, Ivoclar Vivadent, Lichenstein).[6] Some of the latest methods of promoting remineralisation of initial caries lesions involve the use of casein phosphopeptideamorphous calcium phosphate (CPPACP), unstabilised (ACP) and a bioactive glasscontaining calcium sodium phosphosilicate, etc.[7] The combination of 5 % sodium fluoride (NaF) and CPP-ACP (Recaldent®) is extensively studied and proven to be an effective way to remineralise teeth (MI varnish®, GC, Tokyo, Japan).[7]

In the last decade interest in detecting and monitoring WSL and subclinical precavitated lesions has increased. One of the available non-invasive methods to measure early enamel demineralisation is laser fluorescence (LF).[8]

Therefore, the aim of this study was to compare the efficacy of two topically applied fluoride varnish formulations on remineralisation potential of WSL using LF.

Methods

The study was approved by the Ethics Committee of Institute of Dentistry Banja Luka (registration number 01-343-3/17), and Ministry of Education and Culture of the Republic of Srpska, Bosnia and Herzegovina (registration number 07.041/052-7273/17).

Inclusion criteria

Fifty-six children (M: 36, F: 20) ranging from 12 to 13 years of age with regular hygiene habits (brushing teeth everyday) were screened for this study. The inclusion criteria were: children with two or more WSL on the buccal surface of permanent teeth, whose parents/guardians/legal representatives signed an informed consent form. Children with dental caries in the form of small cavities or restorations and/or developmental enamel alterations (hypoplasia, fluorosis) and/or periodontal disease in the selected teeth, presence of orthodontic devices, and children under medical treatment or taking any kind of medicine were excluded from this study. Thirty children (M: 12, F: 18) that met the inclusion criteria were recruited for this study.

Fluoride application

A total of 60 WSL (2 per each child) were present. The selected WSL were randomly divided into two groups according to the fluoride varnish used: G1 = Fluor Protector S®, Vivadent, Lichenstein (n = 30) and G2 = MI varnish®, GC, Tokyo, Japan (n = 30). Following the recruitment, baseline characteristics (plaque and gingival index) were assessed. In all sessions, professional brushing/dental prophylaxis was performed prior to each application of topical fluoride, with the aim of providing clean tooth surfaces for the application of fluoride varnishes and LF evaluation of the WSL. The fluoride varnishes were submitted to three applications: at baseline, four weeks and eight weeks following the baseline, according to the manufacturer's recommendations.

Laser fluorescence

For each WSL, the mineral density of the enamel was measured using LF (DIAGNOdent® 2095, KaVo, Biberach, Germany). DIAGNOdent® 2095 operates with a diode laser having a wavelength of 655 nm and 1 mW peak power. Sound enamel does not fluoresce at this wavelength, but caries and bacteria do.[8] Three measurements were taken and averaged to give the final test value. Measurements were performed at baseline and at fourth, eighth and twelfth week after starting the treatment and LF scores calculated. All measurements were conducted by one pedodontics (RK).

Statistical analysis

The SPSS (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Illinois, USA) Version 11.0 was used for the statistical calculations. The Wilcoxon Signed Ranks test was applied for comparison between LF scores and Mann-Whitney U test was applied for comparisons between varnish groups ($p < 0.05$).

Results

Application of the fluoride varnish from G1 (Fluor Protector S®, Vivadent, Lichenstein) resulted in statistically significant treatment outcomes when compared to baseline (Table 1 and 2).

Table 1: The results of application of the fluoride varnish G1 measured by laser fluorescence

DIAGNOdent	Baseline	4 weeks	8 weeks	12 weeks
Mean	17.41	15.91	14.44	12.94
Standard deviation/SD	3.83	4.30	3.38	3.74
Median	18.00	16.50	14.50	13.00

Table 2: The statistical analysis of the fluoride varnish G1

	Baseline	4 weeks	8 weeks	12 weeks
Baseline		Z = 4.242 p = 0.000	Z = 4.557 p = 0.000	Z = 4.963 p = 0.000
4 weeks			Z = 3.550 p = 0.000	Z = 4.562 p = 0.000
8 weeks				Z = 4.059 p = 0.000
12 weeks				

Table 3: The results of application of the fluoride varnish G2 measured by laser fluorescence

DIAGNOdent	Baseline	4 weeks	8 weeks	12 weeks
Mean	16.48	15.87	15.19	14.26
Standard deviation/SD	4.80	4.98	4.76	4.52
Median	17.00	16.00	15.00	14.00

Table 4: The statistical analysis of the fluoride varnish G2

	Baseline	4 weeks	8 weeks	12 weeks
Baseline		Z = 1.891 p = 0.059	Z = 2.439 p = 0.015	Z = 3.959 p = 0.000
4 weeks			Z =2.018 p = 0.044	Z = 3.638 p = 0.000
8 weeks				Z = 2.894 p = 0.004
12 weeks				

Table 5: The comparison between values of laser fluorescence from two fluoride varnish groups

		Baseline	4 weeks	8 weeks	12 weeks
Fluoride varnish G1	Mean	17.41	15.91	14.44	12.94
	Median	3.83	4.30	3.38	3.74
	Standard deviation/SD	18.00	16.50	14.50	13.00
Fluoride varnish G2	Mean	16.48	15.87	15.19	14.26
	Median	4.80	4.98	4.76	4.52
	Standard deviation/SD	17.00	16.00	15.00	14.00
	p	0.323*	0.939*	0.419*	0.275*

** - there was no statistically significant difference;*

Application of the fluoride varnish from G2 (MI varnish®, GC, Tokyo, Japan) resulted in statistically significant treatment outcomes when compared to baseline (Table 3 and 4). The values of LF between two flouride varnish groups showed no significant difference (Table 5).

Discussion

Tooth decay is affecting 60-90 % of the children. Its level vary between countries and it is strongly related to behavioural and socioeconomic factors (eg, income, education and employment). Consequently, the caries prevalence is very high in the Republic of Srpska, Bosnia and Herzegovina.

The presence of WSL is considered an initial stage of caries and it is really important to detect it in order to prevent further process of demineralisation that will further lead to cavitation. Treatment of WSL with fluoride is considered the widespread measure of primary prevention of caries.[10] The use of fluoride varnishes for caries prevention is supported and recommended by American Academy of Paediatric Dentistry and European Academy of Paediatric Dentistry.[11, 12] The American Dental Association recognises 5 % sodium fluoride (NaF) or 2.26 % fluoride content varnish treatment for the benefit of caries prevention when given at least twice per year to children up to age of 18.[11]

To date, radiographic image is considered the golden standard for the early caries detection.[13] However, non-invasive LF represents a promising method for monitoring enamel demineralisation and progression of caries lesion.[14, 15]

In this study we performed a 3-month comparative study of WSLs with two different fluoride varnishes. On assessing DIAGNOdent scores at baseline, 4, 8 and 12 weeks after treatment initiation, we found that the scores significantly decreased in all groups (Table 1 and 3). Previous studies demonstrated that fluorides remineralised WSL in 63.6 %.[16]

To date, majority of published studies demonstrated superiority of CPP-ACP fluoride varnishes when compared to conventional fluoride varnishes.[17-20] However, in our study the comparison of remineralisation efficacy between the G1 and CPP-ACP compounds fluoride varnish was not significant. it was concluded that both fluoride

treatments were effective in remineralising WSLs, despite their different compositions.

Also, in this study the detected remineralisation after 3 months of follow up failed to show the complete remineralisation evaluated by laser fluorescence. We suppose that major explanation for this drawback was short follow up period.

Conclusion

Within the limitations of this study it can be concluded that fluoride varnishes used in this study were capable of remineralising white spot lesions evaluated by laser fluorescence. No difference was noted in the remineralising efficacy of the varnishes despite their different compositions. The main limitations of this study are small sample size and short follow up period. Therefore, further studies with large sample size and a longer follow up are, however, necessary.

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None.

Conflict of interest

None.

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CONTENT

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REVIEW ARTICLE

Involvement of Phospholipase C in the Norepinephrine-Induced Hypertrophic Response in Cardiomyocytes

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Abstract

Norepinephrine (NE) is known to mediate cardiomyocyte hypertrophy through the G protein coupled α_1-adrenoceptor (α_1-AR) and the activation of the phosphoinositide-specific phospholipase C (PLC). Since the by-products of PLC activity are important downstream signal transducers for cardiac hypertrophy, the role of and the regulatory mechanisms involved in the activation of PLC isozymes in cardiac hypertrophy are highlighted in this review. The discussion is focused to underscore PLC in different experimental models of cardiac hypertrophy, as well as in isolated adult and neonatal cardiomyocytes treated with NE. Particular emphasis is laid concerning the α_1-AR-PLC-mediated hypertrophic signalling pathway. From the information provided, it is evident that the specific activation of PLC isozymes is a primary signalling event in the α_1-AR mediated response to NE as well as initiation and progression of cardiac hypertrophy. Furthermore, the possibility of PLC involvement in the perpetuation of cardiac hypertrophy is also described. It is suggested that specific PLC isozymes may serve as viable targets for the prevention of cardiac hypertrophy in patient population at-risk for the development of heart failure.

Key words: Phospholipase C isozymes; Norepinephrine; α_1-adrenoceptor; Cardiomyocytes; Experimental models of cardiac hypertrophy; Signal transduction.

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Introduction

Cardiovascular disease (CVD) remains the major cause of death worldwide and congestive heart failure (CHF) represents an enormous clinical, societal and economic burden.[1] In fact, according to the World Health Organization,[2] CVD is the leading cause of death globally, with an estimated 17.9 million deaths per year. Furthermore, > 75 % of CVD related deaths are due to heart attacks and strokes and about 30 % of these deaths occurring prematurely in people < 70 years of age. While it was estimated that there were over 37.7 million heart failure cases worldwide in 2016,[3] in 2020, the worldwide prevalence of heart failure was reported to be 64.34 million cases (8.52 per 1,000 inhabitants), accounting for 9.91 million years lost due to disability and 346.17 billion US $ expenditure.[4]

CHF is invariably associated with cardiac hypertrophy and changes in the shape and size of cardiomyocytes (cardiac remodelling) are considered to explain cardiac dysfunction in CHF. While the heart is known to adapt to increased work and haemodynamic load by increasing muscle mass as well as changing the size and shape of the heart, such a remodelling of the myocardium is compensatory at initial stages, but results in cardiac failure at late stages of the development.[4, 5] A moderate

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increase in the level of hypertrophic hormones including norepinephrine (NE) produces beneficial effects during early stages of cardiac hypertrophy, but prolonged exposure of the hearts to an excessive amount of NE produces deleterious actions at late stages of cardiac hypertrophy.[4, 5]

It is now well established that different subcellular organelles including the sarcolemma (SL) membrane, undergo varying degrees of changes in their biochemical composition and molecular structure during the development of cardiac hypertrophy as well as in the transition of cardiac hypertrophy to heart failure.[6-9] This SL remodelling occurs due to alterations in cardiac gene and protein expression as well as activation of different signalling proteins including phospholipases that are associated with the SL membrane.[10-12] The activation of phospholipase C (PLC) has a number of immediate consequences for signal transduction events in cardiomyocytes and thus has an integral role to play in SL and cardiac remodelling during the early stages of cardiac hypertrophy.[10-12] Although there are several hypertrophic agents that can activate PLC including angiotensin II, endothelin 1 and other growth factors, this brief review is intended to describe the involvement of specific PLC isozymes in the NE-induced hypertrophic response in cardiomyocytes and provide evidence that PLC isozymes may play an important role in the initiation of signal transduction processes involved in cardiac hypertrophy. In addition, evidence is provided to show that specific PLC isozymes may potentially be targeted for the prevention of cardiac hypertrophy and its ultimate transition to heart failure in patient population at-risk for the development of heart disease.

Phospholipase C isozymes and their regulation

The phosphoinositide-specific PLC enzyme is expressed in all mammalian cells and is critically involved in various signal transduction processes.[13, 14] Indeed, the activation of different PLC isozymes has been observed to be a key early event in the initiation of various cell functions.[13, 14] There are 13 families of PLC isozymes, which have been categorised into 6 classes. Earlier data on amino acid sequencing from cDNAs revealed the existence of PLC β, δ and γ isozymes,[15] but additional PLC isozymes, namely, ε, ζ, and η were discovered later.[16] Recently, this has expanded to 16 isozymes with the discovery of 3 atypical PLCs in the human genome.[14] It should be noted that there are four PLC β isozymes (β_1 to β_4), three PLC δ isozymes (δ_1, δ_3, δ_4), two PLC γ isozymes (γ_1, γ_2), one PLC ε isozyme, one PLC ζ, isozyme and two PLC η (η_1, η_2); these differ in their expression patterns in a variety of cells.[17] It is also pointed out that all PLC family members are a diverse group of isozymes that exhibit unique structures and cellular functions.[13] However, PLC is known to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce two second messenger molecules, namely inositiol-1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG).[18] IP_3 has been shown to trigger the release of Ca^{2+} from the intracellular stores, whereas DAG is known to activate protein kinase C (PKC) (Figure 1).

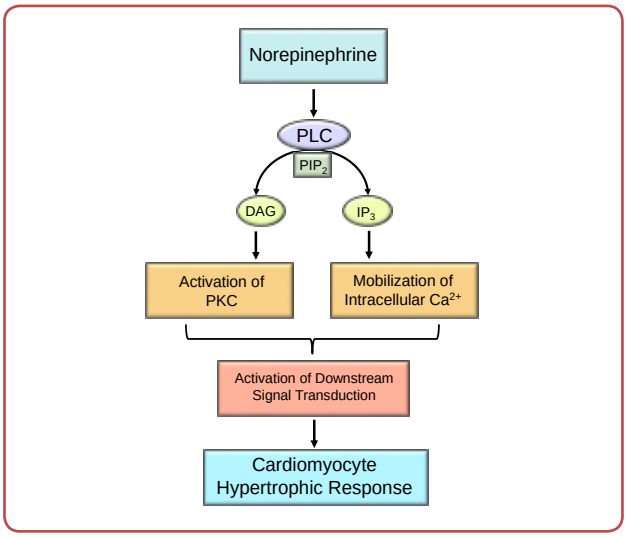

Figure 1: Involvement of phospholipase C signal transduction in the cardiomyocyte hypertrophic response to NE

PLC = phospholipase C; PIP_2 = phosphatidylinositiol-4,5-bisphosphate; DAG = sn-1,2-diacylglycerol; IP_3, inositiol-1,4,5-trisphosphate; PKC = protein kinase C

Indeed, the primary step of the signal transduction pathway for the activation of PKC involves the stimulation of PLC. It should be mentioned that specific PKC isozymes have also been implicated in the regulation of hypertrophic growth of cardiomyocytes.[19-22] Although the PLC family of isozymes signal through the same phospholipid hydrolytic products, each isozyme may contribute to distinct cellular functions.[13, 17] PLC isozymes are activated by a variety of factors including heterometric G proteins, small G proteins, receptor/non-receptor tyrosine kinases and calcium.[23] Among the PLC isozymes, PLC β and PLC γ are stimulated by receptor activation; PLC β by G-protein coupled receptors (GPCRs), including α_1-adrenoceptor (α_1-AR), whereas PLC γ by receptor tyrosine kinases.[24]

PLC β, δ, γ and ε are expressed in adult ventricular cardiomyocytes.[25-30] PLC β family has four types of isozymes (β_1, β_2, β_3 and β_4).[22] While PLC β_1 and PLC β_3 isozymes have been extensively characterised in cardiac tissue, higher PLC β_4 mRNA expression levels as compared to PLC β_1, β_2, and β_3 have been reported in human left ventricular tissue.[31] α_1-AR agonists, including NE are relevant stimulators of PLC β isozymes via the α subunits of the heterotrimeric Gq subfamily;[32] PLC β has been shown to be activated by Gβγ dimer.[33] Interestingly, it was further demonstrated that, similar to the other three PLC β isozymes, PLC β_4 was activated by the α subunit of Gq, but not by the transducin α subunit. However, unlike other PLC β isozymes, PLC β_4 was not responsive to activation by Gβγ subunits.[33] It has recently been reported that the direct activation of PLC β by Gαq and/or Gβγ subunits mediates the signalling by Gq and some Gi couples GPCRs respectively, suggesting that the disruption of autoinhibitory interactions leads to increased PLC β activity.[34] It may also be noted that Gβγ has also been shown to directly interact and activate PLC ε.[35] The most abundant PLC isoform found in the heart, PLC γ_1 is cytosolic and is activated by growth factor receptor tyrosine kinases.[36] A non-tyrosine kinase mediated activation as well as GPCRs via non-receptor tyrosine kinase activation of PLC γ isozymes has also been reported.[37] Although PLC γ is activated through receptor tyrosine kinase, it seems that a reciprocal cross-talk between tyrosine kinase and Gqα may exist in cardiomyocytes,[38] linking α_1-AR with tyrosine kinase associated receptors.

PLC δ_1 is considered the predominant PLC isozyme associated to the SL membrane, because the N-terminal part of the pleckstrin homology domain of PLC δ_1 possesses a critical region rich in basic amino acid residues, which bind with high affinity to the polar head of PIP_2.[39] This property confers on the δ_1 isoenzyme a unique capacity of association with the plasma membrane, which is lost with single basic amino acid replacement by a neutral or acidic amino acid.[40] The α_1-AR initiated events for the activation of PLC δ isozymes are considered to be mediated by the dimeric G_h protein.[41-43] It should be mentioned that the PLC β_1 splice variant PLC β_{1b} and not he PLC β_{1a} associates with a Shank3 complex at the SL membrane via its splice-variant specific C-terminal tail,[44] and it appears that SL membrane localisation is central to the activation of PLC and downstream signalling events in response to the activation of GPCRs.[44]

PLC isozymes and the cardiomyocyte hypertrophic response

The role of PLC in the development of different types of cardiac hypertrophy has been documented; for example, the development of cardiac hypertrophy in stroke-prone spontaneously hypertensive rats has been suggested to involve an increase in the PLC signalling pathway.[45, 46] In addition, studies in neonatal rat cardiomyocytes stimulated with different hypertrophic stimuli, including NE, have shown an increased mRNA expression of PLC β isozymes.[47, 48] Stimulation of signalling pathways via Gqα provokes cardiac hypertrophy in cultured cardiomyocytes and transgenic mouse models overexpressing Gqα,[49-52] that may be linked to the activation of PLC. On the other hand, no correlation of hypertrophy to PLC activation in two other transgenic mouse lines expressing activated Gqα has been demonstrated.[53, 54] Recently, the activation of PLC β_3 mediated signal transduction has been reported in a rat model of cardiac hypertrophy induced by aortic constriction.[55] While the activation of PLC isozymes as an important signalling event in hypertrophy of the adult heart, a loss of PLC ε signalling in PLC ε knock out mice has been reported to sensitise the heart to development of hypertrophy in response to chronic isoproterenol treatment.[56] On the other hand, PLC ε depletion, using siRNA, reduces the hypertrophic response to NE as well as other hypertrophic stimuli in neonatal rat cardiomyocytes.[56] These authors also observed that PLC ε activity was required for hypertrophic development, yet PLC ε depletion did not reduce inositol phosphate production suggesting a requirement for localised PLC activity. It has also been suggested that a PLC ε - dependent component of β-adrenoceptor signalling in cardiomyocytes is responsible for the maintenance of contractile reserve and that loss of PLC ε signalling in PLC ε (-/-) mice sensitises the heart for the development of hypertrophy in response to cardiac stress.[57]

We have previously reported an increase in PLC isozyme gene and protein expression as well as activities in the hypertrophied rat heart subsequent to volume overload induced by arteriovenous shunt.[58, 59] It was demonstrated that specific increases in PLC β_1 and PLC γ_1 were associated with the hypertrophic stage in this model.[59] In contrast, PLC β_1 and Gαq protein levels have been reported to be unchanged during hypertrophy due to pressure overload induced by ligation of the de-

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scending thoracic aorta in the guinea pig.[60] However, translocation of PKC isozymes from cytosol to membranous fractions was elevated. These investigators suggested that PKC translocation occurred without changes in Gαq and PLC-β protein abundance and that it might be due to increases in Gαq and PLC β_1 activity rather than upregulation of expression,[57] but PLC β_1 activity was not determined in this study. It is pointed out that mechanical stress induced by cell stretching in neonatal cardiomyocytes has also been reported to increase PLC activity.[61] However, in this study no attempt was made to identify the PLC isozymes responsible for such responses. Since mechanical stretch is an initial factor for cardiac hypertrophy in response to haemodynamic overload (high blood pressure) and that increases in Gqα and PLC β_1 activities[61] as well as enhanced NE release from sympathetic nerves[62] are involved in pressure-overload hypertrophy, it is likely that α_1-AR activates PLC β isozymes under conditions of mechanical stress. It should also be noted that the involvement of α_1-AR-Gαq-protein-PLC-IP_3 signal transduction pathway in the development of NE-induced cardiac hypertrophy may be complimentary to other well-established mechanisms, namely β-AR-Gs protein-adenylyl cyclase-cyclic AMP for the induction of cardiac hypertrophy by catecholamines.[5, 9, 62]

It is interesting to note that the caveolae have a key role in signal transduction processes including an important role in the development of cardiac hypertrophy.[63, 64] In this regard, the α1-adrenoceptor, Gq, PLC β_1 and PLC β_3 have been found to be located exclusively to the same caveolin microdomain in the caveolar fraction isolated from rat heart.[65] It is pointed out that the NE-induced IP_3 generation in neonatal rat cardiomyocytes has been reported to be primarily due to α_1-AR-mediated activation of PLC β_1.[48] PLC β_1 exists as two splice variants, PLC β_{1a} and PLC β_{1b}, which differ only in their C-terminal sequences of 64 and 31 amino acids, respectively. While PLC β_{1a} is localised in the cytoplasm, PLC β_{1b} targets to the SL, which is enriched in caveolae, where α_1-AR signalling is also localised.[66] Furthermore, in cardiomyocytes, responses initiated by α_1-AR activation involve only PLC β_{1b}, thus the selective targeting of this splice variant to the SL membrane provides a potential target to reduce hypertrophy.[66] In this regard, the overexpression of PLC β_{1b} in neonatal cardiomyocytes was also shown to result in increases cell size and protein/DNA ratio as well as elevated atrial natriuretic factor (ANF) levels, indicating that the hypertrophic response due to the activation of the α_1-AR is mediated by PLC β_{1b} and thus may serve as a viable target for the limitation of cardiac hypertrophy.[67] Additionally, PLC β_4 gene expression levels have been reported to be increased in response to hypertrophic stimuli in mouse HL-1 cardiomyocytes thus indicating that PLC β_4 may also have a role to play in hypertrophic response in cardiomyocytes.[31]

It was reported earlier that NE increases in ANF gene expression and protein synthesis in adult rat cardiomyocytes, which are attenuated by a PLC inhibitor, U73122.[68] It was also observed that the NE-induced increase in ANF gene expression and protein synthesis were inhibited by prazosin, an α_1-AR blocker.[68] Furthermore, both prazosin and U73122 depressed the NE-induced increase in DAG production in cardiomyocytes. Taken together, it was determined that the α_1-AR mediated activation of PLC is involved in the hypertrophic response in cardiomyocytes. An extension to these observations[69] demonstrated that specific PLC isozymes may be involved in the cardiomyocyte hypertrophic response to NE. In this regard, while NE increased the activities as well as the mRNA levels of the predominant forms of PLC expressed in ventricular cardiomyocytes, β_1, β_3, δ_1 and γ_1, pre-treatment of adult rat cardiomyocytes with prazosin resulted in an attenuation of the NE-induced increases in PLC isozyme activities and gene expression (Figure 2).[69]

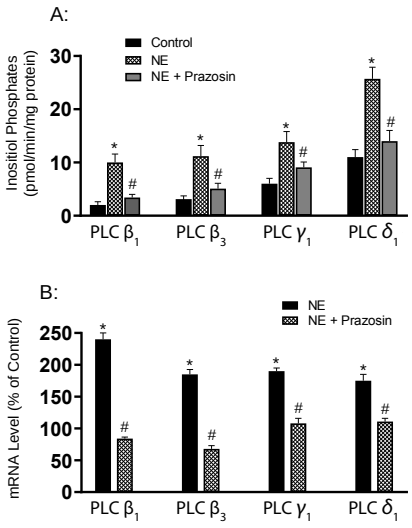

Figure 2: Phospholipase C (PLC) β_1, β_3, γ_1 and δ_1 activities and mRNA levels in cardiomyocytes treated with NE in the presence and absence prazosin

*Adult rat cardiomyocytes were treated with 5 μM NE for 2 h in the absence and presence of prazosin (2 μM). Values are mean ± SE of five experiments performed with five different cardiomyocyte preparations and expressed relative to GAPDH mRNA level. *Significantly different ($P < 0.05$) versus control; #significantly different ($P < 0.05$) versus NE. NE = norepinephrine; Data are based on the analysis of information in our paper[69]*

The involvement of these specific PLC isozymes in the cardiomyocyte hypertrophic response to NE was further substantiated by PLC gene silencing techniques using siRNA. Silencing of PLC β_1, β_3, δ_1 and γ_1, with siRNA resulted in the prevention of the NE-induced increase in ANF expression (Figure 3).[70]

In addition, cardiomyocyte protein synthesis, as evidenced by the incorporation of [^{3}H] phenylalanine, was markedly reduced in cardiomyocytes transfected with PLC isozyme siRNA[70] that was linked to a depression in the NE-induced increases in PLC isozyme activities (Table 1).[70]

The study of PLC and its involvement in cardiac hypertrophy under different pathophysiological conditions is both exciting and intriguing, but complex. It has for a long time been considered that the role of the α_1-AR in cardiac hypertrophy is a contributory factor, however, based on our studies as well as that of others, it seems that the activation of the α_1-AR-PLC signal transduction pathway may be a primary event in the initiation and pathogenesis of cardiac hypertrophy. It should be mentioned that the mechanisms of the regulation of PLC isozymes has also been examined. In this regard, it has been observed that the NE-induced increases in PLC isozyme gene expression occurs via a PKC- and ERK1/2- dependent signalling

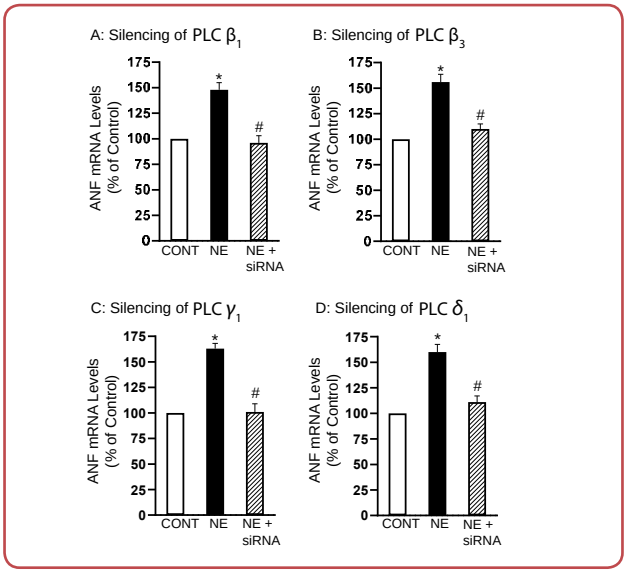

Figure 3: Inhibition of NE - induced increases in ANF mRNA levels in cardiomyocytes transfected with phospholipase C (PLC) isozyme siRNA

*Quantified data showing ANF mRNA levels relative to GAPDH mRNA level in cardiomyocytes transfected with 5 nM PLC isozyme (A) β_1, (B) β_3, (C) γ1 and (D) δ_1 siRNA and treated with NE (5 μM) for 2 hrs. Values are mean ± S.E. of five experiments performed with five different cardiomyocyte preparations. *Significantly different (P < 0.05) versus control; #significantly different (P < 0.05) versus NE. CONT = control; NE = norepinephrine; siRNA = small interfering RNA. Data are based on the analysis of information in our paper[70]*

Table 1: Inhibition of NE - induced increases in protein synthesis and phospholipase C (PLC) isozyme activities in cardiomyocytes transfected with PLC isozyme siRNA

A:	**Protein Synthesis ([^{3}H] phenylalanine incorporation, DPM)**			
Control	9185 ± 820			
NE	21046 ± 1132*			
PLC β_1 siRNA	10171 ± 860#			
PLC β_1 siRNA	11499 ± 778#			
PLC γ_1 siRNA	10049 ± 752#			
PLC δ_1 siRNA	9411 ± 764#			
B:	**Inositol Phosphates (pmol/min/mg protein)**			
	PLC β_1	PLC β_3	PLC γ_1	PLC δ_1
Control	2.3 ± 0.5	2.5 ± 0.7	4.8 ± 0.7	8.1 ± 2.5
NE	10.7 ± 2.1*	13.3 ± 1.5*	15.1 ± 1.5*	22.0 ± 3.1*
siRNA	4.9 ± 1.7#	6.7 ± 2.0#	7.0 ± 2.3#	15.1 ± 2.0#

*Values are mean ± S.E. of five experiments performed with five different cardiomyocyte preparations. *Significantly different (P < 0.05) versus control; #significantly different (P < 0.05) versus NE. CONT = control; NE = norepinephrine; siRNA = small interfering RNA.*

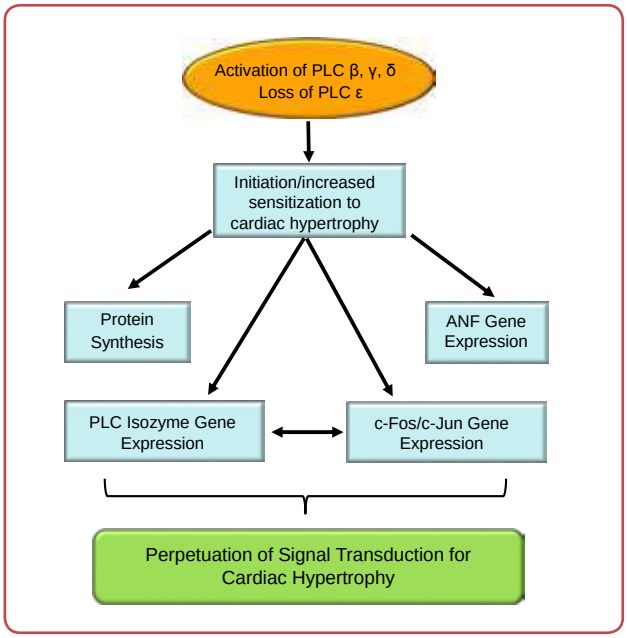

Figure 4: Sequence of events for the perpetuation of the hypertrophic response involving phospholipase C (PLC) isozymes

ANF = atrial natriuretic factor

pathway and involves both *c-fos* and *c-jun* transcription factors.[69] Furthermore, since the PLC activity inhibitor, U73122, attenuated PLC gene expression, it was suggested that PLC isozyme activities may regulate their own gene expression. In addition, a reciprocal regulation of *c-fos* and *c-jun* and PLC isozyme gene expression may exist in cardiomyocytes.[70] Taken together, these observations suggest that PLC may be involved in the perpetuation of the hypertrophic response to NE. Indeed, the specific activation of PLC β, γ and δ isozymes, but loss of PLC ε, may be important elements in the initiation/increased sensitisation for cardiac hypertrophy as depicted in Figure 4.

CONTENT

Conclusion

This review has provided some evidence for the possible involvement of PLC in cardiac hypertrophy as well as identified some of the signal transduction mechanisms involved in the regulation of PLC isozyme gene expression and protein levels in the heart. Most of the available literature has predominantly described the role of PLC β isozymes in cardiac hypertrophy; however, as discussed, there are other PLC isozymes that are expressed in the heart, which may also have a distinct role to play in the cardiomyocyte hypertrophic response. Furthermore, the extent of any overlapping functionality of PLC isozymes, including the presence of PLC splice variants in cardiomyocytes needs to be explored. The role of PLC δ_1 and PLC γ_1 as well as their activation in cardiac hypertrophy also require further investigation, particularly since the cardiac specific overexpression of G_h results in a unique hypertrophy phenotype that is independent of GPCR- induced activation of PLC.[71] While some studies have shown prazosin in mitigating the progression of cardiac hypertrophy to heart failure[72-77] a selective modulation of PLC (isozyme gene expression, protein contents and activities) and regression of cardiac hypertrophy remains to be established in different types of animal models of cardiac hypertrophy. It is pointed out that losartan, an angiotensin II type 1 receptor blocker, can selectively attenuate the increase in PLC isozyme gene expression (PLC β_1, β_3 and δ_1) during the development of cardiac hypertrophy subsequent to arteriovenous shunt.[59] These changes were associated with a regression of cardiac hypertrophy as evidenced by a reduction in the left ventricle/body weight ratio.[59] It should be mentioned that PLC isozyme activities in this study was not determined, which are the key element of PLC signalling function and thus some caution should be exercised in the interpretation of these findings. However, the regression of cardiac hypertrophy by pharmacological agents can be seen to be associated with the selective inhibition of some PLC isozymes.

While the aforementioned discussion has pertained to the role of PKC as a downstream effector of the PLC-derived DAG in the cardiomyocyte hypertrophic response to NE, it should be mentioned that the other by-product of PLC hydrolytic activity, IP_3, has also been implicated as a key component of the cell signal in cardiac hypertrophy.[78, 79] An interesting role of the Golgi in cardiac hypertrophy has recently emerged[80, 81] suggesting that the PLC-derived phosphoinositide and DAG production is required for the activation of protein kinase D during cardiac hypertrophy and that PIP_2 is not the preferred substrate, unlike the plasma membrane phosphatidylinositol 4-phosphate. Overall, it can be suggested that specific PLC isozymes may be involved in the initiation of signal transduction processes for the development of cardiac hypertrophy and thus might constitute additional therapeutic targets for drug discovery for the treatment of cardiac hypertrophy and its progression to heart failure in at-risk patients.

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Conflict of interest

None.

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CURRENT TOPIC

The Use of Adipose-Derived Stem Cells in Cell Assisted Lipotransfer as Potential Regenerative Therapy in Breast Reconstruction

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Abstract

Breast reconstruction for breast cancer patients is performed as a standard of care to improve patients' quality of life, physical and psychosocial well-being. Stem cell therapy holds a promise in regenerative medicine, including in breast reconstruction. This review explores the potential use of adipose-derived stem cells (ADSCs) in cell assisted lipotransfer (CAL) for reconstruction of the breast. The review of literature was done using electronic databases using appropriate keywords, including "adipose-derived stem cell", "stem cell therapy", "adipose-derived stem cell", "cell-assisted lipotransfer", "regenerative therapy", "breast cancer" and "breast reconstruction", with literatures limited to ten years post publication. Adipose-derived stem cells are multipotent cells with angiogenic and immunomodulatory potential. Several studies reveal ADSCs use in CAL results in long-term breast volume retention suggesting improved fat graft survival. Some conflicting outcomes are also discussed, potentially related to numbers of cells enriched and factors affecting the cells' microenvironment. The use of ADSCs in CAL may be beneficial for therapy of breast reconstruction in breast cancer patients after surgical management. Further investigation would be needed to improve the confidence of its clinical use.

Key words: Adipose-derived stem cell; Breast reconstruction; Breast surgery; Regenerative therapy; Cell assisted lipotransfer; Fat graft; Stem cell therapy.

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Introduction

At present, breast cancer still accounts as the most common cancer cases in the female population. According to the World Health Organization, incidence of breast cancer vary worldwide, in Eastern Africa the incidence is 19.3 per 100,000 women, whilst in Western Europe incidence reaches 89.7 per 100,000 women.[1] In Indonesia, breast cancer accounts for approximately 30.5 % of all cancers diagnosed.[2] Patients with breast cancer are undoubtedly faced with high rate of morbidity and mortality, contributing to a great burden. Patients who died of breast cancer in the year 2018 reached up to 627,000 patients worldwide,[3] whilst in Indonesia it accounted for 21.5 % of deaths in females diagnosed with cancer.[2]

For a majority of patients, surgical management has been widely conducted as a definitive treatment and for some in conjunction with radiation therapy and chemotherapy. Surgeons have used variations of surgical procedures to excise lesion according to different staging of the malignancy, namely breast-conserving surgery such as lumpectomy, partial and segmental mastectomy;

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simple mastectomy, skin-sparing mastectomy, radical/total mastectomy and modified radical mastectomy.

Following surgical excision of lesion, breast reconstruction is commonly opted, performed either immediately after mastectomy or delayed. Reconstruction is desired with the aim to restore cosmesis and structure as much as possible. Considering the morbidity and disfigurement following surgical management, the options of breast reconstruction has been available to all patients as a standard of care. Studies have shown how reconstruction surgery greatly affects the patient's quality of life, physical and psychosocial well-being, amongst others.[4-6]

Stem cell therapy has since dominated the promises in relation to regenerative medicine, including in efforts of reconstructing the breast following breast cancer. Particularly, the potential use of adipose-derived stem cells for this purpose is an exciting focus. Adipose-derived stem cells (ASCs) were first isolated by plastic surgeons, derived from processed lipoaspirate tissue.[7] These cells are identified as multipotent stem cells with the natural capability to differentiate into endodermal, mesodermal and ectodermal cells. To name a few are adipocyte, endothelium, chondrocyte, osteocyte, keratinocyte, hepatocyte, beta islet cell and even neuronal and glial cells.[8-12] Adipocyte derived stem cells are readily available and therefore have been explored in many aspects in regenerative medicine, including in management of wound ischaemia in diabetic patients, bone regeneration, promoting neurogenesis, cardiomyocyte proliferation in heart diseases, among others.[13-16] Understandably, its regenerative properties have made ASCs as a potential novel treatment for application in the complexity of breast reconstruction. This review aims to explore the characteristics of ASCs and its potential in improving reconstruction of the breast following surgical management of breast cancer.

This review of literature was conducted by using electronic databases, namely Pubmed and Ovid. Search terms used were: "stem cell therapy", "adipose-derived stem cell", "adipose-derived stem cell", "cell-assisted lipotransfer", "regenerative therapy", "breast cancer" and "breast reconstruction". In order to emphasise on findings from current research and practices, search results were limited to literatures published after the year 2010.

Results

Stem Cells

Stem cells are cells that have the capability of renewing themselves as well as differentiating into other cell lineages in the body.[8] For this reason, stem cells have been an ever-growing interest in its role in tissue engineering for regenerative therapy purposes. Generally, there are two types of stem cells, embryonic stem cells and adult stem cells. Embryonic stem cells come from embryonic tissue, particularly from the inner cell mass of a blastocyst and have pluripotent characteristic.[17] It means that they are able to form cells or tissues derived from all three germ layers. Whilst adult stem cells have multipotent or unipotent characteristic, which have more limitation in its differentiation, commonly in one germ layer only. Adult stem cells are located in many parts of the body, examples of them are mesenchymal stem cells, haematopoietic stem cells, epidermal stem cells, cardiac stem cells, neural stem cells and many others, which will rise to adult somatic cells.[18] Above all, there are also totipotent stem cells, which can differentiate into cells from all three germ layers as well as extra-embryonic tissues such as the placenta, hence it has the highest potential for differentiation.[17] A zygote is considered as a totipotent cell.

It is possible to create pluripotent cells from adult stem cells or even adult somatic cells to create induced pluripotent stem cells (iPSCs), by way of nuclear transfer or reprogramming.[19] Unfortunately, the therapeutic application of iPSCs have been limited due to ethical issues. Furthermore, conversion of differentiated somatic cells into other types of differentiated cells have been proven to be possible using complex molecular mechanisms involving various transcription factors, a process termed trans differentiation. Such techniques of induced pluripotency or trans differentiation to achieve desired cells have been studied in an array of disorders, such as in neurodegenerative diseases (Parkinson's disease), ischaemic heart disease and vascular diseases, in the hope to create personalised cell therapy for patients.[20-22]

Stem cells have certain specific characteristics according to its type and its lineage. Biomarkers help in identifying different types of stem cells. Markers of pluripotency in human embryonic stem cells namely are Nanog, Sox2 and Oct-4.[23]

These are transcription factors that govern the functions of such cells in preserving their quiescence. Additionally, cell surface markers have also been used to identify embryonic stem cells, such as SSEA-1, SSEA-4, TRA-1-81 and TRA-1-60.[24] Markers for adult stem cells for example are as follows, haematopoietic stem cells: CD34, CD48, CD150, Sca-1; keratinocyte stem cells: K15, Sox9, CD34; neural stem cells: LeX, CD133, Nestin, EGFR, Sox2, Musashi; intestinal stem cells: Lgr5, Bmi1, muscle stem cells: Pax7, CD34, emerin (EMD), LMNA, VCAM1; adipocyte stem cells: CD90, CD13, CD29, CD44, CD105, CD34, CD73, CD10, CD166, CD59, CD49e, HLA-ABC and STRO-1.[25-29]

Functions of stem cells are greatly affected by the niche in which they reside. Stem cell niche are specialised microenvironment that controls stem cell regulation through cell signalling by way of autocrine, paracrine and systemic pathways, as well as through interaction with extracellular matrix components and other signalling.[30, 31] It may represent substantial starting point in the therapeutic modulation of stem cell activity. Thus, it is important to understand how different niches would control the behaviour of stem cells used in therapeutic purposes.

Adipose-derived stem cells

Adipose-derived stem cells (ADSCs) are essentially mesenchymal stem cells. Mesenchymal stem cells are adult stem cells that were first found in the bone marrow, however have been found in other tissues in the body including in adipose tissue, as well. Other sources of mesenchymal stem cells are in the skin, peripheral blood, skeletal muscle, cartilage, pancreas, heart, lung, dental pulp, cord blood, trabecular bone and periosteum.[32, 33] Adipose tissue is a great source of these cells for therapeutic intentions due to its ease in harvest, in larger quantities, whilst leaving less morbidity in the donor site.

The adipose tissue consists of mature adipocytes that form lobes and stromal vascular fraction (SVF). The exact location of ADSCs in adipose tissue has been unclear, however some sources have speculated it to be concentrated in the vasculature or perivascular area.[34] Adipose-derived stem cells can be retrieved, in the SVF portion of adipose tissue using cellular isolation techniques, alongside other cellular components such as endothelial progenitor cells, keratinocytes, macrophages, lymphocyte and smooth muscle cells.[35]

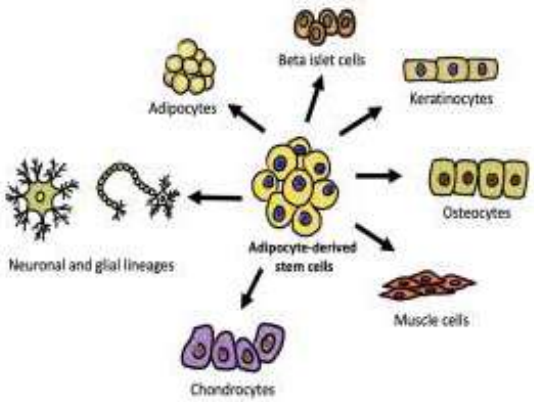

Figure 1: Adipocyte derived stem cell - mesenchymal stem cell with the ability to differentiate into several other cells from all three germ layers

Biomarkers for ADSCs namely are CD90, CD13, CD29, CD44, CD105, CD34, CD73, CD10, CD166, CD59, CD49e, HLA-ABC and STRO-1.[25] They should also be absent of markers such as CD34, CD14, CD11b, CD79a, CD19, CD45, as well as HLA-DR.[8]

Both *in vivo* and *in-vitro,* ADSCs have been proven to be able to differentiate into cells originating from all three germ layers, such as adipocyte, endothelium, osteocyte, chondrocyte, keratinocyte, hepatocyte, beta islet cell, neuronal and glial cells. In itself, ADSCs are essential mesodermal in origin.[8-12] *In vitro* procedure guidelines to induce differentiation of ADSCs into various cell lineages have been studied extensively, using specific induction factors and culture conditions.[10, 36, 37] To illustrate, induction of adipogenic differentiation from ADSCs has been explained by Naderi et al,[38] essentially by cultivating the cells in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % foetal bovine serum (FBS) solution, dexamethasone, insulin, hydrocortisone, indomethacin and 3-isobtyl-1-methylxanthine. Followed by formation of microtissue in hanging drops, its differentiation process assessed using light microscope and lipid staining using Oil Red O, which should show development of lipid droplets in the mature adipocyte. As previously mentioned, stem cells reside in specific niches that regulates the activity and behaviour of these cells. Niche of ADSC is said to be in the perivascular region of adipose tissue, as well as on adventitial vasculogenic zone in blood vessel wall.[39] The niche of ADSCs work similarly to preserve its stemness and clonogenicity, regulating proliferation and differentiation as needed by the tissue by way of interaction

between cells, ECM, growth factors, transcription factors, cytokines and other cell signalling pathways. Jiang et al explained the role of peroxisome proliferator-activated receptor gamma (PPARg) in ADSCs, it leads to activation of platelet-derived growth factor receptor beta (PDGFRß) and vascular endothelial growth factor (VEGFR), which subsequently results in vascular development and stem cell affinity towards the vessel niche.[40] Furthermore, it has been suggested that adenosine receptors has a role in regulating cellular differentiation towards adipogenesis.[41] Changes in glycosaminoglycans expression such as heparan sulphates in ECM and cell surfaces have also shown to affect stem cells' fate from self-renewal to differentiation, through processes that involve protein ligands interactions.[42, 43]

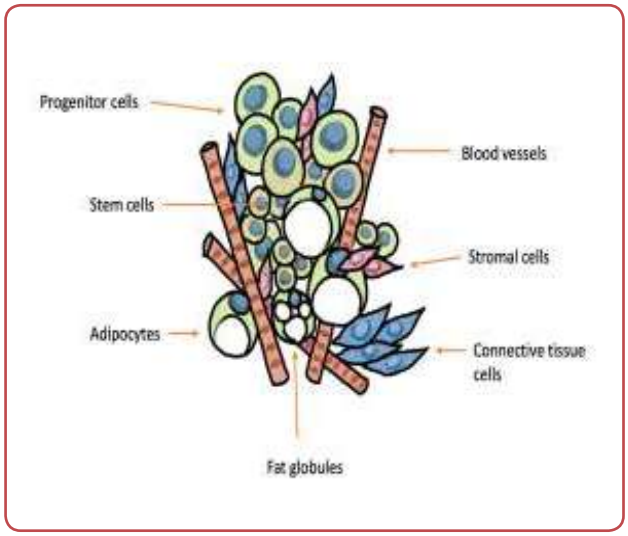

Figure 2: Adipose-derived stem cell niche

The role of ADSCs in relation to wound healing has been studied exponentially. This is generally due to its ability to differentiate into keratinocyte, endothelial cells and fibroblasts, as well as producing cytokines which aids in wound healing.[32] Differentiation into keratinocyte has been observed *in vivo* and *in-vitro*. Culture of ADSCs in collagen matrix along with keratinocyte conditioned media and co-cultured with primary keratinocyte shows differentiation of ADSCs into cobblestone-like structure, suggesting keratinocyte-like cell differentiation.[44] Ebrarhimian et al[45] demonstrated how GFP-positive ADSCs injected in the wound tissue of mice eventually show expression of keratinocyte markers, namely K5 and K14. Fibroblast, a vital component of tissue remodelling in wound healing, was generated from human ADSCs and have shown to produce robust ECM containing collagen 1, fibronectin and elastin *in vitro*.[46] The use of such fibroblast differentiation was used in canine vocal fold injury demonstrating secretion of elastin, hyaluronic acid, decorin, fibronectin and collagen as ECM properties, which is beneficial in wound healing.[47] Finally, vascularisation is important in tissue regeneration and wound healing. Endothelial cell differentiations from ADSCs increase neovascularisation by way of angiogenesis in ischaemic tissue. Some angiogenic factors secreted by endothelial cells from ADSCs include VEGF and hepatocyte growth factor (HGF) which are essential in vasculogenesis.[48]

The ability of ADSCs to secret an array of cytokines, chemokines and growth factors play an important role in tissue healing and regeneration. These substances essentially help in many stages of wound healing, from induction of cell proliferation and migration, promoting angiogenesis and generation of epithelial cells, as well as remodelling. Besides VEGF and HGF, ADSCs are able to secrete angiogenic cytokines such as PDGF, GM-CSF, bFGF, SDF-1, TGF-ß, IL-8, IL-6, FGF2 and MMP.[48, 49] Additionally, ADSCs also produce cytokine that regulates proliferation and migration of fibroblasts, such as VEGF, bFGF, EGF and PDGF-AA.[50]

Cell Assisted Lipotransfer in Breast Reconstruction

Isolation of ADSCs is done by initially performing liposuction or direct excision of fat tissue in the trunk area or extremities such as the thigh and buttocks (Coleman technique), followed by isolation of cells from the stromal vascular fraction using enzymatic processes and cellular centrifugation.[51] Lipotransfer using autologous fat graft is then conducted using fat tissue that has been enriched with the isolated ADSCs or SVF. The high regenerative and proliferative potential of these cells are expected to support the graft survival against fat absorption and encourage wound healing.[52] Several types of cell-assisted lipotransfer (CAL) techniques are commercially available to be used by plastic surgeons.

Mesenchymal stem cell from adipose tissue has shown yield of number of stem cells that is higher than in the bone marrow per gram of tissue.[53] The clonogenic ability of ADSCs, as well as potential to differentiate into adipocytes, endothelial and epithelial cells warrants its therapeutic use in regenerating the breast structure. The angiogenic properties is expected to enhance regeneration of

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blood vessel in the fat tissue, potentially improve graft survival and reduce postoperative absorption.[54]

Domenis et al demonstrated *in vitro* differentiation of isolated ADSCs into endothelial cells, adipocytes, even smooth muscle cells and skeletal muscle cells using several types of CAL. Afterwards, patients treated with CAL for breast reconstruction post breast cancer showed improvement in subcutaneous tissue thickness and 1 year follow up showed significantly reduced thickness loss in medial breast compared to lipoaspirate without ADSCs enrichment.[52] A case report brought by Tsekouras et al[55] revealed improvement in contour of the post mastectomy breast, up to 22 months follow up using ADSCs in CAL. Furthermore, the amount of cells used in CAL may affect breast volume retention, in which higher number of cells used in enrichment displayed higher volume retention of fat graft compared to using lower number of cells or lower dose of SVF. Subsequently improved long-term volume retention is maintained.[56] The first clinical trial for ADSCs use in breast reconstruction was done and results showed majority of patients feeling satisfied with the result after 1 year. Perez-Cano et al[57] demonstrated the improvement in breast contour deformity with minimal complication the form of cyst due to injection and no cancer recurrence of the breast was reported. Successful restoration of the breast contour has also been reported by Gentile et al,[58] in which CAL proved superior compared to traditional lipotransfer, with 63 % and 39 % volume retention after 1 year, respectively. Interestingly in some cases that has been reported, on top of improvement in reducing volume loss, the area of skin overlying it also showed noticeable rejuvenation.[59]

Implementation of CAL has been attempted at not just post-mastectomy reconstruction, but also breast augmentations. Jung et al revealed that breast augmentation in healthy patients using CAL and SVF had shown a decrease in breast volume by 47 % one-year post procedure. Similarly, Wang et al demonstrated 51 % fat resorption 6 months post CAL breast augmentation of healthy patients.[60] On the contrary, Kamakura et al showed improved breast measurement post CAL breast augmentation that is stable after 9 months follow up, indicating graft viability, whilst with minimal complication in the form of benign cyst.[61] This may demonstrate the complexity in clinical use of SVF and may be due to inadequate numbers of ADSCs in the SVF and skin tension affecting fat absorption.[62]

The ADSCs population with potential of facilitating wound healing and remodelling through formation of fibroblast and generating vascular supply could be a key component in the fat graft long term volume retention. Moreover, ADSCs ability in self renewal, differentiation and in secreting angiogenic factors such as VEGF and HGF as well as modulating local inflammatory response is known to be more robust in hypoxic condition. Immunomodulatory properties of ADSCs were found to be similar with mesenchymal stem cells derived from the bone marrow; they are able to reduce proliferation of mononuclear cells and differentiation of immature dendritic cells.[63] Immunomodulatory cytokine release by ADSCs was explained previously in this review. Recalling the essential role of stem cell niche and the various cell signalling that governs ECSs regulation, certain microenvironment conditions largely affects their survival and function.[64]

Conclusion

The review of literature suggests that cell assisted lipotransfer using adipose-derived stem cells may be a beneficial therapeutic management for breast reconstruction post breast cancer surgical management. The enrichment of autologous ADSCs into grafted fat tissue may result in improved long-term graft retention that supports its clinical advantage. These are due to their inherent properties of multipotent characteristic, angiogenic and immunomodulatory potential. Although a number of studies support this conclusion, some studies also show conflicting result, possibly due to differences in cell isolation methods or number of cells enriched in the graft. Additional studies using a control technique and longer follow up time is encouraged to further investigate the advantages of CAL in maintaining graft survival in breast reconstruction. This is particularly interesting considering the area is prone to microvascular damage and compromised wound healing following radiation therapy.

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Conflict of interest

None.

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CASE REPORT

A Case Report of Malignant Peripheral Nerve Sheath Tumour of the Left Thigh and Popliteal Fossa With Lungs, Spleen, and Brain Dissemination Related to Neurofibromatosis Type 1

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Abstract

A malignant peripheral nerve sheath tumour (MPNST) is a highly aggressive sarcoma. This disease develops in a number of people with neurofibromatosis type 1 (NF1), which is a common genetic disease. The paper presents a patient with typical manifestations of a malignant tumour of the peripheral nerve sheath, in the form of a large tumour of primary localisation in the distal part of the left thigh and left popliteal fossa and with significant dissemination into the lung parenchyma, which was accompanied by respiratory risk. The first operation of the tumour was done four years earlier, after which the patient did not come for regular check-ups. Nine cycles of chemotherapy were performed by Doxorubicin / Ifosfamide / Mesna protocol with clinical improvement and stabilisation, but without a significant impact on the dynamics of the disease and the overall survival was 14 months. It is of utmost importance to early recognise clinical presentation of the malignant form of this tumour and active supervision of a patient with a benign form by experts. In this way, it is possible to apply the optimal treatment modality in a timely manner.

Key words: Malignant peripheral nerve sheath tumour; Lung metastases; Neurofibromatosis type 1; Sarcoma.

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Introduction

A malignant peripheral nerve sheath tumour (MPNST) is a highly aggressive sarcoma. These tumours arise *de novo* or by the transformation of plexiform neurofibromas which are one of the manifestations in patients with an uncommon variant of neurofibromatosis type 1 (NF1). NF1 is an autosomal dominant inherited disease that is caused by a mutation in the NF1 gene. The clinical presentation of NF1 is diverse: white coffee-coloured skin spots (*Café au lait*), pigmented nod-

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ules in the iris, pseudoarthrosis, scoliosis, various cosmetic problems. NF1 affects an estimated 1 per 3,500 people, making it a common genetic disorder.[1, 2]

People with this mutation have an increased risk of developing benign but also malignant tumours, such as optic gliomas (15-20 %), pheochromocytoma (0.1-5.7 %), gastrointestinal stromal tumours (GIST) (4–25 %), rhabdomyosarcoma (1.4–6 %), MPNST (8–13 %), a seven times higher risk of developing leukaemia and a five-times higher risk of developing breast tumours.[3] Almost one quarter (20-25 %) of patients with NF1 develop plexiform neurofibroma (PNF), most often at an early age, which has a high tendency of malignant alteration into MPNST.[4]

Poor prognostic factors are deeper tumour localisation, locally advanced disease and R2 resection, which are more common in patients with NF1-associated MPNST as opposed to the sporadic onset of the disease.[12] Treatment of the local disease is primarily surgical. Recurrence of the disease, after surgical treatment, can be expected in one-third to one-half of patients. The most common sites of dissemination are the lungs, followed by the bones and visceral organs of the abdomen, primarily the liver and peritoneum, central nervous system and the least common lymph nodes. Chemotherapy and radiotherapy are used in advanced and systemic diseases.[5, 6]

Case presentation

In May 2019, a 35-years old male was examined at the Oncology Clinic of the University Clinical Centre of Republic of Srpska, due to prior radiologically discovered multiple soft tissue lesions of the lung parenchyma bilaterally. At the time, the patient was respiratory insufficient, ECOG PS (Eastern Cooperative Oncology Group, ECOG, Performance Status, PS) was 3. The main difficulties were persistent productive cough, shortness of breath and weight loss with preserved appetite. Anamnestic data showed that he underwent surgery in 2015, during which the tumour was removed from the distal part of the left thigh and left popliteal fossa. A pathohistological finding of resected tissue confirmed that it was neurofibromatosis (non-malignant). Additional anamnestic data were acquired from the patient, which described a more significant number of relatives in the close family with the same skin nodules.

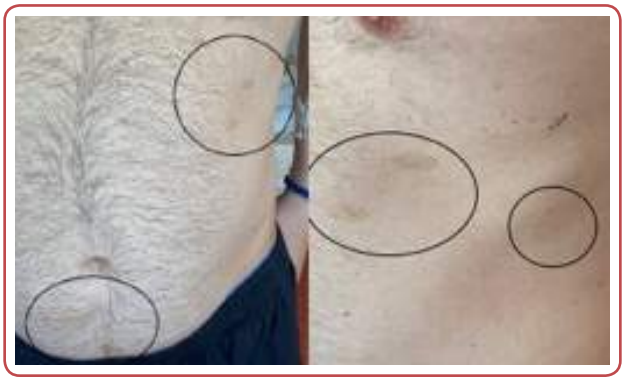

Figure 1: White coffee stains (Café au lait)

Clinical examination revealed several white coffee-coloured spots on the anterior abdominal wall (Figure 1) and a large number (> 10) of soft tissue subcutaneous nodular lesions of various sizes (neurofibromas). In the area of the previous operation, there was now a large tumour whose diameter exceeded the scope of the ultrasound field of the probe. It was decided to perform computed tomography (CT) guided percutaneous needle biopsy of available lung lesion.

Furthermore, the revision of the pathohistological findings of the material obtained by the operative procedure from 2015 was requested. The pathologist's report confirmed that the tumour tissue obtained by needle biopsy from the lungs and the revision report of the previous pathohistological finding were the same type of tumour which correspond to MPNST (Figures 2 and 3).

Histological characteristics in the primary tumour and metastasis in the haematoxylin and eosin (HE)-stained preparations were identical, while immunohistochemistry was different. In primary tumour, focal positivity of S100 and α-smooth muscle actin (SMA) was noted, while metastasis tissue was S100 negative and SMA positive. The difference in immunophenotype may be since staining in the primary tumour was done on a large section of the tumour, while metastasis sample was much smaller obtained by needle biopsy in which part of the tumour expressing S100 was not involved because expression in the primary tumour was focal (Figure 2).

The patient underwent additional diagnostics. Chest computerised tomography (CT) scan (Figure 4) showed numerous metastases (over 100) in the lungs, where the largest was measured 7.3 cm on the long axis. Magnetic resonance imaging (MRI) of the left thigh showed a large tumour mass (craniocaudal diameter 13.5 cm) in the dis-

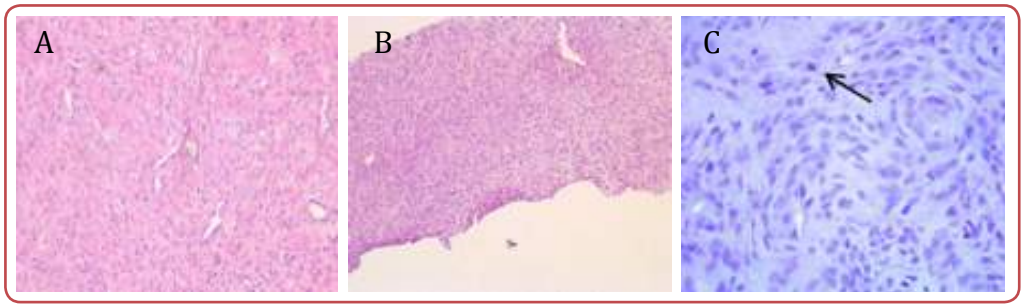

Figure 2: A) Primary tumour (HE 10x); B) lung metastasis (HE 10x); C) primary tumour (HE 40x) (arrow indicates mitosis)

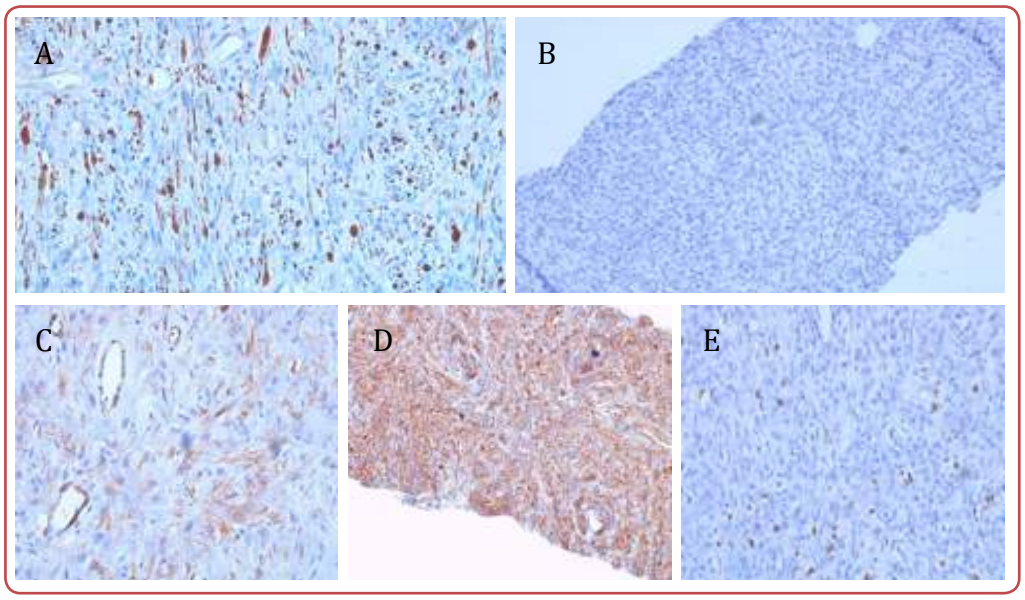

Figure 3: A) Primary tumour (S100 20x); B) lung metastasis (S100 10x); C) primary tumour (SMA 10x); D) lung metastasis (SMA 20x); E) primary tumour (Ki67 20x)

tal posterior thigh region and part of the popliteal fossa. Positron emission tomography-computed tomography (PET-CT) with 18F-FDG confirmed the existence of an expansive metabolically active tumour mass in that region, multiple metabolically active lesions in the lungs and spots of moderately increased metabolic activity in the posterior aspects of the right and left thigh and lower legs.
It was decided to start treatment with chemotherapy according to the Doxorubicin / Ifosfamide / Mesna protocol. After three cycles of chemotherapy, the patient had minimal respiratory distress, ECOG PS 1. Control chest CT scan (Figure 5) showed a moderate decrease in the size of the lung metastases compared to the previous examination.

Treatment was continued according to the same chemotherapy protocol. A total of six cycles were applied by October 2019, with good tolerance, no cycle delay and with the support of Granulocyte Colony-Stimulating Factor (G-CSF). The achieved results were a partial response (PR) of the lungs metastases, followed by clinical improvement of

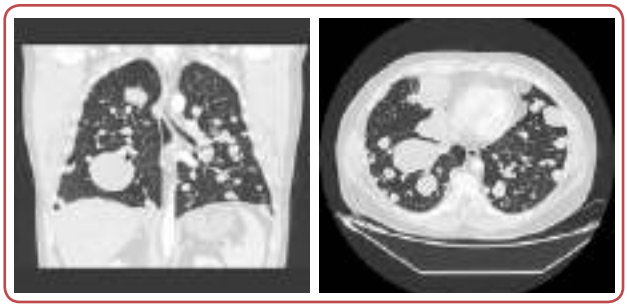

Figure 4: Chest computerised tomography (CT) scan prior to systemic therapy

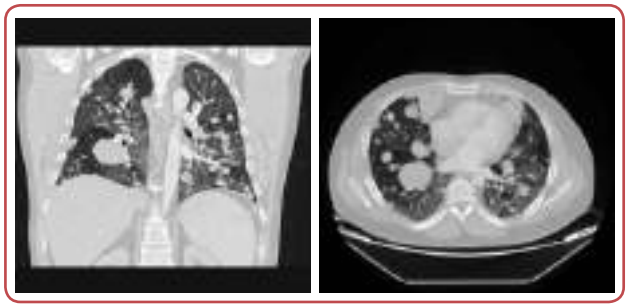

Figure 5: Chest computerised tomography (CT) scan after six cycles of chemotherapy

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the general condition, while there was a local progression of the tumour mass in the distal part of the left thigh and popliteal fossa. The patient was referred for palliative surgery. As a result of the operation, paresis of the left foot was left behind. The patient was admitted to the oncologist again in February 2020 for a regular check-up. Chest and abdominal CT scans showed the progression of the lung metastases and multiple nodules in the spleen, which were highly suspicious for metastases. At that time, the progression-free survival (PFS) period for the patient was about eight months.

A tumour board decided to continue treatment with previously administered chemotherapy (Doxorubicin / Ifosfamide / Mesna), up to a cumulative anthracycline dose. Three more cycles (nine in total) were applied with good tolerability. After that, the patient was monitored regularly. In June 2020, the patient's general condition rapidly declined, accompanied by the onset of neurological symptoms. A head CT scan showed the presence of multiple metastases in the endocranium. Palliative radiotherapy was performed and supportive care was continued. The patient passed away in August 2020. Overall Survival (OS) was approximately 14 months.

Discussion

About 10 % of all soft tissue sarcomas belong to MPNST, whereby 50 % occur in people diagnosed with neurofibromatosis type 1. The disease is more often in male population. The most common localisations are the extremities and the trunk.[7]

This type of sarcoma most often metastasises to the lung, which was the case with the presented patient. Other possible localisations are bones, adrenal glands, liver, diaphragm, brain, mediastinum, ovary, retroperitoneum, and kidneys, which are other authors' conclusions.[8]

The two-year survival of patients with NF1, who develop MPNST, is 21 %, while the five-year survival is 18 %. Tumour size, localisation, sex, as well as choice of treatment have the most significant impact on OS. A poor treatment outcome is associated with the tumour size (> 10 cm) and the presence of NF1.[9]

In metastatic and inoperable diseases, research data has shown shorter OS survival in patients associated with NF1. However, precise reasons were not yet concluded, even though leads are pointing out that chemotherapy sensitivity is lower in this population of patients. The OS for this patient was almost 14 months as previous statistic shows.[12]

Surgical treatment is the cornerstone in the treatment of localised disease with the tendency to achieve negative resection margins.[12] Chemotherapy and radiotherapy have not shown a significant effect on survival.[8] A Dutch national study, published in the European Journal of Cancer last year, showed that primary surgical treatment was performed in 88 % of patients with localised MPNST and in whom R0 resection was achieved in 66.3 % of all patients. Additional radiotherapy was performed in 44.2 % of patients. Postoperative radiotherapy was mainly performed (in 42.5 %), but preoperative radiotherapy was preferred at the end of the study. Chemotherapy was mostly given to patients with retroperitoneal localisation of the primary tumour. In inoperable tumours, treatment options were radiotherapy and chemotherapy, but radiotherapy was generally preferred.[10]

Treatment options for advanced MPNS as well as metastatic disease are very limited and results are modest. The recommendations are mostly the same as for other forms of soft tissue sarcoma. An essential treatment recommendation is chemotherapy Doxorubicin as monotherapy or the Doxorubicin/Ifosfamide combination, with better results being achieved with combination therapy, primarily in PFS (EORTIC study 62012).[11] The other often used cytostatics are vincristine, cyclophosphamide, dactinomycin and etoposide. Preference should always be given to inclusion in clinical studies where experimental drugs are given if this is possible. In the search for new drugs, many studies were performed, mainly phase I and II, where the effects of new targeted therapy were examined, such as Erlotinib, Sorafenib, Imatinib, combinations Everolimus/Bevacizumab (SARC016), Sirolimus/Gantespib (SARC023). Even though studies were negative, they are becoming solid ground for future studies and new generation of drug modalities that will have possible promising results in the time ahead.[13-15]

It is worth mentioning the new drug Selumetinib (MEK1/2 inhibitor). The drug was approved in April 2020 by the FDA for paediatric patients two years of age and older with inoperable plexiform neurofibromas related to NF1. The drug was approved after a phase II clinical trial. The results of

the study showed a high percentage of partial response of about 70 % (reduction in tumour size) and maintenance minimally one year long or even longer, with acceptable tolerance and safety.[16] Selumetinib holds out hope to this group of patients in reducing the risk of developing MPNST, although studies have yet to be conducted to confirm this, because prevention is always the best therapy. Furthermore, there are also promising expectations from study NCT02691026, which examines the use of pembrolizumab in patients with inoperable MPNST. The study should be finalised in 2025.

The therapy applied to the patient presented in the paper is in accordance with the world's leading recommendations. The decision to continue the same protocol after disease progression was made because he initially responded well to it, but also because of the limited treatment options available for these types of tumours in country.

Conclusion

Early recognition of the clinical signs of this genetic disease is essential. Moreover, active expert supervision is very important, primarily to identify all suspicious lesions promptly and apply optimal treatment modalities, in the first place surgical. It is significant not only for the patients but also for their relatives, who should be included in the supervision due to the hereditary nature of this disease. A multidisciplinary approach is needed to ensure an optimal treatment in patients with rare malignancies.

One of the steps in improving the treatment and care of patients with malignant tumours of the peripheral nerve sheath is undoubtedly the establishment of the Centre for Rare Tumours in the Republic of Srpska, where in one place, patients with this but also other rare malignancies would be supervised by experts, applied appropriate diagnostic procedures and implemented optimal treatment modalities.

Acknowledgements

None.

Conflict of interest

None.

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CASE REPORT

Allergic Contact Dermatitis Caused by Dexpanthenol Confirmed With Open Application Test: a Case Report

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Abstract

Topical medications and cosmetic products contain many allergens that can trigger allergic contact dermatitis. One of the most frequent ingredients is dexpanthenol (bepanthen, panthenol). A case of a 19-year-old female patient is presented, with a 2-year history of continuous episodes of contact allergic reactions with positive open application test to dexpanthenol, after other dermatoses and allergens were excluded.

Key words: Allergic contact dermatitis; Dexpanthenol; D-panthenol; Allergen; Dermatology.

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Introduction

Exposure to various materials in our close environment can induce allergic reactions in the form of allergic contact dermatitis (ACD). As a form of a delayed allergic reaction (type 4 hypersensitivity response), it represents a T-cell mediated inflammation of the skin caused by repetitive exposure to allergen which previously led to sensitisation of T-lymphocytes. It consists of two phases - induction and elicitation, with an allergen in the form of hapten which can penetrate the top epidermal layer known as stratum corneum. Considering previous sensitisation, any repeated contact triggers an allergic reaction because of memory T-cells who are also responsible for more serious allergic reaction because of cell division and multiplication. Prevalence of contact dermatitis is about 20 % in Europe,[1] with approximate prevalence of 15 % in 12-16 year old adolescents.[2] Some forms of ACD are labelled as occupational diseases because of high prevalence in specific population such as hairdressers, cleaners, painters and health care workers.[1] Also, recent studies show that ACD is found less in people who suffer from psoriasis. Some of the most frequent sensitisers are metals (gold, nickel, mercury, chromium, essential oils, acrylates and food additives, hair dyes, preservatives and textile chemicals). Clinical features of ACD include skin lesions, most commonly 2-3 days after the exposure to the allergen. Description of skin efflorescence usually vary from erythema or a light rash to papules and vesicles that can ooze or drain and are also accompanied by pruritus, swelling or pain. A gold standard for diagnosing ACD is a patch test. Treatment involves strict avoidance of the allergen if it is known, while topical corticosteroids are used in an acute phase with oral antihistamines or systemic corticosteroids and topical immunomodulators in severe cases.[2] Resolution is expected in 3-4 weeks, without treatment. Sometimes, causes and exacerbating factors are misidentified because of unusual clinical presentation and rare frequency.

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Case history

A 19-year-old female patient presented with bilateral erythema on upper and lower eyelids, along with pruritus. She was prescribed topical steroids by her family physician. Information gathered by thorough anamnesis revealed that previously mentioned rash emerged for the first time five months previously, with gradually worsening with every repeated episode. After being examined at our dermatology department, the rash was described as erythematous, pruritic patches with papules and vesicles followed by localised oedema and following lichenification. Eyelid oedema was severe on several occasions such that the patient was admitted to the emergency room because angioedema was suspected. Systemic corticosteroids and antihistamines were administered. With continuing episodes of resolution and recurrence, detailed tests were carried out. A skin prick test for inhalant and food allergens was negative and total immunoglobulin E – ELISA was in reference range. Tests for systemic and infective diseases also came back as negative. Patch test with European standard series was done and it was negative. The recurrence of rash worsened throughout one and half years, spreading from eyelids and affecting cheeks and chin.

Figure 1: Bilateral erythema and oedema after exposure to dexpanthenol

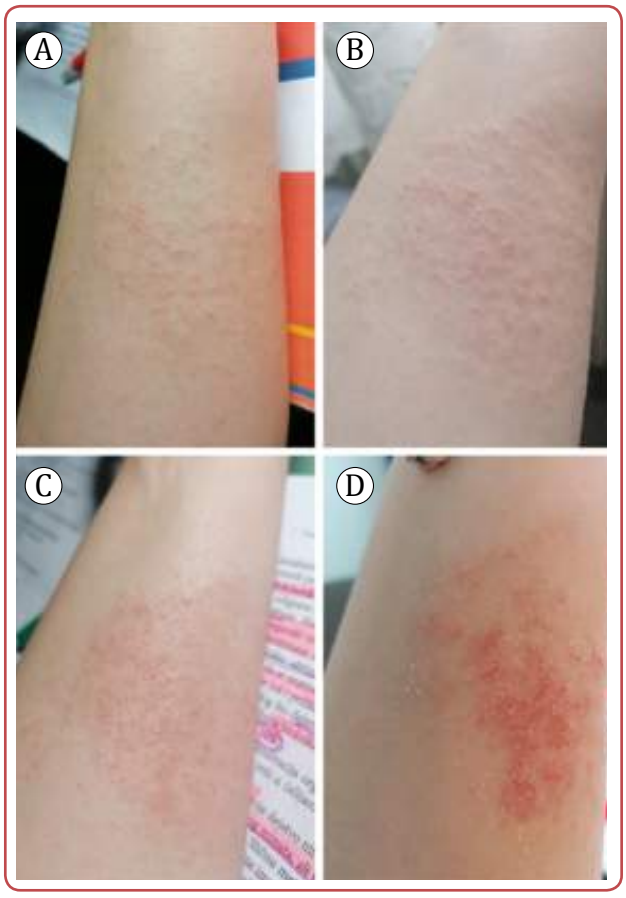

Figure 2: Open application test with dexpanthenol liquid solution: (a) after 48 hours (b) after 72 hours (c) after 96 hours (d) after 15 days

Topical steroids were prescribed, such as alclometasone and mometasone, with oral antihistamines and topical immunomodulators (pimecrolimus). The patient responded well, but recurrence was frequent, without any known cause. As a side effect of treatment, she developed steroid rosacea. An important moment was development of rash during the treatment to a prescribed product that contained panthenol. Patient reported that she used homemade hair shampoo, which mostly contained panthenol solution. After quitting the shampoo, the eyelid rash and oedema did not reoccur. Considering the localisation of the rash, it was possible that ACD was caused by dripping shampoo down from the hair, because rinse-off products such as shampoo can cause generalised presentation.

Over a period of two years, it was noticed that every product that contained dexpanthenol caused the same allergic reaction. Our patient reported a rash caused by hand and face cream, lip balm, face masks, wet wipes, body lotion and make-up. It was suggested that an open application test should be performed to confirm the doubts because there were little reported and confirmed

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cases. Repeated open application test (ROAT) is indicated in patients where new allergens are involved or they are not thoroughly explored. It is performed by applying the suspected allergen to the volar surface of the forearm two times a day for seven days.[3] A 5 % liquid solution of dexpanthenol was applied on the patients' forearm. After 24 hours, a mild erythema began to appear, followed by papular lesion in the next 24 hours. The rash worsened and it was followed by itching. Corticosteroid creams, specifically alclometasone, were applied on the area of evolving dermatitis because it did not resolve spontaneously after the end of the exposure to the allergen.

Discussion

Dexpanthenol is used frequently in moisturisers[4] and various cosmetics, including hair products, wipes, makeup and facial cleansers.[5] Even though it is considered to be rare, ACD is reported in adults[6] and in children[5] and should be considered as a possible allergen. Because of moisturising effects, it is often prescribed to patients in treatment of dermatitis. Bearing in mind that it has potential to trigger allergic reactions, consideration should be given to advising patients in using products that contain dexpanthenol. If it is not carefully approached to ACD of unidentified cause, the patient could enter in *circulus vicious* with progressive worsening of ACD with every next exposure. This also means that patients with chronic ACD also have certain side effects of treatment with corticosteroids, including thinning and cracking of the skin, skin dryness and steroid-induced rosacea. Impact on mental health should also be mentioned, especially if we are talking about teenagers and young adults with facial dermatitis or other visible parts of the body. Additionally, patient should be given an advice on how to avoid allergen, with recommendations on what to pay attention to such as different names of allergen or even using an online tool such as *SkinSAFE.*[7]

Compared with several other cases, it is concluded that symptoms of exposure include generalised or localised eczema. As it can be noticed in a patient that was treated with creams that contained panthenol because of venous stasis dermatitis[8] and a patient that underwent radiotherapy for basal cell carcinoma,[8] where they both developed generalised eczema that resolved after corticosteroid therapy. In these cases it can be observed that it can often be an iatrogenic condition. It was described even in 1965 in a 56 year-old patient that was using a panthenol ointment because of post thrombotic leg ulcer and dermatitis.[9]

Even one or two episodes of eczema should be an alert for a possible rare cause when other allergens are not positive. Facial wipes that are labelled as "hypoallergenic" can also be the cause, where facial eczema can occur 24 hours after removing make-up with previously mentioned wipes.[5] It is often disregarded in diagnosing dermatitis, especially when patients enter in chronic phase, where authors have described development of multiple scaly lesions on face and nummular eczema-like lesions on trunk, where the patient used baby cream containing panthenol.[10] Unusual clinical presentation of panthenol-caused dermatitis is pustular form, where pustules all over erythematous and eczematous bases can be described.[11]

Phases in ACD often take turns, from latency to dermatitis, especially if patients use anti-inflammatory therapy. This means that patients' history of dermatitis are long, years or decades, where intermittent pruritic patches on face, trunk or extremities are often treated symptomatically. With positive patch testing to one allergen or more, patients often use hypoallergenic products where panthenol is one of the main ingredients.[12] That is the main reason why ACD caused by panthenol is hard to diagnose, mainly because cosmetical and pharmaceutical products often contain this allergen, so it is often overlooked.

Conclusion

Documenting and reporting certain allergens is of great importance because of possible misdiagnosis and treatment errors. Considering that dexpanthenol is found in almost every skin and hair products, especially in ones used for skin repairing, healing and calming that are also a part of ACD treatment, it should be noted that it is a potential allergen, especially in chronic dermatitis of unknown cause, where implementation of patch test series that contain panthenol should be considered. Open application test, which was crucial for diagnosis in this case, should be advised to patients when one is not able to identify the cause.

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None.

Conflict of interest

None.

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