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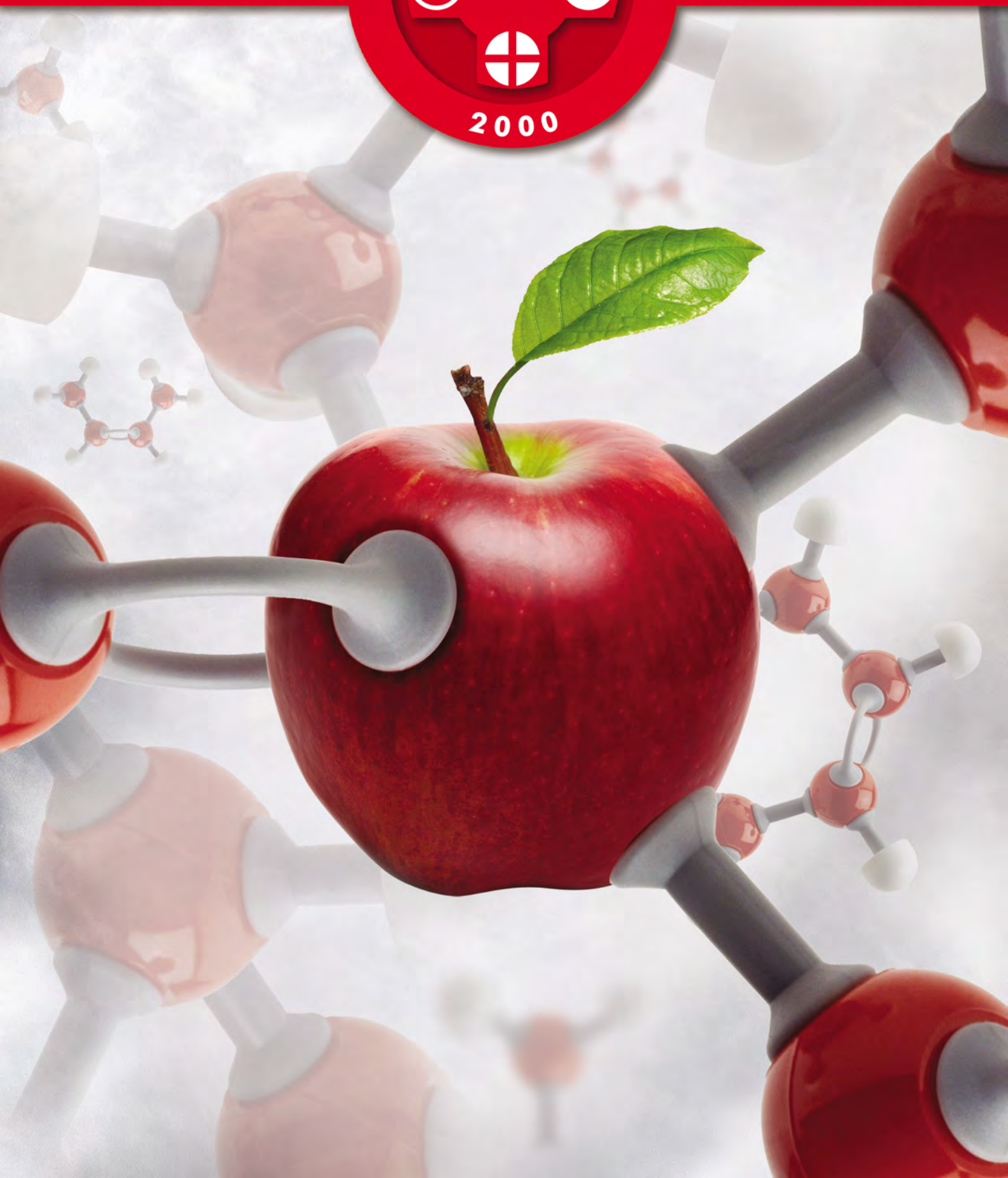

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STEM CELLS: NEW HOPE FOR SPINAL CORD INJURY

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MATIČNE ĆELIJE: NOVA NADA ZA POVREDE KIČMENE MOŽDINE

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ABSTRACT

Stem cell therapy offers several attractive strategies for spinal cord repair. The regenerative potential of pluripotent stem cells was confirmed in an animal model of Spinal Cord Injury (SCI); nevertheless, optimized growth and differentiation protocols along with reliable safety assays should be established prior to the clinical application of hESCs and iPSCs. The therapeutic effects of mesenchymal stem cells (MSCs) in SCI result from neurotrophin secretion, angiogenesis, and antiinflammatory actions. Several preclinical SCI studies have reported that the occurrence of axonal extension, remyelination and neuroprotection occur after the transplantation of olfactory ensheathing cells (OECs). The transplantation of neural stem cells NSCs (NSCs) promotes partial functional improvement after SCI because of their potential to differentiate into neurons, oligodendrocytes, and astrocytes. The ideal source of stem cells for safe and efficient cell-based therapy for SCI remains a challenging issue that requires further investigation.

SAŽETAK

Terapija matičnim ćelijama pruža nekoliko atraktivnih mogućnosti za lečenje povreda kičmene moždine. Regenerativni potencijal pluripotentnih matičnih ćelija je potvrdjen u animalnim modelima povrede kičmene moždine, medjutim, protokoli za kultivaciju i diferencijaciju ovih ćelija kao i testovi za potvrdu njihove bezbednosti tek moraju biti ustanovljeni kako bi se hESCs i iPSCs primenile u kliničkoj praksi. Terapijski efekat MSCs u povredi kičmene moždine se zasniva na sposobnosti ovih ćelija da sekretuju neurotrofne i antiinflamatorne faktore, kao i da promovišu angiogenezu. U nekoliko predkliničkih studija su pokazani rast aksona, remijelinizacija i neuroprotektivno delovanje OECs. Transplantacija NSCs doprinosi funkcionalnom oporavku nakon povrede kičmene moždine diferencijacijom NSCs u neurone, oligodendrocite i astrocite. Otkrivanje idealnog izvora matičnih ćelija za efikasnu i bezbednu terapiju povrede kičmene moždine i još uvek je izazov i zahteva dalja istraživanja.

INTRODUCTION

Spinal cord injury (SCI) is a devastating condition with permanent lifelong consequences (1). Epidemiological data show that the incidence of traumatic SCI in the US ranges from 27 to 83 per million while in Europe it is approximately 10–30 new cases per million (1). SCI usually results in sudden and long-lasting locomotor and sensory neuron degeneration below the injury (2).

The pathophysiological processes that underlie SCI comprise the primary and secondary phases of injury. During the primary phase, because of the direct mechanical trauma of the spinal cord by fractured and displaced bone fragments and disc material, there is massive axonal damage as well as neuronal and glial cell losses (3, 4, 5). During the secondary phase of injury, further tissue damage occurs mostly from ischemia, electrolyte imbalance, inflammatory response, oxidative stress and excitotoxicity (3). Despite major advances in the medical and surgical care of SCI patients, there are currently no effective therapies for the treatment of traumatic SCI in humans (2). Stem cell therapy offers several attractive strategies for spinal cord repair. Stem cells may play an important role in the replacement of damaged neuronal and glial cells, axonal regeneration and remyelination, the restoration of neuronal circuitry, and the production of neurotrophic factors, anti-inflammatory cytokines, and other molecules that promote tissue repair and neovascularization.

In this review, we will evaluate the therapeutic role of human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), and olfactory ensheathing cells (OECs) for treating SCI, and we will cover some of the clinical trials that aim to translate laboratory stem cell research into clinical practice.

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Table 1. Therapeutic potential of stem cells for treatment of spinal cord injury

Stem cell source	Advantages	Disadvantages
hESCs	differentiation into neurons and glia, modulation of local immune response, activation of endogenous neurogenesis.	ethical issues, immune rejection, potential for tumor formation.
iPSCs	differentiation into neurons, glia, and neural progenitor cells.	potential for tumor formation.
MSCs	providing trophic support to damaged neurons by secreting angiogenic and neurotrophic factors, modulation of local immune response.	no universal consistency in cell sourcing, and the optimal administration method.
NSCs	differentiation into oligodendrocytes and astrocytes.	immune rejection, formation of glial scars.

hESCs, human embryonic stem cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; NSCs, neural stem cells.

Human Embryonic Stem Cells

hESCs are derived from the inner cell mass of human blastocysts; they have the ability to proliferate by maintaining both their pluripotency and their ability to differentiate into nearly all cell types, including neuronal and glial cells (6). Improved protocols have been developed to differentiate hESCs into motoneuron progenitors (MPs) and oligodendrocyte progenitors (OPCs) (7, 8). The transplantation of hESC-derived MPs and OPCs can efficiently recover locomotor function in both contusion and transection animal models of SCI (7, 8). The regenerative mechanism of hESC therapy for SCI depends on the potential of hESC-derived OPCs and MPs to differentiate into neuronal and glial cells and the immunomodulatory characteristics of transplanted hESC-derived OPCs (7, 1).

Based on promising preclinical data from hESC-derived OPC transplants in rodent SCI models, the Food and Drug Administration (FDA) approved the first hESC clinical trial in 2009. The Geron Company attempted to test the safety of hESC-derived OPCs in human SCIs. Two million hESC-derived OPC cells (GRNOPC1) within the acute phase were transplanted directly into the spinal cord of four ASIA A patients with complete thoracic SCI. In 2011, Geron discontinued this trial for financial reasons. The preliminary results indicated that GRNOPC1s do not cause any harm, but the debate about the efficacy of these cells still continues.

Concerns about the transplantation of hESC-derived neural cells to treating SCI are related to the ethical issues of cell derivation, the immune rejection of transplanted cells, the use of differentiation protocols that still involve mediums, growth factors, and supplements of animal origin, and the possibility of teratoma formation from incomplete or aberrant differentiation resulting in the formation of non-neural cells (6, 9).

Induced Pluripotent Stem Cells

iPSCs were originally generated by the ectopic expression of four transcription factors called, namely Oct4, Sox2, Klf4, and c-Myc in fibroblast cells by Takahashi and Yamanaka in 2006 (10). iPSCs show morphological, transcriptional, epigenetic, and phenotypic similarity to hESCs and can differentiate towards any cell in the human body including neurons, glia, neural progenitor cells (NPCs), and motoneurons (11).

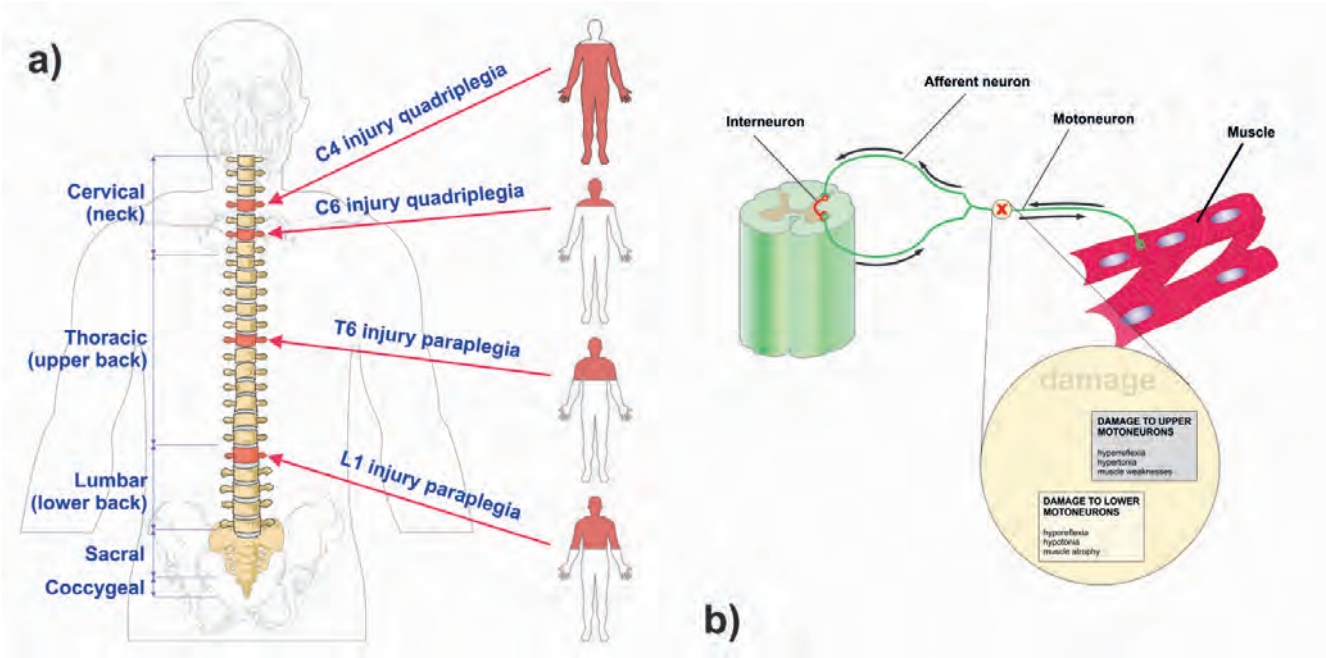

Figure 1: Spinal cord injury: a) clinical signs and b) site of injury

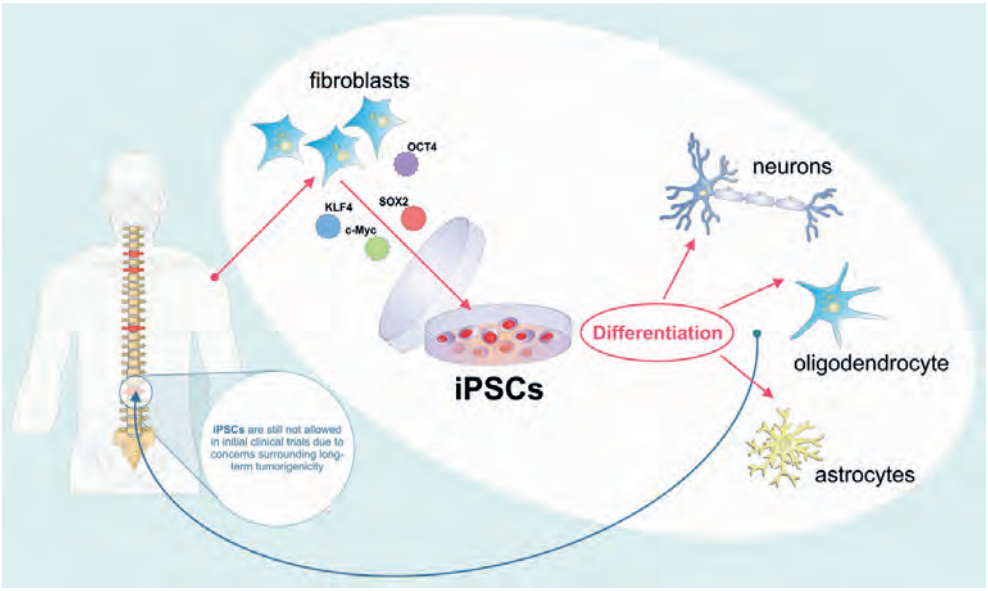

Figure 2: Method for derivation of iPSCs from adult somatic cell by introducing OCT4, SOX2, KLF4 and c-Myc. iPSCs can differentiate toward neurons, oligodendrocytes and astrocytes.

Patient-specific iPSCs derived from somatic cells through the ectopic expression of a defined set of factors do not present ethical and immnunological concerns (11). The primary concern about the use of these cells in clinical trials was with the reprogramming technology that involved viral vectors and their tumourigenicity (2). Some of the reprogramming issues are solved by the deriving iPSCs bythrough nonviral methods such as mRNA or chemicals and small molecules (12, 13). The regenerative potential of iPSCs was confirmed in a rodent model of SCI (14, 15); nevertheless, optimized growth and differentiation protocols and reliable safety assays should be established prior to the clinical application of iPSCs.

Mesenchymal Stem Cells

MSCs are adult, self-renewable, multipotent cells that can be found in almost all postnatal tissues (16). In addition to their stem/progenitor properties, MSCs have been shown to possess broad immunomodulatory abilities (16). The therapeutic effects of MSCs in SCI result from neurotrophin secretion, angiogenesis, and antiinflammatory actions, rather than direct translineage conversion to functional oligodendrocytes or neurons (17, 18, 19, 20, 21). Engrafted MSCs act as neuroprotectors by secreting brain-derived neurotrophic factor (BDNF), glia cell line-derived neurotrophic factor (GDNF),

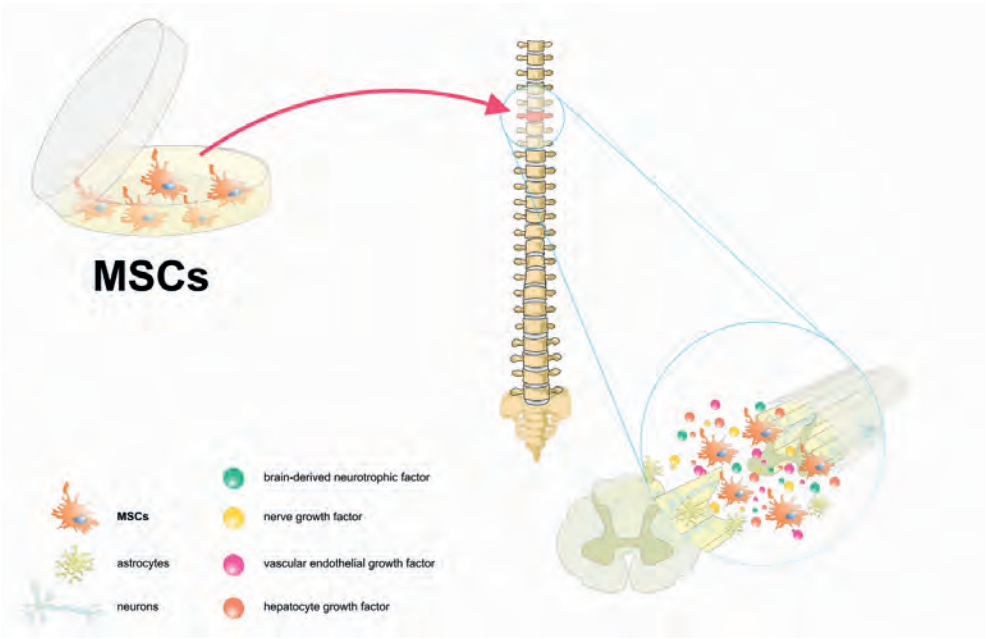

Figure 3: Transplanted MSCs act as neuroprotectors in spinal cord injuries by producing growth factors and anti-inflammatory cytokines.

nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) (22, 23).

The number of clinical trials that employ MSCs for SCI treatment is increasing, indicating that despite several questions that still need to be addressed at pre-clinical levels, MSCs are considered potentially beneficial for translational studies (24). The pathological processes that occur at the lesion site in SCIs evolve over time, from the acute to subacute to chronic phases; therefore, transplantation at different times post-lesion may have varied effects (25). Sykova et al. suggested that performing MSC transplantation within a therapeutic window of 3-4 weeks following SCI is critical for the success of MSC-based therapy (26). Yoon et al. studied the effects of autologous bone marrow MSC transplantation in combination with the administration of granulocyte-macrophage colony-stimulating factor to 35 patients with complete SCIs at acute, subacute and chronic stages (27). No serious complications were reported, and 30.4% of patients who received MSCs at acute and subacute stages showed significant improvements in their ASIA scale position (27). Few clinical studies have shown neurological improvements in MSC-treated patients who during the chronic stage of SCI, when the glial scar is already present (28, 29, 30, 31, 32). In these studies, MSCs were transplanted directly into the lesion intrathecally, intravenously and intrathecally (simultaneously) at once, and intraarterially.

Although the clinical study results are promising, there are important issues that should be addressed to achieve successful MSC-based therapy, that is, the universal consistency in cell sourcing and culture conditions, the ideal cell quantity and the optimal administration method (24, 1).

Olfactory Ensheathing Cells

OECs are a unique population of macroglia found in the lamina propria of olfactory mucosa, around the olfactory nerve fascicles and in the two outer layers of the olfactory bulb. OECs have the dual nature of astroglial cells and Schwann cells (33). Several preclinical SCI studies have reported the occurrence of axonal extension, remyelination and neuroprotection after OEC transplantation (34, 35, 36, 37, 38). OECs migrate to injured sites and secrete a large number of factors that are necessary for the growth, development, differentiation, and maturation of different types of neurons and reduce astrocyte activity and glial scar formation (39, 40). Spinal cord regeneration and functional recovery depend on the nature and source of OECs, the injury model, the graft cell preparation, the time of transplantation, and the transplantation procedures (25).

Feron et al. performed a single-blind phase I clinical trial in which three patients with SCI (chronic injuries) received autologous OECs. The feasibility of the procedure and the safety of these cells were reported, but there was no evidence of clinical efficacy (41, 42). Lima et al. reported that the transplantation of minced olfactory mucosa in patients with chronic SCI was not significantly efficient (44). By contrast, recent clinical studies suggested that there was a neurological improvement in SCI patients after OEC transplantation (44, 45).

Neural Stem Cells

NSCs are multipotent cells with the potential to differentiate into neurons, oligodendrocytes, and astrocytes, and they can be efficiently propagated *in vitro* (46).

NSCs can be found in the periventricular subependymal layer, in the subgranular zone of the dentate gyrus, and in the ependymal regions lining the central canal (47). The activation of resident ependymal stem cells following SCI is not sufficient to promote recovery because the cells differentiate mostly into actrocytes and oligodendrocytes (48, 49). Several preclinical studies confirmed that the transplantation of NSCs promotes a partial functional improvement after SCI (50, 51). The transplantation of OPCs that had differentiated from ependymal stem cells efficiently recovered the locomotor function of an SCI animal model (47). The source of NSCs, the methods of cell isolation and preparation, the time of transplantation, the chosen

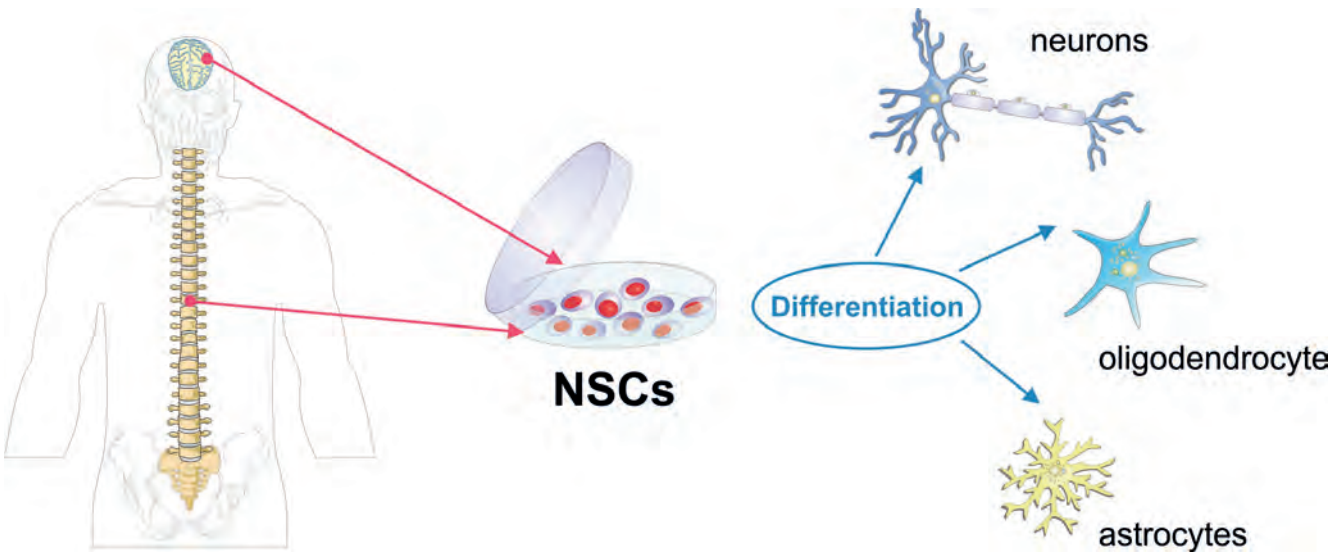

Figure 4: Potential uses of NSCs which were isolated from the adult brain and spinal cord as a source of neurons, oligodendrocytes, and astrocytes.

immunosuppression, and the type of injury (contusion vs. transection) are important issues for achieving successful NSC-based therapy after SCI (49). Human NSCs have been isolated from foetal brains, and spinal cords have been isolated from aborted foetuses. Unlike adult NSCs, foetal-derived NSCs generate neurons in addition to glia in the injured spinal cord (1).

Currently, two human trials involving allogeneic NSCs for SCI are ongoing. The primary objectives of these studies are to determine the long term safety and preliminary efficacy of NSC transplantation in subjects with thoracic spinal cord trauma.

CONCLUSIONS

Numerous preclinical studies suggest that stem cells are able to enhance recovery following SCI. However, the ideal source of stem cells for the efficient and safe cell-based therapy of SCI remains a challenging issue that requires further investigation and continuous cooperation between clinicians, researchers, and patients.

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ST2 DEFICIENCY AMELIORATES HIGH FAT DIET-INDUCED LIVER STEATOSIS IN BALB/C MICE

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DELECIJA GENA ZA ST2 U BALB/C MIŠEVA UBLAŽAVA STEATOZU JETRE INDUKOVANU DIJETOM SA VISOKIM SADRŽAJEM MASTI

Nemanja Jovičić [1,2*], Ilija Jeftić [1,3*], Marina Miletić Kovačević [2], Irena Tanasković [2], Nebojša Arsenijević [1], Miodrag L. Lukić [1], Nada Pejnović [1,3*]
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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is strongly associated with obesity, but the molecular mechanisms of liver steatosis and its progression to non-alcoholic steatohepatitis and fibrosis are incompletely understood. Immune reactivity plays an important role in the pathogenesis of NAFLD. The IL-33/ST2 axis has a protective role in adiposity and atherosclerosis, but its role in obesity-associated metabolic disorders requires further clarification. To investigate the unresolved role of IL-33/ST2 signalling in NAFLD, we used ST2-deficient ($ST2^{-/-}$) and wild type (WT) BALB/c mice maintained on a high-fat diet (HFD) for 24 weeks. HFD-fed $ST2^{-/-}$ mice exhibited increased weight gain, visceral adipose tissue weight and triglyceridaemia and decreased liver weight compared with diet-matched WT mice. Compared with WT mice on an HFD, ST2 deletion significantly reduced hepatic steatosis, liver inflammation and fibrosis and downregulated the expression of genes related to lipid metabolism in the liver. The frequency of innate immune cells in the liver, including $CD68^+$ macrophages and $CD11c^+$ dendritic cells, was lower in HFD-fed $ST2^{-/-}$ mice, accompanied by lower TNFα serum levels compared with diet-matched WT mice. Less collagen deposition in the livers of $ST2^{-/-}$ mice on an HFD was associated with lower numbers of profibrotic $CD11b^+Ly6c^{low}$ monocytes and $CD4^+IL\text{-}17^+$ T cells in the liver, lower hepatic gene expression of procollagen, IL-33 and IL-13, and lower serum levels of IL-33 and IL-13 compared with diet-matched WT mice.

Our findings suggest that the IL-33/ST2 axis may have a complex role in obesity-associated metabolic disorders. Although it is protective in HFD-induced adiposity, the IL-33/ST2 pathway promotes hepatic steatosis, inflammation and fibrosis.

Key words: *Obesity, steatosis, non-alcoholic steatohepatitis, liver fibrosis, immune cells*

SAŽETAK

Nealkoholna masna bolest jetre je najčešće udružena sa gojaznošću, ali su molekularni mehanizmi razvoja steatoze i progresije u stetaohepatitis i fibrozu jetre nedovoljno razjašnjeni. Imunski mehanizmi imaju važnu ulogu u razvoju nealkoholne masne bolesti jetre. IL-33/ST2 signalni put ima zaštitnu ulogu u gojaznosti i aterosklerozi, ali je njegova uloga u razvoju metaboličkih poremećaja udruženih sa gojaznošću nedovoljno ispitana.

U ovom istraživanju ispitivali smo ulogu IL-33/ST2 signalnog puta u nealkoholnoj masnoj bolesti jetre na mišjem modelu gojaznosti indukovane primenom dijete sa visokim sadržajem masti u trajanju od 24 nedelje na ST2 deficijentnim ($ST2^{-/-}$) i miševima divljeg soja BALB/c.

$ST2^{-/-}$ miševi na dijeti sa visokim sadržajem masti su imali veći prirast telesne težine, veću težinu visceralnog masnog tkiva i više serumske nivoe triglicerida, dok je težina jetre bila manja u pređenju sa miševima divljeg soja na istoj dijeti. Nadalje, delecija $ST2^{-/-}$ gena je značajno smanjila steatozu jetre, inflamaciju i fibrozu jetre što je bilo praćeno sniženom ekspresijom gena uključenih u metabolizam lipida u jetri. Zastupljenost ćelija prirodne imunosti u jetri, $CD68^+$ makrofaga i $CD11c^+$ dendritskih ćelija i serumski nivo TNFα su bili niži kod $ST2^{-/-}$ miševa. Manje izražena fibroza jetre u $ST2^{-/-}$ miševa je bila povezana sa sniženom zastupljenošću profibrotskih $CD11b^+Ly6c^{low}$ monocita i $CD4^+IL\text{-}17^+$ T limfocita u jetri, sniženom ekspresijom gena za prokolagen, IL-33 i IL-13 i sniženim serumskim nivoima IL-33 i IL-13 u poredjenju sa miševima divljeg soja.

Dobijeni rezultati ukazuju na kompleksnu ulogu IL-33/ST2 signalnog puta u metaboličkim poremećajima udruženim sa gojaznošću. Iako protektivan za razvoj gojaznosti, IL-33/ST2 signalni put pospešuje steatozu, inflamaciju i fibrozu jetre.

Ključne reči: *gojaznost, steatoza, nealkoholni steatohepatitis, fibroza jetre, imunske ćelije*

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ABBREVIATIONS

ABCA - ATP-binding cassette transporter
BSA - bovine serum albumin
CD - cluster of differentiation
CDNA - complementary DNA
FCS - foetal calf serum
FFAs - free fatty acids
Gal-3 - galectin 3
HFD - high fat diet
IFNγ - interferon-γ
IL - Interleukin
LXRα - Liver X receptor alpha
NAFLD - non-alcoholic fatty liver disease
NASH - non-alcoholic steatohepatitis
PBS - phosphate-buffered saline
PPARγ - peroxisome proliferator-activated receptor gamma
qRT-PCR - quantitative real-time polymerase chain reaction
RNA - Ribonucleic acid
TNFα - tumour necrosis factor alpha
VAT - visceral adipose tissue
VLDL - very low density lipoprotein
WT - wild type
α-SMA - alpha smooth muscle actin

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in developed countries and comprises a wide spectrum of liver pathologies, from benign liver steatosis to non-alcoholic steatohepatitis (NASH), eventually causing liver cirrhosis that may lead to hepatocellular carcinoma (1). Approximately one third of the individuals with simple steatosis develop NASH, and among them, up to 20% will progress to liver cirrhosis over the period of years (2).

NAFLD is considered as a hepatic manifestation of metabolic syndrome, which is a cluster of interrelated metabolic disorders, including obesity, hypertension and atherosclerosis, insulin resistance and diabetes, and dyslipidemia and fatty liver. The central features of metabolic syndrome are related to lipotoxicity, glucotoxicity and chronic low-grade inflammation leading to insulin resistance (3,4), for which immune mechanisms and complex cytokine network coordinate the inflammatory responses and metabolic disturbances (3,5). Genetic and environmental factors play a role in the development of obesity (6,7), but the cellular and molecular mechanisms involved in obesity-associated metabolic disorders are incompletely understood.

The hallmark of NAFLD is hepatocyte triglyceride accumulation. Hepatic steatosis represents excessive fat accumulation in hepatocytes and occurs as a result of multiple metabolic pathways, including increased fat delivery, increased fat synthesis, reduced fat oxidation, and/or reduced fat export in the form of VLDL (8). Increased circulating fatty acids (FFAs) and de novo lipogenesis from glucose are important determinants of hepatic steatosis. Adipose tissue dysfunction in obesity is thought to increase plasma FFAs, which are the major lipid providers in hepatic steatosis, and ectopic lipid accumulation in liver and muscle tissues, leading to insulin resistance. The mechanisms that lead to excessive plasma FFAs include increased lipolysis in adipose tissue, increased dietary fatty acids and newly synthesized fatty acids in the liver that are esterified into triglycerides and either stored in hepatocyte lipid droplets or secreted as plasma VLDLs (9).

The molecular mechanisms involved in the progression of benign liver steatosis to liver inflammation and fibrosis in NAFLD are incompletely understood. Hepatic lipid accumulation may promote the inflammatory response characterized by activated resident tissue macrophages (Kupffer cells), the increased infiltration of myeloid and lymphoid cells within the liver and the subsequent release of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, all of which enhance the progression of NASH to fibrosis. Moreover, the most recent study demonstrated that development of hepatic steatosis requires IL-1 signalling, which promotes hepatic lipogenesis (10). Other members of the IL-1 superfamily, including the IL-1 receptor antagonist, IL-18 and IL-33, together with IL-1 have been implicated in various pathological conditions, but their roles in obesity-associated metabolic disorders are unclear.

IL-33 is a member of the IL-1 cytokine family, a multifunctional cytokine involved in the pathogenesis of various inflammatory and autoimmune diseases (11). IL-33 is a pleiotropic cytokine that binds to its plasma membrane receptor complex comprising ST2 and the IL-1R accessory protein (11) and generally promotes Th2-type immune responses. IL-33 appears to exert protective metabolic effects in obesity and atherosclerosis (12). IL-33 promotes liver fibrosis through the activation and expansion of liver-resident innate lymphoid cells, which produce profibrotic IL-13 (13). In the fibrotic liver, IL-33 is present in activated hepatic stellate cells, which are key cellular mediators of liver fibrosis (14).

The role of the IL-33/ST2 axis in obesity-associated metabolic disorders requires further clarification. We aimed to investigate the role of IL-33/ST2 signalling in the development of hepatic steatosis, inflammation and fibrosis in a model of high fat diet (HFD)-induced obesity using ST2-deficient ($ST2^{-/-}$) mice on the BALB/c background.

MATERIALS AND METHODS

Experimental mice and study design

Eight-week-old, male mice were used in the experiments. ST2-deficient mice ($ST2^{-/-}$ mice) on the BALB/c background were generated by targeted disruption of the

mouse ST2 gene (15). $ST2^{-/-}$ mice were kindly provided by Dr McKenzie (University of Cambridge, UK). ST2-deficient (ST2-/-) and wild-type (WT) BALB/c mice were accommodated in our animal facilities under standard laboratory conditions in a temperature-controlled environment with a 12-h light/dark cycle. Mice received water and standard chow (10% calories from fat, Mucedola, Milano, Italy) or a high fat diet (60% calories from fat, Mucedola, Italy) *ad libitum*. Animals were sacrificed after 24 weeks of feeding, and blood samples and liver and visceral adipose tissues were collected for further analyses. All animal procedures were approved by the Ethical committee of the Faculty of Medical Sciences, University of Kragujevac.

Metabolic parameters

Body weights and fasting blood glucose levels were measured periodically, every 4 weeks. Before the measurements, mice were fasted for 4 h, and glucose levels (mmol/L) were determined using the Accu-Chek Performa glucometer (Roche Diagnostics, Mannheim, Germany). Serum concentrations of total cholesterol, triglycerides, AST and ALT were measured using the Olympus AU600 Chemistry Immuno Analyzer (Olympus, Tokyo, Japan). Fasting insulin levels in sera were measured using the Mouse Insulin ELISA Kit (Alpco, Salem, NH, USA).

Liver histological analysis

Livers were excised, fixed in 10% buffered formalin and embedded in paraffin. Tissue sections, 5-μm-thick, were stained with haematoxylin and eosin and picrosirius red as previously described (16). The quantification of red-stained collagen in liver sections stained with picrosirius red was performed on 10 fields of a section at 10X magnification, as previously described (17).

We performed Oil Red O staining on 5-μm-thick liver tissue cryosections. Tissue sections were fixed in paraformaldehyde (10%), rinsed with 60% isopropanol and stained with freshly prepared a working solution of Oil Red O for 10 minutes. After rinsing with 60% isopropanol, sections were counterstained with Mayer's haematoxylin and mounted using water-based mounting medium. The quantification of red-stained lipids in mouse liver sections stained with Oil Red O was performed on 10 fields of a section at 100X magnification by digital image analyses, as previously described (18).

The quantification of liver tissue inflammatory cell infiltration was performed in blinded fashion by two independent observers. Analysis was performed on 10 fields of a section at 10X magnification. Inflammatory cell infiltration was graded as follows: 0=no foci; 1=<2 foci/field; 2=2-4 foci per field; and 3=>4 foci per field. Then, a mean score was calculated (19). Histological analysis was performed on tissue sections using light microscope (BX51; Olympus) equipped with a digital camera.

Immunohistochemistry

For immunohistochemical staining, we used paraffin-embedded liver tissue sections (5-μm-thick). After performing heat-mediated antigen retrieval in citrate buffer (pH=6.0), deparaffinized tissue sections were incubated with primary mouse anti-α-SMA antibody (ab7817, Abcam, Cambridge, UK) or mouse anti-CD68 antibody (ab49777, Abcam). Staining was visualized using the Mouse-specific HRP/DAB Detection IHC Kit (ab64259, Abcam), and sections were counterstained with Mayer's haematoxylin. Sections were photomicrographed with a digital camera mounted on a light microscope (Olympus BX51, Japan), digitized and analysed. Analysis was performed on 10 fields of a section at 40X magnification. The results are presented as the mean count of positively stained cells per field.

Isolation of liver mononuclear cells

The mice were euthanized, and their livers were removed, thoroughly dissected and passed through a 100-μm nylon cell strainer (BD Biosciences); isolated cells were then suspended in complete RPMI-1640 medium containing 10% foetal calf serum (FCS). Cell suspensions were centrifuged at 507 rpm for 1 minute, and the supernatants enriched for mononuclear cells were collected and centrifuged at 1500 rpm for 10 minutes, as previously described (20). Cell pellets were then resuspended in complete RPMI-1640 medium.

Flow cytometry

Liver mononuclear cells were stained with combinations of either fluorochrome-labelled primary Abs or isotype controls for 30 min at 4°C. For intracellular staining, cells were activated with PMA/ionomycin and processed as previously described (21). Cells were labelled with the following fluorochrome-conjugated monoclonal antibodies: anti-mouse CD3, CD11b, CD45, CD4, (BD Biosciences), F4/80, CD11c (BioLegend, San Diego, CA), and Ly-6C (Life Technologies, Carlsbad, CA). The cells were analysed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

Expression of genes related to lipid metabolism and fibrosis in the liver

RNA was extracted from frozen mouse liver tissue using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). qRT-PCR was performed using Power SYBR MasterMix (Applied Biosystems) and miRNA-specific primers for procollagen, αSMA, IL-33, CD36, IL-13, TGF-β, Abca-1, LXRα, and PPARγ as well as for β-actin, as

Table 1: Primers used for qRT-PCR analysis

	Sense (5' to 3')	Antisense (5' to 3')
Procollagen	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
α-SMA	ACTGGGACGACATGGAAAAG	CATCTCCAGAGTCCAGCACA
IL-33	TCCTTGCTTGGCAGTATCCA	TGCTCAATGTGTCAACAGACG
IL-13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
TGF-β	ATACAGGGCTTTCGATTCAGC	GTCCAGGCTCCAAATATAGG
mLXR-α	ATCGCCTTGCTGAAGACCTCTG	GATGGGGTTGATGAACTCCACC
PPAR-γ	CCCAATGGTTGCTGATTACAAA	GAGGGTGTTAGAAGGTTCTTCATGA
Abca-1	CGCAGTGACCAGAAAACAATGTG	TATCAATGAGGCAAGGGTGTGG
CD36	TCCAGCCAATGCCTTTGC	TGGAGATTACTTTTTCAGTGCAGAA

a housekeeping gene (Table 1). qPCR reactions were initiated with a 10-minute incubation time at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). The fold change of miRNA gene expression was calculated by the equation $2^{-\Delta\Delta Ct}$, described by Livak and Schmittgen (22), where Ct is the cycle threshold. ΔCt was calculated by subtracting the Ct values of the endogenous control from the Ct values of the miRNA of interest. ΔΔCt was then calculated by subtracting ΔCt of the control from ΔCt of the calibrator.

Cytokine measurements

Cytokine levels in sera were measured using mouse Duoset ELISA kits for IL-6, IL-10, IL-13, IL-33, TGF-β, IFN-γ and TNF-α (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS 22.0. Data are presented as the means ± SEM. Statistical significance was assessed by the Mann-Whitney U test, and, if appropriate, independent sample Student's t test. Statistical significance was assumed at $p<0.05$.

RESULTS

Metabolic analysis in WT and ST2$^{-/-}$ BALB/c mice exposed to an HFD

At the beginning of the experiment, WT and ST2$^{-/-}$ BALB/c mice had similar body weights. After 24 weeks of feeding with either chow or an HFD, no differences in body weights were observed between WT and ST2$^{-/-}$ mice. However, the weight gain and the weight gain expressed as a percentage of the initial body weight were significantly higher in ST2$^{-/-}$ mice on an HFD compared with diet-matched WT mice (Fig. 1A).

The visceral adipose tissue (VAT) weight and the VAT weight expressed as a percentage of total body weight were significantly higher in HFD-fed mice of both genotypes compared with chow-fed mice. The visceral adipose tissue weight was significantly higher in HFD-fed ST2$^{-/-}$ mice compared with HFD-fed WT mice (Fig. 1A). The liver weight and the liver weight expressed as a percentage of the total body weight were significantly lower in ST2$^{-/-}$ mice on an HFD compared with HFD-fed WT mice (Fig. 1A). Fasting blood glucose levels and the HbA1c percentage did not differ in HFD-fed mice of both genotypes, whereas fasting serum insulin levels were significantly lower in ST2$^{-/-}$ mice compared with WT mice (Fig. 1B).

Serum triglycerides were significantly higher in HFD-fed ST2$^{-/-}$ vs. WT mice as well as compared with chow-fed ST2$^{-/-}$ mice. An HFD significantly increased total cholesterol serum levels in both genotypes, with no significant differences observed between ST2$^{-/-}$ and WT mice (Fig. 1C). ALT activity was significantly increased in HFD-fed WT and ST2$^{-/-}$ mice compared with chow fed mice, with no differences found between the genotypes (Fig. 1D). AST levels did not differ between chow- or HFD-fed ST2$^{-/-}$ and WT mice.

Liver steatosis, inflammation and fibrosis in WT and ST2$^{-/-}$ BALB/c mice

Semiquantitative analysis of lipid deposition in liver tissue sections stained with Oil Red O demonstrated that HFD increased liver steatosis in WT and ST2$^{-/-}$ mice compared with chow-fed animals. However, liver steatosis was significantly lower in ST2$^{-/-}$ mice on an HFD compared with diet-matched WT mice (Fig. 2A). Liver inflammation, as evaluated by the inflammatory cell infiltrate score, was significantly lower in ST2$^{-/-}$ mice on an HFD compared with diet-matched WT mice (Fig. 2B). The degree of liver fibrosis, quantified by staining collagen with picrosirius red, was significantly higher in both genotypes of mice fed an HFD compared with chow-fed mice. Notably, the degree of collagen deposition was significantly lower in HFD-fed ST2$^{-/-}$ mice compared with HFD-fed WT mice (Fig. 2C).

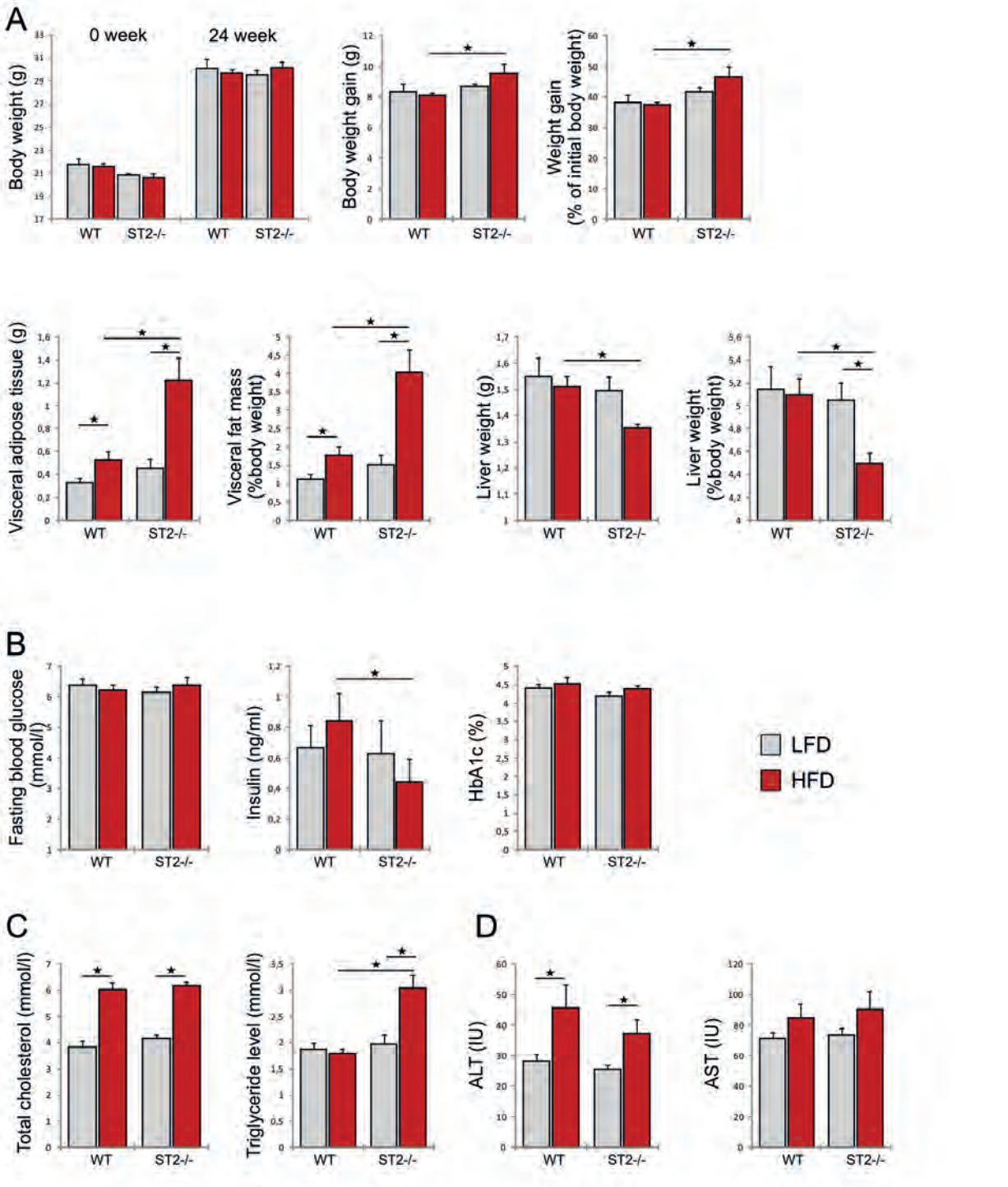

Figure 1. Metabolic parameters

A. Body weight at the beginning of the experiment and after 24 weeks. Body weight gain as well as weight gain expressed as a percentage of the initial body weight were significantly higher in ST2$^{-/-}$ mice on a high-fat diet compared with diet-matched WT mice. The visceral adipose tissue weight and its weight expressed as a percentage of the total body weight were significantly higher in high-fat diet-fed vs. chow-fed groups of mice of both genotypes. The visceral adipose tissue weight was significantly higher in HFD-fed ST2$^{-/-}$ mice compared with HFD-fed WT mice. The liver weight and the liver weight expressed as a percentage of the total body weight were significantly lower in ST2$^{-/-}$ mice fed an HFD compared with diet-matched WT mice.

B. There was no difference in fasting blood glucose levels between groups. Fasting serum insulin levels was significantly lower in ST2$^{-/-}$ mice compared with WT mice, both fed an HFD.

C. Total cholesterol levels in the sera were significantly higher in the HFD-fed groups compared with the respective chow diet-fed groups. ST2$^{-/-}$ mice fed an HFD had significantly higher levels of serum triglycerides compared with ST2$^{-/-}$ as well as WT mice fed a chow diet.

D. ALT activity was significantly increased in WT and ST2$^{-/-}$ mice fed an HFD compared with the respective chow-fed groups. There were no differences in AST activity between groups.

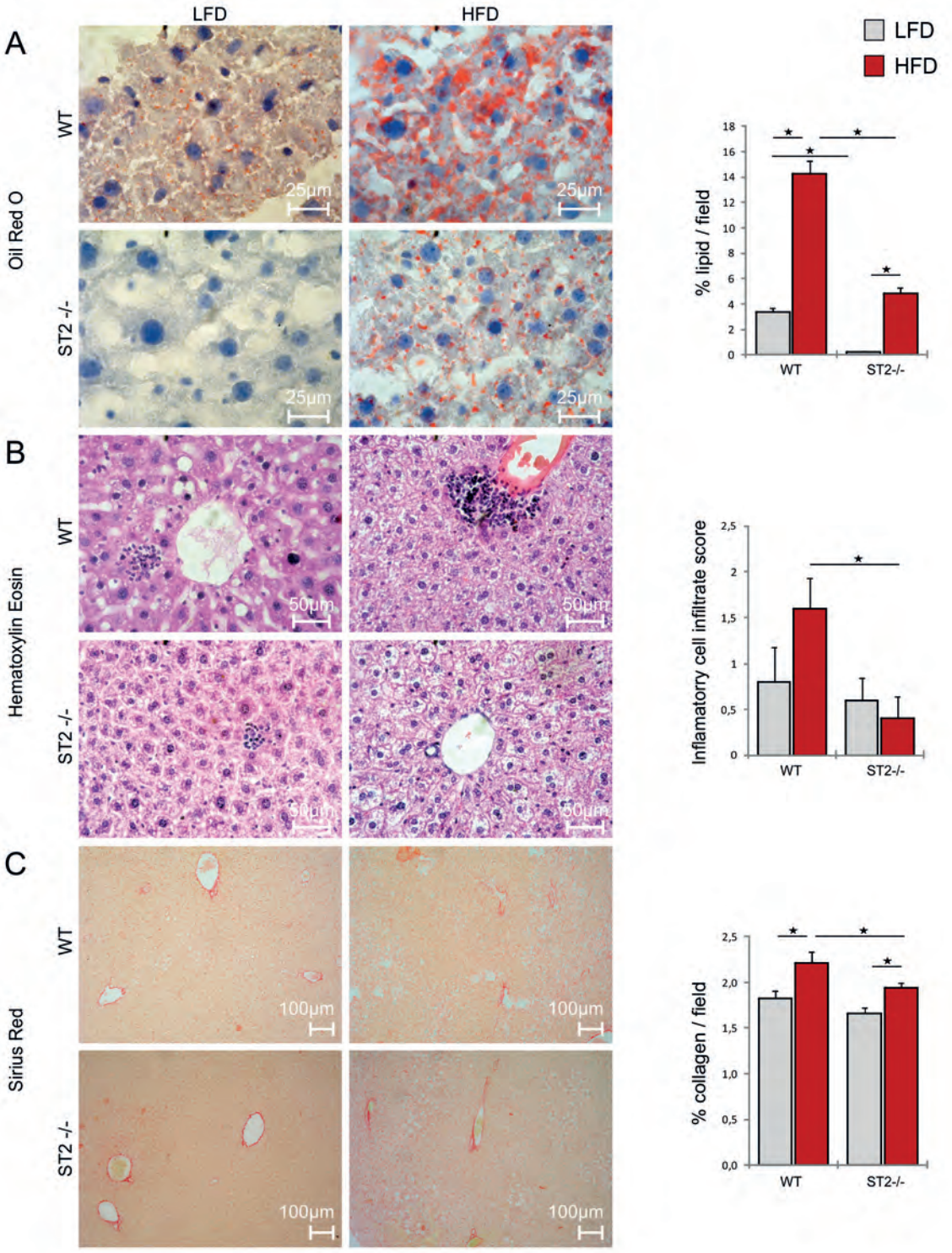

Figure 2. Liver steatosis, inflammation and fibrosis

A. Significantly higher liver steatosis was observed in WT mice fed an HFD compared with WT mice fed chow. Significantly higher liver steatosis was observed in ST2$^{-/-}$ mice fed an HFD compared with ST2$^{-/-}$ mice fed chow. Liver steatosis was significantly lower in ST2$^{-/-}$ mice than in WT mice, both fed an HFD.

B. Liver inflammation, as evaluated by the inflammatory cell infiltrate score, was significantly lower in HFD-fed ST2$^{-/-}$ mice compared with HFD-fed WT mice.

C. The degree of liver fibrosis was significantly higher in mice fed an HFD compared with mice fed a chow diet in both genotypes. The extent of liver fibrosis was significantly lower in ST2$^{-/-}$ mice fed an HFD compared with diet-matched WT mice.

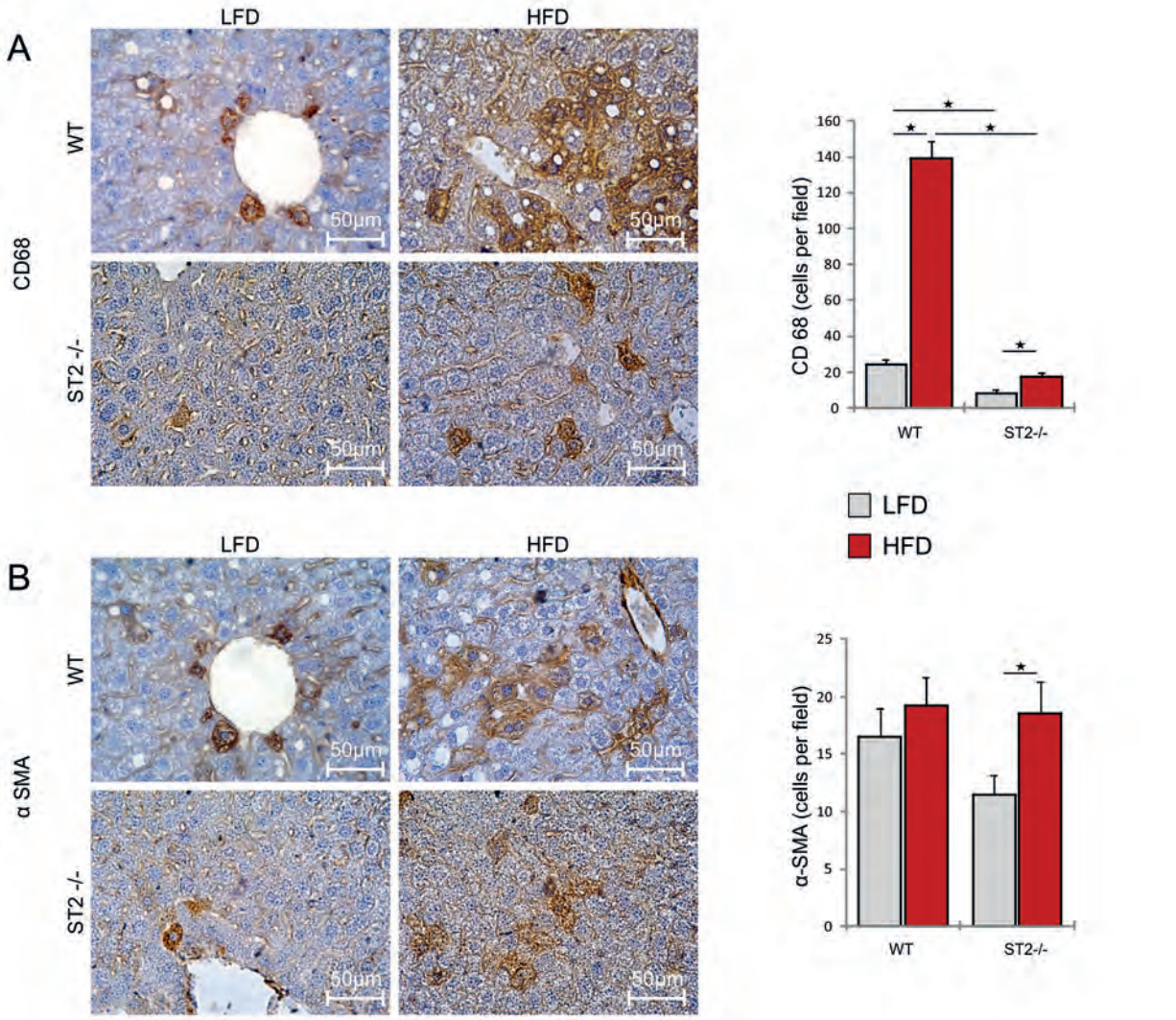

Figure 3. Immunohistochemical staining in liver

A. Number of αSMA positive cells was significantly lower in ST2-/- mice on chow diet compared to ST2-/- mice on HFD, with no differences between the genotypes of mice

B. The number of CD68+ macrophages was significantly lower in ST2-/- mice on chow diet compared to ST2-/- mice on HFD as well as WT mice on chow. ST2-/- mice on HFD had significantly lower number of CD68+ macrophages compared to WT mice on HFD. Wild type mice on chow had significantly lower number of CD68+ macrophages compared to WT mice on HFD.

HFD significantly increased the number of $CD68^+$ macrophages in livers in WT and $ST2^{-/-}$ mice. However, the number of $CD68^+$ macrophages was significantly lower in chow- or HFD-fed $ST2^{-/-}$ mice compared with diet-matched WT mice (Fig 3A). HFD feeding significantly increased the number of αSMA-positive myofibroblasts in the livers of $ST2^{-/-}$ mice only; there was no difference in the number of αSMA-positive cells between the two genotypes of mice fed chow or an HFD (Fig. 3B).

Immune cell composition in the livers of WT and $ST2^{-/-}$ BALB/c mice

We analysed several populations of innate immune cell and lymphocyte subpopulations. HFD feeding increased the percentage of $F4/80^+$ macrophages in the livers of WT mice; in contrast, no significant differences in the proportion of these cells were found between WT and $ST2^{-/-}$ mice on an HFD. The percentage of $CD11c^+F4/80^-$ dendritic cells (DCs) was significantly higher in both genotypes fed an HFD compared with chow-fed mice. $CD11c^+$ DCs were significantly lower in the livers of $ST2^{-/-}$ mice fed either chow or an HFD compared with diet-matched WT mice (Fig. 4A).

HFD feeding significantly increased the percentage of $CD11b^+Ly6C^{low}$ cells in WT mice compared with HFD-fed $ST2^{-/-}$ mice. In contrast, HFD increased the percentages of $CD11b^+Ly6C^{high}$ cells in both genotypes, and the proportion of these cells was significantly higher in both chow- and HFD-fed $ST2^{-/-}$ mice compared with diet-matched WT mice (Fig. 4B).

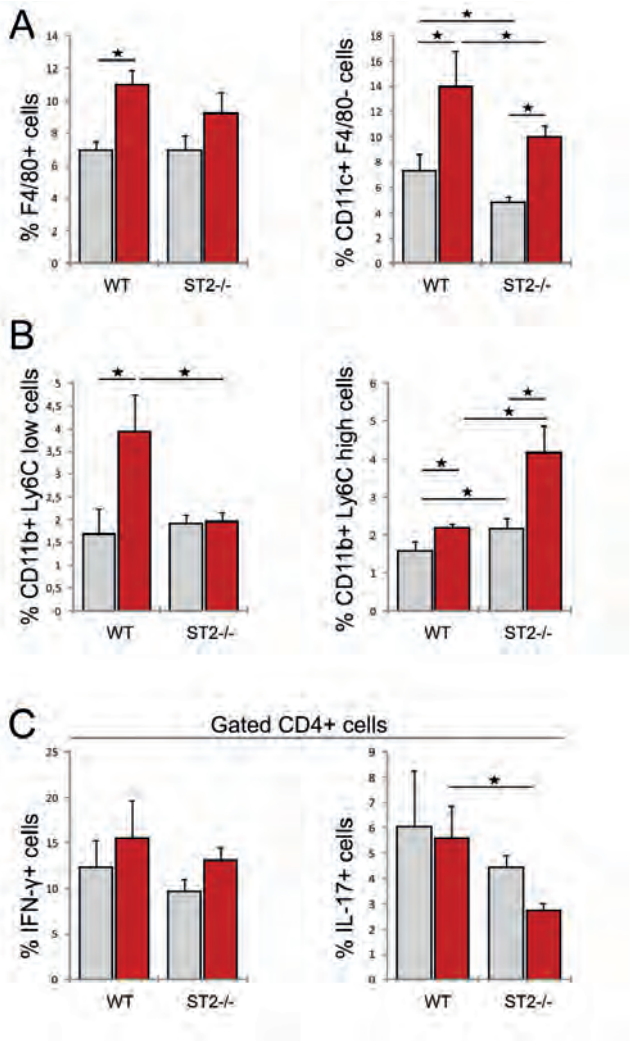

Figure 4. Phenotypic analysis of immune cells in the liver

A. The percentage of F4/80^{+} macrophages was significantly higher in WT mice fed an HFD compared with WT mice fed a chow diet. The percentage of CD11c^{+}F4/80^{-} cells was significantly higher in groups fed an HFD compared with the chow-fed groups. The percentage of CD11c+F4/80^{-} cells was significantly lower in ST2$^{-/-}$ mice fed a chow diet compared with WT mice fed chow and was also significantly lower in ST2$^{-/-}$ mice fed an HFD compared with WT mice fed an HFD.
B. The percentage of CD11b^{+}Ly6C^{low} cells was significantly higher in WT mice fed an HFD compared with WT mice fed chow as well as with ST2$^{-/-}$ mice fed an HFD. The percentage of CD11b^{+}Ly6C^{high} cells was significantly higher in both the chow- and HFD-fed ST2$^{-/-}$ mice compared with diet-matched WT mice.
C. Among the gated CD4+ cells, no difference in the percentages of IFN-γ producing cells was found. The percentage of IL-17-producing CD4+ cells was significantly lower in ST2$^{-/-}$ mice fed an HFD compared with WT mice fed an HFD.

Among gated CD4^{+} T cells, no difference in the percentage of IFN-γ producing cells was observed among the genotypes of mice fed either chow or an HFD. However, the percentage of IL-17-producing CD4^{+} T cells was significantly lower in HFD-fed ST2$^{-/-}$ mice compared with HFD-fed WT mice (Fig. 4C).

The expression of genes related to liver lipid metabolism and fibrosis and cytokine profiles in the sera of WT and ST2$^{-/-}$ BALB/c mice

HFD significantly increased the expression of ATP binding cassette sub-family A member 1 (Abca-1), CD36 and oxysterol receptor LXR-alpha (LXR-α) genes in the livers of WT mice compared with chow-fed animals; no differences were observed in the mRNA levels of peroxisome proliferator-activated receptor gamma (PPAR-γ) (Fig. 5A). LXRα and PPAR-γ expression was significantly lower in HFD-fed ST2$^{-/-}$ mice compared with diet-matched WT mice.

HFD significantly increased expression of the liver fibrosis-related genes collagen alpha 1 chain precursor (procollagen), and alpha smooth muscle actin (αSMA) and the profibrotic genes IL-33 and IL-13 in the livers of WT mice compared with chow-fed animals (Fig 5B). In ST2$^{-/-}$mice, HFD feeding led to significantly increased αSMA expression. The expression of procollagen, IL-33 and IL-13 was significantly lower in ST2$^{-/-}$ vs. WT mice, both fed an HFD. TGF-β precursor expression was not influenced by HFD feeding in both genotypes, although its expression was significantly higher in chow-fed ST2$^{-/-}$ mice compared with diet-matched WT mice (Fig. 5B).

We also analysed serum proinflammatory and profibrotic cytokine levels in WT and ST2$^{-/-}$ mice fed to an HFD for 24 weeks, as indicated in Figure 5. The levels of TNF-α, IL-13 and IL-33 were significantly lower in the sera of ST2$^{-/-}$ mice compared with WT mice (Fig. 5C).

DISCUSSION

Mice on a BALB/c background are relatively resistant to HFD-induced obesity (23). However, we demonstrate that HFD-fed ST2-deficient mice had significantly higher body weight gain compared with wild type mice. It appears that ST2 deletion may partially attenuate the resistance to diet-induced obesity. This result is in accordance with previous studies, which suggested that

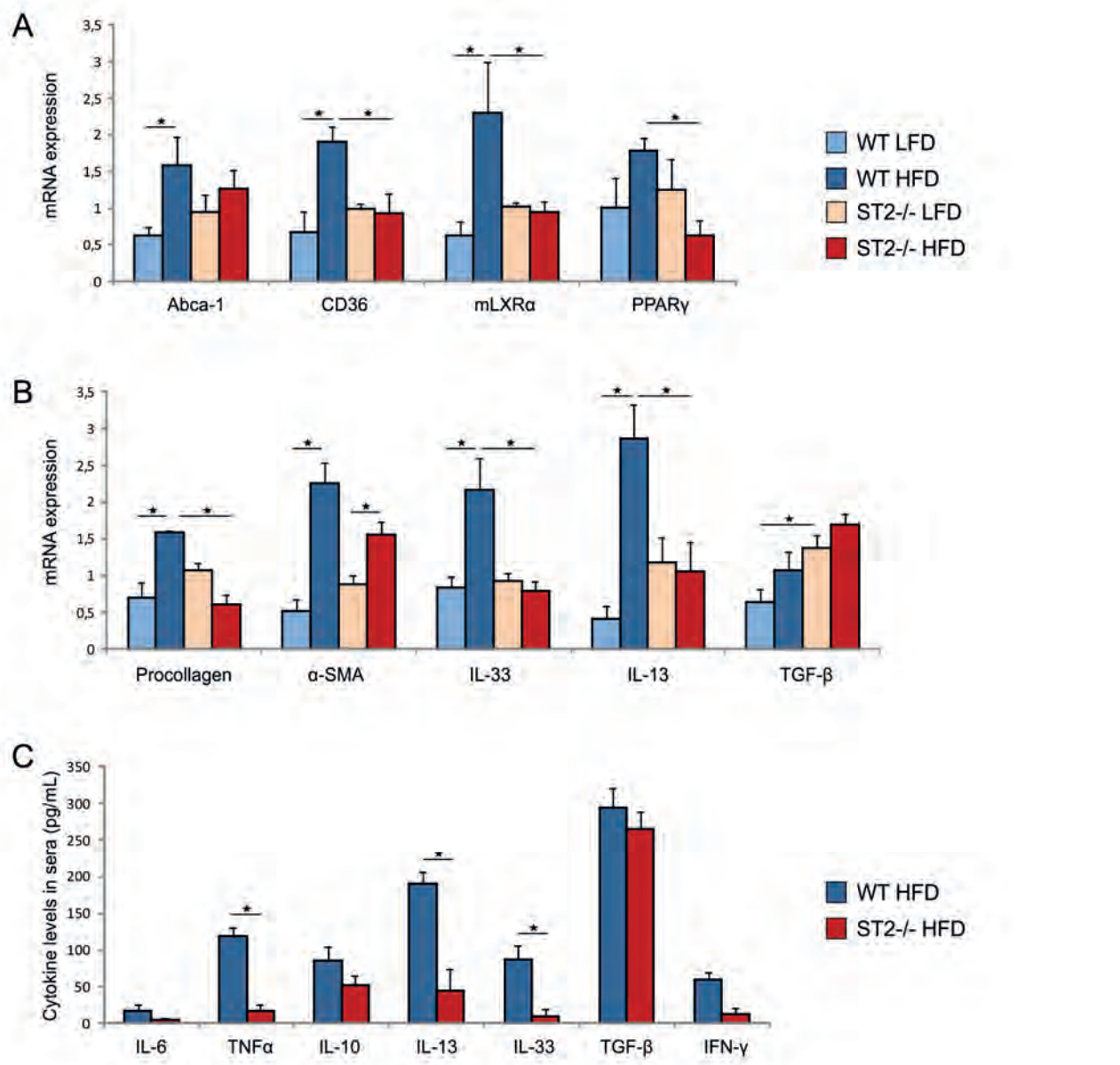

Figure 5. Liver gene expression and cytokine profiles in sera

A. There were no significant differences in the expression of genes related to lipid metabolism in the livers of ST2$^{-/-}$ mice fed chow and an HFD. Significantly increased expression of the Abca-1, CD36 and LXR-α genes was observed in W T mice fed an HFD compared with WT mice fed a chow diet. The expression of CD36, LXRa and PPAR-γ was significantly lower in ST2$^{-/-}$ mice fed an HFD compared with diet-matched WT mice.

B. Expression of the procollagen, alpha smooth muscle actin, IL-33 and IL-13 genes was significantly increased in the livers of WT mice fed an HFD compared with WT mice fed chow. The expression of αSMA was significantly higher in the livers of ST2$^{-/-}$ mice fed an HFD compared with ST2$^{-/-}$ mice fed chow. The expression of IL-33 and IL-13 was significantly lower in ST2$^{-/-}$ mice fed an HFD compared with WT mice fed an HFD. Expression of the TGF-β precursor was significantly higher in ST2$^{-/-}$ mice fed chow compared with diet-matched WT mice.

C. The levels of TNF-α, IL-13 and IL-33 were significantly lower in the sera of ST2$^{-/-}$ mice fed an HFD compared with diet-matched WT mice.

IL-33 plays a protective role in obesity (12). Miller et al. have previously demonstrated that exogenous IL-33 exerted protective effects on adiposity and inflammation, and Pantic et al. showed that ST2 deletion enhanced visceral adiposity and inflammation in BALB/c mice. In line with these studies (12,24) we also demonstrate the higher amount of visceral adipose tissue in ST2$^{-/-}$ mice maintained on an HFD. Our main objective in this study was to investigate the unresolved role of the IL-33/ST2 axis in the development of hepatic steatosis. We demonstrate here that HFD-induced steatosis was ameliorated in ST2$^{-/-}$ mice compared with wild-type mice. Furthermore, the extent of liver steatosis was also significantly lower in ST2$^{-/-}$ mice on a standard diet compared with diet-matched WT mice. The lack of IL-33/ST2 signalling resulted in increased visceral fat weight and hypertriglyceridemia and attenuated liver steatosis in mice fed an HFD. This finding was somewhat unexpected consider-

ing that enhanced lipolysis in enlarged adipose tissues in obesity was shown to be the main contributing factor in the development of liver steatosis (9, 25). Adipose tissue lipolysis is the catabolic process leading to the breakdown of triglycerides stored in fat cells and the release of fatty acids and glycerol (26). Our findings imply that the protective effect of IL-33 in the obesity-associated enlargement of visceral adipose tissue is not exerted on hepatic steatosis. The role of IL-33/ST2 signalling in hepatic steatosis has not been investigated. Recent report suggests that IL-33/ST2 expression may promote maternal lipolysis during pregnancy (27). It could be speculated that the discrepancy between the protective effects on HFD-induced adiposity and enhanced liver steatosis may be related to the presumption that IL-33 promotes lipolysis and the "relocation" of fatty acids in the liver. Furthermore, we demonstrated that WT mice fed an HFD have increased expression of genes associated with lipid metabolism in the liver. The expression of fatty acid translocase (CD36/FAT) was significantly higher in WT mice than in $ST2^{-/-}$ mice, both fed an HFD. When fatty acids are released from adipose tissue stores, they enter the circulation as FFAs. CD36 is a molecule involved in the uptake of fatty acids by cells (28). We show markedly lower LXRα expression in HFD-fed $ST2^{-/-}$ mice compared with HFD-fed WT mice. Liver X receptor alpha (LXRα) is oxysterol-activated nuclear receptor that is expressed in the liver and in other tissues and that regulates inflammation and lipogenesis. It has been demonstrated that LXRα activation has potentially deleterious effects by promoting hepatic steatosis and insulin resistance (29,30). As opposed to $ST2^{-/-}$ mice, WT mice fed an HFD had increased expression of peroxisome proliferator-activated receptor (PPARγ), which has been associated with exacerbated steatosis when overexpressed in hepatocytes (31).

Our findings point to an important role for IL-33/ST2 signalling in obesity-associated changes in lipid metabolism in the liver. A recent study showed that the development of hepatic steatosis requires IL-1 signalling (10), and considering that the IL-33/ST2 axis shares similar downstream molecules with the IL-1 pathway, IL-33/ST2 signalling should be further explored in hepatic lipogenesis. Recently, the direct role of IL-17 in liver steatosis and fibrosis has been demonstrated (32,33). In accordance with this finding, we showed that IL-17-producing $CD4^+$ cells were less numerous in the livers of HFD-fed $ST2^{-/-}$ mice compared with diet-matched WT mice. Liver inflammation was attenuated in $ST2^{-/-}$ mice fed an HFD, as evaluated by lower inflammatory scores and lower numbers of $CD68^+$ macrophages and percentages of $CD11c^+$ DCs in the livers of these mice compared with diet-matched WT mice. Liver damage due to pronounced steatosis in WT mice has been coupled with on-going fibrosis. In contrast to HFD-fed $ST2^{-/-}$ mice, higher gene expression of procollagen, IL-33 and profibrotic IL-13 observed in the livers of HFD-fed WT mice supports the notion of the profibrotic role of IL-33/ST2 signalling in the liver. This was accompanied by increased collagen deposition in steatotic livers of HFD-fed WT mice and increased sera levels of IL-33 and IL-13. In addition, we also demonstrated that $CD11b+Ly6c^{low}$ monocytes, which are cells with profibrotic or M2-type functions in the liver (34,35,36), were more numerous in the livers of HFD-fed WT mice compared with diet-matched $ST2^{-/-}$ mice.

Conclusion

In summary, our findings are compatible with the notion that the IL-33/ST2 (IL-33R) axis may play multiple roles in obesity-associated metabolic disorders and NAFLD. IL-33/ST2 signalling attenuates adiposity and inflammation in visceral adipose tissue but promotes liver steatosis, inflammation and fibrosis, most likely by modulating cell trafficking and the metabolic pathways that link adipose and liver tissues in obesity.

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Disclosure

The authors declare that they have no competing interests or other interests that might be perceived to influence the results and discussion reported in this paper.

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SIMPLE AND COMPLEX COGNITIVE FUNCTIONS UNDER EXERTIONAL HEAT STRESS

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UTICAJ TOPLOTNOG STRESA I FIZIČKE AKTIVNOSTI NA KOGNITIVNE SPOSOBNOSTI

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ABSTRACT

Heat stress is a significant problem in the military services. This study investigated the effects of exertional heat stress on cognitive performance.

Forty unacclimated male soldiers performed exertional heat stress tests in cool (20 °C) and hot environments (40 °C). Cognitive performance was assessed using a computerized battery before and immediately after tests. Physical strain in cool conditions induced mild but significant deficits in accuracy in complex tests. The number of correct answers in the Matching to Sample Visual Search was reduced (92,18% correct answers before vs. 88,64 after; p<0,05) and also in the spatial part of the Pattern and Spatial Recognition Memory Test (85,25 vs. 8,75%; p<0,05). These decreases were more pronounced in hot conditions (92,38 vs. 84,31% in before and 84,21 vs. 73,42% in the latter test; ps<0,01 and <0,001, respectively). Exertional heat stress also impaired more simple cognitive functions. A significant decrease in accuracy (95,74 vs. 93,89%) and an increase in reaction time (300,32 vs. 315,00 ms) was observed in the Reaction Time test.

Strenuous physical activity in a hot environment induces mild cognitive deficits, especially in more complex tasks.

SAŽETAK

Toplotni stres predstavlja značajan problem u vojnoj službi. U ovom istraživanju se ispituje uticaj toplotnog stresa i fizičke aktivnosti na kognitivne sposobnosti.

40 neaklimatizovanih vojnika muškog pola podvrgnuto je testu toplotnog stresa i fizičkog napora u termoneutralnoj (20 °C) i toploj sredini (40 °C). Kognitivne funkcije su ispitivane kompjuterizovanom baterijom neposredno pre i nakon testa. U termoneutralnim uslovima, fizički napor je izazvao blagi pad preciznosti pri rešavanju složenih zadataka u testu Matching to Sample Visual Search (92,18% vs. 88,64; p<0,05), i prostornoj komponenti testa Pattern and Spatial Recognition Memory (85,25 vs. 78,75%; p<0,05). Pad preciznosti je naglašeniji u toplim uslovima (92,38 vs. 84,31%; p<0,01, odnosno 84,21 vs. 73,42%; p<0,001), gde je, osim toga, došlo i do oštećenja jednostavnijih kognitivnih funkcija: u testu Reaction Time zabeležen je pad preciznosti (95,74 vs. 93,89%), kao i produženje vremena reakcije (300,32 vs. 315,00 ms).

Intenzivan fizički napor u toploj sredini dovodi do blagih kognitivnih deficita, pogotovo pri vršenju složenih zadataka.

ABBREVIATIONS

CANTAB - Computerized Cognitive Attention Battery
EHS - Exertional Heat Stress
EHST - Exertional Heat Stress Test
HR - Heart Rate
Tty - Tympanic temperature
WBGT - Wet Bulb Globe Thermometer

INTRODUCTION

Heat stress is a significant problem in the military services. Common preventive measures (e.g., restriction of physical activity, removal of clothing and moving into shade) are often impossible to achieve. Additional disadvantages include the burden of heavy equipment, ballistic vests, and other heavy items that must be worn during training and regular activities, and impermeable protective suits, which may inhibit evaporative cooling. Heat stress can impair physical and mental performance (1,2). Studies of cognitive performance under hot conditions are

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difficult to evaluate because of variations in methodological design, exposure duration, skills and acclimation level. Human tolerance to heat stress also depends on fitness levels. Individuals with higher aerobic capacity show better adaptation to the combination of physical activity and hot environments (3).

Computerized batteries are applied to minimize bias in assessments of cognitive performance during physical exertion and/or heat stress (4, 5). CANTAB (computerized Cambridge Neuropsychological Test Automated Batteries) is a set of neuropsychological tests that are specifically designed for comparative assessments of cognition. The tests are graded in nature, with standardized feedback and a detailed recording of accuracy and speed.

Our study investigated the effects of strenuous physical activity in combination with a hot environment on cognitive performance in young fit male soldiers using computerized test batteries.

SUBJECTS AND METHODS

The participants were 40 male soldiers, aged 20±0,9 years, with similar anthropometric and ergometric characteristics (Table 1). Subjects signed an informed consent form after a short briefing on the nature and purpose of the experiment. The Ethical Committee of the Military Medical Academy approved the experimental protocol. The procedures corresponded to international standards of thermal strain evaluation (6, 7). The subjects were randomly divided in two equal groups. The experimental group (H group) performed the exertional heat stress test (EHST) in a hot environment (40 °C, 29 °C WBGT), and the control group (C group) performed the same test in a cool environment (20 °C, 16 °C WBGT). Trials were conducted in a climatic chamber in the Military Medical Academy, Institute of Hygiene, Department of Physiological Strain, during November and December 2011. Parameters of heat stress (tympanic temperatures and heart rates) were automatically monitored and recorded in real-time using a physiological data monitoring system (MP150SKT100C, BIOPAC Systems Inc., USA), and the Q4500 Exercise Test Monitor (Quinton Instruments, USA) respectively. The test and measurement protocols were described in detail in our previous studies (8, 9). Tests were limited to 90 minutes because of the temperature conditions and level of physical exertion. The following criteria were used for early termination: Tty 39,5 °C, HR 190 bpm, or intolerable subjective discomfort. Cognitive performance was assessed using CANTAB, version 2,0, which consisted of 5 different tests that measure selective, divided, and sustained attention, information processing, working memory and recognition (10). Tests were administered using a computer with a touch-sensitive screen, and a response key was used for reaction timing. Cognitive examinations were administered to each soldier before and immediately after EHST, and each examination included the following tests: Motor Screening (MOT), Reaction Time (RTI), Matching to Sample Visual Search (MTS), and Pattern and Spatial Recognition Memory, which includes visual and spatial components (PSRp and PSRs). Accuracy (percentage of correct answers or number of errors) and speed in ms (reaction time, movement time, or attention time) were recorded for each test.

The Statistical Package for the Social Sciences (SPSS 20.0) was used to perform all statistical tests. Continuous variables are presented as means ± standard deviation. Normal distribution was tested using the Kolmogorov-Smirnov test. Differences between performances before and after EHST were tested using repeated measures *t*-test. Significance was accepted at $p<0,05$.

Table 1. Anthropometric and ergometric parameters in both investigated groups

	H	C	*p* (H vs. C)
Body height (cm)	180,63 ± 5,71	179,55 ± 4,57	0,595081
Body weight (kg)	74,85 ± 9,66	78,13 ±5,32	0,656162
Body Mass Index (kg/m²)	22,9 ± 2,62	24,29 ± 1,95	0,803931
Percentage of body fat (%)	16,86 ± 3,62	17,06 ± 4,47	0,710612
VO_{2max} (ml/min/kg)	59,55 ± 10,64	56,60 ± 5,91	0,235727

VO_{2max} - Maximal oxygen consumption

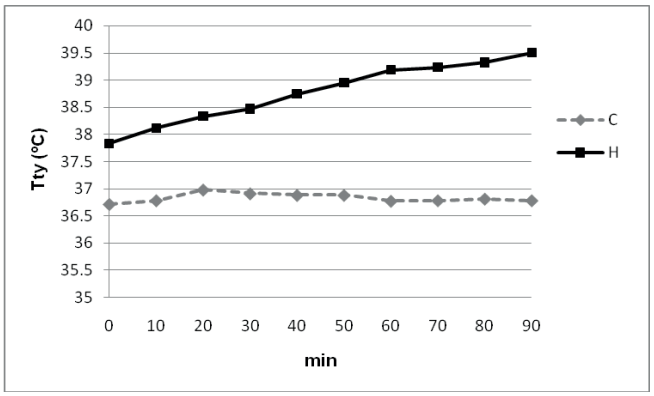

Figure 1. Average tympanic temperatures during the tests in both investigated groups

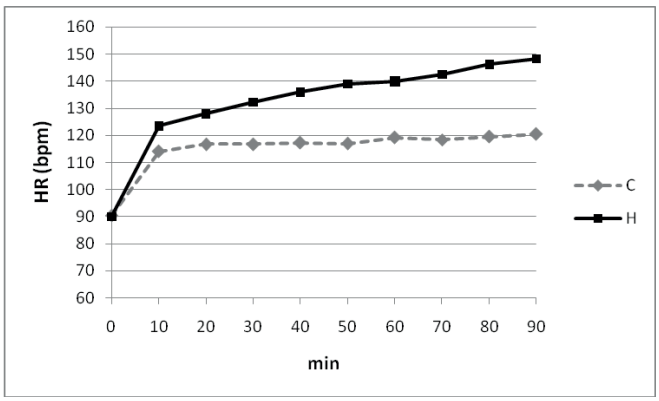

Figure 2. Average heart rates during the tests in both investigated groups

Table 2. Cognitive performance in both investigated groups

Test	Parameter	H		C	
		Before	After	Before	After
MOT	Latention (ms)	525,00 ±60,88	624,37 ±197,10	560,00 ±99,32	625,15 ±146,67
	Errors (N)	27,00 ±7,17	26,42 ±8,19	29,75 ±13,52	25,35 ±11,76
RTI	Correct answers (%)	95,74 ±3,52	93,89 ±3,43 *	96,77 ±3,59	96,82 ±3,04
	Reaction time (ms)	300,32 ±31,53	315,00 ±33,77 *	308,70 ±50,15	308,9 ±35,52
	Movement time (ms)	367,42 ±102,95	373,21 ±117,67	400,20 ±112,37	394,25 ±128,15
MTS	Correct answers (%)	92,38 ±4,90	84,31 ±9,51 **	92,18 ±5,14	88,64 ±6,95 *
	Latention (ms)	1564,47 ±507,41	1694,89 ±529,68	1604,80 ±526,77	1579 ±522,37
PSRp	Correct answers (%)	90,56 ±5,71	87,94 ±9,70	93,54 ±5,14	90,98 ±7,41
	Latention (ms)	1436,37 ±287,91	1371,42 ±312,63	1380,80 ±262,08	1325,75 ±254,29
PSRs	Correct answers (%)	84,21 ±8,54	73,42 ±10,01 ***	85,25 ±7,52	78,75 ±15,55 *
	Latention (ms)	1558,79 ±428,23	1653,79 ±444,71	1524,05 ±342,89	1481,65 ±380,81

* p<0,05 after vs. before
** p<0,01 after vs. before
*** p<0,001 after vs. before

RESULTS

Table 1 shows the anthropometric and ergometric characteristics of the subjects. Both groups were similar in all investigated features. No soldier showed any symptoms of heat exhaustion or other severe heat illness during or after EHST. All participants in the C group completed the tests, but 3 soldiers in the H group terminated tests between 60 and 85 minutes because of subjective intolerable strain or the reaching of the Tty limit. Figure 1 displays comparable reviews of Tty values during EHST. The mean Tty was significantly higher in the H group at the end of EHST (i.e., 90th minute) (39,5±0,16 °C vs. 36,78±0,11 °C in the C group; p=0,0006). The total increase in Tty values (average difference between Tty at the beginning and end of EHST) was also significantly higher in the H group (1,56±0,26 °C vs. 0,07±0,03 °C in the C group; p=0,009). Figure 2 displays comparable reviews of HR values during EHST. Mean HR at the end of EHST was significantly higher in the H group (148,90±5,90 vs. 120,50±7,60 bpm; p=0,0000). The limit of 180 bpm was never reached, and the maximum recorded hart rate was 165 bpm. Table 2 summarises performance task means. There was a significant decrease in the percentage of correct answers in the C group in the MTS test after EHST (88,64±6,95 after EHST vs. 92,18±5,14% before; p=0,038) and the PSRs test (78,75±15,55 vs. 85,25±7,52%; p=0,037). Decreases in the accuracy of both of these tests were more significant in the H group (MTS 84.31±9,51 vs. 92,38±4,90% and PSRs 73,42±10,01 vs. 84,21±8,54%; ps=0,002 and 0,0006, respectively). There was also a statistically significant decrease in performance in the RTI test in the H group. A decrease in the percentage of correct answers (93,89±3,43 vs. 95,74±3,52%; p=0,41) was accompanied by a significant increase in reaction time (315,00±33,77 vs. 300,32±31,53 ms; p=0,048). There were no significant differences between responses for latency or number of errors before and after EHST in other tests, regardless of ambient conditions.

DISCUSSION

Heat strain impairs working efficiency and induces cognitive deficits. These abilities are particularly important in military services, where a dose-dependent relationship between heat stress and the number of errors in helicopter pilots is well established in laboratory and terrain conditions (11,12). Banta and Braun also found that Navy helicopter pilots wearing individual cooling suits during the Gulf war to mitigate the detrimental effect of heat stress on cognition showed improved vigilance and accuracy (13). The possible beneficial effects of microclimate cooling were also of interest in the Army of Serbia. Jovanovic et al. demonstrated the efficiency of individual microclimate cooling systems based on phase-change materials, which alleviated the physical

exertion caused by uncompensable heat stress (8, 9). They investigated male soldiers who performed EHST wearing impermeable protective suits and various types of microclimate cooling vests in a simulated environment (climatic chamber) and external conditions. They concluded that the use of cooling vests under protective suits during physical activity in hot conditions reduced sweating and alleviated heat stress, which was measured by increased core temperature and heart rate values. These cooling vest-induced physiological changes directly improved heat tolerance and hydration state, decreased the risk of heat illness, and extended the duration of soldiers' exposure to hot conditions. Dehydration itself profoundly influences cognitive performance (14), and the use of microclimate cooling systems may represent a promising solution to maintain vigilance and accuracy in soldiers exposed to heat stress.

Our results indicate that physical strain itself induced mild cognitive deficits, which was shown in the increased number of errors during the performance of complex tasks (i.e., MTS and the spatial part of the PSR test). These tests are designed to investigate working memory and sustained attention, and the latter test also investigates spatial orientation, which is a very specific and advanced function that is highly important in military services. The generally good performance of our soldiers in cool conditions is consistent with the results reported by other authors, who found that an acute boost of exercise may improve cognition (15). Mild deficits in our investigation may be caused by the longer duration and more strenuous physical activity compared to the study cited above.

The combination of the same intensity of physical strain and heat stress in our investigation led to more pronounced cognitive impairments in the same complex test, but it also imposed additional effect on attention, which are tested in the RTI. Soldiers in hot conditions showed a significant decrease in accuracy and a significant delay in reaction time in the RTI. The only function that was unaffected in this test was movement time. This mild cognitive deficit can likely be attributed to heat stress.

Hancock and Vasmatzidis stated in their review (16) that there are major difficulties in the interpretation of data on the influence of heat stress on cognitive functions. These difficulties are the consequences of various testing procedures, task demands and categorisation, duration of heat exposure, and subjects' skills and experience. These authors suggest that simple mental tasks show little, if any, decrements following heat exposure, and these tasks are frequently enhanced during brief exposures. However, substantial heat stress (30-33 °C WBGT) may lead to impairments in the performance of more complex tasks. The obtained results are consistent with the high WBGT (29 °C) in our trial.

Most of our subjects who performed tests in a hot environment suffered intensive physical strain, which was recorded by the high values of core temperature (up to 39,5 °C) and heart rates for a substantial time. However, these subjects did not show signs of serious cognitive deficits. Their relative resistance to heat exertion may be attributed to their high aerobic capacity. This hypothesis is supported by the facts that some aspects of heat stress-induced cognitive impairment are explained by cerebral changes (17,18), blood oxidative stress (19), and acute inflammatory reactions (20,21). Several investigations (22,23) confirmed that regular aerobic activity significantly induces beneficial adaptive effects on oxidative stress responses and inflammation parameters to acute exhausting endurance exercise in the heat, and our results may be interpreted from this point of view.

CONCLUSIONS

This study demonstrated the effects of strenuous physical activity in a hot environment on physiological parameters, which showed higher values of tympanic temperature and heart rate compared to cool conditions. This physiological heat stress enhanced the cognitive impairment caused by physical exertion itself. Performance deficits in a cool condition were mild and present only in complex tasks, but simpler tasks were relatively unaffected. Performance deficits in hot conditions were more pronounced and present in both complex and simpler tasks, such as reaction time.

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PERIODONTAL DISEASE AND RISK FOR PRE-TERM BIRTH: A CASE-CONTROL STUDY

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PARODONTOPATIJA I RIZIK ZA NASTAJANJE PREVREMENOG PORAĐAJA: STUDIJA SLUČAJ-KONTROLA

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ABSTRACT

Maternal periodontal infection has been recognized as a risk factor for preterm and low birth weight infants. It is hypothesized that pathogens causing periodontal disease might translocate to the amniotic cavity and contribute to triggering an adverse pregnancy outcome. The growing evidence that an infection remote from the foetal-placental unit might have a role in preterm delivery has led to an increased awareness of the potential role of chronic bacterial infections in the body. The aim of this study was to evaluate whether the presence of chronic periodontitis might influence the incidence of preterm labour and preterm birth.

This study was designed as a hospital-based case-control study. Seventy pregnant women aged 18-40 years, with a single live pregnancy were recruited from the Department of Gynaecology and Obstetrics of a general hospital in Sibenik, Croatia, from March 2013 to March 2014.

The case group included: 30 pregnant women who were hospitalized with signs of preterm labour. The control group included 40 normal pregnancy patients, who were analysed for up to 48 h after the delivery of a term baby having a birth weight of more than 1500 g. A full-mouth periodontal examination was performed on all the patients. Information was collected on the demographics, health behaviours, and obstetric and systemic diseases that might have an influence in preterm delivery.

The presence of chronic periodontitis tended to be higher in women with a preterm delivery (the case group), with 20 cases (66%), than in the women in the, control group, in which chronic periodontitis was found in 14 cases (35%); this difference reached statistical significance (p≤0.01). The PTB cases had a significantly worse periodontal status than the controls (p≤0.001). From the PTL group, 18 patients delivered preterm, and chronic periodontitis, found in 15 cases (83%), was more prevalent than in the control group. The risk of women having periodontitis or attachment loss ≥ 4 mm developing PTB showed an OR of 3.7 (95% CI: 1.91 to 4.86; P< 0.001).

SAŽETAK

Infekcija parodoncijuma majke se smatra faktorom rizika za prevremeni porođaj i malu telesnu masu novorođenčeta. Pretpostavlja se da patogeni koji uzrokuju parodontopatiju mogu da dospeju u amnionsku šupljinu i da doprinesu pokretanju događaja koji za posledicu imaju naželjeni ishod trudnoće. Sve veći broj dokaza koji ukazuje na činjenicu da infekcije udaljene od feto-placentalne jedinice mogu da imaju ulogu u nastajanju prevremenog porođaja su izazvali povećanje interesovanja za potencijalnu ulogu hroničnih bakterijskih infekcija u organizmu. Cilj ove studije je procena povezanosti hronične parodontopatije i incidencije prevremenog porođaja.

Studija je dizajnirana kao hospitalna slučaj-kontrola studija. 70 trudnica, starosti od 18 do 40 godina, sa prvom uspešnom trudnoćom, su ispitivane između marta 2013. i marta 2015. godine, na Odeljenju ginekologije i akušerstva Opšte bolnice u Šibeniku, Hrvatska.

Grupa „slučajeva": Ovu grupu je činilo 30 trudnica hospitalizovanih sa znacima prevremog porođaja. Kontrolna grupa: Kontrolnu grupu su činile trudnice sa normalnom trudnoćom, koje su ispitivane 48 h nakon porođaja, čije je dete rođeno u terminu i imalo težinu na rođenju iznad 2500 g. Svim pacijentkinjama je izvršen pregled parodoncijuma celih usta, a prikupljeni su i demografski podaci, informacije o zdravstvenim navikama, akušerskim i sistemskim oboljenjima koje mogu da utiču na porođaj.

Hronični parodontitis je bio više zastupljen kod žena koje su imale prevremeni porođaj (grupa „slučajeva") – 66% (20 žena) u poređenju sa ženama koje su se porodile u teminu (kontrolna grupa) – 35% (14 žena), i ova razlika je statistički značajna (p≤0,01). Žene u PTB grupi su imale značajno gore stanje parodoncijuma u odnosu na kontrole (p≤0,001). 18 žena iz PTL grupe je rodilo nedonesenu decu, a hroinčni parodontitis je bio češći – 83% (15 žena), u odnosu na kontrolnu grupu. Rizik za nastajanje prveremenog porođaja za žene koje imaju parodontitis ili gubitak vezivnog tkiva ≥4 mm je pokazao OR (odds ratio) od 3,7 (95%CI: 1,91 do 4,86; p<0,001)

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The study shows a significant association between periodontal chronic disease and an adverse pregnancy outcome. Periodontal disease represents a strong, independent risk factor for preterm births, and periodontal prevention and therapy should be a part of preventive prenatal care.

Keywords: *periodontal disease, preterm labour, preterm delivery*

Studija pokazuje značajnu povezanost između hroničnе parodontopatije i neželjenog ishoda trudnoće. Parodontopatija predstavlja jak, nezavistan faktor rizika za prevremeni porođaj. Usled toga prevencija parodontopatije i terapija bi trebalo da budu deo prvencije prvermenog porođaja.

Ključne reči: *parodontopatija, prevremeni porođaj, prevremeno rođenje*

INTRODUCTION

Oral health is an integral component of the general health and well being of an individual. The knowledge regarding the link between periodontal disease and systemic health is increasing rapidly (1). Recently, many epidemiological, clinical and laboratory studies have provided irrefutable evidence that periodontitis is a risk factor for various systemic diseases, such as cardiovascular diseases, atherosclerosis, diabetes mellitus, and pulmonary diseases (2, 3). Periodontal disease refers to a group of endogenous polymicrobial infections that cause inflammation and destruction of the supporting structures of teeth. No overt pathogen has been identified; however, its aetiology is strongly associated with anaerobic Gram-negative bacilli (4). In response to these gram-negative bacteria, the human body activates its host immune response, and immune cells secrete inflammatory mediators. Of particular importance in periodontal disease are cytokines, prostaglandins and matrix metalloproteinases. The destruction of periodontal tissues, including the breakdown of the gingival connective tissue and alveolar bone, results mainly from activation of the immune cells (5). The inflammatory mediators that have been discussed in relationship to incidences of preterm birth include cytokines, prostaglandins and matrix metalloproteinases. Several cytokines play an important role in the initiation and progression of periodontal disease. These cytokines include interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor (TNF-a). Prostaglandins are responsible for most of the alveolar bone destruction observed in periodontal disease, particularly prostaglandin [E. sub. 2] (PGE 2). Matrix metalloproteinases (MMP) are a family of enzymes that work together to destroy connective tissue. It is istheorized that these inflammatory mediators from subgingival plaque are able to enter the bloodstream and travel to the maternal-foetal interface, thus contributing to preterm labour (6). During pregnancy, PGE 2 plays an important role in regulating the onset of labour, contractions and delivery. (PGE 2) levels rise throughout gestation, and when a critical threshold level is reached, labour is induced (7). These associations explain the basic theories for the link between the presence of maternal periodontal disease and the risk for adverse pregnancy outcomes.

However, accumulating evidence demonstrates that oral bacteria might translocate directly into the uterus in-during pregnancy, causing localized inflammation and an adverse pregnancy outcome. Studies in humans and animals have demonstrated that oral bacteria could translocate to the uterus in pregnancy through haematogenous transmission. These recent discoveries shed new light on our understanding of pregnancy complications. (8, 9) Although infections of any type in pregnant women represent a risk for adversities in the developing foetus, periodontal disease might have a significant role in the pathogenesis of adverse pregnancy outcomes.

The term periodontal disease encompasses a variety of diseases ranging from gingivitis to periodontitis. Standard diagnostic tools that are used to determine the presence of periodontal disease include full- mouth periodontal examinations, measurements of the pocket depths (PD), the clinical attachment levels (CAL), gingival recession (GR), visible signs of gingival inflammation (such as bleeding on probing (BOP)) and dental radiographs. No dental radiographs were taken in this study because of the special conditions of the patients.

OBSTRICAL OUTCOMES

Preterm birth (PTB) and low birth weight (LBW) are leading perinatal problems worldwide and have evident public health implications because their incidence does not decrease in spite of the many attempts to prevent perinatal problems. Preterm birth, defined as birth before 37 weeks of gestation, is the leading cause of neonatal mortality, infant morbidity, and long-term sequelae. PTB is a major medical, social, and economic concern. Preterm infants are 75 times more likely to experience early death, and PTB, the leading direct cause of neonatal death, is responsible for 27 per cent of neonatal deaths worldwide, comprising over one million deaths annually (10). The long-term disabilities for surviving preterm infants include pulmonary abnormalities, asthma, cerebral palsy, poor motor skills and neurological or developmental disabilities (11). Human observational studies have identified a number of risk factors for preterm delivery, and some are reversible, whereas others are permanent (12). The risk factors occur in combination, and, therefore, developing effective preventive strategies could be challenging.

Infections and inflammation, maternal or foetal stress, uterine bleeding, and stretching of the uterus are isrecognized as the most common reasons for PTB. Intrauterine infections and maternal bacterial vaginosis are well known risk factors; however, distant infections, even subclinical infections, could induce preterm births. Periodontal disease is associated with infections and inflammation. The presence of periodontal disease and the maternal physical changes that occur during pregnancy enable bacteria to enter the blood stream, leading to "placental seeding" (13, 14). The significance of the relationship of periodontal disease and PTB is evident when the number of affected women and children is considered. At least 20% of the women aged 30 to 50 years have periodontitis. In addition, 12% of all the births worldwide are premature (delivered at less than 37 weeks of gestation); this problem affects many women of child-bearing age in our country, with lasting consequences to their past, present and future children.

The objective of this paper is to examine, from an evidence-based perspective, whether periodontal disease might contribute to the risk for PTB.

MATERIAL AND METHODS

A case control study was conducted in the Department of Obstetrics and Gynaecology at General Hospital Sibenik, R. Croatia, from March 2013 to March 2014. The study was approved by the Institutional Ethics Committee of General Hospital, Sibenik. Informed and written consent was obtained from all of the subjects for this study.

Material

The study population included the following: a case group of 30 pregnant women from 28 to 36+6 weeks of gestation, with clinical signs of preterm labour, who were hospitalized in our department; and a control group of 40 pregnant women with normal pregnancies, who delivered, at term (37-42 g.w), a baby with a birth weight over 2500 gr.

The inclusion criteria included pregnant women, aged 18-40 years, with a single live pregnancy, who signed written informed consent forms.

The Exclusion criteria included: multiple gestation, polyhydramnios, uterine anomalies, a history of second trimester abortion, a history of cervical surgery, cerclage in the present pregnancy, a previous preterm delivery, substance abuse, smoking, an acute symptomatic vaginal infection, and acute oral infection.

Methods

The gestational age of the subjects was determined with the best obstetrical estimation methods using the definitive menstrual history and ultrasonography performed in the first trimester. The diagnosis of preterm labour is generally based on the clinical criterion of regular painful uterine contractions (4 every 20 minutes or 8 every 60 minutes), accompanied by cervical changes, including cervical effacement of at least 80 per cent or cervical dilatation greater than 2 cm. All the subjects with signs of preterm labour were followed and routinely treated in our department, and they were managed according to the hospital protocol for preterm labour.

One dentist performed a full-mouth periodontal examination on all of the patients at the time of their inclusion in the study (the case group, at the time of hospitalization, and the control group, within 2 days of delivery). The periodontal examination was performed with the patient supine on a hospital bed, using artificial light. The following data were recorded: bleeding on probing (BOP), the probing depth (PD) at the floor site per tooth, gingival recession and the clinical attachment level (CAL) on all of the present teeth. The patients were diagnosed with periodontal disease if they exhibited a PD of 4 mm or greater in four or more teeth and a CAL greater or equal to 3 mm at the same site. The BOP was recorded; however, it was not used to determine a diagnosis of periodontal disease. Demographic, socioeconomic and medical data on known risk factors were obtained from the charts of the patients, and an examiner-administered questionnaire was completed. This questionnaire was designed to gather the demographic details, pregnancy and medical history, health behaviour and dental condition. The aim of the questionnaire was to collect information on as many of the known risk factors for an adverse pregnancy outcome as possible.

Statistical analysis:

The sample size was calculated using a statistical package (SPSS Version 10.0, SPSS, Inc., Chicago, IL, USA). Univariate and bivariate analyses were performed. The chi-square test or Fischer's Exact test were used for analysing the categorical variables. To compare the mean values, we performed the t-test. The Spearman correlation coefficient was used to reveal the correlation between the variables. The unadjusted and adjusted ORs were calculated with 95% confidence intervals. Multivariate logistic regression models were developed and performed stepwise to identify the risk factors for PTB; a p value < 0.05 was considered as the cut-off point for the level of significance.

RESULTS

The main demographic and obstetrical data of all the subjects are summarized in Table 1.

A total of 70 pregnant women were included 40 in the control group and 30 in the preterm labour group (PTL). In the case group (PTL), 18 patients did not respond to therapy and delivered a preterm baby (PTB) in our hospital. The age of the subjects was distributed normally, without statistical significance between the mean values ($p= 0.72$). Most of the women had secondary levels of education. There was no significant difference in the marital status or in the pre-natal care. None of the obstetrical risk variables displayed a significant association with PTB in this study.

Table1. Demographic and obstetric information in the control and case groups

Demographic and obstetric information	Control group (n=40)	Group with ptl (n= 30)	Subgroup ptb (n= 18)
Mean age ± SD	26.6 ± 6.0	25.9 ± 4.2	25.4 ± 1.6
First pregnancy [n / (%)]	16 (40%)	11(36,7%)	7 (38,8%)
Second pregnancy	18 (45%)	17 (56.6%)	10 (55,6%)
≥3 pregnancies	6 (15%)	2 (6,7%)	1 (5,6%)
Prenatal care			
No pre-natal care	2 (5%)	0	0
< six visits	11(27.5%)	7 (23,3%)	6 (33,4%)
Six + visits	27(67,5%)	23 (76.7%)	12 (66,6%)
Marital status [n / (%)]			
Single	3(7,5%)	2 (6,7%)	0
Married	22(55%)	18(60%)	10(55,5%)
Non-married couple	15(37,5%)	10(33,3%)	8(44,5%)
Education level [n / (%)]			
Elementary school	2(5%)	3(10%)	2(11,1%)
Secondary school	33(82,5%)	24(80%)	14(77,8%)
University	5(12,5%)	3(10%)	2(11,1%)

ptl- preterm labour; ptb -preterm birth

Table 2. Periodontal condition in patients with preterm labour and the control group subjects

Periodontal condition	Control group (n=40)	Case group (n= 30)	P- Value
Healthy	26 (65%)	10 (33,3%)	< 0.01
Chronic periodontitis	14 (35%)	20 (66,7%)	<0.01
Clinical parameters (mean ± SD)			
PD (mm)	3.1±0.06	4.1±0.09	<0.001
CAL (mm)	2.9±0.04	4.0±0.08	<0.001

PD- probing depth, CAL- clinical attachment level

The periodontal condition is presented in Table 2.

Periodontal disease was diagnosed in 66,7% (20 cases) of the patients with preterm labour and in 35% (14 cases) of the patients in the control group, and this difference reached statistical significance ($p \leq 0.01$). In the group of 18 patients who delivered preterm, chronic periodontitis was more prevalent in 83% (15 cases) more cases than in the control group ($p \leq 0.001$), which is considered to be very statistically significant. The risk for women having periodontitis or an attachment loss ≥ 4 mm to develop a PTB showed an OR of 3.7 (95% CI: 1.91 to 4.86; P< 0.001). Additionally, more CAL was found in the PTB group than in the control group. The only risk factor for preterm delivery that showed statistical significance at 95% CI was periodontal disease.

DISCUSSION

The results of this study support the hypothesis that chronic periodontal infection increases the risk of preterm labour and delivery. The multivariate analysis showed a significant association between PTB and periodontal disease. The main levels of PD and CAL tended to be higher in the preterm labour group (the case group) than in the term delivery group (the control group); the differences were statistically significantly higher in the women with preterm births than in the woman with term deliveries, adjusting for the baseline levels. This study showed a significant risk of preterm delivery in the women with periodontal disease than in the women without. Similar positive associations between periodontal infection and preterm birth were reported by Boggess et al (15). and Sharlene W. J. Afrika (16). This finding suggests that pregnant woman with preterm labour and chronic periodontal infection more often delivered a preterm baby. Gibbs et al (17). provided an excellent outline of the possible association between infections and adverse pregnancy outcomes in their review article. In their hypothesis, microorganisms and their products enter the uterine cavity during pregnancy by an ascending route from the lower genital tract or by a blood-borne nongenital route, causing preterm birth. The growing evidence that infection remote from the foetal-placental unit might have a role in the preterm delivery of LBW infants has led to an increased awareness of the potential role of chronic bacterial infections in the body. For approximately 15 years, maternal periodontal disease has been implicated in a poor pregnancy outcome. In 1996, Offenbacher and his group reported a seven-fold increased risk of a mother with periodontal disease delivering a PTLBW baby (18), and many studies on the subject have been completed, with varying results (19, 20). Lopez et al. confirmed periodontal disease as an independent risk factor and observed that periodontal therapy significantly reduces the incidence of preterm birth with low birth weight in a population of women with periodontal disease (21). A recent meta-analysis confirmed a significant risk of preterm delivery for pregnant women with periodontitis (overall risk ratio: 1.70) and a significant risk for having a low birth weight infant (overall risk ratio: 2.11) (22).

Maternal periodontal infection could directly and/or indirectly influence the health of the foetal-maternal unit (23, 24, 25). In a study evaluating the relationship between foetal inflammatory and immune responses to oral pathogens and the risk for PTB, umbilical cord blood specimens were examined for the presence of foetal immunoglobulin M (IgM) antibody against oral pathogens and levels of C-reactive protein, IL-1, IL-6, TNF-alfa and PGE2. The results showed that the presence of IgM antibodies to oral pathogens and increased levels of TNF-alfa were associated with increased rates of PTB and that the combined effects of foetal IgM, C-reactive protein, TNF-alfa and PGE2 resulted in a significantly increased risk for PTB (26). Hill demonstrated that *Fusobacterium*

nucleatum could be isolated from amniotic fluid cultures of women with preterm labour and intact membranes, suggesting that the transient bacteraemia had originated from the mouth via haematogenous spread and infection of the amniotic fluid through the placenta (27). More recently, Han et al. reported, by association, a term stillbirth case caused by F. *nucleatum*, and she had previously demonstrated that *F. nucleatum* induced premature and term stillbirths in pregnant mice (28, 29). The majority of these species has been associated with pregnancy complications in humans, although their sources of infection were not previously known. The oral cavity might be a significant yet previously overlooked source of infection inside the womb.

Two meta-analyses of case-control studies reported that periodontal diseases in pregnancy significantly increase the risk of a subsequent preterm birth or LBW, and the estimated OR was 1.78 (CI 95%: 1.58, 2.01) for preterm births, 1.82 (CI 95%: 1.51, 1.20) for low birth weight infants and 3.00 (CI 95%: 1.93, 4.68) for preterm low-birth weight infants (30, 31). Studies on the effect of periodontal disease treatment on the pregnancy outcome showed varying results. A study reported that there was a lack of association of periodontitis and preterm birth in a multivariate analysis, which supports the report of a meta-analysis showing a lack of effect of periodontal disease treatment on the preterm birth rate (32). Conflicting results were reported from India showing a significant association of periodontal disease treatment with preterm delivery (33).

Future studies should investigate these mechanisms to understand the host susceptibility to oral-uterine transmission. Only when a thorough understanding of the mechanism is achieved could meaningful intervention studies be designed to utilize effective therapies, target the appropriate populations, and measure the relevant outcomes.

CONCLUSION

It is urgent to determine the extent to which chronic periodontitis affects pregnancy outcomes. Infection of the gingiva and periodontium by gram-negative anaerobic bacteria provide a reservoir for microbial products and sufficiently challenge the host to produce responses that might be deleterious to the pregnant mother and foetus. Future research should focus on the reasons why some women develop adverse pregnancy outcomes because of an oral inflammatory burden whereas others do not. In the future, we hope to identify the women at risk for developing oral bacteria-associated pregnancy complications so that preventive measurements could be taken to manage each case individually. An in-depth knowledge of the disease mechanism is the basis of ispersonalized medicine. Only if there was a clear understanding of the causes would it be possible to develop therapeutic and preventive measures to identify the women at risk as well as to improve the birth outcome, and, ultimately, the quality of lives.

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EARLY CYTOKINE PROFILE CHANGES IN INTERSTITIAL AND NECROTIC FORMS OF ACUTE PANCREATITIS

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RANE PROMENE NIVOA CITOKINA KOD INTERSTICIJALNIH I NEKROTIČNIH OBLIKA AKUTNOG PANKREATITISA

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ABSTRACT

Acute pancreatitis (AP) is a common, potentially lethal, acute inflammatory process with a highly variable clinical course. The aim of this study was to analyse early changes in the serum concentrations of pro- and anti-inflammatory cytokines in the peripheral blood of patients with the interstitial form of acute pancreatitis (IAP) and necrotic acute pancreatitis (NAP), especially in those patients who had lethal outcomes.

The prospective study enrolled 52 patients who were divided into IAP (65.38% of patients) and NAP (34.62% of patients) groups. The serum levels of interleukins (IL) 6, 8 and 10, together with tumour necrosis factor (TNF)-alpha were measured on the 1st and 3rd day of hospitalisation. Significantly higher values of IL-6, IL-8 and IL-10 were found on day 1 and 3 in NAP than in IAP. IL-6 was significantly higher on both days of measurement, whereas IL-10 on the first day and IL-8 on the third day were significantly higher in the group of patients who did not survive in comparison with patients who had the interstitial form of AP.

In conclusion, the data from this study showed that immune suppression and excessive immune stimulation in the first three days after admission could indicate the development of NAP and a potentially lethal outcome.

Key words: *cytokines; pancreatitis; acute; necrotising; survivors*

SAŽETAK

Akutni pancreatitis (AP) je učestao, potencijalno letalni inflamatorni proces sa vrlo varijabilnim kliničkim tokom. Cilj ovog istraživanja je bio da analizira rane promene u serumskim koncentracijama pro- i anti- inflamatornih citokina u perifernoj krvi bolesnika sa intersticijskom formom akutnog pankreatitisa (IAP) i nekrotičnim oblikom akutnog pankreatitisa (NAP), posebno kod onih bolesnika koji su umrli u toku praćenja.

Prospektivna studija je uključila 52 bolesnika podeljenih u grupe IAP (65.38%) i NAP (34.62%). Serumski nivoi interleukina (IL) 6, 8 i 10, kao i tumorskog nekrotišućeg faktora (TNF)-alfa određivani su u prvom i trećem danu hospitalizacije. Vrednosti IL-6, IL-8 i IL-10 bile su značajno više prvog i trećeg dana u NAP nego u IAP grupi. IL-6 je bio značajno povišen u oba dana merenja, dok su IL-10 prvog i IL-8 trećeg dana dostigli značajno više vrednosti u grupi bolesnika sa letalnim ishodom u poređenju sa grupom pacijenata koji su imali intersticijsku formu AP.

U zaključku, podaci iz naše studije pokazuju da imunska supresija i preterana imunska stimulacija u toku prva tri dana posle prijema u bolnicu mogu da ukažu na razvoj nekrotične forme akutnog pankreatitisa i potencijalno letalni ishod.

Ključne reči: *citokini; pankreatitis, akutni, nekrotični; preživeli*

INTRODUCTION

Acute pancreatitis (AP) is a common, potentially lethal, acute inflammatory process with a highly variable clinical course. AP progresses to a severe form in approximately 10-20% of patients, resulting in systemic inflammatory response syndrome (SIRS), multiple organ failure and a prolonged hospitalisation with significant morbidity and mortality (1).

Previously, AP was considered to be a disease of the pancreas. Currently, however, there is strong evidence for systemic effects of the disease. Localised inflammation in the pancreas is the body's initial physiologic protective response, which is generally strictly controlled at the site of injury. Loss of the local control results in excessive uncontrolled activation of inflammatory cells and mediators, which is called SIRS (2).

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Systemic inflammation in AP is concomitantly associated with rapidly strengthening compensatory anti-inflammatory response syndrome (CARS) (3). There are many mediators included in this interplay between SIRS and CARS. In the clinical setting, early diagnosis and, if possible, assessment of the prognosis of AP is a major interest for the clinician.

The principal cells that modulate immune responses are T helper (Th) lymphocytes, which orchestrate the function of other immune cells by cytokine production. Thus, the aim of this study was to analyse the early changes in the serum concentrations of pro- and anti-inflammatory cytokines in the peripheral blood of patients with necrotic and interstitial AP, especially in those patients who had lethal outcomes.

PATIENTS AND METHODS

Patients

We conducted a prospective study that included 52 subjects who were admitted to the Surgical Intensive Care Unit (SICU) of the Clinical Center of Kragujevac, Serbia from October 2011 to July 2013. All ethical approvals were obtained by the local Ethics Committee of the institution, and the research was conducted in accordance with the regulations governing Good Clinical and Laboratory Practices. Informed consent was obtained from all patients before enrolment in the study.

The diagnosis of AP was based on two of the following three criteria: abdominal pain characteristic of AP, serum amylase and/or lipase ≥ 3 times the upper limit of the normal values, and characteristic findings of AP on a CT/US scan. Patients with underlying chronic pancreatitis, those with acute postoperative pancreatitis, pregnant women with acute pancreatitis, patients transferred from other hospitals or other wards to the SICU of the Clinical Center of Kragujevac after more than 48 hours from disease onset, as well as those under 18 years of age were excluded from this study.

Patients were recruited within 24 hours of the time of hospital admission. Clinical data relating to the severity of the disease, the development of organ dysfunction and/or septic complications were prospectively collected in a standardised fashion according to the revised Atlanta Classification (4). For the purpose of detection of (peri) pancreatic necrosis, a single highly experienced radiologist evaluated and interpreted computerised tomography (CT) scans in a blinded manner with respect to the disease outcome, taking into consideration CT examinations that had been performed at least three days after admission to the SICU.

All patients were divided in two groups: interstitial non-necrotic pancreatitis (IAP) and necrotising AP (NAP). The diagnoses were verified by the findings of non-enhancement in the pancreatic parenchyma after contrast administration exceeding 3 cm in size or 30% of the total gland area and/or by heterogeneous fluid collection within the peripancreatic space containing solid material on the abdominal CT scan.

METHODS

Blood samples were collected following patient enrolment in the study within 30 hours of the onset of pain (1st day of admission) and on the third day of the disease course. Blood clots were cut and centrifuged to separate the serum, and all serum samples were kept at -20°C before use. The serum levels of cytokines were measured using sensitive enzyme-linked immunosorbent assay (ELISA) kits specific for humans (R&D Systems, Minneapolis, MN). The serum levels of tumour necrosis factor (TNF)-alpha, interleukin (IL)-6, IL-8 and IL-10 were determined at the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac.

Statistical analysis

The results are reported as the mean and standard error (SE) for continuous variables or as frequencies and percentages for categorical data. The differences between compared groups in the means of continuous variables were analysed by an independent T test, a Kruskal-Wallis test with Mann-Whitney U test, or a Wilcoxon test for paired samples (depending on the actual data distribution assessed by the Kolmogorov-Smirnov test for normality), whereas the categorical variables were compared using a Chi-squared test for frequencies. For all analyses, the level of statistical significance was set at an alpha value of 0.05. The statistical analyses were performed using SPSS 13.0 software.

RESULTS

Demographic and clinical features of the subjects

Fifty-two patients (34 male and 18 female) diagnosed with AP were included in the study (table 1). Necrotic acute pancreatitis (NAP) was observed in 18 (34.62%), patients and the interstitial form of AP was observed in 34 (65.38%) subjects enrolled in our study. There was no difference in the mean age (58.667 ± 18.493 years for NAP and 58.618 ± 14.6 years for IAP), whereas we observed male predominance in both forms of the disease.

During the disease course, 7 (13.46%) patients died, and in 9 (17.308%) subjects, we observed infected pancreatic necrosis as a consequence of a severe form of the disease.

Table 1. Sex distribution in different forms of acute pancreatitis

	Interstitial form (%)	Necrotising form (%)
Male	22	12
Female	12	6
Total	34 (65.38)	18 (34.62)

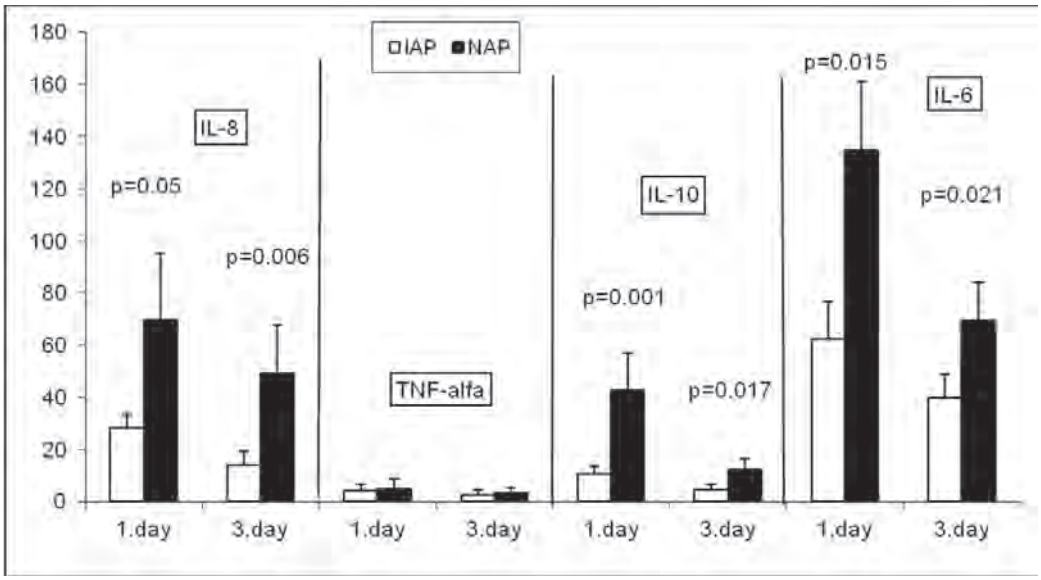

Figure 1. IL-8, TNF-alpha, IL-10 and IL-6 cytokine levels in interstitial and necrotising acute pancreatitis
IAP- interstitial acute pancreatitis
NAP- necrotising acute pancreatitis
p values presented in the figure refer to IAP vs. NAP
Presented cytokine levels are the mean ± SE

Although the patients who died were older than those with the IAP form (64.571 ± 17.175 years for lethal outcome, and 58.618 ± 14.6 years for the interstitial form), the difference in age was not statistically significant.

Cytokine profile

All four measured cytokine levels were significantly higher at the 1st day of AP compared to the values of the 3rd day of the disease course (Wilcoxon test for paired samples). The highest value was for IL-6, which dominated the other cytokine levels, whereas the lowest value was the TNF-alpha concentration (figure 1).

All of the measured pro-inflammatory cytokines, IL-6, IL-8 and TNF-alpha, showed peak serum concentrations on day 1 after the onset of symptoms in both forms of AP, with significantly higher values of IL-6 and IL-8 in necrotising than in interstitial AP. These mediators gradually decreased on the third day of the disease course, but IL-6 and IL-8 remained significantly higher in the NAP group of patients.

Regarding the anti-inflammatory cytokine, IL-10, we found significantly higher values of this cytokine in NAP compared to interstitial AP on both days of the disease course (figure 1). Whereas the IL-6, IL-8 and IL-10 levels were 2-3 times higher in the necrotic form of AP in comparison to interstitial AP, TNF-alpha was only moderately increased. Anti-inflammatory IL-10 was significantly lower on the third day in the NAP group of patients compared to the concentrations of the pro-inflammatory cytokines IL-6 and IL-8 ($p<0.01$ and $p=0.041$, respectively).

Subsequently, we compared the early cytokine levels of the 7 patients who died during the disease course with the group of patients who had only the interstitial form of AP. As shown in figure 2, all pro-inflammatory cytokines, IL-6, IL-8 and TNF-alpha, showed peak serum concentrations on day 1 after the onset of symptoms and were gradually decreased on the third day of the disease course, with significantly higher values of IL-6 and IL-8 in the lethal form of AP compared to IAP.

Anti-inflammatory cytokine IL-10 was significantly higher on the first day of the disease course in lethal AP compared to IAP (figure 2). However, its concentration decreased and became significantly lower in comparison with the pro-inflammatory IL-6 and IL-8 levels on the 3rd day of the disease course in patients who died ($p=0.001$ and $p=0.04$, respectively).

DISCUSSION

Acute pancreatitis is no longer considered only a disease of the pancreas because there is strong evidence for systemic effects of the disease. Localised inflammation in the pancreas is the body's initial physiologic protective response. Loss of the local control results in excessive uncontrolled activation of the pro-inflammatory response (hyperinflammation), which is called systemic inflammatory response syndrome or SIRS (2). SIRS can result in host defence failure, expressed by multiple organ failure (MOF) and a lethal outcome. During the SIRS phase, there was up-regulation of the pro-inflammatory factors, such as TNF-alpha, IL-6 and IL-1beta. Systemic inflammation in AP is concomitantly associated with compensatory anti-inflammatory response syndrome or CARS (3). An anti-inflammatory response may be sufficient to control SIRS, and the patient may survive without complications. However, CARS may be excessive, leading to immune suppression and the activation

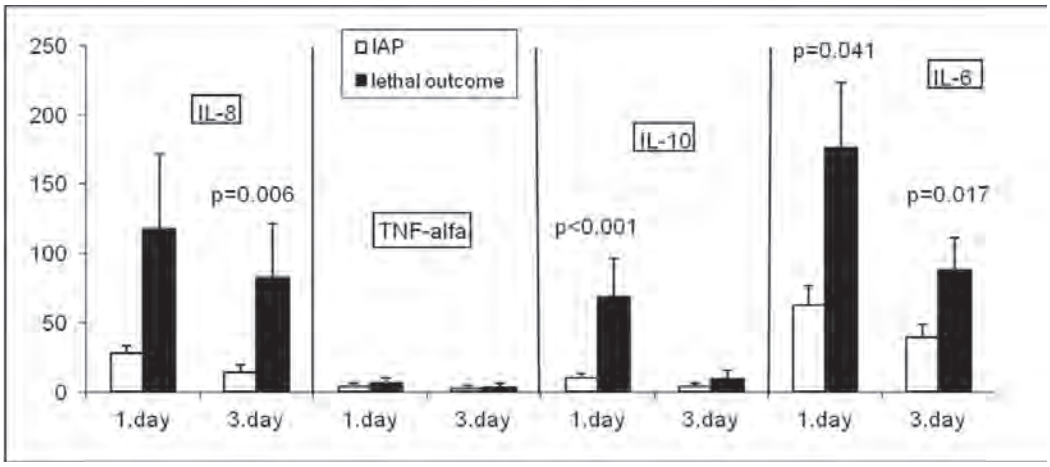

Figure 2. IL-8, TNF-alpha, IL-10 and IL-6 cytokine levels in the IAP group of patients and AP patients with a lethal outcome
IAP-interstitial acute pancreatitis
p values presented in the figure refer to survivors vs. lethal outcome

of circulating cells of the immune system, including monocytes and CD4+ T helper lymphocytes that shift to Th2 response (5-8).

There are many pro- and anti-inflammatory mediators included in this interaction between SIRS and CARS. In this paper, we showed that early changes in the serum cytokine profile could distinguish NAP from IAP and could also indicate a lethal outcome. In the clinical setting, early diagnosis and, if possible, assessment of the prognosis of AP, is a major interest for the clinician. Indeed, early aggressive treatment in patients who will develop the necrotising form of the disease could potentially change the outcome.

In our study, all examined cytokines were higher in the necrotising compared to the interstitial form of AP. Their levels gradually decreased on the third day of the disease course, with significantly higher values of IL-6, IL-8 and IL-10 in the NAP group (figure 1).

Our results are similar to the findings in patients with acute alcoholic and severe biliary pancreatitis, in which the serum IL-6 level reached its peak on admission and was significantly higher in the severe form during the whole observational period (9,10). In the study conducted by Dambrauskas Z, et al. (11), a severe form of AP was associated with a typical SIRS and a 2-5-fold increase in the expression of pro-inflammatory cytokines (IL-6, IL-8, macrophage inhibitory protein-MIF), together with the induction of a compensatory and regulatory mechanism (IL-10). The study also revealed that the serum IL-6 concentration is a good predictor of the necrotising form of the disease and systemic complications (SIRS, MOF). This marker can also be utilised for the stratification of patients with necrotising AP and those with a possible fatal outcome.

Regarding TNF-alpha, we did not find a significant difference between the necrotising and interstitial forms in our AP patients. TNF-alpha plays a pivotal role in NAP, acting early in the disease course, and is quickly cleared. As a result of its rapid clearance, the TNF-alpha serum levels are less useful than downstream cytokines (e.g. IL-6) as biomarkers of early events (12).

We examined the early changes of another pro-inflammatory cytokine, IL-8, produced by macrophages and epithelial cells, which exerts chemotactic activity on neutrophils. In turn, activated neutrophils are a significant source of IL-8 (13). In our study, patients with NAP had significantly higher IL-8 levels on both days, whereas those with a lethal outcome had significantly higher levels on the third day of the disease course, indicating the persistence of hyperinflammation (figures 1 and 2). This result was in agreement with the previous studies that showed significantly higher mean values of IL-8 and neutrophil elastase in patients with complicated pancreatitis (10,14). In another clinical study, the role of serum IL-8 in predicting lethal AP was confirmed (15).

In the study conducted by Li, et al. (16), there was an increase of Th2 cells (IL-4+) in the beginning of NAP, especially in the first week. They also showed decreased monocyte surface expression of HLA-DR antigen in the first week of the disease course, and this decreased expression correlated positively with decreasing levels of TNF-alpha, IL-6 and IL-10 during that period of NAP. IL-10, the most potent anti-inflammatory cytokine, could be responsible for the decreased monocyte HLA-DR expression in these subjects with increased risk of secondary infections and MODS (17). A high level of anti-inflammatory cytokine IL-10 may follow the increase in the pro-inflammatory factors TNF-alpha and IL-6, which might be a part of the CARS (18). In our study, statistically higher levels of IL-10 and IL-6 on the admission day and at the third day of the disease course were found in the NAP group as well as in a group of patients who had lethal outcomes (figures 1 and 2), which confirms the previous results.

The network of various cytokines and other molecules participating in the regulation of the inflammatory process-

es is very complex, and the precise timing of the release and activation of these mediators is not known. SIRS and CARS at a certain stage of the disease might even develop simultaneously (11). In our study, pro- and anti-inflammatory cytokines were significantly higher on the 1st day of acute pancreatitis in the NAP group and in the group of patients with lethal outcomes compared to the values in the IAP group (figures 1 and 2). Anti-inflammatory IL-10 decreased more than IL-6 and IL-8 and was significantly lower than pro-inflammatory cytokines on the 3rd day of the disease course in the necrotising AP form and in the group of patients who died. These findings could indicate that if SIRS and pro-inflammatory cytokines dominate the immune response, the patients will develop the necrotising form of acute pancreatitis with a potentially lethal outcome.

This is the first study in which these cytokines (IL-6, IL-8, IL-10 and TNF-alpha) were tested on the same group of patients. We found that IL-6, IL-8 and IL-10 are good indicators of the presence of necrosis and also of a potentially lethal outcome.

More studies are needed to identify the immunological phenomenon that may lead to the development of immunomodulatory treatments in necrotising AP. A better understanding of the physiology of AP is necessary to accurately identify those patients with necrotising AP in need of monitoring and treatment in intensive care units.

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COMPARISON OF MEDIAL ARCH-SUPPORTING INSOLES AND HEEL PADS IN THE TREATMENT OF PLANTAR FASCIITIS

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POREĐENJE ULOŽAKA ZA PODUPIRANJE MEDIJALNOG LUKA STOPALA I PETNIH ULOŽAKA U LEČENJU PLANTARNOG FASCITISA

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ABSTRACT

Plantar fasciitis is a disorder caused by inflammation of the insertion point of the plantar fascia over the medial tubercle of the calcaneus. Foot orthotics are used to treat plantar fasciitis. Heel pads medialise the centre of force, whereas medial arch supporting insoles lateralise the force. We assessed the clinical results of the treatment of plantar fasciitis with silicone heel pads and medial arch-supported silicone insoles.

We retrospectively reviewed 75 patients with heel pain. A total of 35 patients in the first group were treated with medial arch supporting insoles, and 40 patients in the second group were treated with heel pads. The patients were evaluated with the Visual Analogue Scale (VAS) and the Foot and Ankle Ability Measure (FAAM) at the first and last examinations.

The mean VAS score in the first group was 8.6±1,2 (6-10); the FAAM daily activity score was 66.2±16 (41.2-95.0), and the sporting activity score was 45.4±24,4 (0.1-81) before treatment. At the last follow-up in this group, the mean VAS score was 5.3±1,5 (0-9); the FAAM daily activity score was 83,0±15,1 (55,9-100), and the sporting activity score was 73,5±26,2 (25-100). The mean VAS score in the second group was 8,6±0,9 (7-10); the FAAM daily activity score was 66.4±17 (41.4-95.2), and the sporting activity score was 45.8±24,2 (0.8-81, 3) before the treatment. At the last follow-up in this group, the mean VAS score was 5.5±1,2 (0-9); the FAAM daily activity score was 83.4±14,9 (60, 2-100), and the sporting activity score was 73.8±26 (28-100).

There was no significant difference in the clinical results of both groups. The force distribution by the use of silicone heel pads and medial arch-supported silicone insoles had no effect on the clinical results of the treatment of plantar fasciitis.

Keywords: *Plantar fasciitis, Medial arch-supporting insoles, Heel pads*

SAŽETAK

Plantarni fascitis je poremećaj koji nastaje usled zapaljenja pripoja plantarne fascije za medijalni tuberkulum petne kosti. Za lečenje plantarnog fascitisa se koriste ortoze za stopala. Petni ulošci pomeraju mesto opterećenja ka unutra, dok ulošci za podupiranje medijalnog luka pomeraju mesto opterećenja upolje. Klinički rezultati se bave ispitivanjem lečenja plantarnog fascitisa silikonskim petnim ulošcima i silikonskim ulošcima za podupiranje medijalnog luka stopala.

Retrospektivno je ispitano 75 pacijenata sa bolom u peti. 35 pacijenata u prvoj grupi su lečeni sa ulošcima za podupiranje medijalnog luka stopala. 40 pacijenata u drugoj grupi je lečeno petnim ulošcima. Pacijenti su ocenjivani pomoću Visual Analogue Scale (VAS) i Foot and Ankle Ability Measure (FAAM) na prvom i na poslednjem pregledu.

Pre lečenja, srednja vrednost VAS skora u prvoj grupi je bila 8,6 ± 1,2 (6 - 10), FAAM skora adnevne aktivnosti 66,2 ± 16 (42,1 – 95,0) i skora sportske aktivnosti 45,4 ± 24,4 (0,1 - 81). Srednje vrednosti na poslednjem pregledu VAS skora su iznosile 5,3 ± 1,5 (0 - 9), FAAM skora adnevne aktivnosti 83,0 ± 15,1 (55,9 – 100) i skora sportske aktivnosti 73,5 ± 26,2 (25 - 100). Pre lečenja, srednja vrednost VAS skora u drugoj grupi je iznosila 8,6 ± 0,9 (7 - 10), FAAM skora adnevne aktivnosti 66,4 ± 17 (41,4 – 95,2) i skora sportske aktivnosti 45,8 ± 24,2 (0,8 – 81,3). Na poslednjem pregledu srednja vrednost VAS skora je bila 5,5 ± 1,2 (0 - 9), FAAM skora adnevne aktivnosti 83,4 ± 14,9 (60,2 - 100) i skora sportske aktivnosti 73,8 ± 26 (28 – 100).

Nema statistički značajne razlike u kliničkim rezultatima između grupa. Raspodela sile pritiska upotrebom silikonskih petnih uložaka ili silikonskih uložaka za podupiranje medijalnog luka stopala nema uticaj na kliničke rezultate lečenja plantarnog fascitisa.

Ključne reči: *plantarni fascitis, ulošci za podupiranje medijalnog luka stopala, petni ulošci*

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INTRODUCTION

Plantar fasciitis is a musculoskeletal disorder caused by inflammation of the insertion point of the plantar fascia over the medial tubercle of the calcaneus; the inflammation is followed by degeneration. Clinically, the disorder is characterised by pain in the medial calcaneal area (1, 2). Morning pain is an important diagnostic criteria (3). Intrinsic and extrinsic factors are hypothesised to play an etiological role. Obesity, a decrease in ankle dorsiflexion and plantar arch variations are the leading intrinsic factors in plantar fasciitis (4-10).

Plantar fasciitis causes pain and disability in walking (2, 4, 5). Anatomical shortening causes chronic bone traction by mechanical stimulation (2, 6). Although calcaneal spurs are common, heel pain is not necessary for the diagnosis (7). Plantar fasciitis is generally treated with rest, non-steroidal anti-inflammatory drugs (NSAIDs), stretching of the plantar fascia, physical rehabilitation and heel pads (8). According to Scranton et al. heel pads medialise the centre of force, whereas medial arch-supporting insoles lateralise the force (9).

In our study, we aimed to compare the relationship between the force distribution and the clinical results of the treatment of plantar fasciitis with silicone heel pads and medial arch-supported silicone insoles.

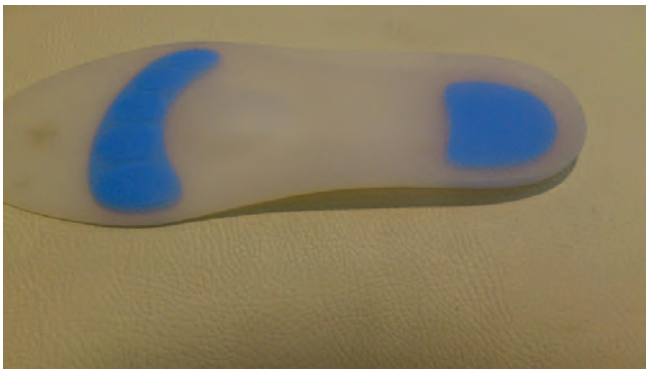

Figure 1. Medial arch-supporting insole

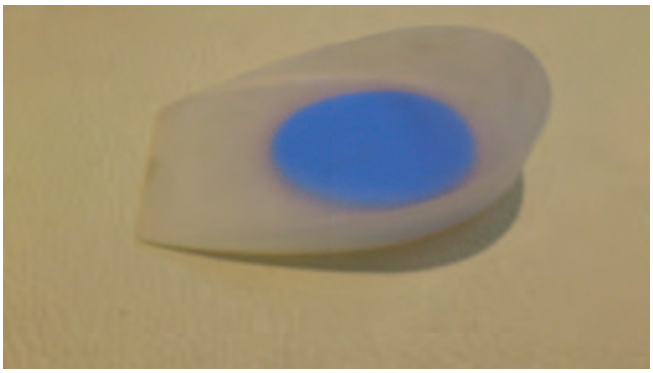

Figure 2. Heel pad

MATERIAL AND METHODS

We received ethical approval for our research from the Medipol University Ethics Committee, under number 10840098-313. We retrospectively reviewed the patients with heel pain, who were admitted to our clinic, the Medipol University Medical School Departments of Orthopaedics and Traumatology. Patients with foot deformities, such as a pes cavus or pes planus deformity, were not included in our study. Patients diagnosed with plantar fasciitis who had received treatment including foot orthotics or a local steroid injection before admission to our clinic were not included in our study. The participants in the study were sedentary, non-athletic patients, and 75 patients were included in our study. The diagnosis of plantar fasciitis was based on the physical examination and radiographic findings. The patients were evaluated with the Visual Analogue Scale (VAS) and the Foot and Ankle Ability Measure (FAAM) (10). The patients were randomly divided into 2 groups. The 35 patients in the first group were treated with prefabricated medial arch-supporting insoles (figure 1), NSAIDs, and stretching exercises. In the first group, 29 patients were female, and 9 patients were male. The mean age was 45,5±10,3 (26-63) years. The mean follow up period was 9,6±1,8 (8-14) months. The 40 patients in the second group were treated with prefabricated heel pads (figure 2), NSAIDs and stretching exercises. In this group, 23 patients were female, and 17 patients were male. The mean age was 50,3±12,5 (28-70) years. The mean follow up period was 9,9±1,3 (8-12) months. Non-steroidal anti-inflammatory drugs were recommended for use as needed, and 75 mg of diclofenac sodium was prescribed for the NSAID therapy. The patients independently performed plantar fascia stretching exercises, which were defined by DiGiovanni et al (11). The patients in both groups were evaluated with the VAS and Foot and Ankle Ability Measure (FAAM) at the last follow-up. A calcaneal spur was detected in 14 patients in the first group and in 19 patients in the second group.

The VAS scores and FAAM scores were statistically compared in both groups before and after treatment. The non-parametric Mann Whitney U test was used to compare the VAS and FAAM scores in the medial arch supporting insole and heel pad treatments. The post treatment VAS and FAAM scores were analysed with the non-parametric Wilcoxon test with 95% confidence interval ($p<0.05$).

RESULTS

The mean VAS score of all patients was 8.6±1 (6-10). The mean FAAM daily activity score was 66.3±15 (41.2-95.2), and the mean sporting activity score was 45.6±24,2 (0.1-81.3) before the treatment. At the last follow-up, the mean VAS score was 5.4 ±1,5 (0-9); the mean FAAM daily activity score was 83.2±15,3 (55.9-100.0), and the sporting activity score was 73.7±26,2 (25.0-100.0).

The mean VAS score in the first group was 8.6±1,2 (6-10); the mean FAAM daily activity score was 66.2±16 (41.2-95.0), and the mean sporting activity score was

45.4±24,4 (0.1-81) before the treatment. At the last follow-up in this group, the mean VAS score was 5.3±1,5 (0-9); the mean FAAM daily activity score was 83,0±15,1 (55,9-100), and the mean sporting activity score was 73,5±26,2 (25-100). The mean VAS score in the second group was 8,6±0,9 (7-10); the mean FAAM daily activity score was 66.4±17 (41.4-95.2), and the mean sporting activity score was 45.8±24,2 (0.8-81,3) before the treatment. At the last follow-up in this group, the mean VAS score was 5.5±1,2 (0-9); the mean FAAM daily activity score was 83.4±14,9 (60,2-100), and the mean sporting activity score was 73.8±26 (28-100). (Tables 1-2)

One patient in each group reported a total remission of pain after the treatment and a VAS score of 0. There was a statistically significant difference between the pre- and post-treatment VAS and FAAM scores. ($p<0,05$) However, there was no statistically significant difference between the scores of the two groups.

DISCUSSION

The ethiology of plantar fasciitis is not well understood, and mechanical overloading could be an important factor. Orthoses commonly reduce the tensile forces and demonstrate a therapeutic effect (12, 13)

A relationship between pain and an increased medial longitudinal arch index has been hypothesised. Some studies have reported that the medial longitudinal arch is higher in plantar fasciitis patients, which could be explained by an increase in the plantar arch to maintain the arch structure in the static phase. As this posture is maintained, it causes micro trauma in the plantar fascia (14). In our study, there was no statistically significant clinical difference between the treatment with a medial arch supporting insole and the treatment with a heel pad; our results were not correlated with the hypothesis that an increase in the medial arch index is related to pain.

In plantar fasciitis patients, ankle dorsiflexion is limited, and the flexibility of the triceps surae and toe extension is decreased (15, 16, 17). Thus, release of the medial arch could be helpful in the treatment of plantar fasciitis, and for this reason, we used a medial arch-supporting insole in the first group. However, there was no statistically significant difference between the groups. The significant difference between the pre- and post-treatment scores of the medial arch-supporting insole therapy clinically supports this theory.

Scranton et al showed that heel pads medialise the centre of force, whereas the medial arch supporting insoles lateralise the force (9). We predicted a better clinical result using medial arch-supporting insoles. However, there was no statistically significant difference in the scores, which indicates that the mechanical and clinical studies could differ in terms of the results.

In a study with a population of non-athletes, a low medial arch was detected in 82 patients with a symptomatic calcaneal spur, which was hypothesised to be related to the development of plantar fasciitis (18). Foot deformities play an important role in the ethiology of plantar fasciitis; however, because patients with foot deformities were not included in the study, our study is limited in terms of the relationship between foot deformities and plantar fasciitis.

When thickening of the plantar fascia exceeds 4 mm, it causes pain and a functional limitation (19). The pre-treatment and post-treatment plantar fascia thickness measurements could be an effective method for evaluating the treatment results. The lack of measurement of the thickness of the plantar fascia is another limiting factor of our study.

CONCLUSION

There was no difference in the clinical results of conservative treatment modalities of plantar fasciitis with heel pads and medial arc-supported insoles. The force distribution by the use of silicone heel pads and medial arch-supported silicone insoles had no effect on the clinical results for the treatment of plantar fasciitis. Both foot orthotics could be used in plantar fasciitis treatment.

Table 1: FAAM scores of the two treatment groups

	Pre-treatment FAAM daily activity score	Pre-treatment FAAM sporting activity score	After treatment FAAM daily activity score	After treatment FAAM sporting activity score
Group 1 (medial arch supporting insoles)	66.2±16 (41.2-95.0)	45.4±24,4(0.1-81)	83,0±15,1(55,9-100)	73,5±26,2(25-100)
Group 2 (heel pads)	66.4±17(41.4-95.2)	45.8±24,2(0.8-81,3)	83.4±14,9(60,2-100)	73.8±26(28-100)

Table 2: VAS scores of the two treatment groups

	Pre-treatment VAS score	After treatment VAS score
Group 1 (medial arch supporting insoles)	8.6±1,2 (6-10)	5.3±1,5(0-9),
Group 2 (heel pads)	8,6±0,9(7-10)	5.5±1,2 (0-9)

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MACROECONOMIC POLICY IMPACT ON ONCOLOGY-RELATED PUBLIC EXPENDITURE IN AN EMERGING EUROPEAN MARKET – SIGNS OF EARLY RECOVERY

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UTICAJ MAKROEKONOMSKE POLITIKE NA JAVNA IZDVAJANJA ZA ONKOLOGIJU NA RASTUĆEM EVROPSKOM TRŽIŠTU – ZNACI RANOG OPORAVKA

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ABSTRACT

Healthcare financing in Serbia has faced many challenges over the past few decades. One of the most severe challenges is a global macroeconomic recession whose far-reaching consequences deserve particular attention from policymakers in cases of the most demanding major prosperity diseases, such as cancer. The objective of the study was to assess the precise cost matrix of oncology medical care and its chronological evolution during the key years of the macroeconomic recessionary period during 2010-2013.

A retrospective database of hospital discharge invoices was analysed, encompassing 37, 978 hospital admissions and 12, 505 patients during a four-year period. Insight into microeconomic patterns of consumption across groups of medical services was provided. A payer's perspective and one-year time horizon have been adopted.

Total hospital direct medical costs of cancer diagnostics and treatment in the observed tertiary care facility decreased from €7, 411, 446 in 2010 to €5, 715, 884 in 2012 and then increased to an extraordinary €8, 536, 364 in 2013. The costs of oncology nursing care, imaging diagnostics and radiotherapy have increased considerably while those of pharmaceuticals and surgery have decreased radically - completely transforming the resource allocation landscape of public cancer care.

The financial burden of cancer in Serbia is considerable and, unfortunately, expected to increase further in the coming years. Worldwide economic recession and consecutive domestic policy constraints of reimbursement limitations have heavily affected the affordability of cancer treatment for ordinary citizens. Promising signs of market recovery are clearly visible in 2013, which will likely improve both access and equity of medical care in Serbian oncology clinics.

Keywords: *Worldwide Crisis; Recession; Cancer; Costs; Economics; Health Financing; Health Policy; Reimbursement; Hospital; Serbia*

SAŽETAK

Cilj studije je analiza trendova u javnim izdvajanjima za onkolosku zdravstvenu zaštitu u godinama duboke ekonomske recesije u svetu i na Balkanu. Ostali ciljevi su utvrditi finu strukturu troškova u ovoj kliničkoj disciplini kao i eventualno prisustvo korelacije obima potrošnje na dijagnostiku i lečenje malignih neoplazmi sa makroekonomskim kretanjima i zdravstvenom politikom u Srbiji.

Primenjena je retrospektivna studija slučaja, kojom je obuhvaćen period od cetiri godine (2010-2013.) iz perspektive finansijera zdravstvene zastite i sa usvojenim vremenskim horizontom od godinu dana. Studija je izvedena na osnovu izvoda baze podataka o 37 978 epizoda bolničkog lecenja i 12 505 pacijenata sa klinički potvrdjenim kancerom pri regionalnom Centru za onkologiju i radioterapiju Kliničkog Centra Kragujevac.

Ukupni direktni medicinski troskovi dijangostike i lecenja kancera u posmatranom tercijernom centru su pali sa €7, 411, 446 u 2010 na €5, 715, 884 u 2012 i iznova snažno skočili na €8, 536, 364 u 2013. Glavni domeni troškova koji su najviše doprineli ukupnom obimu potrosnje su bili onkoloska medicinska nega, radioterapija i lekovi.

Finansijski teret kancera u Srbiji je ogroman i nažalost izgledno je da ce nastaviti da raste zahvaljujući nizu činilaca poput starenja populacije, boljeg preživljavanja, naraslih očekivanja građanstva o pravu na pristup naprednim metodama lečenja kao i dugoročno izvesnog rasta pokrivenosti stanovništva zdravstvenom zaštitom. Posebno masivni faktori su širenje dostupnosti metoda radioterapije i refundacija skupih bioloških lekova. Obećavajući znaci oporavka nacionalnog tržista će se, nadamo se, pretočiti u napore na poboljšanju pristupa i priuštivosti onkološke nege običnom građaninu.

Ključne reči: *Svetska ekonomska kriza; recesija; kancer; troškovi; ekonomija; finansiranje zdravstva; zdravstvena politika; refundacija; bolnice; Srbija*

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INTRODUCTION

Cost-of-illness analyses of the key prosperity diseases remain rather infrequent in Eastern Europe and the Balkans region (1). Over the past decade, a few pioneering assessments were published, laying the ground for the informed decision making of local health policymakers (2). These findings reflected the workload and financial burden imposed by the diagnostics and treatment of chronic obstructive pulmonary disease, community-acquired pneumonia, alcohol dependence, diabetes mellitus, hepatitis C, risky pregnancies and others (3-8). Another set of contributions revealed the considerable budgetary impact of some key medical technologies, such as medical imaging (9), radiotherapy (10) and monoclonal antibodies, applied in oncology (11-12). These and further on-going efforts on domestic, local health economic estimates are essential to improve the financial efficiency of our health system (13). This claim is supported by the well-known fact that similar estimates from high-income markets are not straight- forwardly applicable to clinical settings across Eastern Europe and the Balkans due to their substantially different histories, traditions and socioeconomic milieus (14-15).

Oncological morbidity deserves a particularly high place among the leading noncommunicable prosperity diseases. The diagnostics, treatment and rehabilitation associated with cancer are commonly much more demanding in terms of medical technology use and physician consultation time and frequency compared to other major illnesses. Additionally, the clinical outcomes of these interventions are far less predictable, with illness itself resulting in a heavily reduced life expectancy, quality of life and working ability of an individual citizen. Cancer's economic burden to the community is enormous, and the issue of treatment affordability remains high on the policy agendas of even the richest countries worldwide (16). Pioneering assessments in Serbia were published only recently and confirmed the aforementioned facts evidenced elsewhere (17).

Unfortunately, both global and European cancer prevalence and incidence are increasing (18). Serbia exhibits a slightly higher incidence compared to the EU average, which remains somewhat lower compared to the European average that includes the CIS countries and the Russian Federation (19-20). Among the most frequently cited reasons for higher oncological mortality in Serbia are poverty, unhealthy lifestyles and ecological contamination due to the Chernobyl disaster and wars in Yugoslavia during the 1990s (21-22). However, throughout Eastern Europe, poor implementation of screening procedures (23) and limited affordability of some innovative treatment technologies remain powerful contributors (24).

The global macroeconomic crisis has caused severe instability among the Western Balkan economies bordering the EU (25). The recession has compromised financial sustainability within the health sector (27), and its far-reaching consequences deserve particular attention from policymakers in cases of the most demanding major prosperity diseases, such as cancer (28). The core research question of this study was the assessment of the macroeconomic recession's impact on public medical spending mediated by national policy (29). Early signs of economic recovery, which have been present in Serbia since 2013, are likely to improve access to and the affordability of medical care for patients suffering from cancer. So far, targeted biological therapy reimbursement has remained one of the hottest domestic policy issues (30).

PATIENTS AND METHODS

To address the aforementioned research question, a retrospective, bottom-up, case series study design over a one-year horizon and payer's perspective was implemented (31). The tertiary care University of Kragujevac clinic allowed selective examination of their electronic database of discharge invoices. All patients whose cancer diagnosis was confirmed by clinical, imaging, laboratory and pathohistology findings and who were admitted and treated at the regional Oncology and Radiotherapy Centre were processed. Key cost drivers and determinants of resource consumption during oncological inpatient care were identified. Personal data remained protected during the study consistent with positive legislation on biomedical research in human subjects in Serbia via anonymous handling of patient files. A fine cost matrix was produced through stratification of the Republican Health Insurance Fund (RFZO) "Blue Code Book" of all medical goods and services provided within the national health system.

The patient sample recruitment period was January 2010-December 2013 and included inhabitants of this central Serbian region. Total sample size was 12, 505 patients or 37, 978 hospital admissions with assigned oncology treatment protocols during the 2010-2013 period. These years were selected because the 2010-2012 years were marked by the heavy impact of worldwide economic crisis, while 2013 was a year of slow but steady recovery of the national economy. To the authors' best knowledge, this sampling method and approach to longitudinal data is standard and common in the discipline of health economics (32).

RESULTS

Total hospital direct medical costs of cancer diagnostics and treatment at this tertiary university hospital fell from €7, 411, 446 in 2010 to €5, 715, 884 in 2012 and then increased to an extraordinary €8, 536, 364 in 2013. Costs of oncology nursing care, imaging diagnostics and radiotherapy have greatly increased while pharmaceuticals and surgery followed at a much slower pace, completely transforming the resource allocation landscape of public cancer care.

Most major service groups follow this general pattern, while pharmaceuticals and surgery differ from the dominant trend. Drug acquisition represented 56, 4% of total

costs in 2010 and 53, 3% in 2012. Cytostatics and immunosuppressants experienced a particularly steep decline in value-based turnover of 55, 4%, decreasing from €1, 699, 164 in 2010 to €758, 490 in 2012. Antibiotics, antiemetics, bone marrow stimulating factors and analgesics followed the same pattern. Among the few drugs that increased during the 2010-2012 period were monoclonal antibody costs, which rose by 20, 3% (from €1, 350, 235 in 2010 to €1, 624, 245 in 2012).

Radiotherapy costs also increased during the 2010-2012 period by 28, 1% (from €416, 193 to €533, 303), and this increase is unfortunately mostly a consequence of a higher workload produced by more frequent outpatient visits and inpatient admissions.

Decreased surgery-related costs should be attributed to the market decreases in the prices of consumables.

The radiology imaging, contrasts and films budget impact changed substantially from 3, 515, 050 RSD (€33, 319) in 2010 to only 1, 168, 519 RSD (€10, 276) in 2012, which is a nearly 70% decrease. Net savings acquired this way should be attributed to the new information system installed in the diagnostic services and clinic, which has eliminated the need for traditional roentgen films in most examination techniques. The value-based turnover across major cost domains over these four years is shown in detail in Table 1.

DISCUSSION

As indicated by the data above, some service groups show a sudden and clear upward trend in 2013, while others have followed at much slower pace. Thus, the big picture of resource allocation to cancer diagnostics and treatment in large hospitals in Serbia has evolved according to the macroeconomic landscape, market circumstances and official policies of the national authorities (13, 15, 29).

The 2010 dominance of drug acquisition costs (primarily conventional cytostatic, antiemetic, analgesic, hormonal and antibiotic drugs) is overtaken in 2013 by expanding radiotherapy, oncology nursing care and imaging diagnostics. This pattern is consistent with previously published evidence on the region stating that particularly serious budget impacts were imposed by over-utilization of high-tech radiology imaging procedures (11, 17, 24).

Pharmaceuticals were greatly influenced by novel, expensive biological treatments, such as monoclonal antibodies (mAbs) and protease inhibitors. This pattern is only small portion of far larger changes. One recently published study analysed official annual data from the national medicines agency (ALIMS) since 2004 (33). Total public expenditure on drugs with primary oncology-related indications increased by approximately five times during the period 2004-2012. During that same decade, public consumption of mAbs increased nearly 20 times due to the aforementioned societal and market changes (12). Although it is favourable that many patients had access to these innovative medicines, the cost-effectiveness of many mAbs is a source of heated international debate (34, 35). The societal affordability of expensive biologicals and willingness to pay thresholds are rather low in Eastern Europe compared to the West (13, 15). Contradictions such as shortages of basic, conventional cytostatics alongside reimbursement of most expensive medicines occur frequently (11, 12).

The downward slope of cytostatic drug acquisition costs from 2010 to 2012 in our regional sample has an underlying cause, which is invisible in the presented data. Unfortunately, due to pharmaceutical market disturbances across Western Balkan economies, during the last quarter of 2011 and the first five months of 2012, continuous hospital supplies of these drugs were severely threatened. These disturbances were mostly caused by delayed payments from state-owned health insurance funds to major multinational manufacturers supplying the region (13, 15). One particularly sensitive issue was the lack of simple 5-fluorouracil, which is itself a quite inexpensive medicine but is an essential part of many expensive and complex treatment protocols that could not be provided for months due to this shortage. Occasional shortages of cytostatic medicines also occurred. Consequently, clinics have experienced sudden decreases in their need for drugs used to treat the most common adverse effects of cytostatics, such as antibiotics, bone marrow growth factors and antiemetics used to treat febrile neutropenia, opportune infections and vomiting (36-38).

Another core influence of the sudden decrease in prescribing and dispensing of cytostatic drugs and their costs is the restrictive reimbursement policy imposed by the Republican Health Insurance Fund of Serbia (RHIFS), which was mostly triggered by the macroeconomic recession (39). The common practice is financial coverage of a particular medicine for select indications, such as narrowly defined malignant tumour clinical types, grades and stages, while the same drug might not be funded for another malignancy. Policies of funding agencies to prioritize interventions within their optimal clinical efficiency and cost-effectiveness were in place in many major markets around the globe (40-41).

The temporary decrease in oncology nursing care costs from 2010 to 2012 occurred immediately following domestic policy measures to allow contracted general practitioners to prescribe and administer opioid analgesics were implemented. This practice was uncommon within the national health system of Serbia. At approximately the same time, an outpatient pain treatment service was founded within this tertiary university clinic. Both measures ultimately resulted in less frequent hospital admissions due to severe metastatic pain, and patients used the opportunity to resolve their symptoms on an outpatient basis and preferred staying at home. That this strategy induces net savings while improving patient satisfaction and quality of life has already been observed elsewhere (42). The financial sustainability of home care in Serbia was recently an objective of thorough consideration (43).

Table 1. Cost matrix of oncology related diagnostics and treatment within tertiary university clinic of Kragujevac 2010-2013

COST DOMAIN	2010	2011	2012	2013
Number of patients diagnosed with confirmed malignancy				
Number of hospital admissions due to cancer				
Oncology related medical care				
Hospital Admission	1,118,074	1,102,027	950,470	988,679
Physician Consultations	50,800	63,924	42,386	215,942
Clinical Pharmacology/ Pharmacist services	27	309	115	
Rehabilitation services	355	2,408	1,462	1,153
Dialysis	868	493	373	754
Psychotherapy	83	12	8	709
Administrative expenses	138	154	135	295
All Other services (social care , transport, counselling, epidemiological	4,183	1,448	2,847	442,627
Total Cost of General - Oncology related medical care	1,174,533	1,170,776	997,796	1,650,161
Pharmaceuticals				
Antineoplastic agents and immunosuppresants	1,691,948	1,200,255	758,490	572,908
Monoclonal antibodies	1,344,501	1,432,796	1,624,245	1,559,090
Analgesics NSAID, opioid, others - pain control medicines	14,268	10,891	7,587	6,000
Antibiotics, antimicotics, antiviral and antiprotozoal drugs	170,376	111,822	78,586	104,546
Antiemetics	190,435	138,018	100,743	110,606
Parenteral and enteral nutritive solutions and systems	118,386	92,833	86,628	91,349
Hematopoietic colony stimulating factors	150,539	79,288	65,552	101,069
Antiandrogens , antiestrogens – therapy of steroid dependent carcinoma	153	10		
Blood and its derivatives – transfusions	155,917	111,031	109,201	60,997
All other drugs	345,498	306,273	218,122	264,412
Total cost for pharmaceuticals	4,182,025	3,483,217	3,049,154	2,870,977
Laboratory Analysis				
Classical Biochemistry and hemathology	296,599	295,276	190,453	291,817
Targeted cancer prevention screenings	71	83	13	1,576
Tumor marker detection			1,010	2,042
Pathohistology tests and cytology examinations	125,808	131,191	105,862	119,742
Immunodiagnostics, genetics, cell culture techniques	77,689	66,720	51,570	73,677
Law medicine and forensic services	15,217	16,586	16,125	31,043
Total cost for laboratory analysis	515385	509,856	365,033	519,897
Surgery				
Surgical Interventions	132,278	157,041	135,291	133,586
Nursing care and consumables	450,585	327,310	179,865	174,116
Total cost for surgery	582,863	484,351	315,156	307,702
Imaging diagnostics				
Classical imaging diagnostics – Röntgen	5,764	6,177	4,568	6,498
Contrasts, films and consumables intended for imaging diagnostics services	33,177	30,512	10,276	15,783
Ultrasound imaging examinations	6,577	7,369	7,295	9,032
Imaging diagnostics	221,555	231,005	174,272	358,947
Magnet resonance imaging	15,795	9,482	9,685	28,378
Nuclear medicine diagnostics and treatment	220,422	201,841	210,978	353,428
Total cost of imaging diagnostics	503,290	486,386	417,074	772,066
Interventional radiology				
Interventional neuroradiology services (both diagnostic and treatment)	1,055	837	1,902	2,378
Cardial interventional radiology	1,476	3,241	3,222	3,296
Urological interventional radiology	2,621	1,474	948	2,005
Vascular interventional radiology	1,160	571	151	357
Interventional radiology -other methods (biopsies, cyst punctuations, nonvascular int. etc)	206	356	13	366
Implants and consumables used in interventional radiology services (stents, tools etc)	32,407	35,032	32,131	44,944
Total cost of interventional radiology	38,925	41,511	38,367	53,346
Radiation treatment				
Teleradiotherapy procedures in Oncology	393,010	406,776	440,524	1,544,853
Brachyradiotherapy (intracavitary) procedures in Oncology	21,416	139,496	92,779	817,362
Total cost of radiation treatment	414,425	546,272	533,303	2,362,215
Total hospital cost (RSD)	785,227,937	703,434,811	650,000,662	978,626,707
Total hospital cost per patient (€)	2,087	1,640	1,547	
Total hospital cost (€)	**7,411,447**	**6,722,370**	**5,715,884**	**8,536,364**

Regardless of the weaknesses of these assessments in the field, it is crucial for policymakers to notice the enormous size and structure of public health expenditures on hospital in- and outpatient cancer care peculiar to the local setting (9-12, 17). Serbia will likely suffer from ongoing economic difficulties, such as increasing foreign debt, currency devaluation, high unemployment rates and inefficient healthcare financing (13, 15). A bold step forward was the introduction of national health accounts in the country based on similar World Health Organization practices in other regions across the globe (44). National health accounts (NHA) allow more precise knowledge of financial flows within the health system and international comparability of data (45). Economic and/or managerial inefficiency recognized via the NHA system might be selectively improved without affecting the availability or quality of services elsewhere in the system (46).

The most effective strategy to cope with the increasing burden of malignancies would likely be investment in population health education targeted to change risky health behaviours (47). Another rewarding investment is broad screening strategies whose cost-effectiveness has been well established in other countries (23). These strategies are particularly fruitful in some of the most prevalent carcinomas, which are curable by simple surgeries if discovered at early stages of clinical evolution (48). The early discovery of malignancies such as cervical, breast, colorectal, skin and gastric carcinomas prevents serious, expensive morbidities (49, 50). The outcomes and success of late treatment of advance disease forms, including surgery, complimentary radiotherapy, cytostatic protocols and occasionally novel biologicals, are highly unpredictable (24). Life expectancy is usually low, and premature mortality has enormous ethical and economic consequences for the community. Unfortunately, we are losing not only elderly citizens but also many people in their productive life stages (51).

Radiation treatments are major contributors to the total costs of care (9-10). Insufficient equipment capacities are common across the region (13, 15). Due to a poor network of facilities across rural and remote regions of the Balkans and difficulty accessing specialist care, many patients seek treatment too late (24). Late treatment involves multiple radiotherapy sessions with modest or poor success (52). Providing palliative, end of life care for advanced stage, metastatic disease is a more frequent practice compared to Western European and high-income settings (53). Absenteeism, decreased working ability and premature death are common (54-56).

It is crucial to emphasize that the aforementioned decreased cost of pharmaceuticals is not due to a decrease in the underlying prevalence and incidence of cancer or to successful public policy (17). Such savings are unfortunately largely a consequence of reimbursement limitations imposed by the national health insurance fund due to the macroeconomic recession (12, 13, 15). The considerable growth of overall resource use in oncology clinical care in 2013 may be a promising early sign of economic recovery (57).

Study limitations

Although representing a pioneering attempt in the field, which is essential for Western Balkan health policymakers, the study weakness slightly limit the generalizability generalisability of the conclusions. No indirect, absenteeism-related costs were calculated in this trial. If Grossman's human capital method was used, lost productivity, home care and premature mortality costs would likely nearly double current assessments (51, 58).

The retrospective approach used in this study was inevitable to acquire a large sample (31). Patient data on resource use (physician consultations, laboratory and imaging examinations, interventional radiology methods, surgical interventions, pharmaceuticals treatment, etc.) were acquired from clinical files. Therefore, important data on patients' clinical background was lacking. These are more likely to be provided within a prospective framework, which would assume much smaller sample (59). Further research should focus on the clinical outcomes of cancer treatment and assessment of cost-effectiveness, especially of medical technologies (60). This was primarily a cost of illness and budget impact estimate, and such efforts were well outside the scope and budget of this study.

CONCLUSION

Serbian public health expenditure on cancer was severely constrained by the reimbursement limitations imposed by authorities due to national consequences of the global economic recession. Slow but steady recovery is clearly visible according to the large increase in oncology related public expenditure in 2013, which was evidenced in a large domestic tertiary care university clinic. Under the assumption that GDP growth accelerates to pre-recession levels, policymakers should dedicate sufficient attention to improving the affordability and timely delivery of medical care to patients suffering from cancer. This is a key issue in a country with sizeable private out-of-pocket spending on healthcare. Properly targeted screening as well as efficient and accessible diagnostic and treatment services would likely achieve better clinical outcomes, such as improved patient longevity and quality of life. Health gains by citizens in need will provide a return on investment to society by enhancing national economic productivity.

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ISOLATED CARPAL DISLOCATION OF THE TRAPEZIUM

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IZOLOVANA KARPALNA DISLOKACIJA TRAPEZOIDNE KOSTI

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ABSTRACT

Trapezium fractures and dislocations of the trapezium are both extremely rare injuries whether they occured with or without fractures of the surrounding bones. Specific radiological images can be difficult to help for the diagnosis. CT scan may be necessary for the diagnosis and adequate treatment. We are presenting an unusual case of volar and radial isolated trapezium dislocation concomitant second metacarpal basis fracture in which is treated by using open reduction and Kirschner wire fixation. In our case, isolated dislocation of trapezium was a result of violent and direct trauma. Different techniques have been proposed to achieve a stable fixation and the treatment outcomes. In our case, open reduction, Kirschner wire fixation and intercarpal ligament repair through dorsal approach are recommended for satisfactory outcomes in similiar cases.

SAŽETAK

Frakture i dislokacije trapezoidne kosti su izuzetno retke povrede bilo da se javljaju udruženo sa prelomima okolnih kostiju ili izolovano. Radiološki snimci su uglavnom nedovoljni za postavljanje dijagnoze, tako da je često neophodno uraditi CT skeniranje za postavljanje dijagnoze i adekvatnu terapiju. U ovom radu je predstavljen neobičan slučaj volarne i radijalne izolovane dislokacije trapezoidne kosti sa pridruženim prelomom baze druge metakarpalne kosti, koji je lečen otvorenim nameštanjem i fiksacijom Kiršnerovim žicama. U slučaju koji smo predstavili, izolovana dislokacija trapezoidne kosti je nastala kao posledica nasilne i direktne traume. Predlažu se različite tehnike za postizanje stabilne fiksacije preloma i adekvatnog ishoda lečenja. U ovom slučaju, kao i u sličnim slučajevima, otvoreno nameštanje, fiksacija Kiršnerovim žicama i reparacija interkarpalnog ligamenta dorzalnim pristupom je preporučljuva za pozitivan ishod lečenja.

INTRODUCTION

Trapezium fractures and dislocations of the trapezium are both extremely rare injuries, whether they occur with or without fractures of the surrounding bones (1, 2). However, they are very important traumas to detect and treat early, given the importance of the trapezium in the carpometacarpal joint in actions such as grip and pinch. Occasionally, there may also be associated ligament damage (anterior oblique ligament, dorsoradial ligament, intermetacarpal ligament, posterior oblique ligament) (3). Specific radiological images help only slightly to obtain a diagnosis. A CT scan may be necessary for diagnosis and adequate treatment (4, 5). We present an unusual case of volar and radial isolated trapezium dislocation concomitant with a fracture of the second metacarpal basis. The case was treated with open reduction and Kirschner wire fixation.

CASE PRESENTATION

A 21-year-old right-handed man, a pastry worker, was seen in our emergency unit for pain, diffuse oedema and functional impairment of the wrist and thumb. An isolated volar and radial dislocation of the trapezium and a fracture of the second metacarpal basis were diagnosed on radiographic images and confirmed by a CT scan (Figure 1a, 1b, 2a, 2b).

A closed reduction of the trapezium was attempted in the emergency clinic. Post-reduction radiographs revealed that the trapezium remained dislocated. The wrist was immobilized in a splint with the thumb in an overlying thumb position, and the patient was admitted to the clinic for surgery.

Under general anaesthesia, a dorsal longitudinal incision was made. A volar and radial dislocation of the tra-

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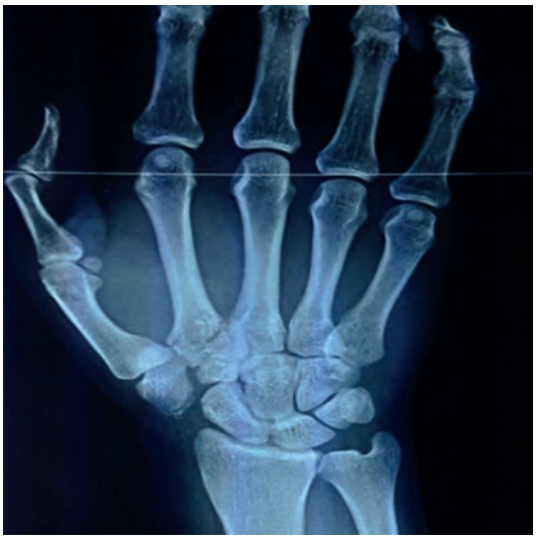

Figure 1a: After trauma anteroposterior X-ray

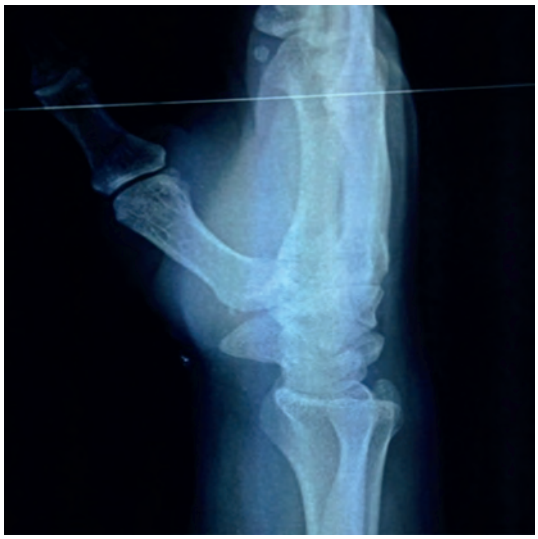

Figure 1b: After trauma lateral X-ray

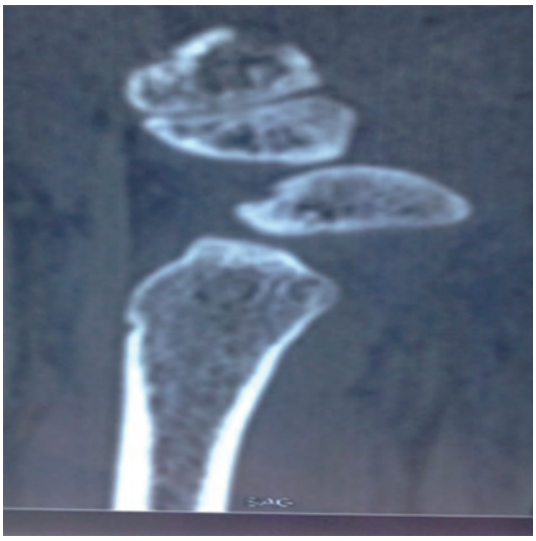

Figure 2a: After trauma CT

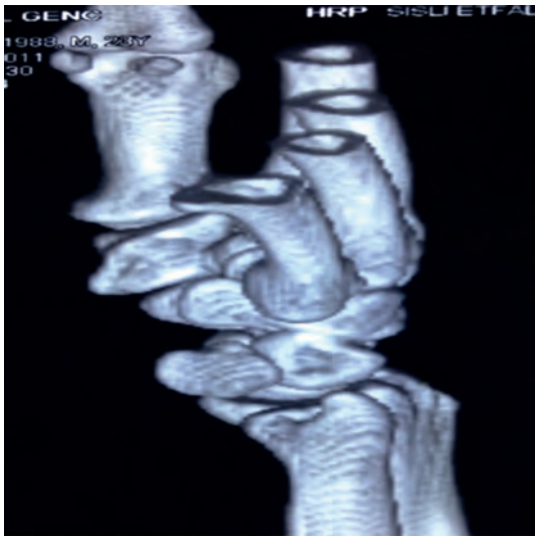

Figure 2b: After trauma CT

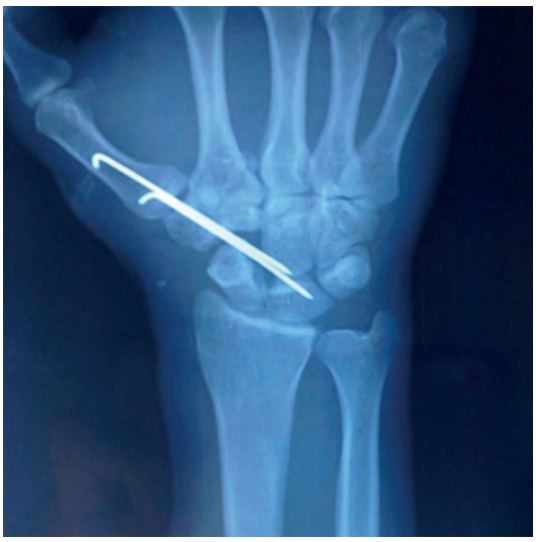

Figure 3a. Post-operative anteroposterior X-ray

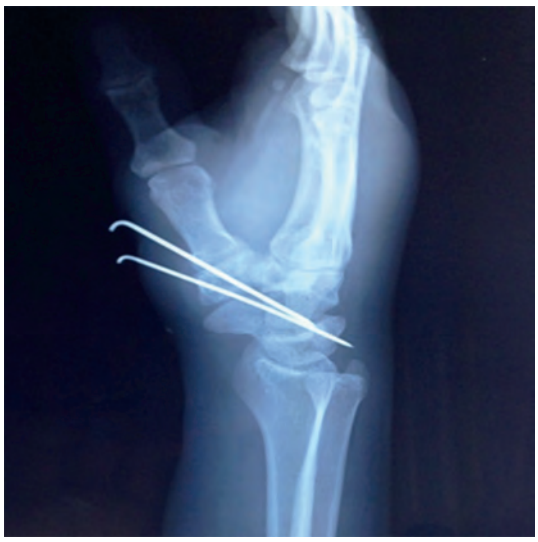

Figure 3b. Post-operative lateral X-ray

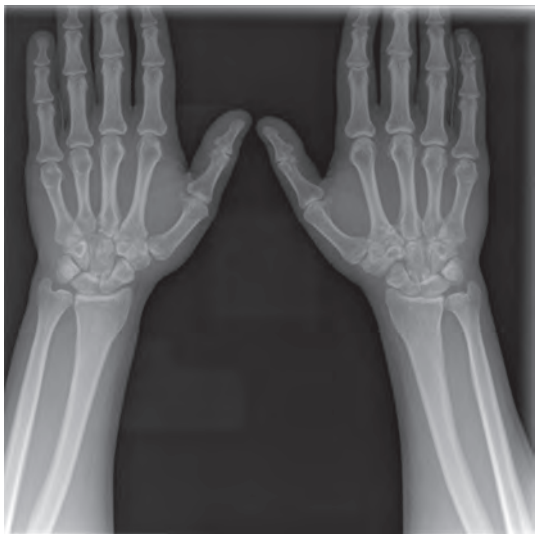

Figure 4a : Antero-posterior view at 36 months

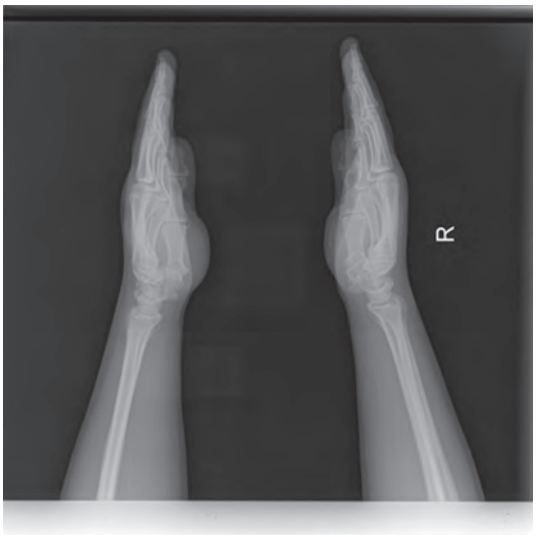

Figure 4b: Lateral view at 36 months

pezium were observed. The intercarpal ligaments between the trapezium and surrounding bones were ruptured. Open reduction was performed through the incision, and the bone was fixed with two Kirschner wires. The operation was completed after the capsule and ligament were repaired (Figure 3a, 3b). The wrist was immobilized in a splint including the proximal phalanx of the thumb. After four weeks, before the free mobilization began, the Kirschner wires were removed. The patient returned to work after 45 days and was satisfied with the result.

At 36 months follow-up, the patient had a painless range of motion. The active and passive motions of his wrist were the same compared with the uninjured side, with normal appositional and oppositional pinch strength. There was no avascular necrosis and no radiological signs of arthrosis (Figure 4a, 4b, 5a, 5b).

DISCUSSION

Dislocation of carpal bones is uncommon and generally occurs as a result of a high-energy injury. The mechanism of injury usually involves either direct dorsoradial impaction or indirect axial loading. Indirect trauma that is transmitted by the thumb may produce an incomplete dislocation of the trapezium. Isolated dislocations and fractures of the carpal bones are rare and are usually associated with other hand or wrist injuries (Bennett's fracture, Rolando's fracture, fracture of the scaphoid, hook of hamate, distal radius and carpometacarpal dislocation) (6, 7). The clinical presentation can be quite variable depending on the displacement of the fracture and the involvement of the carpometacarpal joint. Some patients only complain of minor pain at the base of the thumb without any gross swelling or deformity. Es-

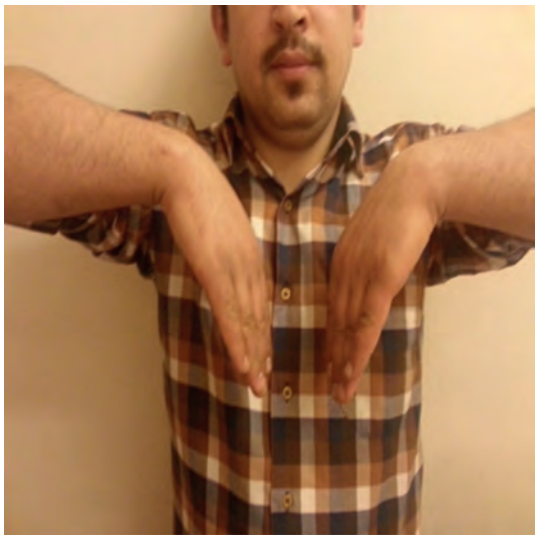

Figure 5a: Flexion at 36 months

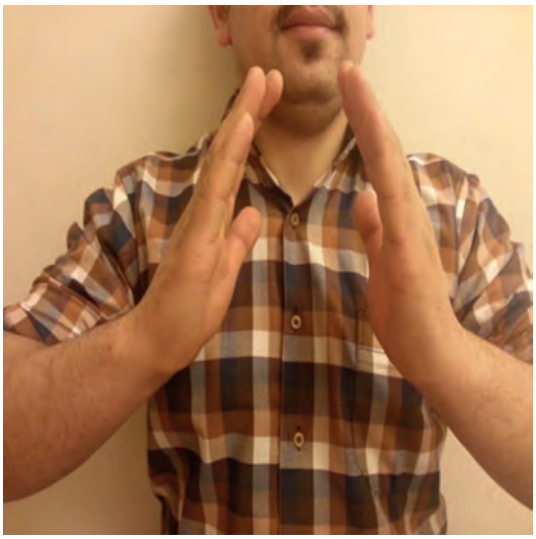

Figure 5b: Extension at 36 ay

pecially in cases with associated dislocation, rupture of the surrounding ligaments and the dorsal joint capsule may result in instability. Once appropriately stabilized, these cases may require repair. Reconstruction of the inter-metacarpal and capsular structures, such as the inter-metacarpal abductor pollicis longus augmentation described by Brunelli et al. may be required, especially in isolated dislocations (3).

Trapezium injuries are likely to be missed on routine radiographs. A CT should be performed if the patient with localized pain has tenderness in the region even if the results of routine radiographs appear to be normal (4, 5). Occult injuries could be identified by using a special view such as a true anteroposterior radiograph (Robert's view) that is excellent for identifying the trapezium and the base of the metacarpal.

Anatomical reduction is recommended because of the importance of the trapeziometacarpal joint of the thumb function. Surgical treatments have been proposed in the literature. Peterson recommended excision of the trapezium following complete dislocation because of the likelihood of avascular necrosis (8). Brunelli reported reconstruction of the intermetacarpal and capsular structures in isolated dislocations (9).

In our case, isolated dislocation of trapezium was a result of violent and direct trauma. Different techniques have been proposed to achieve a stable fixation and the treatment outcomes. In our case, open reduction, Kirschner wire fixation and intercarpal ligament repair through a dorsal approach are recommended for satisfactory outcomes in similar cases.

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SECONDARY HAEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS – THE DIFFERENTIAL DIAGNOSIS DILEMMA IN PAEDIATRICS

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SEKUNDARNA HEMOFAGOCITNA LIMFOHISTIOCITOZA – DIFERENCIJALNO–DIJAGNOSTIČKA DILEMA U PEDIJATRIJI

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ABSTRACT

Secondary haemophagocytic lymphohistiocytosis (SHFLH) is a rare, potentially fatal disorder, most commonly caused by the Epstein–Barr virus. It is characterized by neoplastic proliferation of cells that belong to the monocyte–macrophage system and by varied clinical expression.

A girl aged 3 years and 7 months was hospitalized due to continuing high febricity, yellow skin colouring, hepatosplenomegaly and cytopenia in a complete blood count (CBC). Four weeks before hospitalization, she had a lacunar angina and lymphadenopathy.

A low number of erythrocytes, leukocytes and thrombocytes were noted in CBC, with anaemia and the presence of virocytes in a peripheral blood smear. Biochemical blood analyses indicated hyperbilirubinaemia, increased values of transaminases, lactic dehydrogenase, ferritin, triglycerides, D-dimer, acceleration of the activated partial thromboplastin time and decreased values of fibrinogen, with increased values of C-reactive protein and procalcitonin. Using an ultrasound examination of the abdomen, hepatosplenomegaly was perceived; using echocardiographic examination, pericardium layering was noticed; and using a roentgen graphic picture of the lungs, the presence of pleural effusion was detected. In a bone marrow biopsy, the percentage of blasts did not exceed 25%, and rare chemophagocytes were noticed. Using serologic tests, positivity to Epstein-Barr virus in IgM class was demonstrated.

According to the criteria by Histiocyte Society, there were sufficient criteria to establish a diagnosis of SHFLH. With the exception of symptomatic therapies, according to the protocol for SHFLH treatment, a double antibiotic therapy and IV immunoglobulins were given, to which the patient responded with a clinical and laboratory recovery. Therefore, there was no demand for a treatment protocol with cytostatics or bone marrow transplantation.

To resolve a differential diagnosis dilemma in solving cases of uncertain febrile neutropenia.

Key words: *haemophagocytic lymphohistiocytosis, Epstein–Barr virus, children*

SAŽETAK

Sekundarna hemofagocitna limfohistiocitoza (SHFLH) je redak, potencijalno fatalan poremećaj, pokrenut najčešće Epstein–Barr virusom. Karakteriše se neoplastičnom proliferacijom ćelija monocitno–makrofagnog sistema i različitim kliničkim ispoljavanjem.

Devojčica uzrasta 3 godine i 7 meseci hospitalizovana zbog dugotrajne visoke febrilnosti, žute prebojenosti kože, hepatosplenomegalije i citopenije u kompletnoj krvnoj slici (KKS). Četiri nedelje pre hospitalizacije imala je lakunarnu anginu i limfadenopatiju.

U KKS je uočen nizak broj eritrocita, leukocita i trombocita, uz anemiju i prisustvo virocita u perifernom razmazu krvi. Od biohemijskih analiza krvi detektovana je hiperbilirubinemija, povećane vrednosti transaminaza, laktične dehidrogenaze, feritina, triglicerida, ubrzanje aktiviranog parcijalnog tromboplastinskog vremena, D–dimera i snižene vrednosti fibrinogena, uz povećane vrednosti parametara inflamacije (C–reaktivnog proteina i prokalcitonina). Ultrazvučnim pregledom abdomena uočena je hepatosplenomegalija, ehokardiografskim pregledom perikardno raslojavanje, a rentgenografskim snimkom pluća postojanje pleuralnog izliva. U bioptatu kostne srži procenat blasta nije prelazio 25%, a uočeni su ređi hemofagociti. Serološkim testovima dokazana je pozitivnost na Ebstein–Barr virus u IgM klasi.

Prema kriterijumima udruženja Histiocyte Society, postojalo je dovoljno kriterijuma za postavljanje dijagnoze SHFLH. Pored simptomatske terapije, prema protokolu za lečenje SHFLH ordinirana je dvojna antibiotska terapija i i.v. imunoglobulini, na koje je pacijentkinja odreagovala kliničkim i laboratorijskim oporavkom. Nije zahtevala lečenje protokolima citostatika ili transplantaciju kostne srži.

Diferencijalno–dijagnostička dilema u slučaju rešavanja nerazjašnjenih febrilnih neutropenija.

Ključne reči: *hemofagocitna limfohistiocitoza, Ebstein–Barr virus, deca*

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INTRODUCTION

Histiocytoses are disorders that are characterized by unknown pathophysiological mechanisms of emergence and by neoplastic proliferation and accumulation of cells from the monocyte–macrophage system (1, 2). There are two groups of immunological cells: histiocytes, which are dendritic cells with primary antigen–presenting cell function, and macrophages, with primary phagocytic function (1). Classification of these diseases is difficult, and currently, the most reliable method is histopathological classification according to the international Histiocyte Society. The classification is accepted by the World Health Organization (1, 3). (Table 1)

Haemophagocytic lymphohistiocytosis belongs to the second class of histiocytoses and is divided into primary (familial or hereditary) and secondary form. There are no clinical or laboratory differences between these two forms of the disease. They are differentiated according to genetic examinations (4, 5).

The familial type of the disease is often comorbid with some immunodeficient conditions, such as Chédiak–Higashi syndrome, Griscelli syndrome, and X–linked lymphoproliferative syndrome. It is autosomal recessive and found in 1 in every 30 to 50 thousand newborns. Genes that encode perforin are established, and they are considered to be responsible for the expression of the primary form. These genes include PRF1, UNC13D, STX11, STXBP2 and RAB27A, which are located on the second arm of chromosome 9 and 10 (4–11).

Emergence of secondary haemophagocytic lymphohistiocytosis is primarily attributed to viruses. In approximately 70% of cases it is caused by the Epstein–Barr virus and less commonly Cytomegalovirus, Humane Herpesvirus, HIV, Parvovirus, viruses of Hepatitis A, B or C, and extremely rarely bacteria (Salmonella, Staphylococcus, Mycobacterium tuberculosis, Brucella, Leptospirosis, Rickettsia prowazekii), fungi (Candida albicans) or parasites (Leishmania). The causes of the disease can be malign, rheumatologic and autoimmune diseases (4, 5, 12–26).

The criteria for making a diagnosis of haemophagocytic lymphohistiocytosis are (presence of 5 to 8 criteria is necessary) (1, 4, 5, 27–30):

1. Temperature >38°C for five days, resistant to the application of antipyretics
2. Splenomegaly according to echosonographic criteria, depending on age and sex (31)
3. Cytopenia ≥ 2 cell lines
   - Haemoglobin (Hb) level < 90 g/l (newborns < 4 weeks, Hb<100 g/l)
   - Number of neutrophils (Neu) < 1 x10^9/l
   - Number of thrombocytes (Tr) < 100 x10^9/l
4. Hypertriglyceridaemia and/or hypofibrinogenaemia
   - Triglycerides≥ 3 mmol/l
   - Fibrinogen ≤ 1,5 g/l
5. Hyperferritinaemia – Ferritin ≥ 500 μg/l (32, 33)
6. Serum concentration CD25 (receptor for IL–2) ≥ 2400 U/ml (32, 34, 35)
7. Decreased or absent activity of NK cells (35)
8. Haemophagocytosis seen through a microscope in a peripheral blood smear, bone marrow aspiration, cytological examination of liquor or biopsy of lymph node (36)

Disease prognosis is uncertain, and the treatment must be started as soon as possible, as the goal is to prevent increased inflammatory response (1, 4, 27, 30, 37).

CASE REPORT

A girl aged 3 years and 7 months was directed to the Pediatric Clinic, Clinical Centre Kragujevac because she had an increased temperature of 40°C, which lasted for two days before admission and rarely responded to the application of antipyretics (Paracetamol). Her symptoms were fatigue, loss of appetite, sickness and vomiting of undigested food content 2–3 times per day, as well as watery stools (2–3 times a day), that were light and had visible mucus. Her parents provided information that her urine was darker than normal and had a brown colour. Basic labo-

Table 1. Classification of histiocytosis

Class	Syndrome	Cell type	Diagnostic characteristics of cells
I	Histiocytosis of Langerhans cells (eosinophilic granuloma, Hand Schuller Christian disease, Letterer–Siwe disease)	Langerhans (dendritic) cells	Birbeck granules under electronic microscope, CD1 positive cells
II	Haemophagocytic lymphohistiocytosis - primary (familial) - secondary	Mononuclear phagocytes	Negative Birbeck granules and CD1, positive nonspecific esterase
III	Malign diseases of histiocytes Acute monocytic leukaemia (FAB M5) Real histiocytic lymphoma	Malign cells of monocytic–macrophagic composition	Malign morphologic characteristics of cells, with cell features from class II
IV	Other histiocytic syndromes (benign) Sinus histiocytosis with massive lymphadenopathy (Rosai–Dorfman disease) Juvenile Xanthogranuloma Reticulohistiocytoma	Mononuclear phagocytes	Negative Birbeck granules and CD1, positive nonspecific esterase

ratory analyses were performed. An examination of urine sediment, conducted using a qualitative method, albumins, urobilinogens and bilirubins were detected. Moreover, in a complete blood count, anaemia (Hb 108 g/l), leucopenia (Le 1,45 x10^9/l), and thrombocytopenia (Tr 56 x10^9/l) were noticed. Apart from antipyretics whilst she was highly febrile, she did not use any medications in the 3 weeks before admission.

Personal anamnesis of the child was without any specifications. Four weeks before the appearance of symptoms, the child was treated with penicillin for a period of 7 days due to the lacunar angina and the swelling of lymphatic nodes on her neck. In the family anamnesis, apart from the data regarding members of her family suffering from hypertension and diabetes mellitus type II, the presence of other heredodegenerative diseases was negative.

During the physical examination, it was determined that the girl, body weight 16 kg, body height 106 cm and body mass index 14,29 kg/m^2, was conscious, highly febrile (38,8°C), and considerably adynamic, with a subicteric tint of her skin, which showed a decrease in turgor and elasticity. Additionally, she had icteric sclera, coated tongue, slightly hyperemic pharynx, and the lymph nodes of her neck had normal dimensions for her age. She had no signs of haemorrhagic syndrome. Auscultatory findings on the heart and lungs were normal, SaO2 96%, number of respirations was 24/min, TA was 95/55 mmHg, and pulse was 132 beats/min. Her abdomen was not painfully sensitive during palpation. Her liver was palpated 4 cm below the rib arch and her spleen on the rib arch. Neurological findings, besides mild somnolence, were normal for her age.

Due to the serious clinical outlook and laboratory parameters, especially the decreased number of neutrophils, the girl was hospitalized in the intensive care unit in the isolation department because she was thought to have a septic condition. There, her vital functions were regularly monitored, intravenous rehydration was started, antipyretic therapy was applied every 6 hours, and to correct the intestinal flora, an antidiarrhoeal diet was introduced with a probiotic preparation.

Upon admission, initial laboratory examinations were performed. She had normal electrolyte and acid–base status. According to the complete blood count that was repeated daily during hospitalization, among pathological values, anaemia was determined (Hb 102...98...94 g/l), with a reduced number of erythrocytes (Er 3,72...3,67...3,52 x10^{12}/l), perceived hypochromia Er in the peripheral blood smear, normal percentage of reticulocytes (Rtc 0,9%) and with negative direct and indirect Coombs tests. Distinct leucopenia was persistent (Le 1,4...1,4...1,9 x10^9/l), with predominance of lymphocytes in the leukocyte formula (Ly 1,1...1,2...1,4 x10^9/l, with 10–20% of atypical lymphocytes in the peripheral blood smear), neutropenia to agranulocytosis (neutrophils 0,25...0,18...0,34 x10^9/l), as well as thrombocytopenia (Tr 44...35...46 x10^9/l).

Among biochemical analyses of the blood, values of total (TBill) and conjugated (DBill) bilirubin (72 µmol/l and 44,2 µmol/l), values of transaminases [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] and values of lactic dehydrogenase (LDH) were increased during the hospitalization (AST 123...141 U/l, ALT 296...168 U/l, LDH 1364...1270 U/l). Additionally, we found the acceleration of activated partial thromboplastin time (aPTT 32,4...34,7 s). Fibrinogen levels were decreased, whereas there were high levels of D-dimer (fibrinogen 2,017...1,673 g/l, D–dimer 3884,49 ng/ml). Ferritin values were considerably increased, 2409 g/l. Cholesterol values, including HDL and LDL fractions, were within the limits of normal values. However, hypertriglyceridaemia was determined (4,08 mmol/l). Additionally, hypoalbuminaemia was determined (albumins 22 g/l). Values of glycaemia, urea, creatinine and uric acid were in the referential scope.

Increased values of the following inflammation parameters were detected that persisted in the following days: sedimentation of erythrocytes (SE) 15 mm/lh, C–reactive protein (CRP) 50,2...22,2 mg/l and procalcitonin (PCT) 2,910...1,780 ng/ml [which demanded detailed bacteriological swabs of pharynx and nose, antistreptolysin titre (ASOT), two blood cultures and urinoculture] and virological examinations (Cytomegalovirus, Epstein–Barr virus, HIV, Toxoplasma gondii, Hepatitis A, B and C, and Micoplasma pneumoniae).

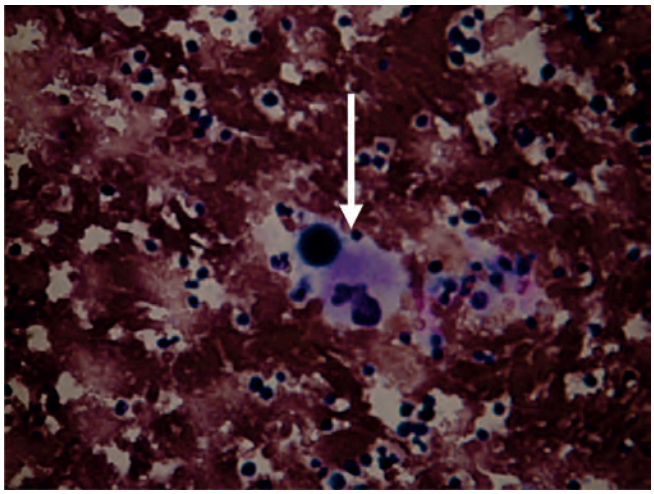
Considering the diagnosis of febrile neutropenia, it was necessary to include a double empirical antibiotic therapy. Therefore, cephalosporin of the third generation was applied – Ceftazidime, with Aminoglicozide and Amikacin (38–40).

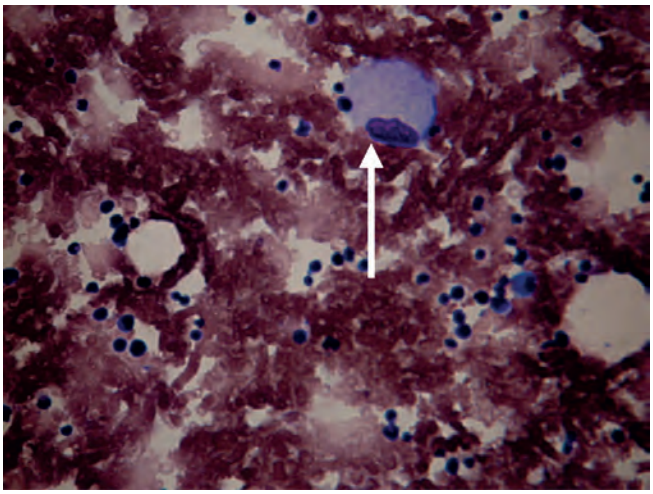
Values of immunoglobulin fractions (IgA, IgM, IgG), as well as of the component complements (C3 and C4) were within the limits of referential values for that age.

Using an ultrasound examination of the abdomen, the presence of hepatomegaly (anteroposterior diameter of right lobe 116 mm) and splenomegaly (cranio–caudal diameter 106 mm) were determined, according to the table of normal dimensions for the patient's age and sex (31).

Bone marrow aspiration was performed, and using the microscopic examination of aspirate, cellularity of III–IV degree was determined along with evident productive megakaryocytes and all forms in the development of red and white blood cells. The percentage of blast did not exceed 25%. After 3 days of hospitalization, bone marrow aspiration was repeated and a detailed microscopic examination showed rare haemophagoctes in several places (Pictures 1A. and 1B.). Both peripheral blood and bone marrow aspirate samples were collected for flow cytometry, which was not performed due to technical reasons.

Although the therapy was applied, during the first 3 days, the girl was still highly febrile (39,5°C), inactive, had a loss of appetite, and her skin, visible mucus, urine and stool were slightly discoloured. Furthermore, she suffered from tachydyspnea, with a number of respirations reaching 36/min. She developed a dry and sensitive cough, in bases with weak, quiet breathing sound during auscultations more to the left side, and low oxygen saturations,





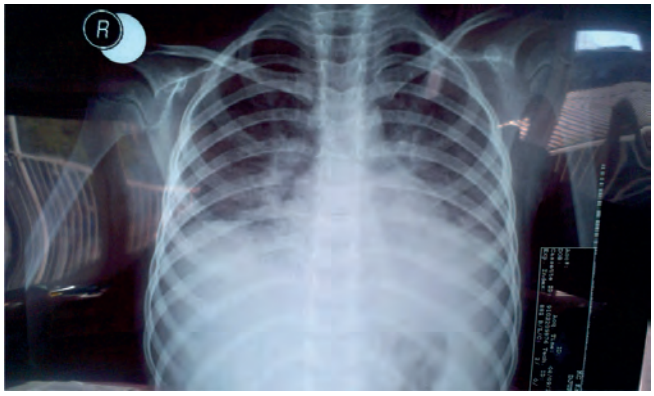
Pictures 1A and 1B. Haemophagocytosis in the preparation of bone marrow aspirate

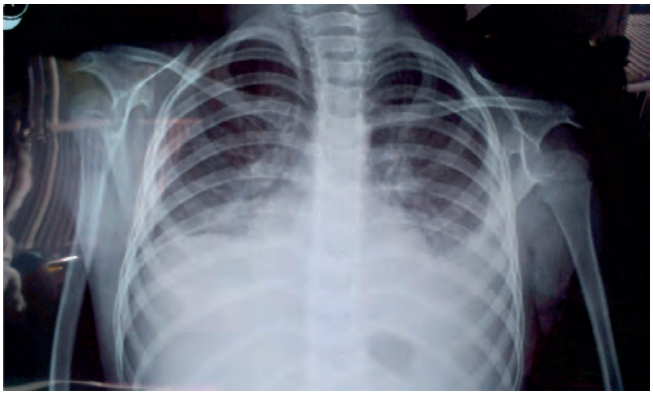
SaO2 91–92%. Therfore, roentgenography of the lungs (RTG) was conducted. Paramediastinally zones of consolidations of lung parenchyma with a tendency to merge were noticed on images. A shadow of effusion in the left costophrenic angle up to the level of the fifth rib was also noticed. A repeated recording of the following day showed that the condition was progressing (Pictures 2A and 2B). In light of this, considering the possibility of the emergence of more serious nosocomial infections, current antibiotic therapy was changed, and secondary antibiotics were given according to the protocol– Meropenem and Vancomycin, with antimycotic Fluconazole, and considering an atypical cause of pneumonia, Azithromycin was also applied (38–42).

Moreover, due to the quiet heart tones during auscultation, electrocardiographic examination (the finding was normal for her age), and laboratory observation of heart enzymes [troponin I, creatine kinase (CK) and CK MB, and N-terminal beta natriuretic peptide (NTproBNP) were all within the limits of referential values) were performed. However, at the echocardiographic examination, besides normal heart morphology and contractility, and an ejection fraction of 66%, light tricuspid regurgitation and hyperechogenicity of the pericardium were determined, with minimal layering of the visceral and parietal leaf of 3 mm.

After placement onto the surface, the results of sterile blood cultures and urinoculture were ready. The presence of normal flora in the nose and pharynx swab was determined, and ASOT were in the limits of referential values. However, virological analyses proved seronegativity to all considered viruses, except for a highly positive titre to Epstein–Barr virus in the IgM class and a less positive one in the IgG class. Therefore, it was confirmed that infection 4 weeks before hospitalization, which was regarded as lacunar angina and treated with beta lactam antibiotics, was actually infective mononucleosis. These findings led to a suspicion that the girl suffers from secondary haemophagocytic lymphohistiocytosis (SHFLH), which was caused by the Epstein–Barr virus.

According to the treatment protocol for SHFLH (43), IV immunoglobulins were introduced in the therapy. The girl was directed to the referential institution, Institute of Mother and Child Care "Dr Vukan Čupić" in Novi Beograd, due to additional medical diagnoses and possible treatment with cytostatic protocol and/or a possible need for bone marrow transplantation.





Picture 2A. RTG lung 04.09.2013.

Picture 2B. RTG lung 05.09.2013.

During hospitalization at the Institute, there was a spontaneous, steady, both clinical and laboratory recovery of the child, with the application of only symptomatic therapy (the continuation of antibiotic therapy started in Clinical Center Kragujevac and IV rehydration). Therefore, IV immunoglobulin therapy, to which the girl adequately responded, was most likely sufficient. She became afebrile, in better general state, regained her appetite, and there was no more colouring of the skin and mucus. The number of cell lines in CBC was normalized, as well as the biochemical analyses of the blood. With such a condition of the child, bone marrow aspiration was not repeated. On the roentgen graphic picture of lungs it was determined that the finding is in considerable regression with no signs of pulmonary parenchymal consolidation, but with the persistence of a small shadow of pleural effusion in the right costophrenic angle. In the ECHO examination of the heart, the presence of an increased amount of pericardium fluid was not determined. Moreover, in the ECHO examination of abdomen, persistence of splenomegaly was determined, with considerably decreased values (cranial–caudal diameter of 96 mm) and no hepatomegaly (anteroposterior diameter of the right lobe 103 mm). A parameter that is also in favour of a SHFLH diagnosis is soluble anti CD25, whose values were increased over 2400 U/ml.

Due to technical reasons, the activity of NK cells were not determined, and genetic examinations of the primary form of the disease were not undertaken because these analyses are not performed in the Republic of Serbia.

Since then, the girl has been in a good general state with regular physical findings and normal laboratory analyses. She is submitted for regular examinations by the haematologist in charge.

DISCUSSION

Haemophagocytic lymphohistiocytosis was first described in 1939 as histiocytic medullary reticulosis (44). In 1952, a familial type of the disease in two newborn twins was described (45), and 1965 after the simultaneous development of the disease in father and son, it was thought that infection can be important in the pathogenesis of the disease (46). The hereditary form usually emerges until 4 years of age, and according to some authors even until the second year of age. In the cases of anamnestically known and/or proven infection 3–4 weeks before the beginning of the disease, the secondary form should be considered. In the cases where there are relatives with the same disease, the familial form should be considered. However, it is often the case that the hereditary form is initiated by some viral influences (47). The primary form is found together with some immunodeficient conditions, whilst it is typical for the secondary form to emerge in children with a normal immunological status, which was the case with our female patient. The secondary form may develop into a spontaneous remission, whilst the primary form always necessitates the application of therapy. Treatment is identical for both forms, which are potentially fatal diseases, and poor prognosis and higher mortality rate are typical of the primary form (1, 29, 30, 48–51).

The Epstein–Barr virus is thought to be responsible for the emergence of secondary haemophagocytic lymphohistiocytosis in approximately 70% of cases. The mechanism of disorder in the proliferation of cytotoxic and helper T lymphocytes is unknown. However, a similar monoclonal proliferation of T lymphocytes was determined in those patients suffering from secondary haemophagocytic lymphohistiocytosis and in EBV+ T cell lymphoma (52). The consequence is a hyper production of proinflammatory cytokines, especially interferon γ, cell factors, tumour necrosis factor α and of various interleukins (IL – 1, 6, 16, 18), decreased inactivation of NK cell functions and to hyper activation and accumulation of macrophages (12, 29, 30, 38, 48–50, 53).

Clinical signs that may indicate the presence of haemophagocytic lymphohistiocytosis are nonspecific. Commonly, high temperature >38°C and fever (in 80% of patients), with a considerably bad general state (fatigue, sleepiness, refusal to eat) are evident. The highly febrile state lasts for several days and is resistant to antipyretics, which was the case with our patient. Sickness, vomiting, and watery stools before hospitalization can be attributed to the general bad condition of the child, to the increase in intra–abdominal pressure due to hepatosplenomegaly and to the influence of mediator released from the monocyte-macrophage system to the cell function of gastrointestinal tract. The girl did not have a generalized lymphadenopathy, that is, besides splenomegaly (in 60%) and hepatomegaly (50%), described in 40% of cases as one of the signs of the diseases (1, 12, 27–30, 37, 50, 51, 54, 55). A common sign that is mentioned is rash, with visible marks on the skin (35–65%), as well as neurological symptomatology in the sense of somnolence, meningismus, ataxia, and even convulsions and epileptic attacks (30%) (56, 57).

Yellow colouring of the skin, a visible mucous membrane, lighter stool and dark coloured urine, and the presence of urobilinogens and bilirubins in the urine indicated hyperbilirubinaemia, which was confirmed in laboratory. The origin of hyperbilirubinaemia and comorbid hepatosplenomegaly was examined. Firstly, a possibility of the existence of a stronger haemolysis of erythrocytes in the enlarged spleen was eliminated by determining the number of Er in CBC, the percentage of reticules in peripheral blood, as well as, direct and indirect Coombs tests. Increased values of transaminases and lactic dehydrogenase indirectly indicated a necrosis of hepatocytes, whilst acceleration of the activated partial thromboplastin time and decreased values of fibrinogens with high values of D–dimer pointed to the decreased synthetic liver function.

In addition to anaemia and thrombocytopenia, in CBC, leucopenia persisted, with a predominance of lymphocytes in leukocyte count, with 10–15% of atypical lymphocytes (so called virocytes), which indicated that it is a viral in-

Table 2. Clinical and laboratory criteria for establishing the diagnosis

Criteria for establishing the diagnosis (7 out of 8)	Auxiliary criteria
1. Temperature above 38,5°C for 9 days 2. Splenomegaly (106 mm) 3. Cytopenia ≥ 2 cell lines Haemoglobin 98 g/l Neutrophils 0,25 x 10^9/l Thrombocytes 45 x 10^9/l 4. Hypertriglyceridaemia and/or hypofibrinogenaemia Triglycerides 4,08 mmol/l 5. Ferritin 2409 μg/l 6. Haemophagocytosis in bone marrow 7. Soluble anti CD25 (receptor for IL–2) ≥ 2400 U/ml	1. IgM EBV positive 2. Pleuritis, pericarditis 3. Decreased values of fibrinogen 4. Increased values of transaminases 5. Increased values of bilirubin 6. Accelerated aPTT 7. Increased values of D–dimer 8. Increased values of LDH 9. Hypoalbuminaemia 10.Hepatomegaly

fection, as well as neutropenia to agranulocytosis. Anamnestic data on non–usage of drugs or on the exposition to toxic materials in the last 3 weeks excluded a possibility of bone marrow aplasia caused by these substances (58).

To exclude the possibility of the emerging malignant haemopathies, in a peripheral blood smear, as well in a microscopic examination of bone marrow biopsy, over 25% developing forms of such cells were not found (59). Moreover, as tumour markers, values of ferritin were increased. However, besides the role of a tumour marker, ferritin was, in this case, considered as an index of inflammation and liver function (60). Additionally, normal levels of uric acid, which is a marker of increased cell degradation of tumour cells (59), were detected. Using an ultrasound examination of the abdomen, the existence of retroperitoneum enlarged lymph cords was not determined. Unfortunately, flow cytometry was not performed due to technical reasons.

Because the girl was regularly vaccinated and had a BCG mark of 3 mm, normal immunological status, was from a good socioeconomic environment, with no evidence of living in a collective group or a possible contact with tuberculosis, the possibility of tuberculosis pleural effusions was not considered. Before and during hospitalization, there was no neurological symptomatology in the female patient apart from light sleepiness that is a consequence of a complete bad condition and high temperature. Therefore, there was no need for diagnostic lumbar puncture, which is also in the diagnostic protocol for SHFLH (30, 37, 50, 51, 56, 57).

The results of sterile blood cultures and urinoculture, which revealed normal flora from the swab of the nose and pharynx and ASOT that was in the limits of referential values, excluded a possibility of a septic state caused by bacterial infection. With a confirmed seronegativity to all considered viruses, except for highly positive titre to Epstein–Barr virus in the IgM class, it was thought that the girl had a case of haemophagocytic lymphohistiocytosis (SHFLH).

To establish a final diagnosis, values of triglycerides that were increased were considered, as well as the values of albumins (according to Huang et al (61), decreased values of albumin will be another criteria for establishing a diagnosis for SHFLH, and it is a good predicative parameter of the disease). Hypertriglyceridaemia and hypoalbuminaemia cannot be considered as the cause of pleural and pericardium effusion, with the emergence of chylothorax in the first, or the decrease in colloid–osmotic pressure of plasma in the second case, but the effusion is most surely a consequence of aseptic serositis, i.e., influence of mediator to epithelial cells of pleura and pericardium and their increased secretion (62–64). Moreover, bone marrow aspiration was performed once more, and a detailed and targeted microscopic search of biopsies revealed rare haemophagocytes in several places.

The clinical and laboratory criteria are summarized in table 2. According to international Histiocyte Society, these criteria as well as the auxiliary diagnostic criteria that we also followed in solving differential diagnosis dilemmas are used to diagnose SHFLH (1, 4, 5, 27–30).

Prognosis of the disease is uncertain, and it depends on the age and immunological status of the patient, degree of organs affected, dissemination of the disease, speed of establishing a diagnosis and the response to therapy. The disease can end with a spontaneous recovery, adequate curative treatment, or it could also result in rapid and progressive death, regardless of the applied therapy. Spontaneous recovery is expected in 20–30% of the patients. With an adequate symptomatic and immunosuppressive treatment, recovery is expected in 60–70% of the patients. That percentage is higher after bone marrow transplantation. The mortality rate, depending on the studies, is approximately 25–60% of patients (1, 4, 12, 13, 50, 51, 65–68).

Apart from the symptomatic therapy (IV rehydration with the correction of electrolytic and acid–base status, antipyretics, oxygen therapy, adequate care and diet, transfusion of deplasmatisized erythrocytes and concentrated thrombocytes, antibiotics and antimycotics), immunosuppressive and immunomodulatory therapy are performed (corticosteroids, immunoglobulins, antithymocytic globulin, cyclosporine A) as well as various protocols with cytostatics (initial cytostatic therapy – Etoposide, intrathecal methotrexate, with corticosteroids, and continuous cytostatic therapy – same medications in smaller dosages, according to the treatment protocol suggested by Histiocyte society). As a final measure, allogenic transplantation of bone marrow is suggested (27, 30, 37–43, 68–71). Some

studies have shown efficiency in the application of biologic therapy (72). Antiviral medications (ege.g., Acyclovir) do not have significance in the treatment (12, 13, 27, 30).

In our example, besides the applied symptomatic and antibacterial therapy, a therapy with IV immunoglobulins was sufficient, and it is described in the works of other authors (43, 66–68).

Based on the facts reported herein, we can conclude that although hemophagocytic lymphohistiocytosis is a rare disease, one should be aware of it, especially in clinically uncertain conditions, such as in cases of febrile cytopenia, when the patients do not respond to the application of standard symptomatic therapy. Timely diagnosis offers higher chances for survival, with a timely beginning of immunosuppressive, immunomodulatory therapy and possible bone marrow transplantation.

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ENHANCEMENT OF DERMAL FIBROBLAST ISOLATION METHOD

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UNAPREĐENJE METODE IZOLACIJE DERMALNIH FIBROBLASTA

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ABSTRACT

Cultivated fibroblasts have been widely used in a large number of in vitro studies. Although they readily proliferate under cell culture conditions, improvements in methods for their isolation are necessary. Here, we present our modified enzyme digestion method and compare its efficiency with commonly used techniques.

Three foreskin samples from young, middle-aged and old donors were used. The classical explant, standard enzyme digestion method with collagenase and our improved enzyme digestion method were compared for efficiency of fibroblast isolation and the time needed to achieve 95% confluence in a 30-mm Petri dish.

The explant method was the slowest to achieve fibroblast confluence, especially with the tissues from the older donors (up to 23 days). With the standard enzyme digestion method, the skin tissue was partially digested, but the fibroblasts reached confluence much faster (the younger donor cells needed approximately 7 days to reach confluence). Our modified "mixed" enzyme digestion method was the fastest (the fibroblasts from the younger donors needed up to 5 days to reach confluence).

For studies requiring the primary isolation and cultivation of dermal fibroblasts, the best method to achieve this goal is the tissue digestion method with the multiple enzyme solution.

Keywords: *dermal fibroblast, isolation, cultivation, enzyme digestion*

SAŽETAK

Kultivisani fibroblasti se često upotrebaljavaju u brojnim „in vitro" studijama. Iako oni relativno lako proliferišu u uslovima ćelijskih kultura, standarne metode primarne izolacija fibroblasta nisu dovoljno efikasne. U ovom radu mi prikazujemo modifikovanu metodu enzimske digestije tkiva i upoređujemo njene rezultate sa standardnim metodama.

U eksperimenu su korišćena tri uzorka dobijena nakon cirkumcizije kod mladih, sredovečnih i starih pacijeneta. Upoređivana je efikasnost primarne izolacije fibroblasta korišćenjem eksplant metode, standardne enzimske metode uz korišćenje kolagenaze i naše modifikovane metode enzimske digestije tkiva. Upoređivano je vreme neophodno za dostizanje 95% konfluencije fibroblasta u 30mm Petri šoljama.

Eksplant metoda je najsporija kada je u pitanju dostizanje konfluencije i to posebno kod starih donora (do 23 dana). Standardna metoda enzimske digestije dovodi do nepotpune disocijacije tkiva humane kože, ali je dostizanje konfluencije fibroblasta bilo znatno brže nego kod eksplanta (kod mladih donora polovinom 7. dana). Naša, modifikovana enzimska metoda sa mešavinom enzima je najbrže dovela do konfluencije ćelija (kod mladih donora 5. dana).

Kada dizajn neke studije zahteva efikasnu izolaciju dermalnih fibroblasta i visok procenat vijabilnosti ćelija, najbolja metoda je digestija tkiva sa mešavinom enzima.

Ključne reči: *dermalni firboblasti, izolacija, kultivacija, enzimska digestija*

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INTRODUCTION

Fibroblasts are the main cell population in the dermal part of the skin. They play a crucial role in the maintenance of the normal histological organisation of the dermis and the repairing of wounded connective tissue. In the dermis of the skin, there are two different layers of connective tissue (1), including the papillary layer, which is mainly composed of loose connective tissue with a large number of biologically active fibroblasts, and the reticular layer, which is largely comprised of dense irregular, connective tissue that contains less fibroblasts with weaker mitotic activity (2, 3). In the cell culture system, fibroblasts readily proliferate in the presence of bovine sera and even a small amount of nutrients in the medium and frequently overgrow other cells in cultivation dishes; thus, they are commonly referred to as "weeds" of cell cultures (4). Considering that cultivated fibroblasts have been widely used in a great number of *in vitro* studies, ranging from examinations of metabolic pathways, cell-to-cell interactions, and drug effects to tissue engineering and genetic investigations, determining a reliable and quick method for the isolation and establishment of healthy primary fibroblast cultures is important (5-10). Although most studies have been performed on fibroblast cell lines purchased from cell factories, experiments with laboratory animals frequently require primary isolation for the confirmation of results obtained from cell cultures. There are few studies in the literature providing data on the improvement of isolation protocols, especially with regard to the shortening of the time necessary to establish healthy primary cultures and improvements in total cell yields (11, 12). Authors have agreed that the main success-limiting factors are the tissue type and the age of the donor. Younger and especially embryonic connective tissues are potent sources of fast-growing fibroblasts. In contrast, dense tissues from older donors may present significant problems for successful fibroblast culture establishment (13). In research laboratories, two major methods of fibroblast isolation are typically used, tissue explant and enzyme digestion with different types of matrix metalloproteinases, particularly collagenase (10-16). The main disadvantage of the explant method is the fact that it generates cells quite slowly (15). On the other hand, enzyme digestion can generate a great number of fibroblasts quickly, but problems that are mainly due to incomplete tissue digestion may occur when a small amount of tissue is to be processed (17, 18). Here, we present an improved protocol for fibroblast isolation and compare its efficiency with those of the standard explant and enzymatic methods.

MATERIALS AND METHODS

Foreskin samples from the three donors were used. All experiments were performed according to the EU (86/609/EEC) and local ethical guidelines with consent from the donors. The ages of the donors were 18, 34 and 68 years for samples one, two and three, respectively. All donors underwent a circumcision procedure, and their skin was found to be absent of pathological changes. The skin samples were kept in sterile saline solution with antibiotics, and all samples were processed within three hours. After the removal of excess tissue, the samples were transferred to a sterile dispase II (20 mg/ml, Sigma-Aldrich, USA) solution overnight at 8°C. Then, the epidermis was peeled off with tweezers. The remaining tissue was finely minced with scissors to obtain small dermal pieces of approximately 1 mm in diameter. The skin tissue was then measured, and 18 grams of tissue from each sample were divided into three equal parts to be used for each isolation method so that the same amount of skin tissue would be evaluated in each case. Three different methods for the isolation of dermal fibroblasts were used as follows: the explant technique, the standard enzymatic dissociation procedure with collagenase II (10 mg/ml (Gibco, USA), overnight at 37°C) and our isolation procedure with a "mixed" enzyme solution (10 mg/ml collagenase II, 5 mg/ml dispase II, 1 mg/ml DNase (Invitrogen, USA), and 10 mM calcium-chloride (Sigma-Aldrich, USA), overnight at 40°C). For the explant method, pieces of tissue from each donor were transferred into three 30-mm Petri dishes, and cell culture medium was added. Tissue lysates obtained by enzyme digestion were inspected for the degree of digestion and cell viability using a fluorescence microscope with an acridine orange/ethidium bromide fluorescent dye. The cells were then isolated by washing in the buffer solution and centrifugation. Isolated fibroblasts from each donor were seeded in six Petri dishes. All cells were cultivated in low-glucose DMEM medium (Invitrogen, USA). The medium was changed every other day, and the time needed for the cells to reach over 95% confluence in the 30-mm Petri dishes was then measured. Near the end of the experiment, transparent millimeter paper was attached under the bottom of the Petri dish and used to assess the degree of confluence. All values were expressed as the mean ± standard deviation (SD). Commercial SPSS version 20.0 for Windows (SPSS version 20.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data distributions were evaluated for normality using the Shapiro-Wilk test. Statistical evaluation was performed by Student's t-test for paired observations or one-way ANOVA, depending on the data distribution. P values of less than 0.05 were considered significant.

RESULTS

The results of the three different fibroblast isolation methods are described as follows. With the explant method, the fibroblasts started to show outgrowth after 4-9 days. The earliest outgrowth was observed with the tissue obtained from sample 1 (mean value of 102±19 hours), while sample 2 showed the same results, but with a delay

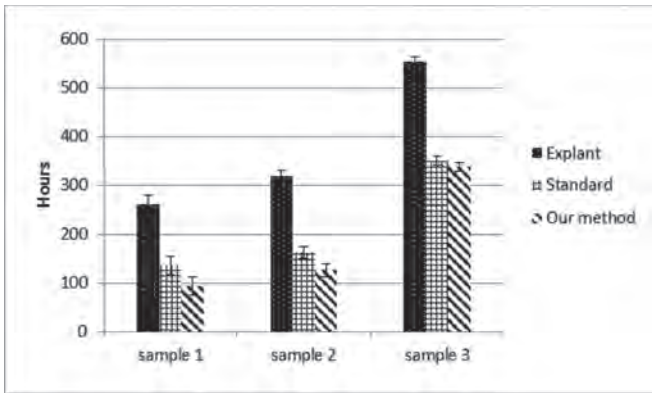

Figure 1: Time needed for 95% fibroblast confluence in relation to donor age and the type of cell isolation method used.

of almost two days (mean value of 138±14 hours). Cell outgrowth of sample 3 (the oldest donor) failed to occur in one Petri dish (after 3 weeks), and the others showed proliferation within 204±12 hours. The desired 95% cell confluence was reached on the 11th day (261±19 hours) for sample 1 and the 14th day for sample 2 (318±26 hours). For sample 3, cells in two Petri dishes did not reach confluence even after 4 weeks, but for the other 3 specimens, confluence was reached on the 23rd day (554±46 hours) (Fig. 1).

With the standard enzyme digestion method, the fibroblasts from sample 1 reached confluence in 6 days (137±13 hours), and confluence was reached in 7 days for sample 2 (163±11 hours) and 15 days for sample 3 (350±43 hours). Tissue digestion was not complete because a significant number of fibroblasts were still trapped inside of the collagen fibre mesh after the treatment (Fig. 2).

Our improved enzyme digestion method with a "mixed" enzyme solution was found to be the most effective. The fibroblasts from the youngest donor reached 95% confluence in 4 days (94±9 hours), while the cells from sample 2 achieved this goal after 5 days (127±12 hours). Interestingly, although the digestion of the dermal tissue from the oldest donor was better with our method than with standard enzyme digestion method, the fibroblasts isolated by our method reached the 95% confluence at roughly at the same time as those isolated by the standard method (338±36 hours) (Fig. 1).

Statistical analysis showed that the fibroblasts isolated by both enzyme digestion methods reached 95% confluence significantly faster ($p<0.05$) than those isolated with the explant method. A comparison of the two enzyme digestion methods showed that for sample 1 (the young donor), our method allowed for the desired number of cells to be obtained significantly faster ($p<0.05$), but this did not occur for the middle-aged and older donors ($p>0.05$).

DISCUSSION

Fibroblasts are the most abundant cells in the human body. Their main role is to produce fibres and components of the extracellular matrix of connective tissues (1, 2). Because they readily proliferate under cell culture conditions, they have been widely used in various *in vitro* studies (5-10). Their primary isolation and cultivation methods have been known for decades (16), but reports of improvements in these techniques are rare and have not been very effective (19). As expected, our study showed that the explant method for the isolation and cultivation of dermal fibroblasts was by far the slowest way to obtain the desired number of these cells compared to the enzyme digestion methods, even when considering the one-day advantage of the explant method (the other methods needed one additional day for digestion). This fact has been well documented in previous publications (15). Although there are some differences between our findings and results from other studies concerning the time that fibroblasts need to start outgrowth from the tissue and proliferate to the desired number, these discrepancies are due to the uses of unequal starting amounts of skin tissue. Nevertheless, we can agree that the explant method should not be used when cultured dermal fibroblasts are needed quickly. This is especially true when the starting tissue originates from older donors because in this case, the explant method is not reliable due to the frequent failure of fibroblast outgrowth that has been observed. In our study, failure occurred in 50% of the cases, if we include the specimens that did not reach 95% confluence. A much faster way to obtain a primary dermal fibroblast culture is the enzyme digestion technique (11, 12, 17). For this technique, the collagenase II enzyme is most commonly used to break up collagen fibres and release fibroblasts from connective tissue. Our results showed that this method is reliable and produces enough cells for a relatively fast cultivation onset. However, in this study, we tried to fully utilise the histological architecture of the skin and enzymatic characteristics to improve the isolation procedure. Considering the two distinct parts of the dermis and especially the structural characteristics of the papillary layer, we assumed that

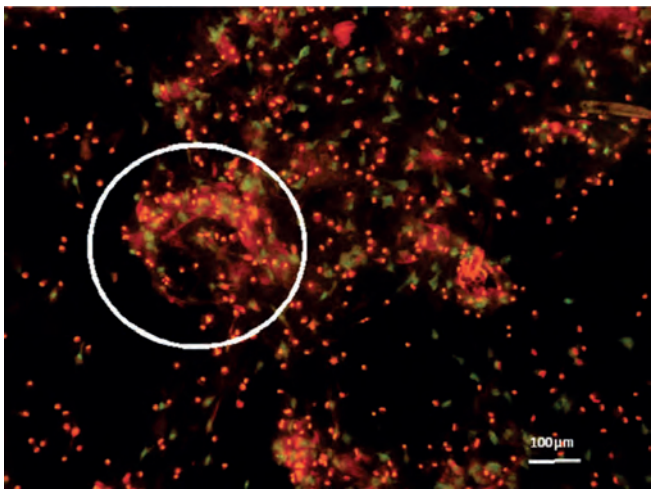

Figure 2: Incomplete digestion. Cells trapped In collagen fiber mash cells (encircled)

proteases, such as dispase II, would enhance cell release by breaking down the attachment of the fibroblast to the collagen fibres because this enzyme hydrolyses peptides bound to non-polar amino acids, which are often found in collagen (20, 21). The results proved that this assumption was correct. The digestion of the young dermal tissue was better with the "mixed" enzyme solution, and the tissue specimens treated with our solution gave rise to more fibroblasts that reached 95% confluence more rapidly than the ones obtained with the standard enzyme digestion method. Furthermore, our method included an elevated calcium concentration and a higher temperature than usual because calcium ions are important for the ability of collagenase to bind to collagen fibres and start digestion, and the activity of this enzyme is higher at slightly elevated temperatures (22, 23). The only major exception occurred when tissue samples from older donors were investigated. In these cases, the "mixed" enzyme solution failed to produce more cells than the standard one, and the fibroblasts reached confluence at virtually the same time. This is surely because of the atrophic changes in the skin of elderly individuals, including reduced cellularity and a significant increase in collagen content (13). Older dermal tissue is denser and less responsive to enzymatic treatment. In addition, fibroblasts lose mitotic potential with age; thus, the overall effect of the digestion solution was diminished. We were somewhat puzzled by the results concerning the middle-aged donors. We expected results similar to those obtained with the younger tissue e.g., the significant improvement of cell isolation, but tissue digestion with the combined enzyme solution was not as complete compared with the young donor. There was some difference in favour of the "mixed" enzyme solution compared to the standard one, but it was not significant. This is probably also due to the structural changes of the skin; therefore, we are in agreement with some authors who have stated that the aging process of the human skin is already apparent during the fourth decade of life (24, 25).

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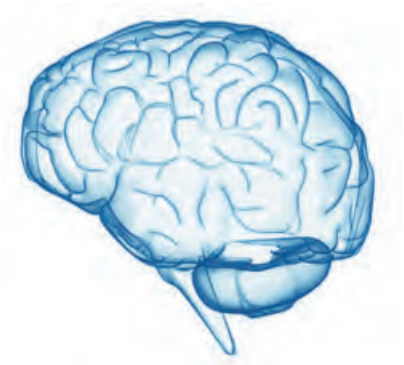

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