ISSN 1820-8665

Vol. 14•No4 • DECEMBER 2013.

Serbian Journal of Experimental and Clinical Research

2000

Serbian Journal of Experimental and Clinical Research

2000

Print
Faculty of Medical Sciences,
University of Kragujevac

Indexed in
EMBASE/Excerpta Medica, Index Copernicus, BioMedWorld, KoBSON, SCIndeks

Address:
Serbian Journal of Experimental and Clinical Research, Faculty of Medical Sciences, University of Kragujevac
Svetozara Markovica 69, 34000 Kragujevac, PO Box 124
Serbia
http://www.medf.kg.ac.rs/sjecr/index.php

SJECR is a member of WAME and COPE. SJECR is published four times circulation 250 issues
The Journal is financially supported by Ministry for Science and Technological Development, Republic of Serbia
ISSN 1820 – 8665

Table Of Contents

PERSPECTIVES ON REGENERATION OF ALVEOLAR BONE DEFECTS

Ljupcho Efremov[1], Tatjana Kanjevac[2], Dušica Ciric[2] and Darko Bosnakovski[1]
[1]Faculty of Medical Science, University Goce Delcev, Stip, R. Macedonia
[2]Faculty of Medical Science, University of Kragujevac, Kragujevac, Srbija

PERSPEKTIVE U REGENERACIJI ALVEOLARNIH KOŠTANIH DEFEKATA

Ljupčo Efremov[1], Tatjana Kanjevac[2], Dušica Ćirić[2], Darko Bosnakovski[1]
[1]Fakultet medicinskih nauka, Univerzitet Goce Delčev, Štip, RepublikaMakedonija
[2]Fakultet medicinskih nauka, Univerzitet Kragujevac, Kragujevac, Srbija

Received / Primljen: 14.01.2014. Accepted / Prihvaćen: 30.01.2014.

ABSTRACT

Bone atrophy of the alveolar process is an important parameter in patients undergoing dental implants. There are several methods for preserving the alveolar process, with the autologous bone graft as the gold standard. Other approaches include the use of allografts, xenografts and synthetic bone grafts.

In recent years, the use of stem cells has increased in importance. The most common type of stem cells used are mesenchymal stem cells from various sources, including bone marrow, adipose tissue and dental pulp. The discovery of induced pluripotent stem cells and the continued research on embryonic stem cells open new possibilities in this field.

However, further research is needed to optimise protocols for isolation, differentiation and transplantation of cells with or without appropriate scaffolds, and to determine the correct clinical and therapeutic implications.

Keywords: *alveolar process atrophy, bone grafts, scaffolds, stem cells.*

SAŽETAK

Atrofija alveolarnog nastavka važan je parametar prilikom planiranja postupka ugradnje stomatoloških implantata. Postoji više načina za očuvanje alveolarnog nastavka, pri čemu se autologni koštani graft smatra zlatnim standardom. Ostali pristupi očuvanja alveolarnog nastavka uključuju upotrebu alograftova, ksenograftova i sintetičkih koštanih graftova.

Poslednjih godina sve više dobija na značaju upotreba matičnih ćelija u ove svrhe. Najčešće korišćeni tip matičnih ćelija jesu mezenhimalne matične ćelije izolovane iz različitih izvora, kao što su koštana srž, masno tkivo i zubna pulpa. Otkriće indukovanih pluripotentnih matičnih ćelija, kao i dalja istraživanja embrionalnih matičnih ćelija, otvaraju nove mogućnosti u ovoj oblasti.

Međutim, neophodna su dalja istraživanja da bi se optimizovali protokoli za izolaciju, diferencijaciju i transplataciju matičnih ćelija sa ili bez upotrebe odgovarajućih skafolda i da bi se utvrdile njihove tačne kliničke i terapijske indikacije.

Ključne reči: *atrofija alveolarnog nastavka, koštani graftovi, skafold, matične ćelije.*

INTRODUCTION

Surgical repair of bone defects remains a major challenge for orthopaedic, reconstructive, dental and craniofacial surgeons, and usually occurs after a traumatic experience. The loss of bone can also occur from infection, neoplasm and congenital disorders.

An important concern in dental medicine are defects that materialise after tooth extraction. Tooth extraction is one of the most common procedures, arising from several conditions, such as severe tooth decay, fractures, periodontal diseases and endodontic lesions. The periodontium is a complex tissue composed mainly of periodontal ligament tissue (PDL), gingival tissue, alveolar bone and cementum. PDL has a deposit of somatic stem cells that could reconstruct the periodontium, although its use in bone reconstruction is still the period under investigation. For successful implant placement into sites with missing dental units, adequate bone regeneration becomes vital in patient management.

Alveolar process and dimensional changes of post-extraction sockets

The main aim of management is to prevent alveolar process atrophy, that can occur after tooth removal. This atrophy starts developing during tooth eruption. The alveolar process supports the tooth socket and begins to resorb following tooth loss [1]. The volume and shape of the alveolar process is determined by the tooth formation, axis

UDK: 602.1:616.314-089.843 / Ser J Exp Clin Res 2013; 14 (4): 145-153
DOI: 10.5937/SJECR5321

Correspondence to: Tatjana Kanjevac, PhD, Assistant Professor, Faculty of Medical Science, University of Kragujevac, Darko Bosnakovski, PhD, Associate Professor, Faculty of Medical Sciences, University "Goce Delcev" Stip, Krste Misirkov bb, 2000 Stip, R. Macedonia, Tel. 389 (0)70 516649, darko.bosnakovski@ugd.edu.mk

of eruption and eventual inclination [2]. From early studies by Amler et al., we have a detailed description of unassisted histological healing of alveoli in healthy humans [3]. When a tooth is removed, a clot forms and is gradually replaced by granulation tissue in the base and the periphery of the alveolus. After the first week, new bone formation is evident, with the osteoid matrix at the alveolus base as noncalcified bone spicules. In 38 days, this osteoid starts to mineralise from the alveolus base in a coronal direction, filling two-thirds of the alveoli. At this point, the first sign of a progressive resorption of the alveolar crest occurs. This process is followed by a continuous re-epithelialisation, which completely covers the socket 6 weeks after extraction. After additional bone fill develops, a maximum radiographic density is achieved around the hundredth day.

The usual outcome after tooth extraction includes is a reduction in the dimensions of the socket due to pathologic and traumatic processes that damage the bone walls of the socket. According to Araujo and Lindhe, notable osteoclastic activity occurred during the first eight weeks after tooth extraction, resulting in resorption of the crestal regions of both the buccal and lingual bone walls [4]. If fibrous tissue invades the empty socket, normal healing and osseous regeneration would be prevented, causing problems for future dental implants [5].

Alveolar process atrophy

Along with an incomplete healing of the socket, progressive bone resorption commences along the residual alveolar process. A reduction in both horizontal and vertical directions has been observed over a 12-month period, with a predominant reduction occurring during the first 3 months. This continual resorption leads to a narrower and shorter alveolar process [6]. Due to this effect, the alveolar process makes a positional change to a more palatal/lingual position. A study showed that the clinical loss in width (3.87 mm) is greater than the loss in height, as assessed both clinically (1.67-2.03 mm) and radiographically (1.53 mm) [2]. With bone grafting techniques, the horizontal and vertical dimensions of the alveolar process can be preserved.

While alveolar atrophy is not a concern for most dentist and surgeons, knowledge about the healing process at the extraction sites, including the change in contour, as caused by bone resorption, is needed for treatment planning. In an effort to restore aesthetics and mastication function, procedures for bone regeneration by filling the extraction sockets have been developed. This has resulted in a satisfactory alveolar process height and width, thus providing sufficient alveolar bone volume for dental implants.

BONE GRAFTS

During dental procedures, large bone defects can be created, which can cause problems associated with aesthetics, function, the healing process, and even jaw bone stability. The application of several materials to the area around these bony defects aids bone regeneration and eliminates the defects or limits their size. These materials may also prove useful in the regeneration of periodontal tissues, the filling of bone defects around an implant, and the augmentation of a deficient alveolar ridge [7].

Bones can regenerate from bone grafts. Bone grafting is a surgical procedure that replaces missing bone with material from the patient's own body, or from an artificial, synthetic or natural substitute. As natural bone grows, the graft material is usually completely replaced, resulting in a fully integrated region of new bone. Clinical outcomes of bone grafting depend on the bone defect and extension, structural properties of the grafting material and the immunologic reaction of the host [8].

The bone grafts should be sterile, non-toxic, non-antigenic, biocompatible and easy to use. Other important properties of bone grafts include the following [9-12]: 1. Oosteointegration (This is the ability to chemically bond to the surface of bone without an intervening layer of fibrous tissue); 2. Osteoconduction (this is the ability to support the growth of bones as a scaffold on which bone cells can proliferate; osteoblasts from the margin of defect are grafted and utilised as the bone graft material as a framew ork upon which to spread and generate new bone); 3. Oosteoinduction (This is the ability to induce proliferation and differentiation of the MSCs from surrounding tissues to an osteoblastic phenotype; Stimulating the osteoprogenitor cells to differentiate into osteoblasts is needed, which begins the formation of new bone); 4. osteopromotion (This is the enhancement of osteoinduction without having any of the osteoinductive properties); and 5. Osteogenesis (Tthe graft material is a reservoir of MSCs and progenitor cells that can form new bone). The interaction between the graft and the surrounding host bone is very important and is the subject of many researchers. Although some grafts will merely act as space fillers, the ideal graft will be osseoconductive and osseoinductive [13].

Bone formation is a complex process that begins with the recruitment and proliferation of osteoprogenitor cells that are then differentiated into osteoblasts, with subsequent osteoid formation and matrix mineralisation. Their ability to attach to a scaffold surface is an important part in the development of new tissue. An ideal bone graft augments this osseous healing by providing a cellular milieu for new bone formation and a structural framework during healing [14]. A bone graft should not support local pathogens or cross-infection and should be resorbable, microporous and easy to handle.

Cancellous grafts have the highest concentration of osteogenic cells, and the particulate form of these grafts has the greatest cell survival ability, due to better diffusion and rapid revascularisation. These grafts must completely undergo a two-phase mechanism of graft healing. Osteoblasts that survive transplantation proliferate and form osteoid. This process is active in the first 2 to 4 weeks,

and the definitive amount of bone formed is related to the quantity of osteoid formed in phase one. Phase two starts at around the second week after grafting, and although it peaks in intensity at approximately 4 to 6 weeks, it continues until the graft matures. The initiation of phase two is marked by osteoclastic cell activity within the graft. Osteoclasts remove minerals, forming Howship's lacunae along the trabeculae. This resorptive process exposes the extracellular matrix of the bone, which is the natural location of the bone-inductive glycoprotein (BMP). Exposure of BMP initiates an inductive process characterised by chemotaxis of the mesenchymal stem cells, proliferation of cells in response to mitogenic signals, and differentiation of cells into osteoblasts. Inducible cell populations may be local or distant from the graft site.

Examples of local cell populations that may contribute to the graft include osteoprogenitor cells in the graft endosteum, stem cells of the transplanted marrow, or cells in the cambium layer of adjacent periosteum. Additional inducible pluripotent cells may arrive at the graft site with budding blood vessels. During phase two, there is progressive osteoclastic resorption of phase one osteoid and nonviable graft trabeculae. This continues to expose BMP, which perpetuates the differentiation of osteoblasts, leading to the formation of mature vascular osteocyte-rich bone [15]. Based on their bone of origin, grafts can be divided into four categories: autografts, allografts, alloplastic grafts and heterografts [16].

Autologous bone grafts (autografts)

The treatment of bone defects and preservation of the socket include autografting and allografting cancellous bone. The intraoral or extraoral autogenous bone graft is readily available and is the first choice of bone grafting material for many clinicians. Autologous bone grafting usually harvests bone from the non-essential bones, such as the iliac crest, mandibular symphysis or anterior mandibular ramus, maxilla, cranium, tibial plateau and ribs. The shape, form, and volume of the graft procured are linked to the defect to be reconstructed. This is considered as a gold standard. Essentially, this graft has less risk of graft rejection or other immunological resistance, provides a scaffold for osteoconduction, growth factors for osteoinduction and progenitor cells for osteogenesis, and permits a fast angiogenic in-growth of vessels [17,18]. The main advantages of autogenous bone graft are biocompatibility, sterility and availability. However, there are several limitations, including limited availability of bone, high surgery cost and post-operational morbidity, such as blood loss, wound complications and chronic pain.

All bones require blood supply in the transplanted site. Depending on the transplant site and graft size, an additional blood supply may be required. For these types of grafts, the extraction of the periosteum parts and accompanying blood vessels with the donor bone is required. This kind of graft is known as a free flap graft [8].

Allogenic bone grafts (allografts)

Allografts are an alternative to autografts. Different sources for bone harvesting can be used, such as and includebone from living or post-mortem donors. The graft may be fresh or fresh-frozen bone, freeze-dried allograft (FDBA) or decalcified freeze-dried bone allograft (DFDBA) and is considered a good source of bone morphogenic protein. The American Association of Tissue Bank standards require that all donors be screened, serologic tests be performed, and all specimens be sterilised and verified by culture prior to release [13].

The increasing number of grafting procedures and the disadvantages of current autograft and allograft treatments (limited graft quantity, risk of disease transmission) drive the need for alternative methods to treat bone defects [19].

Synthetic bone graft (alloplastic grafts)

The use of synthetic bioactive bone substitute materials is of increasing importance in modern dentistry, as alternatives to autogenous or allogenic bone grafts. Due to the shortcomings of the decellularised, deproteinated, biological materials, the quest for a synthetic material with many of the properties of decalcified, decellularised bone has been conducted. Its positive attributes include avoiding a second surgical site with less risk for patient morbidity and minimal risk of transplant rejection. Their physical properties can be manipulated and may be used in combination with bone-promoting molecules to enhance the effect [12, 20]. However, they possess only two properties of an ideal bone graft material, osteointegration and osteoconduction. Most synthetic bone grafts are biocompatible, show minimal fibrotic reaction, undergo remodelling and have similar strength to the cortical/cancellous bone being replaced.

Various alloplastic bone substitution materials of different origins, chemical composition, and structural properties have been investigated over the years. The materials commonly used are ceramics, polymers or composites. These alloplastic materials are either absorbable or non-absorbable and are naturally derived or synthetically manufactured [21]. Various types of biomaterials (minerals and non-mineral based materials and natural and artificial polymers) with different characteristics have been used to study ossification and bone formation. Calcium phosphate ceramics include a variety of ceramics, such as hydroxyapatite, tricalcium phosphate, and calcium phosphate cement. These mentioned ceramics have either excellent biocompatibility, bone bonding or bone regeneration properties [22].

Xenograft (heterografts)

Xenografts or heterografts are bone grafts from a species other than human, such as bovine bone, which can be freeze-dried or demineralised and deproteinised. Xenografts are usually only distributed as a calcified matrix. Attempts at xenograft transplantation (the transmission

of living organs, tissues, or cells from one species to another) were first performed in the early twentieth century. Today, the relative shortage of human organs and tissue available for transplantation has amplified interest in xenografts as alternatives to human-tissue transplants. Xenografts are often used as scaffold and allow for ingrowth, and sometimes replacement, by host tissue while providing structural support for deficient tissue. Although the obvious advantage of xenotransplantation is the almost infinite amount of nonhuman animal tissue that might be considered for transplantation, its major disadvantage is the risk of cross-species disease transmission.

Of all the grafts, bone autografts give the best results. However their use is limited because a second concurrent surgical procedure is required. Therefore, the aforementioned synthetic substitute materials are used instead, and bone regeneration in areas with large bone defects is satisfactorily accomplished. Successful graft incorporation is defined as the ability of the graft and surrounding tissues to function and maintain mechanical integrity [8, 23].

STEM CELLS

One tissue engineering method proposes tissue regeneration with the help of molecules, cells or a combination of these with biocompatible materials to ensure support and enhance physiological healing processes. Tissue engineering may provide functional substitutes for native tissue to serve as grafts for implantation.

Cells are described as pluripotent if they can form all the cell types of the adult organism. These cells are embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Multipotent stem cells can form all the differentiated cell types of a given tissue, for example, mesenchymal stem cells. In some cases, a tissue contains only one differentiated lineage, and the stem cells that maintain the lineage are described as unipotent, .for example, skin stem cells.

By definition, a stem cell is characterised by its ability to self-renew and to differentiate along multiple lineage pathways. Bone marrow contains a large population of multipotent stem cells that are undifferentiated, which are known as stromal cells or mesenchymal stem cells (MSCs). They can be differentiated into several cell types, including osteocytes, chondrocytes, adipocytes and hematopoietic-supportive stroma cells. This ability has been well proven. MSCs can be isolated from the bone marrow, cultured *in vitro* and implanted into bone defects to repair bone loss. These cells could be distinguished from the hematopoietic elements in the marrow by their high adherence to the substrate plastic in tissue culture flasks.

Historically, the use of MSCs in osteogenesis was initiated by Friedenstein et al. in the 1970s [24, 25] and later by Kuznetsov et al. in 1989 [26], who were among the first researchers experimenting with MSCs transplantation beneath the renal capsule in mice and creating growths in diffusion chambers *in vivo* in monolayer culture. Kuznetsov et al. showed that bone formation was characterised with lamellar, long trabeculae and abundant haematopoiesis. However, this study was limited to kidney transplants. Goshima et al. (1991) postulated that a composite of MSCs and ceramics, used as a delivery vehicles for these cells, contributes to accelerated and massive osseous repair [27]. This was shown by harvesting bone marrow cells from rats that were later introduced into tissue culture, and then the cells were mitotically expanded, passaged and placed on small cubes of porous calcium phosphate ceramics. These samples were grafted in the subcutaneous sites of syngenic rats. Bone formation was observed as early as 2 weeks. The study concludes that while bone graft substitutes, such as calcium phosphate ceramics, are biocompatible and osteoconductive, ceramics do not induce bone formation. Only when such substitutes are combined with MSCs can bone formation be observed in the pore regions of the ceramics, in close association with the host vasculature. Kuznetsov et al. (1997) extended the experimentations by showing that individual human MSCs have osteogenic potential [28]. They transplanted human MSCs into the subcutaneous space of immunodeficient mice within vehicles containing hydroxyapatite-tricalcium phosphate ceramic and then proved that after 8 weeks, the transplants derived their bone from the human donor cells. Currently, this type of experiment is regular procedure for evaluating the osteogenic potential of stem cells *in vivo* [29].

The characteristics of an optimal stem cell include the following: no immunorejection, no graft-versus-host disease, no tumorigenicity, immediate availability, availability in pertinent quantities, controlled cell proliferation rate, predictable and consistent osteogenic potential, and controlled integration into the surrounding tissue[10].

MSCs have been shown to heal bone defects in an autologuous setting. Allogenic donor-derived MSCs present an attractive alternative to using autologuous cells. By using donor-derived cells, the need for harvesting and expanding cells for each patient is eliminated. Because billions of cells may be expanded from an individual donor, many devices can be created from rigorously tested and qualified cells. An allogenic MSC-based bone regeneration constructs for the augmentation and repair of alveolar bone were developed by several researchers. As demonstrated by a long-term study, the histological evaluation of grafts in human mandibles after three years show that the regenerated bone is qualitatively a compact type, rather than a cancellous (spongy) type that is physiological for the area. This was explained by the fact that grafted stem cells did not follow the local signals of the surrounding spongy bone [30]. New research shows that low-intensity pulsed ultrasound stimulation could be a positive influence on osteogenic differentiation of the human alveolar bone-derived mesenchymal stem cells, that can be used in tooth tissue engineering [31]. However, as some studies claim, researchers with MSCs need to establish more predictable outcomes and better long-term prognosis to be considered a first-choice treatment [32-35].

An alternative to bone marrow-derived mesenchymal stem cells are the periodontal ligament stem cells (PDLSc) and the dental follicle cells (DFCs), which represent a new approach in reconstructive dentistry for the treatment of damaged periodontium. Restoration of lost periodontium is a challenge because alveolar bone, cementum and periodontal ligament need to be restored to their original form. More research is needed to explore their true potential, although some progress has been made by several research groups [36-40].

THE IMPORTANCE OF SCAFFOLDS

The number of surgical procedures correcting bone defects use autografts, allografts or metallic and ceramic implants, each with its own drawbacks, including donor site morbidity, pathogen transmission, and mismatching material properties with the native bone, respectively. As an alternative to these procedures, tissue engineering has emerged to create *de novo* tissue by growing cells on three-dimensional (3D) scaffolding [41, 42]. Scaffolds play an important role in dental regenerative medicine because conventional tissue replacements, such as autografts and allografts, have a variety of problems that cannot satisfy high performance demands necessary for today's patients. Bone is a nanocomposite that consists of a protein-based soft hydrogel template (collagen, non-collagenous proteins (laminin, fibronectin, and vitronectin), water and hard inorganic components (70% of the bone matrix is composed of nanocrystalline hydroxyapatite). This self-assembled nanostructured extracellular matrix (ECM) in bone closely surrounds and affects the mesenchymal stem cells, osteoblasts, osteoclasts and fibroblast adhesion, proliferation and differentiation [43].

Investigators are searching for the "ideal scaffold" to facilitate the growth, integration and differentiation of stem cells [44]. The best scaffold for engineered tissue should be the ECM of the target tissue in its native state because ECM components specifically modulate MSC adhesion, migration, proliferation and osteogenic differentiation [45, 46]. Scaffolds are artificial structures that should mimic the morphologic structures and function of the surrounding tissue. Scaffolds allow cell attachment and migration, deliver and retain cells and biochemical factors, enable diffusion of the vital cell nutrients and expressed products [47].

Cell and tissue response to a scaffold depends upon the composition of the scaffold, its surface microstructure, and three-dimensional architecture. Scaffolds should provide void volume for vascularisation and new tissue formation during remodelling, provide the shape and mechanical stability to the tissue defect and provide rigidity and stiffness to the engineered tissues [45]. Appropriate porosity and pore structure is needed to accept and organise the types of cells and tissues that regenerate [48]. Mechanical properties that are appropriate for the cells and their macro- and microenvironments are also needed. The cellular microenvironment at the interface between tissue and scaffold is extremely important and must be created to either recruit cells into the scaffold or allow cells to be seeded or transplanted for repair. Scaffolds allow cell attachment and migration, deliver and retain cells and biochemical factors, enable diffusion of the vital cell nutrients and expressed products, and should promote healing and should be easily fabricated [49-51].

Biocompatibility is of the utmost importance to prevent adverse tissue reactions. Because the host cells will interact with the scaffold, biodegradability is a must to facilitate constructive remodelling, which is characterised by scaffold degradation, cellular infiltration, vascularisation, differentiation and spatial organisation of the cells, and replacement of the scaffold by the appropriate tissues [52]. Material scientists can now fabricate biocompatible scaffolds with a wide range of physical parameters, combining mechanical integrity with high porosity to promote cell infiltration and angiogenesis. Currently proposed scaffolds include those made of inorganic materials, organic or synthetic polymers, or mixed materials (composite scaffolds). These materials include natural polymers (collagen, chitin, alginate), synthetic polymers (Polyglycolic acid (PGA), Poly (lactic-co-glycolic acid) (PLGA), Poly (lactic acid) (PLA)), metals (titanium, nitinol), and ceramics, such as calcium phosphates (hydroxyapatite, tricalcium phosphate], calcium sulphates, and biological glass [52, 53]. The biomaterials need to be compatible with the biomolecules and amenable to an encapsulation technique for controlled release of the biomolecules with retained bioactivity [45]. Natural materials offer a high degree of structural strength, are compatible with cells and tissues and biodegradable, but are often difficult to process and afflicted with the risk of transmitting animal-associated pathogens or provoking an immunoresponse. Synthetic polymers provide excellent chemical and mechanical properties and allow greater control over the physicochemical characteristics, such as molecular weight, configuration of polymer chains, and the presence of functional groups. Hydrogels offer numerous properties including high biocompatibility, a tissue-like water content and mechanical characteristics similar to those of native tissue. An ideal scaffold should combine the best properties of each of these groups of biomaterials [44, 54, 55].

Experiments with cell-free scaffolds are especially attractive because they have an easier handling process that eliminates the issues associated with the use of stem cells, their expansion *in vitro*, storage and shelf-life, cost, the immunoresponse of the host and transmission of diseases [53, 56]. However there are some disadvantages in this method: the cells may have low survival rates, the cells might migrate to different locations within the body, leading to aberrant mineralisation patterns. A solution may be to apply the cells together with a scaffold. This would help to position and maintain cell localisation [53, **57**].

The paradigm of bone tissue engineering procedures is in the isolation and expansion of mesenchymal stem cells (MSCs) from the patients and their seeding onto porous biodegradable matrices, and scaffolds. Scaffold morphol-

ogy, in terms of interconnectivity, pore-size and shape is a crucial point for stem cell-biomaterial interaction. High porosity and adequate pore-size are necessary properties for increasing the surface area available for cell attachment and tissue in-growth in order to facilitate the uniform distribution of cells and the adequate transport of nutrients. Small pores on the macropore surface of the scaffolds may also be helpful to improve the biological performance of the porous scaffolds and promote more favourable bioresorption of the material [50, 58].

During the *in vitro* culture period, stem cells are generally exposed to signalling molecules (growth factors and other osteoinductive molecules), supplied as soluble factors and/or released by the scaffold, to drive MSCs toward the osteogenic lineage differentiation. This engineered tissue is implanted into the damaged site to regenerate the new bone as the scaffold degrades [59]. Implantation of *in vitro*-expanded MSCs within the appropriate scaffold resulted in bone regeneration in various animal models. The supporting scaffold plays a very important part by providing an anchorage point for cells.

The use of scaffolds with different types of particles offers the advantage of perfectly adapting to the shape of the defect without interfering with the vascularisation process. Biological granular scaffolds can stimulate vascularisation and tissue integration because of the appropriate spaces between the particles of the inorganic material. Using granular material also accelerates the scaffold resorption process and the replacement of the inorganic material with newly formed bone. Scaffolds containing crystalline beta-tri-calcium phosphate have been proven to lack local and systemic toxicity. Its granular consistency provide an optimal osteoconductive environment for the development of bone tissue. Tricalcium phosphate is extremely hydrophilic, making it easily insertable inside the defect. Tri calcium phosphate scaffolds ensure rapid resorption, which is an advantage for small and medium size defects, and they also release calcium and phosphate ions, assuring rapid mineralisation of the newly formed tissue.

Porous ceramic scaffolds have already been noted as the most suitable material for reproducing the structural integrity of ossified tissues. When combined with the bioactive attributes of calcium phosphate, hydroxyapatite, bioactive glass, or other similar ceramics, the composite material can support progenitor cells and mimic the natural characteristics of bone [52].

.

LATEST RESULTS AND ACHIEVEMENTS

The emerging field of regenerative medicine will require a reliable source of stem cells, biomaterial scaffolds and cytokine growth factors. One study showed massive bone formation when autologous mesenchymal stem cells placed on TCP scaffolds were implanted in the alveolar sockets on a rat animal model. This study used a large number of osteoprogenitor cells on a scaffold, which has proven to accelerate the osteogenesis process, and showed that a concentration of $5x10^4$ cells/ml can induce bone formation, whereas a concentration of 0,5 - 1 x 10^6 cells/ml did not show satisfactory results. The study concludes that implantation of autologous mesenchymal stem cells on specific scaffolds will augment bone repair [60]. New studies have suggested that transplanted bone-marrow derived MSCs can deliver new mitochondria to damaged cells, thereby rescuing the aerobic metabolism [61].

Adipose tissue represents an alternative source of adult stem cells with the ability to differentiate along multiple lineage pathways. To identify this isolated, plastic-adherent, multipotent cell population, these cells are called adipose-derived stem cells (ASCs) according to the International Fat Applied Technology Society. The evidence supporting the claims that adipose tissue contains multipotent progenitor cells start from an inborn metabolic error, the progressive osseous heteroplasia (POH), where ectopic bone can be formed within the subcutaneous adipose layer of the skin in children. Histological analysis shows the presence of osteoblasts, chondrocytes and adipocytes. This implies that adipose-derived stem cell can differentiate along adipogenic, chondrogenic and osteogenic lineages.

Adipose tissue derives from the mesodermal layer of the embryo and develops both pre- and postnatally. Macroscopically, at least 5 different types of adipose tissue exist: bone marrow, brown, mammary, mechanical and white [62]. A study showed that in humans, subcutaneous white adipose tissue in the arm had a greater number of stem cells compared to the thigh, abdomen and breast [63]. The ASCs maintain their telomere length with progressive passage in culture, however with prolonged passage for more than 4 months, human ASCs can undergo malignant transformation [64]. The greatest advantage of using ASCs is that it can be obtained repeatedly in large quantities under local anaesthesia with minimal patient discomfort.

Beside autologous ASCs, the use of allogeneic ASCs is also important. Studies have demonstrated that the passage of human ASCs reduces the expression of surface histocompatibility antigens and no longer stimulates a mixed lymphocyte reaction when co-cultured with allogeneic peripheral blood monocytes [65, 66]. This profoundly affects the field of regenerative medicine. A study showed that after a 3 month healing period, the addition of ASCs to platelet-rich plasma (PRP) enhanced the amount of n-ewly formed dog alveolar bone [67], and other studies came to thesimilar conclusions [68, 69]. One study compared the use of ASCs and autogenous bone grafts in dogs, bone formation in the maxillary alveolar cleft was higher in the autograft group [70]. More long-term experiments examining the safety of ASCs transplantation in appropriate animal models are required before advanced studies in patients.

Another potential source for tissue engineering is embryonic stem cells (ESCs). They are harvested from the inner cell mass of blastocysts. Their pluripotent characteristics enable unlimited self-renewal and differentiation into all cell types. A challenge that needs to be addressed is their tumorigenic potential. Therefore, removing the re-

maining undifferentiated ES cells from the newly formed tissue before implantation is crucial. Further research is needed to develop efficient methods to direct ES cells into therapeutically desired cell lineages, such as osteoblast, while eliminating the pluripotent cells.

The latest trend in tissue engineering is using nuclear reprogramming to convert a somatic cell type into a different, unrelated one through a switch of the gene expression pattern, resulting in the generation of an embryonic stem cell-like pluripotent cells by ectopic overexpression of only four genes in human fibroblasts. These cells are called induced pluripotent stem cells (iPSCs) [71-73]. This research showed that somatic cells, such as fibroblasts or adipocytes, can be directly converted to clinically relevant cell types after ectopic delivery of factors which are involved in the embryonic development of the targeted cell type. The assumption is that factors responsible for the maintenance of the pluripotent state in embryonic stem cells (ESCs) could induce pluripotency in somatic cells after ectopic overexpression. Kazutoshi Takahashi and Shinya Yamanaka identified four factors Oct4, Sox2, Klf4 and c-Myc as being sufficient to reprogram mouse embryonic fibroblasts (MEFs) into a morphology highly comparable with embryonic stem cells, which they named iPSCs [74]. iPSCs could maintain their self-renewal when cultured under ESC conditions and differentiate into cells of all three germ layers [75-77], which proved that they are nearly indistinguishable from ESCs. More recent investigations convincingly support the osteogenic potential of hESCs and iPSCs *in vivo* [78, 79]. Arpornmaeklong and co-workers derived MSCs from the hESC line BG01, characterised by the expression of MSC-specific surface antigens, and further differentiating them into adipogenic, chondrogenic and osteogenic tissue [78].

Transplanting iPS cells uses the patient's own cells, eliminating the need for immunosuppression. Discovering how the pluripotent state can be efficiently induced and maintained by treating cells with pharmacologically active compounds, rather than genetic manipulation, is an important goal [80].

As new surgical techniques develop for replacing non-functional tissues or organs, the need for more artificial means of organ transplants or tissue regeneration will arise. A well-defined pathology, such as alveolar bone atrophy, requires further advances in the field of bone regeneration using stem cells to generate new tissue or regenerate residual tissue. Development in this field will benefit several branches in medicine and dentistry. Techniques for improved growth rate, extent and strength of n-ewly formed bone must be developed in concordance with increased clinical application. Most importantly, researchers need to ensure that any tumorigenic potential is eliminated.

CONCLUSION AND FUTURE DIRECTIONS

Bone tissue engineering can overcome the drawbacks of traditional bone graft materials and offers a novel way for bone repair and regeneration. Scientists have been actively investigating the ideal cell source to regenerate and repair bone for the last four decades. More than 300 articles [81] on bone regeneration using stem cells in animal models have been published. However, only a few studies include human subjects [82]. MSCs derived from the adult bone marrow provide an exciting and promising stem cell population for bone repair. The disadvantages of MSCs are the limited availability of cells for therapy and the non-specific cell surface markers. Therefore, specific markers need to be identified for easier detection in laboratories. E SCs are also a potential source and have an additional advantage of unlimited division and pluripotency. However a reproducible protocol to ensure that ESCs differentiate into functional bone needs to be developed.

ESCs studies need to overcome ethical issues, immune responses and tumorigenic potential. ESCs represent an innovative treatment for many disease conditions, but still require rigorous evaluation for use in clinical applications. IPS cells are currently the most exciting and promising cell population, with ASCs a close second. Both cells populations are at the apex of their popularity within the scientific community, as a supply of readily available cells can truly push the field of regenerative medicine. The field of regenerative medicine should not be entrenched in only stem cells but also expand knowledge in the use of bone grafts and scaffolds. This should be used in a complementary way, if we strive for maximum results in the treatment of diseases.

REFERENCES

1. Pietrokovski J, et al. Morphologic characteristics of bony edentulous jaws. J Prosthodont 2007; 16: 141-7.
2. Van der Weijden F, Dell'Acqua F, Slot DE. Alveolar bone dimensional changes of post-extraction sockets in humans: a systematic review. J Clin Periodontol 2009; 36: 1048-58.
3. Amler MH, Johnson PL, Salman I. Histological and histochemical investigation of human alveolar socket healing in undisturbed extraction wounds. J Am Dent Assoc 1960; 61: 32-44.
4. Araujo MG, Lindhe J. Dimensional ridge alterations following tooth extraction. An experimental study in the dog. J Clin Periodontol 2005; 32: 212-8.
5. Chen ST, Wilson TG Jr, Hammerle CH. Immediate or early placement of implants following tooth extraction: review of biologic basis, clinical procedures, and outcomes. Int J Oral Maxillofac Implants 19 Suppl 2004; 12-25.
6. Pinho MN et al. Titanium membranes in prevention of alveolar collapse after tooth extraction. Implant Dent 2006; 15: 53-61.
7. Howe WR, Dellavalle R, Vitamin D deficiency. N Engl J Med 2007; 357: 1981; author reply 1981-2.
8. Kumar P, Vinitha B, Fathima G. Bone grafts in dentistry. J Pharm Bioallied Sci 2013; 5: 125-7.

9. Moore WR, Graves SE, Bain GI. Synthetic bone graft substitutes. ANZ J Surg 2001; 71: 354-61.
10. Logeart-Avramoglou D, et al. Engineering bone: challenges and obstacles. J Cell Mol Med 2005; 9: 72-84.
11. Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury 2005; 36 Suppl 3: 20-7.
12. Misch CE, Dietsh F. Bone-grafting materials in implant dentistry. Implant Dent 1993; 2: 158-67.
13. Palmer R, Smith B, Howe L, Palmer P. Implants in clinical dentistry. Taylor and Francis e-Library 2002; 131-133.
14. Beaman FD, et al. Bone graft materials and synthetic substitutes. Radiol Clin North Am 2006; 44: 451-61.
15. Peterson, L.J., Peterson's Principles of Oral and Maxillofacial Surgery. 2nd ed. BC Decker, 2004.
16. Fragiskos FD. Oral Surgery. Springer, 2007.
17. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Medicine 2011; 9: 66-76.
18. Arrington ED, Smith WJ, Chambers HG, Bucknell AL, Davino NA. Complications of iliac crest bone graft harvesting. Clin. Orthop. 1996; 329, 300–309.
19. Giannoudis PV, Pountos I. Tissue regeneration. The past, the present and the future. Injur 2005; 36 Suppl 4: 2-5.
20. Shegarfi H, Reikeras O. Bone transplantation and immune response. Journal of Orthopaedic Surgery 2009; 17: 206-11.
21. Burg KJ, Porter S, Kellam JF. Biomaterial developments for bone tissue engineering. Biomaterials 2000; 2: 2347-2359.
22. Kunert-Keil C, Gredes T, Gedrange T. Biomaterials Applicable for Alveolar Sockets Preservation: In Vivo and In Vitro Studies In book: Implant Dentistry - The Most Promising Discipline of Dentistry 2011.
23. Afzali B, Lechler RI, Hernandez-Fuentes MP. Allorecognition and the alloresponse: clinical implications. Tissue Antigens 2007; 69: 545-56.
24. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970; 3: 393-403.
25. Friedenstein AJ, et al. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 1974; 17: 331-40.
26. Kuznetsov SA, Grosheva AG, Fridenshtein A. Osteogenic properties of adhesive cells in Dexter culture of the mouse bone marro. Biull Eksp Biol Med 1989; 108: 236-8.
27. Goshima J, Goldberg VM, Caplan AI. Osteogenic potential of culture-expanded rat marrow cells as assayed in vivo with porous calcium phosphate ceramic. Biomaterials 1991; 12: 253-8.
28. Kuznetsov SA, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 1997; 12: 1335-47.
29. Gang EJ, et al. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood 2007; 109: 1743-51.
30. Giuliani A, et al. Three years after transplants in human mandibles, histological and in-line holotomography revealed that stem cells regenerated a compact rather than a spongy bone: biological and clinical implications. Stem Cells Transl Med 2013; 2: 316-24.
31. Lim K, et al. In vitro effects of low-intensity pulsed ultrasound stimulation on the osteogenic differentiation of human alveolar bone-derived mesenchymal stem cells for tooth tissue engineering. Biomed Res Int 2013; 2013: 269724.
32. Jakobsen C, et al. Mesenchymal stem cells in oral reconstructive surgery: a systematic review of the literature. J Oral Rehabil 2013; 40: 693-706.
33. Trofin EA, Monsarrat P, Kemoun P. Cell therapy of periodontium: from animal to human? Front Physiol 2013; 4: 325.
34. Zigdon H, Levin L. Stem cell therapy for bone regeneration: present and future strategies. Alpha Omegan 2012; 105: 35-8.
35. Zhang Z. Bone regeneration by stem cell and tissue engineering in oral and maxillofacial region. Front Med 2011; 5: 401-13.
36. Maeda H, et al. Promise of periodontal ligament stem cells in regeneration of periodontium. Stem Cell Res Ther 2011; 2: 33.
37. Iwata T, et al. Tissue engineering in periodontal tissue. Anat Rec (Hoboken) 2014; 297: 16-25.
38. Mrozik KM, et al. Regeneration of periodontal tissues using allogeneic periodontal ligament stem cells in an ovine model. Regen Med 2013; 8: 711-23.
39. Khorsand A, et al. Autologous dental pulp stem cells in regeneration of defect created in canine periodontal tissue. J Oral Implantol 2013; 39: 433-43.
40. Chen FM, et al. Stem cell-delivery therapeutics for periodontal tissue regeneration. Biomaterials 2012; 33: 6320-44.
41. Smith LA, et al. The influence of three-dimensional nanofibrous scaffolds on the osteogenic differentiation of embryonic stem cells. Biomaterials 2009; 30: 2516-22.
42. Bosnakovski D, et al. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. Biotechnol Bioeng 2006; 93(6): 1152-63.
43. Zhang L, Webster JT. Nanotechnology and nanomaterials: Promises for improved tissue regeneration. Nano Today 2009; 4: 66-80.
44. Rodríguez-Lozano FJ, et al. Mesenchymal dental stem cells in regenerative dentistry. Med Oral Patol Oral Cir Bucal. 2012; 17: 1062-7.
45. Chan BP, Leong W. Scaffolding in tissue engineering: general approaches nd tissue-specific considerations. Eur Spine J 2008, 17 Suppl 4: 467–479.
46. Egusa H, et al. Stem cells in dentistry – Part II: Clinical applications. Journal of Prosthodontic Research 2012; 56: 229–248.

47. Martino S, Francesco D'Angelo F, Armentano I, Kenny JM, Orlacchio A. Stem cell-biomaterial interactions for regenerative medicine. Biotechnology Advances 2012; 30: 338-51.
48. Chen Q, Roether JA, Boccaccini AR. Tissue Engineering Scaffolds from Bioactive Glass and Composite Materials. Topics in Tissue Engineering 2008; 1-27.
49. Basu B, Katti D, Kumar A. Advanced Biomaterials: Fundamentals, Processing and Applications. John Wiley & Sons, Inc., USA, 2009.
50. Mitra J,Tripathi G, Sharma A, Basu B. Scaffolds for bone tissue engineering: role of surface patterning on osteoblast response. RSC Adv. 2013, 3: 11073–11094.
51. Yuan H, et al. A preliminary study on osteoinduction of two kinds of calcium phosphate ceramics. Biomaterials 1999; 20: 1799-806.
52. Galler KM, D'Souza RN, Hartgerink JD, Schmalz G. Scaffolds for Dental Pulp Tissue Engineering. Adv Dent Res 2011; 23: 333-339.
53. Zaidman N, Bosnakovski D. Advancing with Ceramic Biocomposites for Bone Graft Implants. Recent Patents on Regenerative Medicine 2012; 2: 65-72.
54. Galler KM, D'Souza RN. Tissue engineering approaches for regenerative dentistry. Regen Med 2011; 6:111-24.
55. Willerth SM, Sakiyama-Elbert SE. Combining stem cells and biomaterial scaffolds for constructing tissues and cell delivery. (accessed in december 2013. at http://www.stembook.org/node/450)
56. Laino G. et al. An approachable human adult stem cell source for hard-tissue engineering. J Cell Physiol 2006; 206: 693-701.
57. Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative endodontics: a review of current status and a call for action. J Endod 2007; 33: 377-90.
58. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. Macromol Biosci 2004; 4: 743–65.
59. Siddappa R. et al. Cellular and Molecular Prerequisites for Bone Tissue Engineering. Ph.D Thesis. (accessed in december 2012 at http://doc.utwente.nl/58105/1/thesis_Siddappa.pdf?origin=publication detail)
60. Sava-Rosianu, et al. Alveolar bone repair using mesenchymal stem cells placed on granular scaffolds in a rat model. J. of nanomat. and biostruc. 2013; 8: 303-311.
61. Owen M, Friedenstein AJ, Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 1988; 136: 42-60.
62. Tang W, et al. White fat progenitor cells reside in the adipose vasculature. Science 2008; 322: 583-6.
63. Yu J, et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2009; 324: 797-801.
64. Lemischka IR, Raulet DH, Mulligan RC. Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 1986; 45: 917-27.
65. Pittenger MF, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-7.
66. Undale AH, et al. Mesenchymal stem cells for bone repair and metabolic bone diseases. Mayo Clin Proc 2009; 84: 893-902.
67. Aziz Aly LA, et al. Influence of Autologus Adipose Derived Stem Cells and PRP on Regeneration of Dehiscence-Type Defects in Alveolar Bone: A Comparative Histochemical and Histomorphometric Study in Dogs. Int J Stem Cells 2011; 4: 61-9.
68. Tobita M, Mizuno H. Adipose-derived stem cells and platelet-rich plasma: the keys to functional periodontal tissue engineering. Curr Stem Cell Res Ther 2013; 8: 400-6.
69. Tobita M, et al. Periodontal tissue regeneration by combined implantation of adipose tissue-derived stem cells and platelet-rich plasma in a canine model. Cytotherapy 2013; 15: 1517-26.
70. Pourebrahim N, et al. A comparison of tissue-engineered bone from adipose-derived stem cell with autogenous bone repair in maxillary alveolar cleft model in dogs. Int J Oral Maxillofac Surg 2013; 42: 562-8.
71. Gurdon, J.B. and D.A. Melton, Nuclear reprogramming in cells. Science 2008; 322: 1811-5.
72. Takahashi K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131: 861-72.
73. Yu J, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; 318: 1917-20.
74. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126: 663-76.
75. Cai J, et al. Dopaminergic neurons derived from human induced pluripotent stem cells survive and integrate into 6-OHDA-lesioned rats. Stem Cells Dev 2010; 19: 1017-23.
76. Tateishi K, et al. Generation of insulin-secreting islet-like clusters from human skin fibroblasts. J Biol Chem 2008; 283: 31601-7.
77. Zhang J, et al. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 2009; 104: e30-41.
78. Arpornmaeklong P, et al. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. Stem Cells Dev 2009; 18: 955-68.
79. Kuznetsov SA, Cherman N, Robey PG. In vivo bone formation by progeny of human embryonic stem cells. Stem Cells Dev 2011; 20: 269-87.
80. Silva J, et al. Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol 2008; 6: 253.
81. Meijer GJ, et al. Cell-based bone tissue engineering. PLoS Med 2007; 4: 9.
82. Marolt D, Knezevic M, Novakovic GV. Bone tissue engineering with human stem cells. Stem Cell Res Ther 2010; 1: 10.

LACK OF ST2 ENHANCES HIGH-FAT DIET-INDUCED VISCERAL ADIPOSITY AND INFLAMMATION IN BALB/c MICE

Jelena M. Pantic[1], Nada N. Pejnovic[1,2], Gordana D. Radosavljevic[1], Ivan P. Jovanovic[1], Aleksandar LJ. Djukic[2,3], Nebojsa N. Arsenijevic[1], and Miodrag L. Lukic[1]
[1]Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia;
[2]Institute of Pathophysiology, Faculty of Medical Sciences, University of Kragujevac, Serbia;
[3]Center for Endocrinology, Diabetes, and Metabolic Diseases, Clinical Center Kragujevac, Serbia.

DELECIJA GENA ZA ST2 PROMOVIŠE GOJAZNOST I INFLAMACIJU U VISCERALNOM ADIPOZNOM TKIVU BALB/C MIŠEVA NA DIJETI SA VISOKIM SADRŽAJEM MASTI

Jelena M. Pantić[1], Nada N. Pejnović[1,2], Gordana D. Radosavljević[1], Ivan P. Jovanović[1], Aleksandar LJ. Djukić[2,3], Nebojsa N. Arsenijević[1], and Miodrag L. Lukić[1]
[1]Centar za molekulsku medicinu i istraživanja matičnih ćelija, Fakultet medicinskih nauka, Univerzitet u Kragujevcu,
[2]Institut za patofiziologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu,
[3]Centar za endokrinologiju, dijabetes i bolesti metabolizma, Klinički centar Kragujevac, Srbija

Received / Primljen: 26.12.2013. Accepted / Prihvaćen: 16.01.2014.

ABSTRACT

Obesity and obesity-related disorders are strongly associated with a chronic low-grade inflammation that originates from growing visceral adipose tissue during nutrient excess. Although interleukin (IL)-33 may play a protective role in obesity and atherosclerosis, the impact of the IL-33/ST2 axis on metabolic disorders needs to be further elucidated.

In this study, we investigated the role of the IL-33/ST2 pathway in high-fat diet (HFD)-induced obesity using ST2-deficient ($ST2^{-/-}$) and wild type BALB/c mice.

The deletion of ST2 enhanced systemic and visceral adipose tissue (VAT) inflammation; $ST2^{-/-}$ mice that were fed a HFD for 18 weeks had experienced a significantly increased weight gain and had a higher amount of total VAT. More classically activated M1 macrophages and markedly fewer alternatively activated M2 macrophages were observed in the VAT of the HFD-fed $ST2^{-/-}$ mice. Additionally, the VAT of the HFD-fed $ST2^{-/-}$ mice had an increased percentage of $CD3^+$ T cells but fewer $CD4^+CD25^+FoxP3^+$ T regulatory cells when compared to the VAT of the low-fat diet-fed controls. The numbers of $CD3^+IL\text{-}17^+$ and IL-5 positive VAT-derived mononuclear cells were significantly decreased in the HFD-fed $ST2^{-/-}$ mice. Serum levels of the proinflammatory cytokines IL-1β and IFN-γ were increased in the HFD-fed $ST2^{-/-}$ mice, while the levels of IL-6 and CRP did not differ among the groups. Importantly, the levels of the anti-inflammatory cytokines IL-10 and IL-13 were significantly lower in the sera of the $ST2^{-/-}$ mice than the levelsin the sera of the wild-type controls.

Our findings suggest a protective role of IL33/ST2 signalling in high-fat diet-induced adipose tissue inflammation. ST2 deficiency related to nutrient excess is associated with the polarisation of macrophages toward the M1 phenotype and the induction of a Th1-mediated immune response.

Key words: *obesity, adipose tissue, inflammation, cytokines, macrophages*

SAŽETAK

U osnovi patogeneze gojaznosti i metaboličkih poremećaja povezanih sa gojaznošću je hronična sistemska inflamacija niskog stepena koja nastaje u visceralnom adipoznom tkivu (VAT) u uslovima povećanog unosa nutrijenata. Iako rezultati dosadašnjih istraživanja ukazuju na moguću protektivnu ulogu IL-33 u nastanku gojaznosti i ateroskleroze, uloga IL-33/ST2 signalnog puta u patogenezi ovih bolesti je nedovoljno razjašnjena.

U ovom istraživanju ispitivali smo ulogu IL-33/ST2 signalnog puta u mišjem modelu gojaznosti indukovane primenom dijete sa visokim sadržajem masti u ST2 deficijentnih i miševima divljeg soja BALB/c.

Delecija gena za ST2 promoviše sistemsku inflamaciju i inflamaciju u VAT-u što se ogleda u porastu telesne mase i uvećanju količine VAT-a tokom 18 nedelja primene dijete sa visokim sadržajem masti. Proinflamatorni milje u VAT-u $ST2^{-/-}$ miševa na ishrani bogatoj mastima karakteriše povećana zastupljenost klasično aktiviranih M1 makrofaga, uz smanjeno prisustvo alternativno aktiviranih M2 makrofaga. Pored toga, dijeta sa visokim sadržajem masti značajno je uticala na povećanje zastupljenosti $CD3^+$ T limfocita, dok je prisustvo $CD4^+CD25^+FoxP3^+$ regulatornih T limfocita bilo značajno sniženo u VAT-u $ST2^{-/-}$ miševa u odnosu na $ST2^{-/-}$ miševe na dijeti sa niskim sadržajem masti. Učestalost $CD3^+IL\text{-}17^+$ i IL-5 pozitivnih mononuklearnih ćelija je bila značajno smanjena u VAT-u gojaznih $ST2^{-/-}$ miševa. Iako nije bilo razlike u serumskim nivoima IL-6 i CRP-a, koncentracija proinflamatornih citokina IL-1β i IFN-γ je bila povećana u gojaznih $ST2^{-/-}$ miševa. Važno je istaći da su serumski nivoi anti-inflamatornih citokina, IL-10 i IL-13, bili niži u $ST2^{-/-}$ miševa u poređenju sa miševima divljeg soja.

Rezultati studije ukazuju na protektivnu ulogu IL-33/ST2 signalnog puta u pokretanju inflamacije u VAT-u nakon primene dijete sa visokim sadržajem masti, koju karakteriše polarizacija makrofaga u pravcu M1 fenotipa i indukcija Th1 imunskog odgovora.

Ključne reči: *gojaznost, adipozno tkivo, inflamacija, citokini, makrofagi*

UDK: 613.25:575.224.2 / Ser J Exp Clin Res 2013; 14 (4): 155-160
DOI: 10.5937/SJECR5243

Correspondence to: Dr. Jelena M. Pantic, Faculty of Medical Sciences, University of Kragujevac; Svetozara Markovica 69, 34000 Kragujevac, Serbia;
Phone: +381641550001; e-mail: panticjelena@open.telekom.rs

ABBREVIATIONS

BSA-bovine serum albumin
CRP-C reactive protein
CD-cluster of differentiation
FBS-foetal bovine serum
FoxP3- forkhead box P3
HFD-high-fat diet
IL-interleukin
LFD-low-fat diet
NKT-natural killer T cells
PBS-phosphate-buffered saline
Th-T helper cells
TNF-α-tumour necrosis factor-α
WT- wild-type
VAT-visceral adipose tissue
IFN-γ-interferon-γ

INTRODUCTION

The complex pathogenesis of obesity and obesity-related metabolic disorders are strongly associated with a chronic low-grade inflammation that is characterizised by an increased recruitment of immune cells into the visceral adipose tissue (VAT) [1]. Adipocytes are believed to play a central role in the initiation of the inflammatory response in response to metabolic danger signals during increased caloric intake [2]. The expanding adipose tissue found in obese individuals is predominantly infiltrated with IFN-γ-producing Th1 and NKT cells, followed by an enhanced recruitment of classically activated M1 macrophages with a related decrease in the presence of alternatively activated M2 macrophages [3-6]. Activated macrophages produce pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, which contribute to systemic inflammation and negatively impact insulin sensitivity [7]. This proinflammatory milieu resulting from nutrient excess is additionally characterised by a significantly decreased presence of immunosuppressive regulatory T cells in the visceral adipose tissue [8].

IL-33 is a newly identified member of the IL-1 cytokine family, which includes IL-1 and IL-18 [9]. Several lines of evidence suggest that IL-33 is a pleiotropic cytokine that signals through its receptor ST2 to orchestrate the innate and acquired immune responses [10]. Although IL-33 is primarily involved in the induction of Th2-type responses and can act directly on Th2 cells to increase the secretion of Th2 cytokines, such as IL-5 and IL-13, IL-33 can also promote Th1-type responses under certain conditions [11,12]. Additionally, IL-33 induces the production of pro-inflammatory cytokines and chemokines by mast cells and eosinophils and amplifies the polarisation of alternatively activated M2 macrophages [13].

IL-33 is a multifunctional cytokine involved in the pathogenesis of not only different inflammatory and autoimmune diseases, as well as in the pathogenesis of carcinogenesis [14-17]. Although IL-33 may play a protective role in obesity and atherosclerosis [18,19], the contribution of the IL-33/ST2 axis in metabolic disorders needs to be further elucidated. Aware that the BALB/c mice are relatively resistant to HFDhigh-fat diet (HFD)-induced obesity, we investigated the role of ST2 in HFD-induced obesity using ST2 deficient ($ST2^{-/-}$) and wild-type BALB/c mice.

MATERIAL AND METHODS

Animals

Six-week-old, male ST2 deficient ($ST2^{-/-}$) and corresponding wild-type (WT) BALB/c mice were fed either a high-fat diet (HFD with 60% fat, obtained from Mucedola, Milan, Italy) or a low-fat diet (LFD with 3% fat, obtained from Mucedola, Milan, Italy) and were given free access to food and water. After 18 weeks on the specific diets, the animals were sacrificed, and the targeted tissues were collected for further examination. Blood collected from the abdominal aorta was centrifuged, and the isolated sera were stored at -20°C until further analysis. All animal procedures were approved by the Ethical Committee of the Faculty of Medical Sciences at the University of Kragujevac.

Metabolic parameters

Body weight and fasting blood glucose levels were measured every second week of the month. To evaluate fortest for glycaemia, whole blood was collected via tail vein puncture and assessed using the Accu-Chek glucometer (Roche Diagnostics, Mannheim, Germany). The total visceral adipose tissue was isolated from the peritoneal cavity and measured after sacrifice.

Isolation of mononuclear cells from the visceral adipose tissue

The visceral adipose tissue was minced and washed twice in PBS containing 10% FBS. The tissue was then digested with 1 mg/ml collagenase type II (Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 2% BSA for 1 h at 37°C with vigorous shaking. The digested tissue was passed through a 40 μm nylon cell strainer (BD Biosciences, San Jose, CA, USA), and the red blood cells were lysed using an erythrocyte lysis buffer. The isolated cells were then washed twice and resuspended in a RPMI cell medium (Sigma-Aldrich) containing 10% FBS for flow cytometric analysis.

Flow cytometry

The cells were labelled with the following fluorochrome-conjugated monoclonal antibodies: anti-mouse CD3, CD4, IL-17, IL-5, CD25, FoxP3, F4/80, CD206 and CD11c (all from BD Biosciences). For intracellular staining, the cells were activated using PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) with GolgyStop (BD Biosciences)

for 5 h at 37°C and then stained with the fluorochrome-conjugated antibodies using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. The cells were analysed using a FACS Calibur flow cytometer (BD Biosciences), and the analysis was conducted with the FlowJo software (Tree Star).

Serum cytokines measurement

The sera were assayed for CRP, IL-1β, IL-6, IFN-γ, IL-10 and IL-13 using highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) that were specific for these mouse cytokines; the kits were used in accordance with the manufacturer's instructions.

Statistical analysis

All data are presented as the mean ± SE. The data were analysed with the statistical package SPSS, version 13, by using either a two-tailed Student's t-test or the nonparametric Mann-Whitney test, where appropriate. The results were considered significantly different when $p<0.05$.

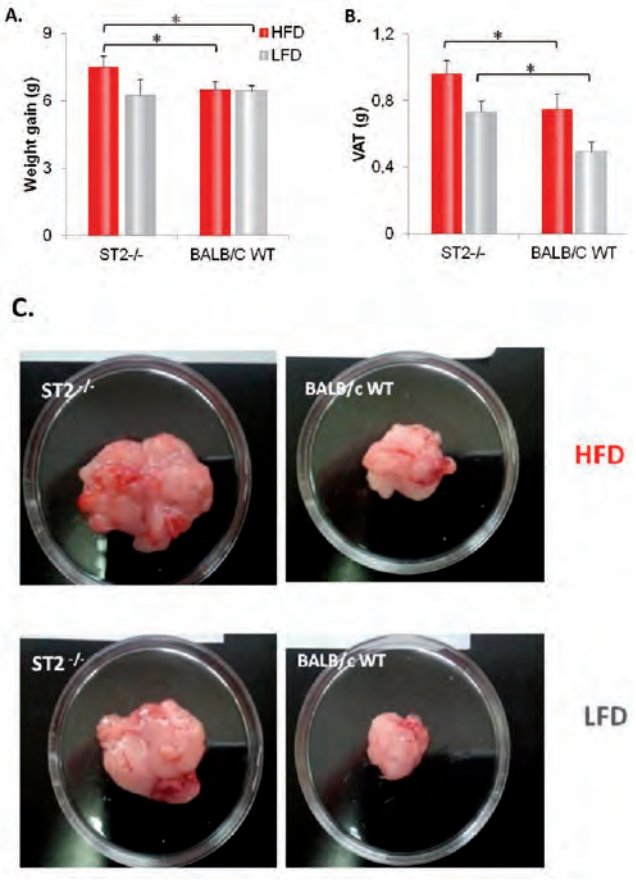


Figure 1. Increased visceral adiposity in HFD-fed ST2 deficient mice. A. Total weight gain was individually determined for each mouse after 18 weeks on the specific diet regimens. B. Total visceral adipose tissue was excised and measured after sacrifice, which occurred 18 weeks after the mice began the specific diet regimens. C. Representative photographs of isolated visceral adipose tissue after sacrifice. The data are presented as the mean ± SE. Statistical significance was determined at *$p<0.05$.

RESULTS

Deletion of ST2 accelerates HFD-induced adiposity

After 18 weeks on the specific diets, we observed a significant weight gain in the HFD-fed ST2$^{-/-}$ mice compared to the WT mice on both diet regimes. Total weight gain was determined as the difference in body weight of each mouse after 18 weeks on a specific diet and the body weight measured on day 0. The body weight did not differ among the groups on day 0 (data not shown). Our data showed that the HFD-fed ST2 deficient mice had a significantly increased total weight gain during the 18 weeks when compared to both the HFD-fed and the LFD-fed WT mice (Figure 1A). We also observed a significantly larger amount of total visceral adipose tissue in the HFD-fed ST2$^{-/-}$ mice than in the corresponding WT animals (Figure 1B, 1C). At the same time, the amount of total visceral adipose tissue isolated from the LFD-fed ST2 deficient mice was significantly higher than that isolated from the LFD-fed WT mice, indicating the relevance of the ST2 molecule in the expansion of the visceral adipose tissue (Figure 1B, 1C).

The adipose tissue of obese ST2$^{-/-}$ mice have an increased percentage of CD3$^+$ T cells , fewer CD3$^+$IL-17$^+$ and IL-5 expressing mononuclear cells and decreased regulatory T cells than the adipose tissue of ST2$^{-/-}$ lean mice

Flow cytometric analysis of mononuclear cells isolated from the visceral adipose tissue showed that the HFD increased the infiltration of CD3$^+$ T cells into the visceral adipose tissue of the ST2 deficient mice, and these cells expressed lower levels of IL-17 than those of the HFD-fed WT mice and

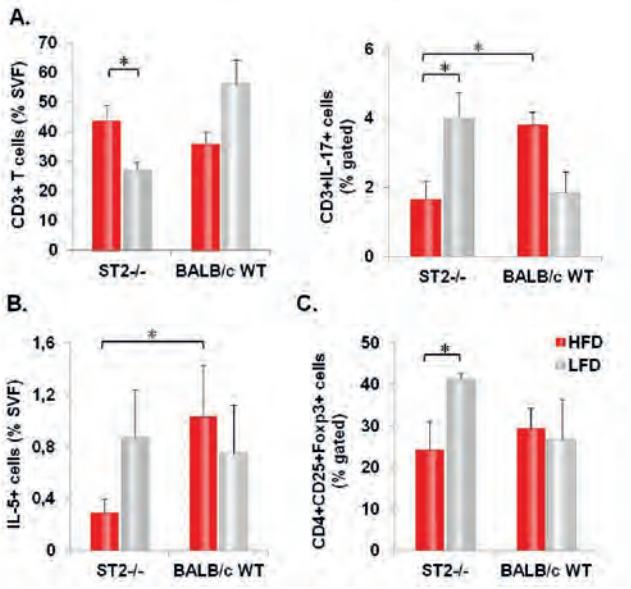


Figure 2. A higher percentage of T cells and a decreased presence of IL-17-producing CD3+ cells, IL-5-positive mononuclear cells and regulatory T cells in the VAT of HFD-fed ST2-/- mice. Mononuclear cells isolated from visceral adipose tissue were labelled with fluorochrome-conjugated anti-mouse antibodies and analysed with flow cytometry. The presence of different cell phenotypes was determined as a percentage of the gated mononuclear cells. The data are presented as the mean ± SE. Statistical significance was determined at *$p<0.05$.

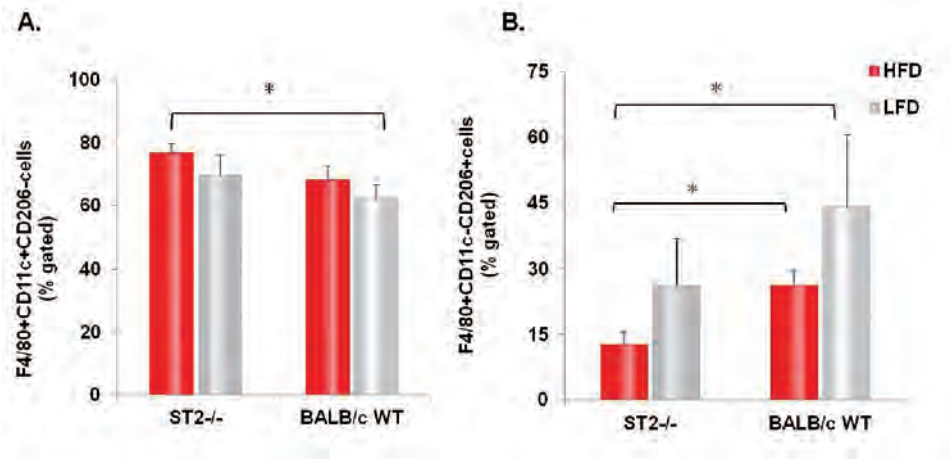


Figure 3. A decreased percentage of alternatively activated M2 macrophages in the HFD-fed ST2-/- mice. The frequencies of different cell phenotypes are presented as percentages of the gated mononuclear cells isolated from visceral adipose tissue using a collagenase digestion protocol. The isolated cells were labelled with fluorochrome-conjugated anti-mouse antibodies and analysed using flow cytometry. The data are presented as the mean ± SE. Statistical significance was determined at *p<0.05.

the LFD-fed ST2$^{-/-}$ mice (Figure 2A). The number of IL-5-producing mononuclear cells was significantly decreased in the visceral adipose tissue of the HFD-fed ST2 $^{-/-}$ mice compared to the number in the corresponding WT animals (Figure 2B). The presence of CD4^{+}CD25^{+}FoxP3^{+} regulatory T cells in the visceral adipose tissue was significantly reduced in the ST2 deficient mice after HFD feeding (Figure 2C).

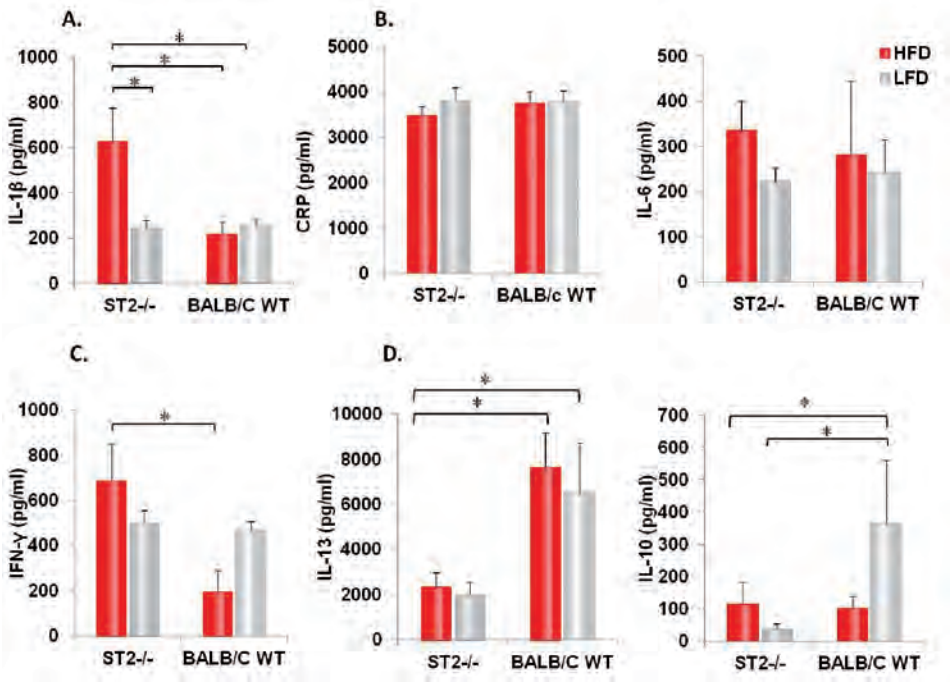


Figure 4. Increased levels of the proinflammatory cytokines IL-β and IFN-γ and decreased levels of the anti-inflammatory cytokines IL-13 and IL-10 in the sera of obese ST2 deficient mice. The levels of cytokines in the serum were measured for each mouse after 18 weeks on the different diet regimens using specific ELISA tests. The data are presented as the mean ± SE. Statistical significance was determined at *p<0.05.

Markedly reduced alternatively activated M2 macrophages in the VAT of obese ST2$^{-/-}$ mice

To further understand obesity-related inflammation in the studied mice after 18 weeks, we investigated the recruitment of macrophages into the visceral adipose tissue and analysed their phenotypes. The number of the proinflammatory F4/80^{+}CD11c^{+}CD206^{+} macrophages was significantly increased in the HFD-fed ST2 deficient mice when compared withto the LFD-fed WT mice (Figure 3A), but this number was not increased compared to the other experimental groups. However, the number of alternatively activated F4/80^{+}CD11c^{-}CD206^{+} M2 macrophages was markedly reduced in the visceral adipose tissue of the HFD-fed ST2$^{-/-}$ mice in comparison with both the diet-matched WT animals and the LFD-fed WT mice (Figure 3B).

Obese ST2$^{-/-}$ mice have increased serum levels of the proinflammatory IL-1β and IFN-γ and lower levels of the anti-inflammatory IL-13 and IL-10

The systemic inflammatory profile of the experimental mice was evaluated by measuring the serum cytokine levels. After 18 weeks on a HFD, the ST2 deficient mice had exhibited markedly elevated levels of the proinflammatory cytokine IL-1β than compared to the diet-matched WT mice and the LFD-fed mice of both genotypes (Figure 4A). At the same time, we did not observe any difference in the serum levels of the C-reactive protein (CRP) and IL-6 among the experimental groups (Figure 4B). However, the serum level of IFN-γ was significantly increased in the HFD-fed ST2$^{-/-}$ mice thancompared to in the corresponding WT mice (Figure 4C). After 18 weeks on the specific diet, both the ST2 deficient mice on the HFD and LFD had a significantly lower systemic levels of IL-13 when compared to the WT mice on the respective diets; additionally, both the HDF-fed and LDF-fed ST2 deficient mice exhibited decreased production of the anti-inflammatory IL-10 than compared to the LFD-fed WT mice (Figure 4D).

DISCUSSION

In this study, we showed that the ablation of ST2 enhances the visceral adiposity of HFD-fed mice, as indicated by a significant increase in weight and a growing amount of visceral adipose tissue. The amount of VAT was significantly increased in both the HFD-fed $ST2^{-/-}$ mice and the LFD-fed $ST2^{-/-}$ mice compared to their diet-matched WT counterparts. The enhanced adiposity of the ST2 deficient mice was characterised by an increased presence of $CD3^+$ T cells, which is in line with previously reported data showing that the infiltration of T cells into the visceral adipose tissue and their polarisation toward a Th1 phenotype played a crucial role in high-fat diet-induced obesity [3,20]. The induction of Th2 cytokine production is a well-established result of the interaction between IL-33 and the ST2 receptor [11]. Multiple cell types, including the adipocytes in the visceral adipose tissue, can most likely produce IL-33, resulting into the maintenance of tissue homeostasis by promoting a Th2 immune response and the production of Th2 cytokines, such as IL-4, IL-5 and IL-13 [21]. According to those findings, a HFD resulted in a significantly decreased the percentage of IL-5 positive mononuclear cells in the VAT of ST2-deficient mice compared to the HFD-fed WT mice. Interestingly, our data showed a significantly lower incidence of IL-17-producing $CD3^+$ cells in the HFD-fed ST2 knockout mice in contrast to the corresponding WT mice and the LFD-fed ST2 deficient mice. Although there is evidence that IL-17 may be a negative regulator of adipose tissue inflammation [22], the decreased expression of IL-17 could be related to the enhanced Th1 immune response duringin obesity [23].

There is evidence that regulatory T cells play an important role in the maintenance of adipose tissue homeostasis and glucose sensitivity [24]. During diet-induced inflammation, the presence of T regulatory cells in the visceral adipose tissue decreases [8], which is in line with our findings that a HFD significantly reduced the incidence of $CD4^+CD25^+FoxP3^+$ regulatory T cells in the ST2 deficient mice.

The polarizisation of infiltrated macrophages toward the classically activated M1 phenotype and a significant reduction in the amount of alternatively activated M2 macrophages is the mechanism underlies the diet-induced inflammation in the visceral adipose tissue [6]. However, IL-33 promotes the phenotypic switch of macrophages to an M2 phenotype during in obesity [18]. We found that the lack of the IL-33 receptor ST2 expression is correlated with the markedly increased presence of the $F4/80^+CD11c^+CD206^+$ M1 macrophages in the visceral adipose tissue of the HFD-fed mice. These M1 macrophages were recently described as the proinflammatory cell subset present duringin diet-induced obesity [25]. At the same time, we found a significantly decreased incidence of the alternatively activated $F4/80^+CD11c^-CD206^+$ M2 macrophages, which is in line with a previous report related to the phenotypic switch of macrophages in diet-induced obesity in mice [26].

Accelerated HFD-induced adiposity in the absence of ST2 was associated with increased systemic levels of proinflammatory cytokines, such as IL-1β and IL-6 [7], and decreased levels of the anti-inflammatory cytokines IL-13 and IL-10 [27, 28]. Although we did not find any differences in the production of IL-6 and CRP between the experimental groups, the systemic level of the proinflammatory IL-1β was significantly increased in the HFD-fed ST2 deficient mice than in the other experimental groups. In the absence of IL33/ST2 signalling, we observed significantly decreased levels of the anti-inflammatory cytokines IL-13 and IL-10, which were strongly correlated with the enhanced obesity in the ST2 deficient mice.

CONCLUSIONS

These findings suggest that IL33/ST2 signalling plays an important protective role in high-fat diet-induced adipose tissue inflammation and could be of therapeutic relevance.

ACKNOWLEDGMENTS

This work was funded by grants from the Serbian Ministry of Science and Technological Development (Grants No. 175071 and 175069), Serbia and The Faculty of Medical Sciences, University of Kragujevac (JP 03-11).

No potential conflicts of interest relevant to this article were reported.

REFERENCES:

1. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444:860-7.
2. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. J Clin Invest 2011; 121:2111-17.
3. Strissel KJ, DeFuria J, Shaul ME, Bennett G, Greenberg AS, Obin MS. T-cell recruitment and Th1 polarization in adipose tissue during diet-induced obesity in C57BL/6 mice. Obesity (Silver Spring) 2010; 18:1918-25.
4. Ohmura K, Ishimori N, Ohmura Y, et al. Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. Arterioscler Thromb Vasc Biol 2010; 30:193-9.
5. Pejnovic N, Pantic J, Jovanovic I, et al. Galectin-3 deficiency accelerates high-fat diet induced obesity and amplifies inflammation in adipose tissue and pancreatic islets. Diabetes 2013; 62:1932-44.
6. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007; 117:175-84.
7. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of

obesity-related insulin resistance. J Clin Invest 2003; 112(12):1821-30.
8. Feuerer M, Herrero L, Cipolletta D, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med 2009; 15:930-9.
9. Liew FY, Pitman NI, McInnes IB. Disease-associated function of IL-33: the new kid in the IL-1 family. Nat Rev Immunol 2010; 10:103-10.
10. Chackerian AA, Oldham ER, Murphy EE, Schmitz J, Pflanz S, Kastelein RA. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. J Immunol 2007; 179:2551-5.
11. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 2005; 23:479-90.
12. Bourgeois E, Van LP, Samson M, et al. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-γ production. Eur J Immunol 2009; 39:1046-55.
13. Kurowska-Stolarska M, Stolarski B, Kewin P, et al. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. J Immunol 2009; 183:6469-77.
14. Volarevic V, Mitrovic M, Milovanovic M, et al. Protective role of IL-33/ST2 axis in Con A-induced hepatitis. J Hepatol 2012; 56:26-33.
15. Milovanovic M, Volarevic V, Ljujic B, et al. Deletion of IL-33R (ST2) Abrogates Resistance to EAE in BALB/C Mice by Enhancing Polarization of APC to Inflammatory Phenotype. PLoS One 2012; 7 (9):e45225.
16. Zdravkovic N, Shahin A, Arsenijevic N, Lukic ML, Mensah-Brown EP. Regulatory T cells and ST2 signaling control diabetes induction with multiple low doses of streptozotocin. Mol Immunol 2009; 47(1):28-36.
17. Jovanovic I, Radosavljevic G, Mitrovic M, et al. ST2 Deletion Enhances Innate and Acquired Immunity to Murine Mammary Carcinoma. Eur J Immunol 2011; 41:1902-12.
18. Miller AM, Asquith DL, Hueber AJ, et al. Interleukin-33 Induces Protective Effects in Adipose Tissue Inflammation During Obesity in Mice. Circ Res 2010; 107:650-8.
19. Miller AM, Xu D, Asquith DL, et al. IL-33 reduces the development of atherosclerosis. J Exp Med 2008; 205:339-46.
20. Kintscher U, Hartge M, Hess K, et al. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol 2008; 28:1304-10.
21. Kang K, Reilly SM, Karabacak V, et al. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. Cell Metab 2008; 7:485-95.
22. Zuniga LA, Shen WJ, Joyce-Shaikh B, et al. IL-17 Regulates Adipogenesis, Glucose Homeostasis, and Obesity. J Immunol 2010; 185:6947-59.
23. Zorzanelli Rocha V, Folco EJ, Sukhova G, et al. Interferon-γ, a Th1 Cytokine, Regulates Fat Inflammation A Role for Adaptive Immunity in Obesity. Circ Res 2008; 103:467-76.
24. Ilan Y, Maron R, Tukpah AM, et al. Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice. Proc Natl Acad Sci USA 2010; 107:9765-70.
25. Nguyen MTA, Favelyukis S, Nguyen A-K, et al. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. J Biol Chem 2007; 282:35279-92.
26. Fujisaka S, Usui I, Bukhari A, et al. Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. Diabetes 2009; 58:2574-82.
27. Hong EG, Ko HJ, Cho YR, et al. Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle. Diabetes 2009; 58:2525-35.
28. Stanya KJ, Jacobi D, Liu S, et al. Direct control of hepatic glucose production by interleukin-13 in mice. J Clin Invest 2013; 123:261-71.

THE REDOX STATE OF YOUNG FEMALE HANDBALL PLAYERS FOLLOWING ACUTE EXERCISE AND A ONE-MONTH PRECOMPETITIVE TRAINING PERIOD

Irena Pusica[1], Zoran Valdevit[2], Sladjana Todorovic[3], Vladimir Jakovljevic[1], Dejan Cubrilo[4], Dragan Djuric[5], Djordje Stefanovic[1], Vladimir Zivkovic[1], Nevena Barudzic[1], Dusica Djordjevic[1]

[1]Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2]Department of Handball, Faculty of Sports and Physical Education, University of Belgrade, Belgrade, Serbia
[3]Department of Health Statistics, Health Center Cuprija, Cuprija, Serbia
[4]Department of Physiology, Faculty of Sport and Tourism, Educons University, Novi Sad, Serbia
[5]Institute of Physiology "Richard Burian", School of Medicine, Belgrade, Serbia

REDOKS STATUS MLADIH RUKOMETAŠICA NAKON JEDNOKRATNOG VEŽBANJA I JEDNOMESEČNOG PREDTAKMIČARSKOG PRIPREMNOG PERIODA

Irena Pušica[1], Zoran Valdevit[2], Slađana Todorović[3], Vladimir Jakovljević[1], Dejan Čubrilo[4], Dragan Đurić[5], Đorđe Stefanović[1], Vladimir Živković[1], Nevena Barudzić[1], Dušica Đorđević[1]

[1]Katedra za fiziologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2]Katedra za rukomet, Fakultet sporta i fizičkog vaspitanja, Univerzitet u Beogradu, Beograd, Srbija
[3]Služba za zdravstvenu statistiku, Dom zdravlja Ćuprija, Ćuprija, Srbija
[4]Katedra za Fiziologiju, Fakultet za sport i turizam, Univerzitet Edukons, Novi Sad, Srbija
[5]Institut za fiziologiju Rihard Burijan, Medicinski fakultet, Univerzitet u Beogradu, Srbija

Received / Primljen: 22.09.2013. Accepted / Prihvaćen: 11.10.2013.

ABSTRACT

Although the relationship between exercise and oxidative stress has been intensively investigated for over 3 decades, there remains a lack of empirical data on exercise-induced oxidative stress in athletes engaged in sporting games, specifically among the population of elite female athletes. Blood samples were taken from female handball players on the Serbian U20 national team at the beginning and end of a one-month preparatory training period, as well as immediately before and after acute treadmill exercise. Levels of superoxide anion radical, hydrogen peroxide, nitric oxide and lipid peroxidation were measured in plasma samples , while levels of reduced glutathione and the activity of superoxide dismutase and catalase were measured in erythrocytes. Both experimental protocols demonstrated significant increases in plasma levels of hydrogen peroxide and decreases in superoxide dismutase activity in erythrocytes. Despite the increase in plasma levels of hydrogen peroxide after both the treadmill exercise and the one-month training period, the levels of the two antioxidants responsible for eliminating H_2O_2 hydrogen peroxide were not significantly different, as may be expected. Moreover, the marker of lipid peroxidation, TBARS, was not significantly increased. These findings suggest that the first line of antioxidative defence was effective in the prevention of oxidative stress among young female handball players.

Keywords: *oxidative stress, redox balance, handball, training, treadmill*

SAŽETAK

Iako se veza između vežbanja i oksidativnog stresa intenzivno istražuje već više od 3 decenije, još uvek postoji nedovoljno naučnih informacija o vežbanjem-izazvanom oksidativnom stresu kod sportista koji se bave sportskim igrama, a naročito u populaciji žena vrhunskih sportistkinja. Rukometašicama reprezentacije Srbije do 20 godina uzeti su uzorci venske krvi na početku i kraju jednomesečnog pripremnog perioda, kao i neposredno pre i nakon akutnog vežbanja. Nivoi superoksid anjon radikala, vodonik peroksida, azot monoksida i lipidne peroksidacije mereni su u plazmi, dok su nivoi redukovanog glutationa, i aktivnost superoksid dismutaze i katalaze mereni u eritrocitima. I akutno vežbanje i jednomesečni trenažni period doveli su do značajnog porasta nivoa vodonik peroksida u plazmi i snižene aktivnosti superoksid dismutase u eritrocitima. Bez obzira na povećanje nivoa vodonik peroksida i nakon jednokratnog vežbanja i nakon trenažnog procesa, ni u jednom slučaju nije došlo do promene nivoa ostalih antioksidanata, kao što bi se moglo očekivati. Takođe, povećan nivo vodonik peroksida nije imao za posledicu povećanje nivoa lipidne peroksidacije ni u jednom slučaju. To ukazuje na mogućnost da je prva linija antioksidativne odbrane bila dovoljna da zaštiti organizam mladih rukometašica od oksidativnog stresa.

Ključne reči: *oksidativni stres, redoks ravnoteža, rukomet, trening, tredmil*

ABBREVIATIONS

ADS - antioxidative defence system;
CAT - catalase;
GSH - reduced glutathione;
RBCs - red blood cells;
RONS - reactive oxygen and nitrogen species;
SOD - superoxide dismutase;
TBARS - thiobarbituric acid reactive substances;
VO_2max - maximal oxygen consumption.

UDK: 616-008.9:[577.344:546.21 ; 616-008.9:796.322.015 / Ser J Exp Clin Res 2013; 14 (4): 161-168
DOI: 10.5937/SJECR4515

Correspondence to: Dusica Djordjevic, Assistant Professor of Methodology of Anthropometry
Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, 34000 Kragujevac, Republic of Serbia
Tel. +381 69 877 66 69, E-mail:dusicadjordjevickg@gmail.com

INTRODUCTION

During exercise, the several-fold rise of energetic needs and oxygen consumption may induce increased production of reactive oxygen and nitrogen species (RONS) and possible disruption of the redox state, i.e., oxidative stress [1]. The first study of the relationship between exercise and oxidative stress was performed in 1978 by Dillard and colleagues [2] and demonstrated that acute aerobic exercise increases lipid peroxidation, which may be mediated by vitamin E consumption. Although intensively researched during the following decades, there is a dearth of scientific data describing exercise-induced oxidative stress, especially among athletes partaking in sporting games such as handball [3]. Recently published studies showed that handball players have significantly higher superoxide dismutase activity [3, 4], higher levels of reduced glutathione and nitric oxide, and lower levels of lipid peroxidation compared to non-athletes [3], while catalase activity was in one study higher [4] and in another lower [3] than in controls. Another study showed that nonspecific intensive activity, such as maximal progressive tests on a cycle ergometer, induced oxidative stress in young handball players [5]. Conversely, after standard handball training, there were no significant changes to pro/antioxidants except for the activity of superoxide dismutase, which suggests that the first line of antioxidant defence was sufficient in preventing oxidative stress [5]. It was also shown that participation in a handball match [6], as well as creatine supplementation in combination with specific endurance training [7], induced the disturbance of the redox state in handball players. Monitoring the redox states of players during the handball season showed increased parameters of oxidative stress in plasma and decreased parameters in erythrocytes during periods of intensive training and competitions [8].

Furthermore, despite hundreds of papers on the effects of exercise on the redox state, there is a lack of studies investigating this relationship in women. The reason for the disproportionate number of studies on exercise-induced oxidative stress in men and women may be due to the complexity of research and interpretation of the redox state due to the hormonal characteristics of women, which may influence the results. Recent studies [9, 10] concluded that elite female football players have efficient and well-regulated antioxidative defence systems (ADS), both endogenous and exogenous, because increased levels of oxidised glutathione in plasma samples taken immediately after a match did not influence the level of lipid peroxidation. Another study of female football players confirmed that adequate nutrition improves ADS by affecting the activity of primary antioxidative enzymes, such as superoxide dismutase and glutathione peroxidase [11]. However, intensified training and matches during the competitive season resulted in increased lipid peroxidation and protein oxidation index among elite female water polo players [12].

The aim of this study was to assess the redox state of young, elite female handball players after both acute exercise sessions and intensive training periods.

MATERIALS AND METHODS

Subjects

This research was carried out among a group of 20 female handball players on the young Serbian national team, which took 4th place in the European U20 handball championship in 2011 and 4th place on the World U20 handball championship in 2012.

All participants were healthy, used no medications or supplements before the beginning of the study, and were non-smokers. Participants were asked to abstain from heavy physical activity 24 h before the test and not to consume alcohol 48 h before the test.

All participants, and their parents if they were younger than 18 years of age, provided written informed consent. The study was performed in accordance with the Helsinki Declaration and was approved by the ethical committee of The Faculty of Medical Sciences, University of Kragujevac.

Protocol

The study was performed during the preparatory period for the 2012 World U20 handball championship. At the beginning of the preparatory period, all players were evaluated in The Republic Institute for Sports by medical examination and motoric and psychological testing. Acute effects of exercise on oxidative stress markers were assessed by taking a blood sample immediately before and after maximal progressive exercise tests on a treadmill, which were performed to assess the maximal oxygen consumption of players. The effects of the one-month training program were assessed by taking a morning (basal) blood sample at the beginning and the end of the one-month preparatory period.

Anthropometrical measurement

Body composition was measured using an apparatus for bioelectrical impedance analysis, the *In Body 720* (Biospace, Korea), whose validity has been previously confirmed [13]. Measurement was performed according to the manufacturer's instructions. Body weight was measured with an accuracy within 0.1 kg, and body fat was measured with an accuracy of 0.1%. Body height was measured by means of an anthropometer (GPM, Switzerland), and the measurements were accurate within 0.1 cm.

Exercise testing

Maximal progressive exercise testing was performed on a treadmill (T 200, Cosmed, Italy), according to the modified Ellestad Memorial Hospital B protocol [14] presented in Table 1. The maximal oxygen consumption (VO_2max) was assessed through usage of an automated metabolic cart (Quark b2, Cosmed, Italy). The participants stated their subjective feeling of exhaustion by using Borg's CR10 exhaustion scale of at least 8 [15]. We hypothesised that

Load		Duration of exercise	Speed km/h	Grade %
0	Rest	0	0	0 %
I	Stage I	180 sec.	2.7	10 %
II	Stage II	180 sec.	4.8	10 %
III	Stage III	180 sec.	6.4	10 %
IV	Stage IV	180 sec.	8.0	10 %
V	Stage V	Until exhaustion	8.0	15 %
R1	Recovery I	90 sec.	6.4	10 %
R2	Recovery II	90 sec.	4.8	10 %

Table 1. Ellestad B protocol - modified version.

the VO_2max was reached when the oxygen consumption plateaued (the point at which increasing workload cannot affect an increase in oxygen consumption) [16].

Training program

The preparatory training program lasted 1 month, during which 31 trainings were held and 4 matches were played. Details about the training program are presented in Table 2.

Nutrition

During the study period, all athletes consumed a standardised menu consisting of 3 main meals and two snacks. For athletes needing to lose weight, a nutrition plan was made according to dietary recommendations made by the Joslin Diabetes Research Center at the Harvard Medical School for the treatment of obesity, metabolic syndrome, and diabetes in 2005 [17]. Although there is some controversy as to whether a low glycemic load diet leads to improved weight loss [18], there is no question that a low glycemic load diet will generate a lower inflammatory burden [19]. Sport nutrition clinical studies clearly demonstrate that low-carbohydrate diets elicit greater decreases in body weight and fat than energy-equivalent low-fat diets, especially over a short duration [20]. Such a proposed anti-inflammatory diet consisted of approximately 1500 calories per day (approximately 50 grams of monounsaturated fat, 100 grams of low-fat protein, and 150 grams of low glycemic load carbohydrates This anti-inflammatory diet has a 1 : 2 : 3 ratio of fat to protein to carbohydrates based on individual weight. The caloric ratio is approximately 30% fat, 30% protein, and 40% carbohydrates. Athletes maintaining their weight consumed the same diet, with a daily caloric intake of 30 Kcal/kg of their body mass. The daily intake of essential macronutrients for those on a weight loss diet is shown in Table 3.

Athletes also took supplements consisting of 200 mg magnesium citrate twice daily; 1000 mg vitamin C 1x a day; 30 mg CoQ10 with 200 IU d-alpha tocopherol twice daily; and vitamin B-50 complex 1x a day. In each training session and every game, athletes drank 500 ml of water

Microcycle	Morning training		Afternoon training	
No. 1 (7 days)	No. of trainings:	6	No. of trainings:	6
	Aim:	STR-COND	Aim:	TE-TA STR & SPE
	Gym:	6 exercises, 3-4 series 3 tr: 40-50% 1RM 2 tr: 80-90% 1RM 1 tr: 90-100% 1 RM	Sports hall:	3 tr: TE-TA + STR 2 tr: TE-TA + SPE 1 tr: training match
	Sports hall:	3 tr: SPE + SPEND 3 tr: END		
Tournament 1	**2 matches**			
No. 2 (8 days)	No. of trainings:	5	No. of trainings:	7
	Aim:	STR-COND	Aim:	TE-TA STR & SPE
	Gym:	6 exercises, 3-4 series 3 tr: 70-80% 1RM 1 tr: 80-90% 1RM 1 tr: 90-100% 1 RM	Sports hall:	2 tr: TE-TA + STR + AG 2 tr: TE-TA + SPE 2 tr: training match
	Sports hall:	2 tr: SPE + SPEND with ball 2 tr: END + TE-TA 1 tr: TE-TA		
Tournament 2	**2 matches**			
No. 3 (5 days)	No. of trainings:	4	No. of trainings:	3
	Aim:	TE-TA STR & SPE	Aim:	TE-TA STR & SPE
	Sports hall:	2 tr: TE-TA + STR + SPE 2 tr: TE-TA	Sports hall:	1 tr: TE-TA + STR + AG 2 tr: TE-TA + SPE + STR

Table 2. One-month training program (TE-TA: technique and tactics. STR-COND: strength and conditioning, tr: trainings, SPE: speed, STR: strength, END: endurance, SPEND: speed endurance, AG: agility)

Meals	Calories (kcal)	Proteins (gr)	Carbs (gr)	Fats (gr)
Breakfast	455	35	45	15
Morning snack	182	14	18	6
Lunch	364	28	36	12
Afternoon snack	182	14	8	6
Dinner	364	28	36	12
Sum	1547	119	143	51

Table 3. The total daily intake of calories and macronutrients amounts distributed over the meals in one day (subjects on weight loss program).

Characteristic	X±SD
Age (years)	19.14±1.10
Height (cm)	170.14±6.48
Weight (kg)	71.74±9.95
Body mass index	23.37±2.50
Fat (%)	19.07±5.36
Muscle (%)	45.55±2.97
Training and competition experience (years)	8.90±2.25
Hours per week of training (h)	12.26±3.12
Maximal oxygen consumption (ml/kg/min)	43.07±4.78

Table 4. Characteristics of the investigated group.

with 20 g dextrose and 10 g of whey protein isolate. After each training session, athletes drank a protein shake (30 g of protein plus 5 g of glutamine in 500 ml of water). A piece of fruit was eaten after each training session (400 g).

Biochemical assays

Blood samples were drawn from an antecubital vein into Vacutainer test tubes containing sodium citrate anticoagulant. Blood samples were analysed immediately. Blood samples were centrifuged to separate plasma and red blood cells (RBCs) . Biochemical parameters were measured spectrophotometrically.

Superoxide anion radical determination

Levels of superoxide anion radical (O_2^-) were measured using nitro blue tetrazolium reaction in TRIS-buffer combined with plasma samples and read at 530 nm [21]. Levels of O_2^- are presented in nmol/ml of plasma.

Hydrogen peroxide determination

The protocol for measurement of hydrogen peroxide (H_2O_2) is based on oxidation of phenol red in the presence of horseradish peroxidase [22]. Samples of 200 µl s were combined with 800 µl phenol red solution and 10 µl horseradish peroxidase (1:20). Plasma levels of H_2O_2 were measured at 610 nm. Levels of H_2O_2 are presented in nmol/ml of plasma.

Nitric oxide determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite (NO_2^-) was determined as an index of nitric oxide production with Griess reagent [23], 0.1 ml 3 N perchloric acid, 0.4 ml 20 mM ethylenediaminetetraacetic acid and 0.2 ml plasma were put on ice for 15 min, then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µl of K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe. Levels of NO_2^- are presented in nmol/ml of plasma.

Index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS)

The degree of lipid peroxidation in plasma was estimated by measuring the thiobarbituric acid reactive substances (TBARS) using 1 % thiobarbituric acid in 0.05 M NaOH, incubated with plasma at 100 °C for 15 min and read at 530 nm. Distilled water was used as a blank probe. Thiobarbituric acid extract was obtained by combining 0.8 ml plasma and 0.4 ml trichloroacetic acid. Samples were put on ice for 10 minutes and then centrifuged for 15 min at 6000 rpm. This method has been previously described in the literature [24]. Levels of TBARS are presented in µmol/ml of plasma.

Determination of antioxidant enzymes

Isolated RBCs washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl and haemolysates containing approximately 50 g Hb/l (prepared according to McCord and Fridovich [25]) were used for the determination of catalase (CAT) activity. Catalase activity was determined according to Beutler [26]. Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin [27]. Then, 50 µl catalase buf-

Parameter	Before exercise test (X±SD)	After exercise test (X±SD)	Significance
O_2^- (nmol/ml)	3.31±2.45	4.18±2.18	P=0.153
H_2O_2 (nmol/ml)	0.96±0.53	2.41±1.40	P=0.001
NO_2 (nmol/ml)	6.06±3.55	6.57±2.74	P=0.740
TBARS (µmol/ml)	4.59±2.26	5.36±1.73	P=0.084
SOD (U/g Hb x 10^3)	3231.58±2510.51	1936.84±1723.27	P=0.031
CAT (U/g Hb x 10^3)	28.20±28.21	25.23±9.57	P=0.463
GSH (nmol/ml of RBCs)	757.30±466.52	745.35±473.10	P=0.936

Table 5. Levels of pro/antioxidants (X±SD) in athletes' blood before and after exercise test.

fer, 100 µl sample and 1 ml 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. Superoxide dismutase (SOD) activity was determined by the epinephrine method of Misra and Fridovich [28]. One-hundred µl lysate and 1 ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470 nm. The activities of SOD and CAT in RBCs are presented in units per gram of haemoglobin x10^3 (U/g Hbx10^3)

Determination of glutathione

The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid, using the Beutler method [29]. The concentration of glutathione is expressed as nanomoles per millilitre of RBCs.

Statistics

The statistical analysis was performed with SPSS 20.0 for Windows. The results are expressed as the means ± standard deviation of the mean. The data distribution was checked with the Shapiro-Wilk test, and depending on the results, appropriate parametric or nonparametric tests were used. The differences between the values of means from two related samples (before and after the maximal exercise test, before and after the one-month training period) were assessed by Paired t-tests or Wilcoxon tests, where appropriate. The differences were considered to be significant when the P value was lower than 0.05 and highly significant when the P value was lower than 0.01.

RESULTS

Morphofunctional characteristics and data about training experience of the investigated sample are presented in Table 4. Subjects had long training and competitive experience, well body composition, and cardiorespiratory fitness that may be classified as good (when compared with age-matched healthy females) or satisfactory for playing predominantly anaerobic sports such as handball.

Results of the biochemical analysis regarding the acute effects of exercise are presented in Table 3, while chronic effects of exercise are presented in Figures 1 and 2.

Acute exposure to the maximal progressive load test on a treadmill significantly increased levels of hydrogen peroxide and decreased activity of superoxide dismutase (Table 3).

As presented in Figure 1, the only pro-oxidant that significantly changed after the training period was hydrogen peroxide (from 0.96±0.53 to 1.69±1.01 nmol/ml; P=0.005). Among antioxidant activity, presented in Figure 2, activity of superoxide dismutase was significantly lower at the end compared to the beginning of the study (3231.58±2510.51 versus 1458.97±1550.56 U/g Hb x 103; P=0.004).

DISCUSSION

The effect of exercise on the redox state of an individual depends on many factors, such as the type of training, training load, individual characteristics including age, and coexisting factors of risk and physical condition [30].

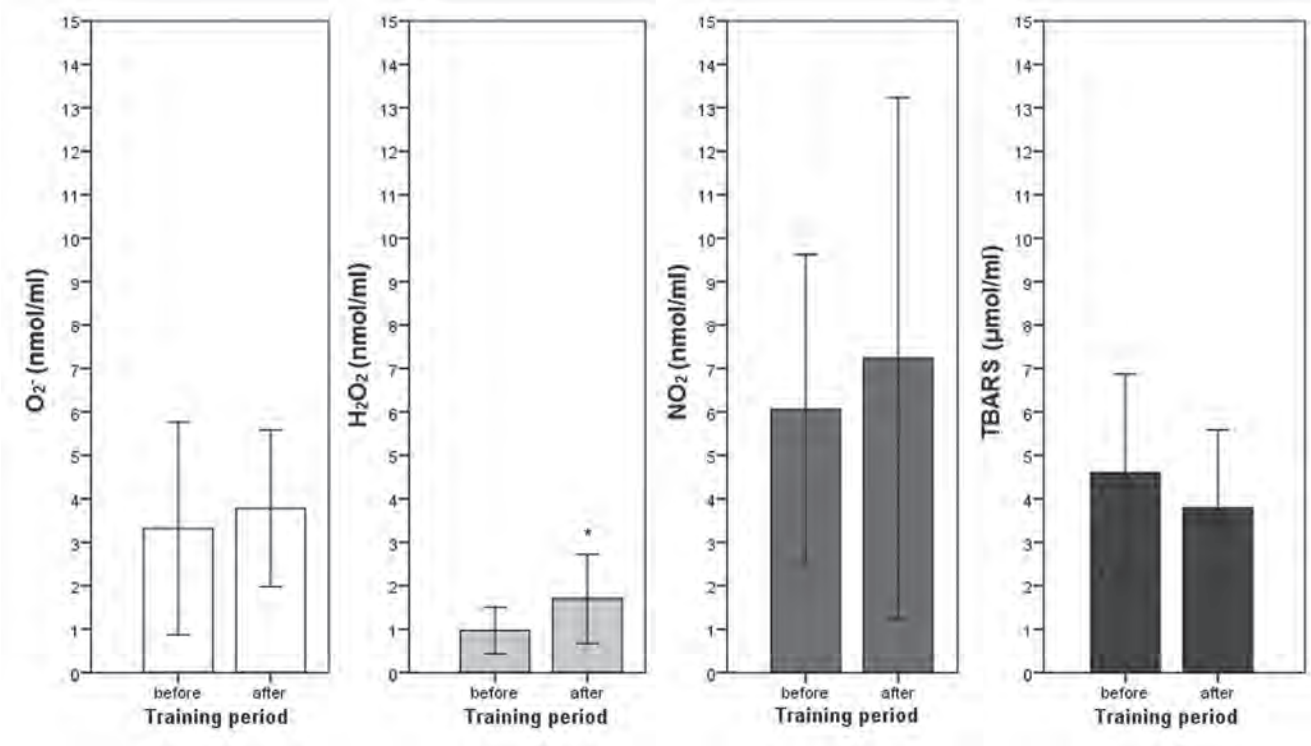


Figure 1. Levels of pro-oxidants (X±SD) in athletes' blood before and after the training period.

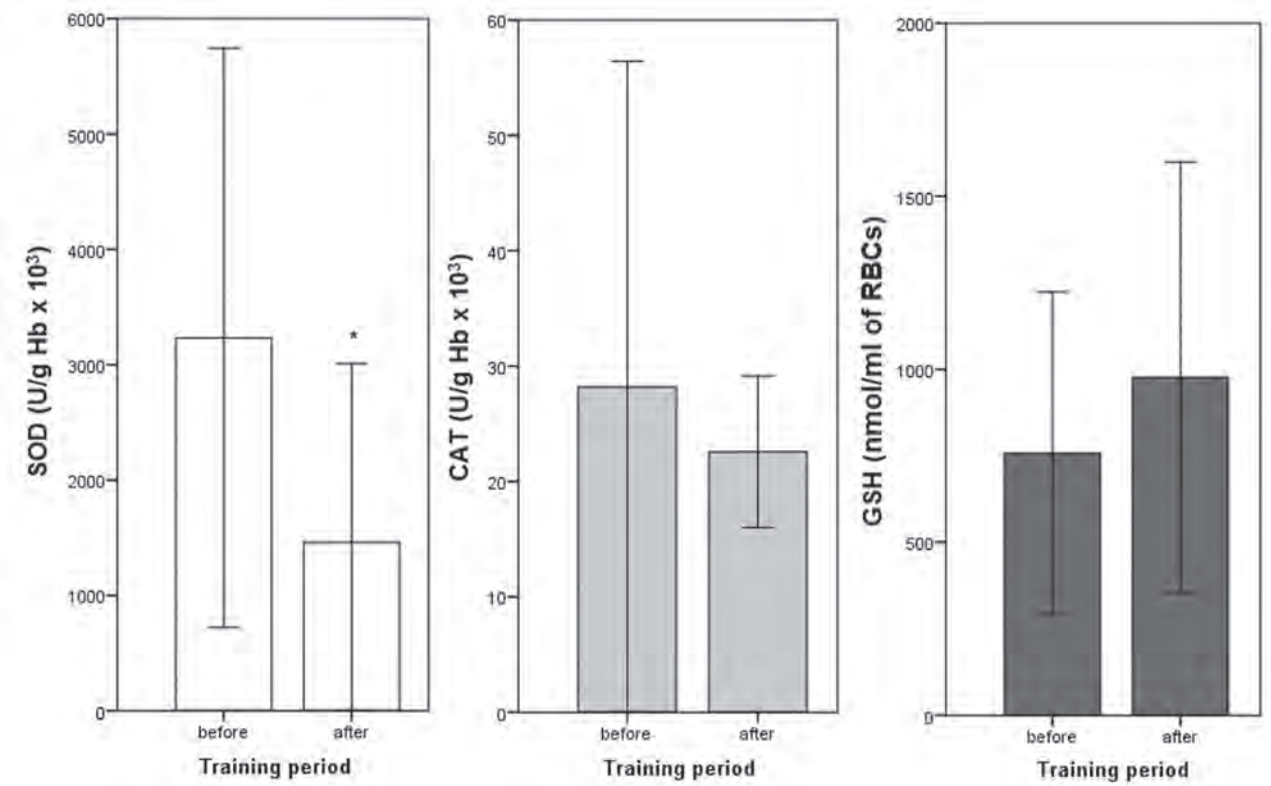


Figure 2. Levels of antioxidants (X±SD) in athletes' blood before and after the training period.

The study contributed to the limited scientific data about exercise-induced oxidative stress in women, adolescent populations, elite athletes, and mixed (aerobic-anaerobic) sports, by assessing changes in redox state of elite young female handball players after acute exposure to an exercise test, as well as after a one-month intensive training period. The results showed significant increase in H_2O_2 levels and decrease in SOD activity both after the treadmill test and after the period of 31 trainings and 4 matches. These findings are consistent with a number of previously published studies. Superoxide dismutase enzyme represents the first line of antioxidant defence and is the enzyme most frequently affected by exercise stimulus in previous studies [31]. However, H_2O_2 is the most stable form of reactive oxygen species produced mainly by O_2^- dismutation by SOD. In vitro studies have shown that elevated H_2O_2 levels inhibit SOD activity [32]. The increase in H_2O_2 levels may explain these results; however, the levels of two other H_2O_2 eliminating antioxidants were not significantly changed, as may be expected. Additionally, the marker of lipid peroxidation, TBARS, was not significantly increased.

Although there are a few studies reporting the effects of treadmill exercise on redox state in football players or non-athletes [1, 33-37], the most adequate comparison of our results is with studies performed on young handball players [4, 5, 31], although the exercise test performed in these studies was cycling ergometer testing. However, because it has been shown that endogenous oestrogen may have a protective role in exercise-induced oxidative stress among female handball players [38] and female non-athletes [39, 40], this comparison is limited due to gender differences and the specificity of the exercise test. After maximal progressive exercise testing on the cycling ergometer, the levels of H_2O_2 among young male handball players were increased and SOD activity decreased, which is consistent with our findings. However, the activity of CAT also decreased and levels of TBARS increased [5]. Interestingly, further analysis showed that increased levels of H_2O_2 and decreased SOD activity were observed only in a group of handball players with the lowest basal SOD activity [31]. Additionally, when comparing handball players with sedentary age-matched controls, exercise testing induced the increase in H_2O_2 levels only in controls who had significantly lower basal SOD activity compared with handball players [4]. This suggests that adaptations of antioxidant defence due to chronic exercise protect athletes from oxidative stress induced not only by an exercise stimulus but also most likely in a number of non-exercise conditions. The decrease in SOD due to exercise testing on an ergometer was also confirmed by Mrowicka et al. [30], who reported that SOD activity was significantly more decreased in athletes compared to non-athletes after cycling ergometry. Another study showed that in non-athletes, SOD activity actually increased in response to any load of exercise on a cycling ergometer [41]. Because female handball players in this study had higher basal SOD activity compared to male handball players in the abovementioned studies, and considering the SOD behaviour in non-athletes during exercise test, we hypothesise that SOD changes are related to the exercise capacity of the subjects, achieved

exercise load and consequently longer duration of the exercise test. Positive correlation between VO_{2max} and H_2O_2 levels was previously established [3].

The second aim of this research was to assess changes in the redox state of our subjects after a one-month preparatory training period. The results of the one-month training period on the redox state were the same as the effects of acute exercise: H_2O_2 levels rise and SOD activity fell at the end of the one-month training period. To the best of our knowledge, only one study monitored the redox state of female handball players over a longer period of time [38]. This recent study reported that CAT activity was the highest during the most intensive period of the handball season, but only in a group of athletes with normal oestrogen levels. Furthermore, levels of lipid peroxidation were significantly lower during the middle and by the end of season, compared to the beginning [38]. In contrast, parameters of oxidative stress in plasma were significantly increased among male handball players during the most intensive periods of trainings and competition, while the opposite was observed in erythrocytes [8]. The observed decrease in plasma-levels of oxidative stress parameters was ossibly most likely due to the significantly increased activity of antioxidant enzymes in erythrocytes [8], confirming the expected adaptation of ADS as a response to programmed training.

The significant increase inof hydrogen peroxide after both treadmill exercise and the one-month training period, without a corresponding increase in the levels of H_2O_2 –eliminating antioxidants or a significant change in the levels of lipid peroxidation, suggests that the first line of antioxidative defence, in the form of superoxide dismutase, is enough to prevent oxidative stress in young female handball players. These results suggest that well-trained elite athletes have upregulated endogenous antioxidative defence systems, which protect them from exercise-induced oxidative stress and that increasing pre-competition training workload should not induce adverse biochemical changes leading to overtraining syndrome.

The limitations of this study include the lack of measurement of oestrogen levels in our subjects and the absence of a control group. However, as the training and nutrition was uniform, some conclusions may be drawn from comparing pre- and post-training values of pro/antioxidants in athletes' blood.

Acknowledgements: This work was supported by junior project 09/2011 of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

REFERENCES

1. Djordjevic D, Jakovljevic V, Cubrilo D, Zlatkovic M, Zivkovic V, Djuric D. Coordination between nitric oxide and superoxide anion radical during progressive exercise in elite soccer players. Open Biochem J 2010; 4: 100-6.
2. Dillard CJ, Litov RE, Savin WM, Dumelin EE, Tappel AL. Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. J Appl Physiol 1978; 45(6): 927-32.
3. Djordjevic D, Cubrilo D, Macura M, Barudzic N, Djuric D, Jakovljevic V. The influence of training status on oxidative stress in young male handball players. Mol Cell Biochem 2011; 351(1-2): 251-9.
4. Djordjevic DZ, Cubrilo DG, Barudzic NS, et al. Comparison of blood pro/antioxidant levels before and after acute exercise in athletes and non-athletes. Gen Physiol Biophys 2012; 31(2): 211-9.
5. Djordjevic DZ, Cubrilo DG, Puzovic VS, et al. Changes in athlete's redox state induced by habitual and unaccustomed exercise. Oxid Med Cell Longev 2012; 2012: 805850.
6. Marin DP, dos Santos Rde C, Bolin AP, Guerra BA, Hatanaka E, Otton R. Cytokines and oxidative stress status following a handball game in elite male players. Oxid Med Cell Longev 2011; 2011: 804873.
7. Percário S, Domingues SP, Teixeira LF, Vieira JL, de Vasconcelos F, Ciarrocchi DM, et al. Effects of creatine supplementation on oxidative stress profile of athletes. J Int Soc Sports Nutr 2012; 9(1): 56.
8. Marin DP, Bolin AP, Campoio TR, Guerra BA, Otton R. Oxidative stress and antioxidant status response of handball athletes: Implications for sport training monitoring. Int Immunopharmacol 2013; 17(2): 462-70.
9. Andersson H, Karlsen A, Blomhoff R, Raastad T, Kadi F. Active recovery training does not affect the antioxidant response to soccer games in elite female players. Br J Nutr 2010; 104(10): 1492-9.
10. Andersson H, Karlsen A, Blomhoff R, Raastad T, Kadi F. Plasma antioxidant responses and oxidative stress following a soccer game in elite female players. Scand J Med Sci Sports 2010; 20(4): 600-8.
11. Gravina L, Ruiz F, Diaz E, et al. Influence of nutrient intake on antioxidant capacity, muscle damage and white blood cell count in female soccer players. J Int Soc Sports Nutr 2012; 9(1): 32.
12. Varamenti EI, Kyparos A, Veskoukis AS, et al. Oxidative stress, inflammation and angiogenesis markers in elite female water polo athletes throughout a season. Food Chem Toxicol 2012. doi: 10.1016/j.fct.2012.12.001. (in press)
13. Lim JS, Hwang JS, Lee JA, et al. Cross-calibration of multi-frequency bioelectrical impedance analysis with eight-point tactile electrodes and dual-energy X-ray absorptiometry for assessment of body composition in healthy children aged 6-18 years. Pediatr Int 2009; 51(2): 263-8.
14. Ellestad MH, Wan MK. Predictive implications of stress testing: follow-up of 2700 subjects after maximum treadmill stress testing. Circulation 1975; 51(2): 363-9.
15. Borg GA. Psychophysical bases of perceived exertion. Med Sci Sports Exerc 1982; 14(5): 377-81.
16. Howley ET, Bassett DR, Welch HG. Criteria for maximal oxygen uptake: review and commentary. Med Sci Sports Exerc 1995; 27(9): 1292-301.

17. Joslin Diabetes Research Center Dietary Guidelines, http://www.joslin.org/docs/nutrition guideline graded.pdf.
18. Ebbeling CB, Leidig MM, Feldman HA, Lovesky MM, Ludwig DS. Effects of a low-glycemic load vs low-fat diet in obese young adults: a randomized trial. JAMA 2007; 297(19): 2092–102.
19. Pittas AG, Das SK, Hajduk CL et al. A low-glycemic load diet facilitates greater weight loss in overweight adults with high insulin secretion but not in overweight adults with low insulin secretion in the CALERIE trial. Diabetes Care 2006; 28(12): 2939–41.
20. Cook CM, Haub MD. Low-carbohydrate diets and performance. Curr Sports Med Rep 2007; 6(4): 225-9.
21. Auclair C, Voisin E. Nitroblue tetrazolium reduction. In: Greenwald RA, ed. Handbook of methods for oxygen radical research. Boka Raton, FL: CRC Press, 1985: 123-132.
22. Pick E, Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods 1980; 38(1-2): 161-70.
23. Green LC, Wagner DA, Glogowski J, Skipper PI, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. Anal Biochem 1982; 126(1): 131-8.
24. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95(2): 351-8.
25. McCord JM, Fridovich I. The utility of superoxide dismutase in studying free radical reactions. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. J Biol Chem 1969; 244(22): 6056-63.
26. Beutler E. Catalase. In: Beutler E, ed. Red cell metabolism, a manual of biochemical methods. New York: Grune and Stratton, 1982: 105-6.
27. Tsuchihashi M. Zur Kernntnis der blutkatalase. Biochem Z 1923; 140: 65-72.
28. Misra HP, Fridovich I. The role of superoxide-anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; 247(10): 3170-5.
29. Beutler E. Reduced glutathione (GSH). In: Beutler E, ed. Red cell metabolism, a manual of biochemical methods. New York: Grune and Stratton, 1975: 112-14.
30. Mrowicka M, Kędziora J, Bortnik K, Malinowska K, Mrowicki J. Antioxidant defense system during dosed maximal exercise in professional sportsmen. Med Sport 2010; 14(3): 108-113.
31. Djordjevic D, Cubrilo D, Zivkovic V, Barudzic N, Vuletic M, Jakovljevic V. Pre-exercise superoxide dismutase activity affects the pro/antioxidant response to acute exercise. Serbian Journal of Experimental and Clinical Research 2010a; 11(4): 147-55.
32. Blum J, Fridovich I. Inactivation of glutathione peroxidase by superoxide radical. Arch Biochem Biophys 1985; 240(2): 500-8.
33. Ajmani RS, Fleg JL, Demehin AA, et al. Oxidative stress and hemorheological changes induced by acute treadmill exercise. Clin Hemorheol Microcirc 2003; 28(1): 29-40.
34. Elokda AS, Shields RK, Nielsen DH. Effects of a maximal graded exercise test on glutathione as a marker of acute oxidative stress. J Cardiopulm Rehabil 2005; 25(4): 215-9.
35. Demirbağ R, Yilmaz R, Güzel S, Celik H, Koçyigit A, Ozcan E. Effects of treadmill exercise test on oxidative/antioxidative parameters and DNA damage. Anadolu Kardiyol Derg 2006; 6(2): 135-40.
36. Seifi-Skishahr F, Siahkohian M, Nakhostin-Roohi B. Influence of aerobic exercise at high and moderate intensities on lipid peroxidation in untrained men. J Sports Med Phys Fitness 2008; 48(4): 515-21.
37. Kłapcińska B, Kempa K, Sobczak A, Sadowska-Krepa E, Jagsz S, Szołtysek I. Evaluation of autoantibodies against oxidized LDL (oLAB) and blood antioxidant status in professional soccer players. Int J Sports Med 2005; 26(1): 71-8.
38. Weber MH, da Rocha RF, Schnorr CE, Schröder R, Moreira JC. Changes in lymphocyte HSP70 levels in women handball players throughout 1 year of training: the role of estrogen levels. J Physiol Biochem 2012; 68(3): 365-75.
39. Akova B, Sürmen-Gür E, Gür H, Dirican M, Sarandöl E, Küçükoglu S. Exercise-induced oxidative stress and muscle performance in healthy women: role of vitamin E supplementation and endogenous oestradiol. Eur J Appl Physiol 2001; 84(1-2): 141-7.
40. Joo MH, Maehata E, Adachi T, Ishida A, Murai F, Mesaki N. The relationship between exercise-induced oxidative stress and the menstrual cycle. Eur J Appl Physiol 2004; 93(1-2): 82-6.
41. Daud DM, Karim AAH, Mohamad N, Hamid NAA, Wan Ngah WZ. Effect of Exercise Intensity on Antioxidant Enzymatic Activities in Sedentary Adults. Malaysian Journal of Biochemistry and Molecular Biology 2006; 13(1): 37-47.

USEFULNESS OF BASE DEFICIT IN THE ASSESSMENT OF SERUM LACTATE LEVELS IN CRITICALLY ILL PATIENTS ON MECHANICAL VENTILATION

Milos Novovic, Filip Zunic, Marija Jankovic and Jasna Jevdjic
Center for Anesthesia and Resuscitation, Clinical center Kragujevac, Kragujevac, Serbia

KORISNOST BAZNOG DEFICITA U PREDIKCIJI VREDNOSTI SERUMSKIH LAKTATA KOD KRITIČNO OBOLELIH BOLESNIKA NA MEHANIČKOJ VENTILACIJI

Miloš Novović, Filip Žunić, Marija Janković i Jasna Jevđić
Centar za anesteziju i resuscitaciju, Klinički centar Kragujevac, Kragujevac, Srbija

Received / Primljen: 21.10.2013. Accepted / Prihvaćen: 24.11.2013.

ABSTRACT

Background/aim: *Acid-base disturbances are common in critically ill patients. Some of the most commonly used markers of metabolic acidosis are base deficit and lactate. The aim of this study was to evaluate the correlation between base deficit and serum lactate and the utility of base deficit in the assessment of serum lactate levels in critically ill patients on mechanical ventilation.*

Methods: *This study was designed as a retrospective, analytical study. We reviewed all arterial gas analyses (base deficit and lactate levels) of patients on mechanical ventilation. The correlation between base deficit and lactate was assessed by calculation of the Pearson correlation coefficient (r) and coefficient of determination (R^2). Receiver operating characteristic (ROC) curves were created for base deficit to detect the presence of hyperlactatemia. The SPSS 12.0 software package (Chicago, Illinois) was used for statistical analyses.*

Results: *One hundred forty-two patients participated in the study: including survivors (n=68) and non-survivors (n=74). The mean value of base deficit was 0.512 ± 6.10 mmol/L, and the mean value of serum lactate was 2.04 ± 2.07 mmol/L. There was no difference in lactate and base deficit values between the groups (p=0.101, p=0.106, respectively). Hyperlactatemia was observed in 44 patients (30.98 %). In ROC curve analysis, the area under the curve for base deficit to detect hyperlactatemia was 0.527.*

Conclusion: *This study indicates that base deficit is not an appropriate marker in the evaluation of real serum lactate values.*

Keywords: *Base deficit, Serum lactate, Critically ill, Metabolic acidosis*

SAŽETAK

Uvod/Cilj: *Acido-bazni poremećaji su uobičajeni kod kritično obolelih pacijenata. Neki od najčešće korišćenih markera metaboličke acidoze su bazni deficit i serumski laktati. Cilj ove studije je bio da se ispita povezanost između baznog deficita i serumskih laktata, kao i korisnost baznog deficita u procenjivanju vrednosti serumskih laktata kod kritično obolelih pacijenata na mehaničkoj ventilaciji.*

Metode: *Studija je dizajnirana kao retrospektivna, analitička studija. Pregledali smo sve arterijske gasne analize pacijenata na mehaničkoj ventilaciji, kojima su određivani bazni deficit i laktati. Korelacija između baznog deficita i laktata procenjivana je pomoću Pearsonovog koeficijenta korelacije (r) i koeficijenta determinacije (R^2). ROC krive su konstruisane za bazni deficit radi procene prisustva hiperlaktatemije. Za statističku obradu podataka korišćen je SPSS 12.0 (Čikago, Ilinois) softverski paket.*

Rezultati: *142 pacijenta, preživeli (n=68) i umrli (n=74). Srednja vrednost baznog deficita bila je 0.512 ± 6.10 mmol/L; srednja vrednost serumskih laktata bila je 2.04 ± 2.07 mmol/L. Nije bilo statistički značajne razlike između grupa za laktate i bazni deficit (p=0.101, p=0.106 redom). Hiperlaktatemija je primećena kod 44 pacijenta (30,98%). ROC površina ispod krive za bazni deficit da otkrije hiperlaktatemiju bila je 0.527.*

Zaključak: *Ova studija ukazuje da bazni deficit nije adekvatan marker u evaluaciji stvarnih vrednosti serumskih laktata.*

Ključne reči: *Bazni deficit, Serumski laktati, Kritično oboleli, Metabolička acidoza*

ABBREVIATIONS:

APACHE II- Acute Physiology And Chronic Health Evaluation;
AUC- Area under the curve;
BD- Base deficit;
ICU- Intensive care unit;
ROC- Receiver operating characteristic

We are grateful to Valentina Rizvić on technical assistance in the preparation of manuscript.

UDK: 616.152.11 / Ser J Exp Clin Res 2013; 14 (4): 169-173
DOI: 10.5937/SJECR4708

Correspondence to:
Milos Novovic, Milesevske partizanske cete 26, 31300 Prijepolje, telephone number: +38162291696, email: milos.novovic@yahoo.com

INTRODUCTION

Acid-base disturbances are common in critically ill patients. Understanding these disorders is of great importance in intensive care medicine. Early identification and correction of acid-base disorders play a major role in the treatment of critically ill patients [1]. Some of the most commonly used markers of metabolic acidosis are base deficit (BD) and lactate [2,3].

Calculation of BD is performedcarried out with a blood gas analyser using measured $PaCO_2$ and pH values as applied to a standard nomogram, and BD represents the number of milliequivalents (mEq) of additional base that must be added to a litre of blood to normalise the pH. An elevated BD represents the presence of unmeasured anions and this is usually taken as a surrogate marker of lactic acidosis [4,5]. BD is a sensible and easy tool to disclose clinically significant metabolic acidosis [6]. Many authors have shown that BD values correlate with the development of multiple organ failure and mortality [7,8,9]. Other authors have shown that BD should be used with caution as a marker of shock and resuscitation of shock [10].

Metabolic parameters such as lactate are minimally invasive measures of systemic oxygen transport. Hypoxia and hypoperfusion in critically ill patients leads to lactic acid production, with consequent lactic acidosis, which is why lactate is often considered as a marker of tissue hypoxia [3,10,19]. Additionally, serum lactate levels may be elevated as a result of inflammation and increased rates of glycolysis caused by stress [3,11]. It has been shown that hyperlactatemia (lactate values greater than 2 mmol/L) was associated with increased mortality in patients with shock, trauma, and sepsis [12,13,14,15,16]. However, many hospitals still have blood gas analysers that analyse only BD and not lactate (18). Considering that the value of BD can be reversed by therapeutic procedures, such as the administration of large amounts of saline solution, BD does not always correlate with serum lactate levels in critically ill patients [10,17].

The aim of this study was to evaluate the correlation between BD and serum lactate and to determine the utility of BD in the assessment of serum lactate levels in critical ill patients on mechanical ventilation.

MATERIAL AND METHODS

This study was designed as a retrospective, analytical study. It involved a subpopulation of critically ill patients on mechanical ventilation that were admitted to the intensive care unit, Clinical Centre Kragujevac, during the period between January 2012 and October 2012. The intensive care unit was equipped with 17 beds, screens for continuous monitoring of vital functions, 10 devices for mechanical ventilation, and one blood gas analyser. The intensive care unit is a polyvalent type and, at the same time, is a primary place in the hospital for providing medical help in the case of polytrauma and especially in the case of neurotrauma after initial treatment in the emergency centere. The study was approved by the ethics committee of the Clinical Centre Kragujevac. Criteria for including patients in the study were the following: patients that needed mechanical ventilation and intensive monitoring of vital parameters (e.g., EKG monitoring, body temperature, and arterial blood pressure). It was necessary that arterial gas analyses and biochemical analyses were performed on the admission date at the intensive care unit. Exclusion criteria were as follows: patients under 18 years of age, patients admitted due to poisoning, and patients diagnosed with cancer.

According to outcomes, patients were divided into two groups: survivors and non-survivors. To establish the mortality rate, all of the patients were monitored during a 28-day period from the moment of admission to the intensive care unit. Demographic data, admission diagnosis, APACHE II score values within first 48 hours of admission (Acute Physiology And Chronic Health Evaluation) and treatment outcome (survivor or non-survivor) were collected from case histories and discharge notes of patients involved in the study. Venous blood was collected through a cannula introduced for therapy application. Different veins in the forearm were used for blood draws. Arterial blood was sampled from the radial artery. Arterial puncture was performed with a syringe and needle (24-26) which were covered with heparin as anticoagulant. All samples were analysed with a gas analyser (GEM Premier 3000). Biochemical parameters were analysed with a biochemical analyser (Ilab 600) in the laboratory of the Clinical Centre Kragujevac.

Sample size was calculated using the Sample Size Correlation Program, based on results obtained from the study performed by Chawla in 2010 [17]. An adequate sample size to detect an assumed correlation of 0.27 with power $1-\beta=0.8$ and error level $\alpha=0.05$ was calculated as 83 patients.

Descriptive and analytical statistical methods were used in the study. The following descriptive methods were used calculation of absolute and relative numbers, central trend measures (arithmetic mean and median), and dispersion measures (standard deviation [SD]). The following analytical methods were used: T-test and Mann-Whitney U test. The correlation between BD and lactate was assessed by calculating the Pearson correlation coefficient (r) and the coefficient of determination (R^2). Receiver operating characteristic (ROC) curves were created for BD to detect the presence of hyperlactatemia. The SPSS 12.0 software package (Chicago, Illinois) was used for statistical analyses.

RESULTS

There were 142 subjects enrolled in the study including (67 men and 75 women). Ninety-one (64.1%) of these patients

N	142
Age (mean, SD)	60.4 ± 16.98
Sex (male)	67 (47.2%)
Reasons for ICU admission:	
Postoperative neurosurgical	33 (23.2%)
Postoperative surgical	53 (37.3%)
Polytrauma	11 (7.7%)
Neurological	13 (9.1%)
Respiratory failure	17 (11.9 %)
Sepsis	10 (7.1 %)
Post-cardiac arrest	5 (3.5 %)
Survivors/Non-survivors	68(47.9%)/74(52.1%)
APACHE II (mean, SD)	16.23 ± 6.44
FiO_2 % (mean, SD)	42.2 ± 12.97
PaO_2 kPa (mean, SD)	1.77 ± 3.85
$PaCO_2$ kPa (mean, SD)	5.98 ± 1.85
Hct (mean, SD)	0.31 ± 0.067
MAP mm Hg (mean, SD)	86.05 ± 34.63
Na^+ mmol/L (mean, SD)	140.27 ± 9.07
K^+ mmol/L (mean, SD)	3.91 ± 0.81
pH (mean, SD)	7.36 ± 0.103
HCO_3 mmol/L(mean, SD)	22.34 ± 5.68
BD mmol/L (mean, SD)	0.512 ± 6.10
Lactates mmol/L	2.04 ± 2.07
Hyperlactatemia n (%)	44 (31%)
Albumin g/l (mean, SD)	26.54 ± 6.19

Table 1. Demographic data and average values
APACHE II- Acute Physiology And Chronic Health Evaluation; FiO_2- Fraction of inspired oxygen; PaO_2- partial pressure of oxygen; $PaCO_2$- partial pressure of carbon-dioxide; Hct- hematocrit; MAP- mean arterial pressure; BD- Base deficit; HCO_3- Bicarbonate; SD- Standard deviation.

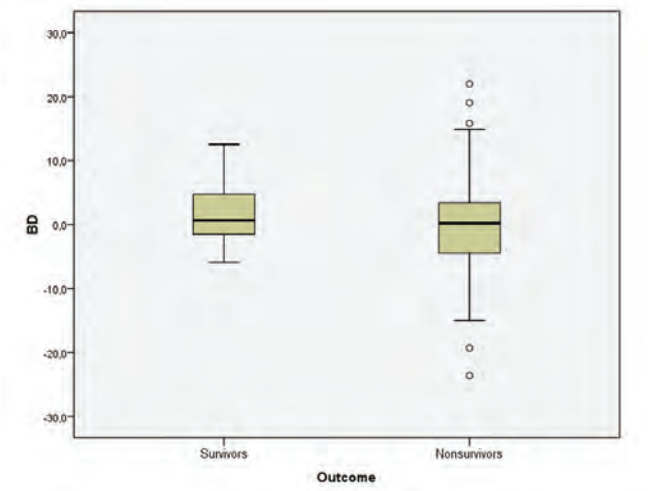


Figure 1. Boxplot of base deficit (BD) levels in the survivor and non-survivor groups.

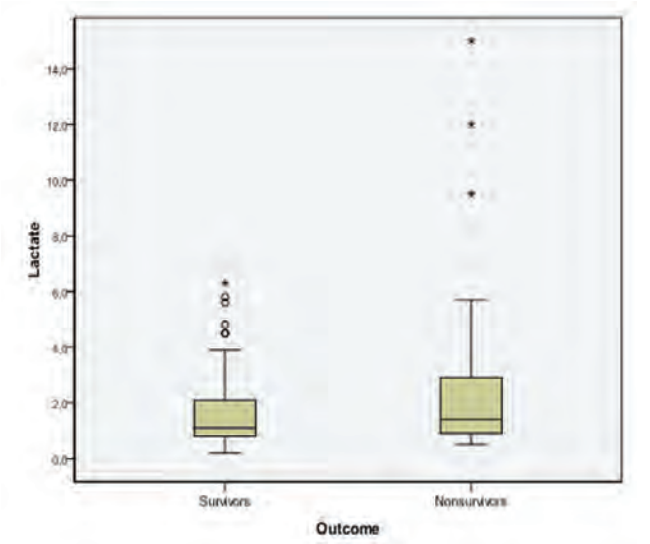


Figure 2. Boxplot of lactate levels in the survivor and non-survivor groups.

were admitted to the ICU after surgery, and 51 (35.9%) patients were admitted with other urgent medical conditions. The APACHE II score on admission to the intensive care unit for the entire sample was 16.23 ± 6.44. There was a statistically significant difference in APACHE II scores (t=5.802; p<0.001) between the groups. Detailed information about average values and demographic data are given in Table 1.

The mean BD value was 0.512 ± 6.10, and the mean serum lactate value was 2.04 ± 2.07. There was no difference in lactate levels (U=2115.5; Z=-1.638; p=0.101) and BD values (U=2120; Z=-1.617; p=0.106) between the groups. The mean BD values and lactate levels in the survivor and non-survivor groups are shown in Figures 1 and 2. Of the 142 patients that were included in the study, hyperlactatemia was observed in

		BD	Lactate	pH	HCO3
BD	Pearson Correlation Sig. (2-tailed)		0.053 0.532	0.188* 0.025	0.442** 0.000
Lactate	Pearson Correlation Sig. (2-tailed)	0.053 0.532		-0.518** 0.000	-0.396** 0.000
pH	Pearson Correlation Sig. (2-tailed)	0.188* 0.025	-0.518** 0.000		0.549** 0.000
HCO_3	Pearson Correlation Sig. (2-tailed)	0.442** 0.000	-0.396** 0.000	0.549** 0.000	

Table 2. Correlations
BD- base deficit; HCO3- bicarbonate; *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed).

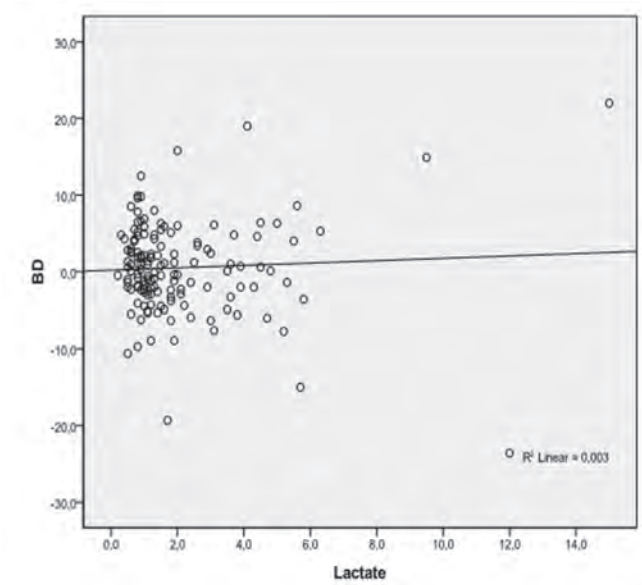


Figure 3. Scatterplot of base deficit (BD) vs lactate level

44 patients (30.98 %). The results of Pearson's correlation between studied variables are presented in Table 2. The correlation of BD compared with lactate is shown in Figure 3. In the ROC curve analysis, the area under the curve for BD to detect hyperlactatemia was 0.527 (Figure 4).

DISCUSSION

This study explored the correlation between the two most commonly used markers of acid-base disturbances in critically ill patients: the value value of BD and serum lactate. Metabolic acidosis remains one of the most common acid-base disorders and often marks the beginning of tissue hypoxia and organ hypoperfusion [6, 7, 10, 18]. Serum lactate levels have been used to detect the presence of tissue hypoperfusion and as a prognosticating marker in different subgroups of patients [3, 8, 10, 12, 13].

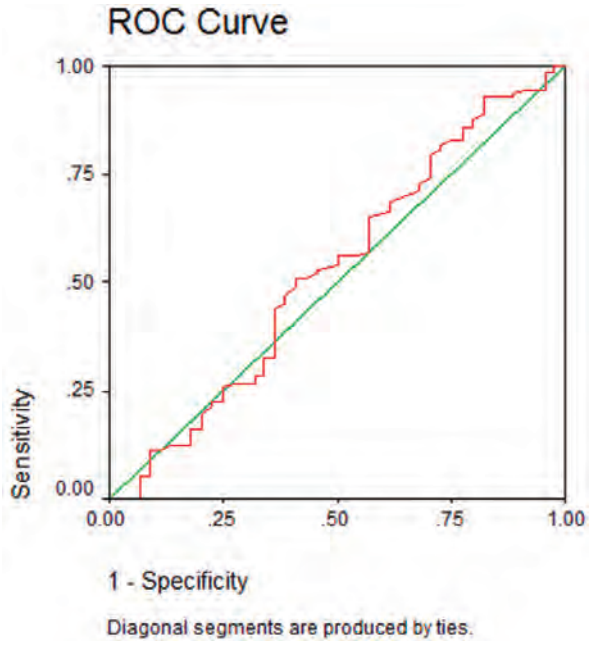


Figure 4. Receiver operating characteristic (ROC) curve for the prediction of hyperlactatemia by the base deficit (BD) level. Area under curve (AUC)=0.527.

In the present study, the incidence of hyperlactatemia on admission to the intensive care unit was 30.98%, but there were no statistically significant differences in lactate and BD levels between the survivor and non-survivor groups (p>0.01). In a population of patients that were admitted to a surgical intensive care unit, Matthew et al. showed that lactic acidosis was present in 41% of the patients. In their study, non-survivors had higher lactate and BD values than survivors. They also showed that an increased BD level had no predictive value if the lactate level was normal [19]. In the study conducted by Chawla et al., in the group of patients undergoing general anaesthesia, hyperlactatemia was observed in 40% of the patients. It was shown that the use of BD can often mislead the clinician as to the actual serum lactate concentration [17].

However, we found that the ROC area under the curve for BD in predicting hyperlactatemia was 0.527, which implies it is a very weak tool as a substitute for serum lactate. These results support the results of other similar studies in which the BD was evaluated as a surrogate marker for hyperlactatemia in critically ill patients [3, 6, 17, 19]. It should be mentioned that this study is the first focused on critically ill patients on mechanical ventilation exclusively. Some studies have shown that, despite a normal BD and lack of acidosis, significant hyperlactatemia and dangerous hypoperfusion states can exist. This was explained by the variety of mechanisms underlying hyperlactataemia in critically ill patients [12, 18, 20].

Our study had certain limitations. We did not have accurate data about types and amount of fluids that were used for resuscitation before admission to the ICU. Additionally, the scope of different diagnoses in this study was very heterogeneous. We suggest that future studies dealing with this phenomenon should focus on patients with a clearly defined diagnosis (e.g., surgical, neurosurgical, neurological or internist-determined) and precisely identified type and amount of fluids that were used in resuscitation efforts.

In conclusion, we demonstrated that hyperlactatemia is a common finding in critically ill patients on mechanical ventilation. The aim of any therapy is restitution of global tissue hypoxia. A decrease in lactate values to normal levels allows this restitution. As shown in this study, the use of BD instead of serum lactate is not desirable. This study indicates that BD is not appropriate in the evaluation of real values of serum lactate.

REFERENCES

1. Wilson RF, Sibbald WJ. Approach to acid-base problems in the critically ill and injured. J Am Coll Emerg Phys 1976;5:515–22.

2. Siggaard-Andersen O: The van Slyke equation. ScandJ ClinLab Invest Suppl 1977; 37:15-20.
3. Aduen J, Bernstein WK, Miller J, et al. Relationship between blood lactate concentrations and ionized calcium, glucose, and acid-base status in critically ill and noncritically ill patients. Crit Care Med 1995;23:246 –52.
4. Balasubramanyan N, Havens PL, Hoffman GM. Unmeasured anions identified by the Fencl-Stewart method predict mortality better than base excess, anion gap, and lactate in patients in the pediatric intensive care unit. Crit Care Med 1999;27:1577– 81.
5. Fencl V, Jabor A, Kazda A, Figge J. Diagnosis of metabolic acid-base disturbances in critically ill patients. Am J Respir Crit Care Med 2000;162:2246 –51.
6. Park M, Taniguchi LU, Noritomi DT, Libório AB, Maciel AT, Cruz-Neto LM. Clinical utility of standard base excess in the diagnosis and interpretation of metabolic acidosis in critically ill patients. Braz J Med Biol Res. 2008;41:241-9.
7. Maciel AT, Park M. Differences in acid-base behavior between intensive care unit survivors and nonsurvivors using both a physicochemical and a standard base excess approach: a prospective, observational study. J Crit Care. 2009;24:477-83.
8. Ouellet JF, Roberts DJ, Tiruta C, Kirkpatrick AW, Mercado M, Trottier V, Dixon E, Feliciano DV, Ball CG. Admission base deficit and lactate levels in Canadian patients with blunt trauma: are they useful markers of mortality? J Trauma Acute Care Surg. 2012;72:1532-5.
9. Sauaia A, Moore FA, Moore EE, et al. Early predictors of postinjury multiple organ failure. Arch Surg 1994;129:39–45.
10. Husain F, Martin M, Mullenix P, Steele S, Elliott D. Serum lactate and base deficit as predictors of mortality and morbidity. *Am J Surg*. 2003;185:485-491.
11. Gutierrez G, Wulf ME. Lactic acidosis in sepsis: another commentary. Crit Care Med. 2005;33:2420-2422.
12. Juneja D, Singh O, Dang R: Admission hyperlactatemia: causes, incidence, and impact on outcome of patients admitted in a general medical intensive care unit. J Crit Care. 2011;26:31-20.
13. Fuller BM, Dellinger RP. Lactate as a hemodynamic marker in the critically ill. Curr Opin Crit Care. 2012 Jun;18(3):267-72.
14. Trzeciak S, Dellinger RP, Chansky ME, et al. Serum lactate as a predictor of mortality in patients with infection. Intensive Care Med 2007; 33:970–977.
15. Mikkelsen ME, Miltiades AN, Gaieski DF, et al. Serum lactate is associated with mortality in severe sepsis independent of organ failure and shock. Crit Care Med 2009; 37:1670–1677.
16. Fall PJ, Szerlip HM. Lactic acidosis: from sour milk to septic shock. J Intensive Care Med 2005; 20:255–271.
17. Chawla LS, Nader A, Nelson T, Govindji T, Wilson R, Szlyk S, Nguyen A, Junker C, Seneff MG. Utilization of base deficit and reliability of base deficit as a surrogate for serum lactate in the peri-operative setting. BMC Anesthesiol. 2010;10:16.
18. Smith I, Kumar P, Molloy S, Rhodes A, Newman PJ, Grounds RM, Bennett ED. Base excess and lactate as prognostic indicators for patients admitted to intensive care. Intensive Care Med. 2001;27:74-83.
19. Martin MJ, FitzSullivan E, Salim A, Brown CV, Demetriades D, Long W. Discordance between lactate and base deficit in the surgical intensive care unit: which one do you trust? Am J Surg. 2006;191:625-30.
20. Mikulashek A, Henry SM, Donovan R, et al. Serum lactate is not predicted by anion gap or base excess after trauma resuscitation. J Trauma 1996;40:218–22.

CONGENITAL HEPATIC FIBROSIS

Zorica Raskovic, Biljana Vuletic, Zoran Igrutinovic, Slavica Markovic
Clinical Centre Kragujevac, Pediatric Clinic

KONGENITALNA FIBROZA JETRE

Zorica Rasković, Biljana Vuletić, Zoran Igrutinović, Slavica Marković
Klinički Centar Kragujevac, Pedijatrijska klinika

Received / Primljen: 19.08.2013. Accepted / Prihvaćen: 06.11.2013.

ABSTRACT

Congenital hepatic fibrosis (CHF) is a rare developmental disorder of the portobiliary system and most commonly associated with polycystic kidney disease. The pattern of inheritance of this disorder is autosomal recessive. The eexact prevalence of CHF is unknown (estimated from 1:10000 to 1:20000). Sequelae of CHF and portal hypertension have been found in less than half of the all CHF patients and were associated with age.

We present a case study of a boy with CHF complicated by portal hypertension, splenomegaly and hypersplenism. This patient was diagnosed with cholestatic syndrome as a neonate. A transcutaneous liver biopsy was performed and repeated at the age of 9 months. Diagnosis of cholestatic syndrome was made based on the findings of a histopathological examination. The ultrasound examination showed polycystic kidneys; however, global renal function remained normal. At the age of 8 years and 6 months, portal hypertension was confirmed by Doppler ultrasonography, and endoscopic examination revealed oesophageal varices of second and third grade, which was also observed in the splenic portography. Thrombocytopenia due to hypersplenism was identified by a platelet count of 75.2 x 10^3. To prevent variceal bleeding, a a splenorenal shunt and a partial spleen resection were performed. The differential of cholestatic syndrome in infants should include CHF. This type of disease may suggest early developing complications of CHF, such as portal hypertension and hypersplenism. Portosystemic shunt surgical treatment is justified in CHF cases with cholestatic syndrome .

Keywords: *portal hypertension, hypersplenism, splenorenal shunt*

SAŽETAK

Kongenitalna fibroza jetre (KFJ) je redak autozomno-recesivni poremećaj razvoja portobilijarnog sistema često udružen sa policističnim promenama na bubregu. Tačna prevalenca poremećaja nije poznata (procenjuje se na 1: 10 000 do 1: 20 000). Sekvele KFJ i portne hipertenzije se razvijaju u manje od polovine bolesnika tokom vremena.

Prikazujemo dečaka sa KFJ komplikovanom portnom hipertenzijom, splenomegalijom i hipersplenizmom. Bolest je prepoznata pod slikom holestaznog sindroma, a dijagnoza bazirana na karakterističnom patohistološkom nalazu pri pregledu tkiva jetre dobijenog perkutanom biopsijom u devetom mesecu po rodjenju. Pored toga, ultrasonografskim pregledom nadjene su i policistične promene na bubrezima. Globalna renalna funkcija je bila normalna. Dijagnoza portne hipertenzije je potvrdjena Doppler ultrasonografijom, splenoportografijom i endoskopskim nalazom varikoziteta drugog i trećeg stepena u području kardije a hiperspenizma na osnovu trombocitopenije (Tr 75,2 x 10^9). U cilju prevencije krvarenja učinjen je splenorenalni šansa parcijalnom splenektomijom. U diferencijalnoj dijagnozi holestaznog sindroma u dojenačkoj dobi treba razmotriti i KFJ. Ovakav oblik bolesti može karakterisati rana pojava portne hipertenzije i hipersplenizma kao komplikacije. Hirurško lečenje izvođenjem portosistemskog šanta opravdano je u ovim slučajevima.

Ključne reči: *portna hipertenzija, hipersplenizam, splenorenalni šant*

ABBREVIATIONS:

CHF - congenital hepatic fibrosis **KFJ** - kongenitalna fibroza jetre

UDK: 616.36-002.17-053.2 / Ser J Exp Clin Res 2013; 14 (4): 175-179
DOI: 10.5937/SJECR4339

Correspondence: Dr Zorica Raskovic; At work: Pediatric clinic, Clinical Centre" Kragujevac", Zmaj Jovina 30, 34000 Kragujevac, Serbia
At home: Lepenički bulevar 11/19, 34000 Kragujevac, Serbia
Tel. at clinic +38134 505174, +38134505175, Fax: +38134370213, Mob. Tel. +381643173246, E-mail: zorica.raskovic0@gmail.com

INTRODUCTION

Congenital hepatic fibrosis (CHF) is a rare, inherited, autosomal-recessive disease that is characterised by periportal fibrosis with irregularly shaped biliary duct proliferation resulting in intrahepatic portal hypertension and oesophageal varices. The disease is sometimes associated with impaired renal function. Hepatic manifestations of the disease were first described in 1856, and the term "congenital liver fibrosis" was adopted in 1961 [1]. The literature describes a small number of cases; therefore, the incidence and prevalence of CHF are unknown (estimated from 1:10000 to 1:20000) [2]. One quarter of newborns and young infants born with predominant renal disease die [1]. Mortality is attributed to cholangitis [1]. Symptoms of the disease include renal and/or hepatic manifestations. When renal manifestations are dominant, patients develop renal failure that is often fatal. If the lesions of the liver are dominant, the disease manifests as portal hypertension, gastrointestinal bleeding of different intensity and splenomegaly with more or less severe signs of hypersplenism. Haemodynamic consequences of impaired blood flow in the portal vein system include oesophageal varices and varices of the stomach veins proximal to the cardia.

CASE REPORT

A boy, eight years and 6 months of age with a body height 130 cm (P25) and weight of 27.2 kg (-0.5 kg, - 1.9%), presented with icteric discoloration of the skin and visible mucous membranes and few telangiectatic changes on the face. The sharp, low edge of his liver was palpable approximately 2 cm below the right rib cage and was enormously enlarged. The patient's spleen was hard and palpable up to 7 cm below the left rib cage. Other physical findings were normal.

Personal anamnesis showed the patient was the first child from a normal, controlled pregnancy, with a birth weight of 3750 g, length of 56 cm and an APGAR score of 10. The patient was regularly vaccinated and fed adapted milk formula from birth. Due to the unclear etiology of conjugated hyperbilirubinemia in the neonatal age, a percutaneous liver biopsy was performed. Histopathological examination showed gigantocellular transformation of hepatocytes and an initial fibrous process with signs of centrilobular cholestasis. Echosonography findings of the extrahepatic biliary tract were normal, while left kidney cystic changes were noted. A percutaneous liver biopsy was repeated at the age of 9 months because of persistent cholestasis. Histopathological examination of the percutaneous liver biopsy confirmed diffuse fibrosis with proliferation of irregular and branched bile capillaries (ductal plate malformation). Abdominal ultrasound and CT examination showed an easily enlarged liver and hyperechogenic parenchyma with periodic beaches of normal tissue transonicity. Gallbladder and bile ducts appeared normal.

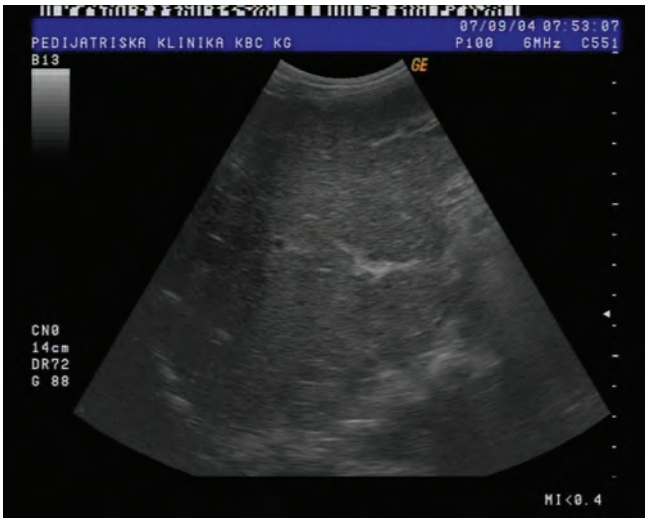


Figure 1. Ultrasound of irregular hepatic structure.

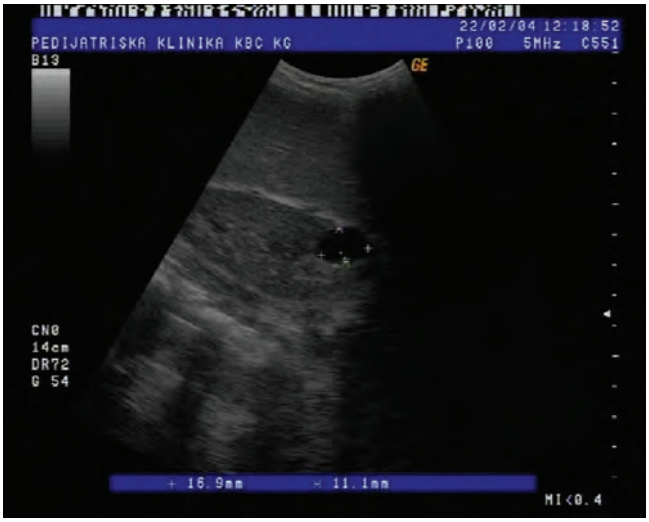


Figure 2. Ultrasound of the left kidney with cysts and large spleen.

Cystic formation was observed in the lower third of the left kidney. Renal function was preserved. In accordance with these findings, and based on the clinical course of the disease, the diagnosis of congenital liver fibrosis was made.

At the time of this visit, laboratory testing found normal levels of ESR and other inflammation markers, normal morphology of red blood cells, a reduced number of platelets ($75.2x10^3$/ml), proper homeostasis, normal renal function and preserved synthetic and homeostatic liver function. Hepatic lesions were associated with permanent cholestasis confirmed by biochemical markers, including increased levels of liver enzymes AST 204 u/l, ALT 262 u/l, ɣGT 513 u/l and alkaline phosphatase 171.1 u/l and a total bilirubin count of 66 mol/l with a conjugated component of 41mol/l. Based on these findings proximal endoscopy was performed. We found numerous oesophageal varices in the distal third of the oesophagus and cardia inconsistent with signs of reflux esophagitis. Abdominal ultrasound identified an enlarged liver with a pronounced periportal binder and ligaments in echo structure (Figure 1).

Biliary ponds up to 100 mm in the form of cysts were observed peripherally in the liver, and Doppler sonography found the portal vein trunk diameter to be 9.5 mm with a slower hepatofugal blood flow of 10-12 mm/s. The spleen vein was also modified (trunk diameter 8 mm) with multiple tortuous collaterals in the region of the cardia and slower blood flow up to 12 mm/s. The spleen longitudinal diameter was approximately 16 cm (the lower edge reaching almost to the iliac bone). The kidneys were in the correct position without dilatation of the pyelocaliceal system and with cysts of 16.9 x 11.1 mm in diameter on the upper pole of the right kidney (Figure 2).

Splenic portography was proposed and confirmed an enlarged spleen and portal vein and displayed retrograde coronary veins with second and third degree varicose changes (Figure 3).

To relieve difficulties in blood flow caused by portal vein hypertension and to reduce the risk of bleeding from oesophageal varicose veins, a splenorenal shunt with partial splenectomy was performed (using the method of Warren (T-L)). The patient was haemodynamically and haemostatic stable. The postoperative course was uneventful. Doppler ultrasonic diagnostic method confirmed normal functioning of the shunt. Two weeks after surgery, platelets showed a significant increase of 107 x 10^9/ml, while the laboratory findings were without significant changes compared with previous findings. Throughout the course of the disease, the function of the kidney was preserved. The child was discharged from the hospital in good condition.

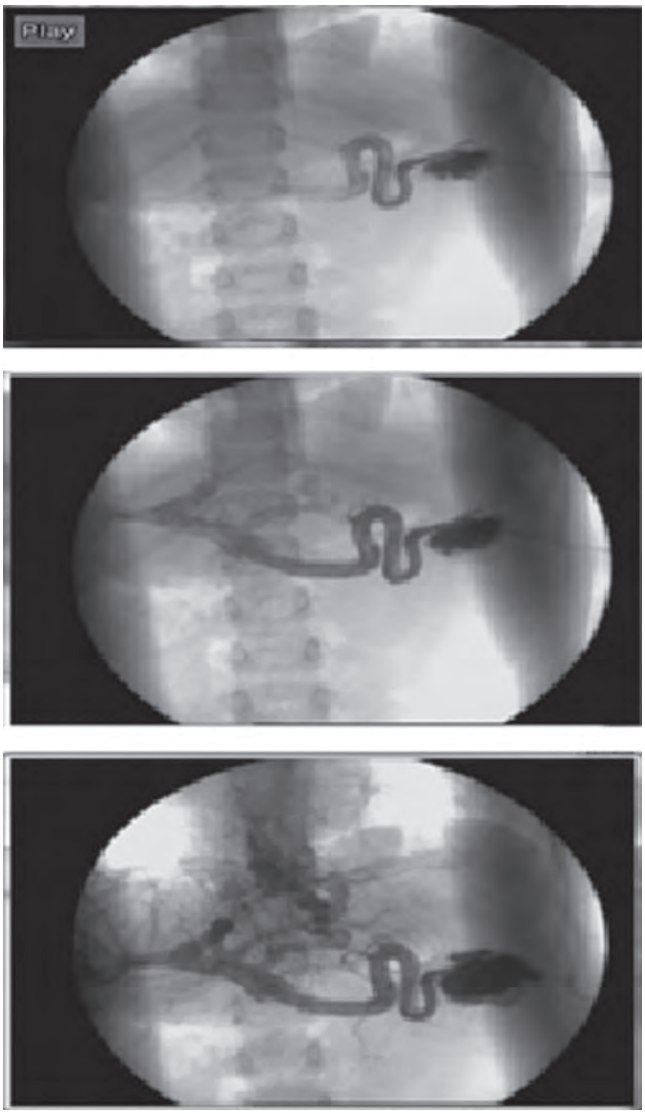


Figure 3. Splenic portography revealed second and third grade oesophageal varices and third grade.

DISCUSSION

Symptoms of congenital liver fibrosis can be manifested in the early infant period, later in childhood and in adolescence. Similarity with other diseases can lead to a delayed or incorrect diagnosis of CLF congenital liver fibrosis. In the initial stage of the disease, a liver biopsy showing diffuse periportal fibrosis and the presence of less than or greater dilation of bile ducts thick bands of fibrous connective tissue are indicative of indicate CLFcongenital liver fibrosis. Although histopathological findings are the gold standard for diagnosis, abnormal hepatic echogenicity and splenomegaly cannot be detected at an early age because the portal fibrosis and portal hypertension develop and progress with age. Hepatomegaly is present in almost all patients presenting with CLFcongenital liver fibrosis[3]. Upon palpation of the liver, the organ is hard; its surface rough and nodular. Ultrasonography greatly aids in diagnosis; finding that echogenicity of the liver tissue is changed, or cystic formation is visible with or without changes in the parenchyma.

Sonographic evaluation includes the Doppler method, with a focus on portal circulation and portal hypertension. With the progression of portal hypertension, the spleen increases and the platelet count decreases due to hypersplenism, developing portosystemic collateral circulation and creating oesophageal and gastric varices. The risk of bleeding increases with the increase in varice size [4, 5, 6]. Variceal bleeding can occur at any age, but is more common in older children and adults. Consequently, endoscopy is indicated in all patients with CHF, especially in those with baseline anaemia and hematemesis. Endoscopic findings confirm the existence of varices, erosions and ulcerations, and facilitate sclerotherapy. Jung and Brancateli use magnetic resonance, i.e., MRCP or magnetic cholecystopancreatography, in the diagnosis of congenital fibrosis of the liver with typical findings of cystic and fusiform dilatations of an irregular intrahepatic biliary duct, an abnormally enlarged liver with dilatation of the extrahepatic bile ducts, gall bladder enlargement, and a markedly enlarged spleen with fibrocystic changes in the kidney [7, 8]. Because renal disease is often associated with CHF, many patients are subjected to a renal evaluation. Fonk et albelieve believe that in patients with primary renal failure, liver biopsy is not necessary; therefore, and that diagnosis

can be based entirely on clinical findings [6]. Congenital fibrosis of the liver often causes great differential diagnostic dilemmas. Confusion often causes suspicion of cirrhosis of the liver because it is present in CHF due to extensive hepatic fibrosis and portal hypertension. Non-cirrhotic portal hypertension is more difficult to distinguish from CHF compared with cirrhotic hypertension. Proper diagnosis is based on medical history, clinical findings, laboratory tests and imaging techniques. The most essential difference between CHF and non-cirrhotic portal hypertension is preserved synthetic liver function [9]. Diseases of the bile ducts (primary biliary cirrhosis and primary sclerosing cholangitis) that evolve to liver cirrhosis are extremely rare in children. Both are followed by the significant increase of liver enzymes, alkaline phosphatase and yGT, whichthat are significantly higher compared with those found in CHF patients and do not have the same visualisation techniques for extra testing [10]. Cystic changes in the liver and in primary biliary cirrhosis antimitochondrial antibodies were not found in any of these diseases. The similarity of CHF with primary sclerosing cholangitis is a phenomenon ascending cholangitis, and sometimes biliary strictures and dilatation, which is seen in primary sclerosing cholangitis, may be misinterpreted and represent the dilated extrahepatic bile ducts or even intrahepatic cysts in CHF. Other causes of cirrhosis, such as viral hepatitis, autoimmune hepatitis, alpha 1 antitrypsin deficiency and Wilson's disease are excluded on the basis of history and laboratory tests. Special attention in the examination of patients may identify liver cysts, which can create further differential diagnostic dilemmas. If liver cysts are observed in autosomal dominant polycystic liver disease, the cysts can lead to portal hypertension but are not typical of CHF, as they vary across a range of time and occupy a substantial part of the liver parenchyma [11,12]. Macroscopic cysts of the liver, in continuity with the biliary ducts, are a typical and very common finding among patients with CHF and are indicative of Caroli's Syndrome. Almost all rare cases of isolated forms of CHF occur without Caroli's Syndrome . The genes responsible for these isolated forms of CFH are unknown. The results of genetic engineering have shown that most infants with the severe perinatal form of diseaseed CHF associated with Caroli's Syndrome have two mutations in the PKHD1 gene, and the majority of survivors in the neonatal period have at least one mild mutation [13]. This syndromic phenomenon certainly should be distinguished from congenital liver disease (Caroli's Syndrome), which that is characterised by cystic dilatation of only the intrahepatic bile ducts[14]. Congenital hepatic fibrosis is a disease for which there is no specific therapy to repair the primary ductal plate malformation or to recover fibrotic changes and abnormalities of the biliary tree. Pharmacological treatment with antibiotics is indicated only in cases of cholangitis. Portal hypertension and oesophageal varices require a special type of treatment. Primary prevention (before the onset of bleeding) includes giving non-selective beta-blockers, in accordance with the measured pulse and blood pressure. Secondary prevention of bleeding varices (in case the bleeding has already happened) requires bending ligation and sclerotherapy. A portosystemic shunt surgical intervention has the least side effects and contraindications [15,16]. Alvares et al describe a population of 27 children with CFL in which a portosystemic shunt was performed in 16 patients aged 3-16 years [17]. During a 3 month to 12 year follow-up, liver functioning did not worsen, and signs of hepatic encephalopathy were absent. Prognosis is,; in fact, considerably better in the infantile form of the disease [18]. Consideration of the need for surgery (portal system shunt) in a patient who has never had bleeding from varices may be reasonable if the portal hypertension gets progressively weaker and threatens the liver synthetic function. The use of a surgical shunt may also be considered for patients who did not have episodes of bleeding when there is no possibility of emergency response and safe, secure care if bleeding occurs. Our patient had never bled from oesophageal varices, although they were third degree. In line with potential risk, and to prevent serious complications, the splenorenal shunt method was selected with a minimum of contraindications. Therefore, the increase in the degree of varices was prevented, and the possibility of bleeding was reduced to a minimum. Further monitoring of the patient involved the analysis of synthetic, homeostatic and haemostatic liver function, cholestasis and renal function control as well as oesophageal varices condition.

ACKNOWLEDGMENTS

The authors would like to thank Prof Dr Nedeljko Radlovic for significant assistance. The authors would also like to thank the University Children's' Hospital of Belgrade surgical team, including Prof Dr Zoran Krstic, Ass Dr Aleksandar Sretenovic, and the radiologists Prof. Ddr Dragan Sagic, Dr Zeljko Smoljanic, and the clinicians from the Institute for Mother and Child's Health in Belgrade who performed the initial liver biopsy.

REFFERENCES

1. Nazer H.Nazer, D.Congenital Hepatic Fibrosis. Available on http//www.eMedicine.com/ped/topic459.htm, taken 23.01.2010.
2. Meral Gunaz-Aygin, William A. Gahi, TheoHeller. Congenital Hepatic Fibrosis Overview. Gene Reviews; 2008. Available on : http//www.ncbi.nlm.nih.gov./bookself/book=gene&part=hepatic-fibrosis, taken 27.01.2010.
3. Vuletic B, Radlovic N, Lekovic Z. ,Sretenovic A, Krstic Z, Smoljanic Z, Raskovic Z. Congenital Fibrosis of the Liver. Pediatric days in Serbia and Montenegro; September 2004. ; Nis;172-177 (in Serbian)
4. Kerr DN, Okonkwo S,Choa RG.Congenital hepatic fibrosis: the long-term prognosis. Gut 1978;19 :514-20.

5. Summerfield JA, Nagafuchi Y, SherlocksS, CanafalchJ, ScheuerPJ. Hepatobiliary fibropolycystic disease: a clinical and histological review of 51 patient. J Hepatol. 1986;2:141-156.
6. FonkC, Chauveau D, Gagnadoux MF, PirsonY. Grunfield JP. Autosomal recessive polycystic kidney disease in adulthood. Nephrol Dial Transplant. 2001;16:1648-52.
7. Jung G, Benz-Bohm G, Kuge IH, Keller KM, Querfield U. MR cholangiography in children with autosomal recessive polycystic kidney disease. PediatrRadiol. 1999;29:463-6.
8. BrancatelliG, FederieMP, VilgrainV, Vullierme MP, MarinD, LagallaR. Fibropolycystic liver disease; CT and MR imaging findings. Radiographics. 2005;25:659-70.
9. Sarin SK, Kumar A.Noncirrhotic portal hypertension. Clin Liver Dis. 2006; 10:627-51.
10. KumagIT, AlswatK, HirchfieldGM, HeatcholetJ.New insights into autoimmune liver diseases. Hepatol Res. 2008;38:745-61.
11. DemetrioTamiolakis, Panagiotis Prassopoulos, Athanasia Kotini, Kiriaki Avgidou Constatine Simopoulos and Nikola Papadopulos.Intrahepaticcystic disease with congenital fibrosis (Caroli's complex disease). A case report and review of the literature. Acta Clin Croat. 2003;42:233-236.
12. DrenthJP, MartinaJA, Van de Kerkhof R, BonifacinoJS, JansenJB. Polycystic liver disease is a disorder of contraslational protein processing. Trends Mol Med. 2005;11:37-42.
13. Bergmann C, Flegauf M, Bruchie NO, Frank V, Olbrich H, Kirchner J et al.Loss of nephrocystin -3 function can cause embryonic lethality, Meckel-Gruber like syndrome, situs inversus, and renal-hepatic-pancreatic dysplasia. J Am Soc Nephrol. 2003;14:76-89.
14. Caroli J. Diseases of the intrahepatic biliary tree. Clin Gastroenterol. 1973;2:147-161.
15. Bosh J, Berzigotti A, Garcia- Pagan Abraldes JG. The management of portal hypertension :rational basis, available treatments and further options. J Hepatol. 2008;48:S68-9.
16. Schneider BL, Magid MS. Liver diseases in autosomal recessive polycystic kidney disease. Pediatr. Transplant 2005;9:634-9.
17. Alvarez F.Bernard O, Brunelle R et al. Congenital hepatic fibrosis in children. J Pediatr. 1981;99:370.
18. Riviero MJ, Roman E, Cilleruel ML, Sanchez E.Barrio. Caroli's syndrome. Report of case beginning in childhood with favorable course. An Esp Pediatr. 2000;53:59-61JC.

SEPSIS AND CARDIORENAL SYNDROME: ETIOPATHOGENESIS, DIAGNOSIS AND TREATMENT

Dejan Petrovic[1], Željko Mijailovic[2], Biljana Popovska[2], Vladimir Miloradović3, Predrag Djurdjević4
[1]Clinic for Urology and Nephrology, Clinical Center Kragujevac, Kragujevac
[2]Infectious Diseases Clinic, Clinical Center Kragujevac, Kragujevac
[3]Clinic for Cardiology, Clinical Center Kragujevac, Kragujevac
[4]Clinic for Haematology, Clinical Center Kragujevac, Kragujevac, Serbia

SEPSA I KARDIO-RENALNI SINDROM: ETIOPATOGENEZA, DIJAGNOSTIKA I LEČENJE

Dejan Petrović[1], Željko Mijailović[2], Biljana Popovska[2], Vladimir Miloradović[3], Predrag Đurđević[4]
[1]Centar za urologiju i nefrologiju, Klinika za urologiju i nefrologiju, KC Kragujevac
[2]Klinika za infektivne bolesti, KC Kragujevac, Kragujevac
[3]Klinika za kardiologiju, KC Kragujevac, Kragujevac
[4]Klinika za hematologiju, KC Kragujevac, Kragujevac, Srbija

Received / Primljen: 24.08.2013. Accepted / Prihvaćen: 12.09.2013.

ABSTRACT

Introduction: *Sepsis is the most common cause of acute renal failure in intensive care units.*

Aim: *This study aimed to analyse the etiopathogenesis of sepsis and the clinical significance of early detection and timely treatment of sepsis in intensive care units.*

Method: *We analysed literature and clinical studies addressing the pathogenesis, diagnosis and treatment of sepsis syndrome.*

Results: *There was a 1.5% increase in the number of patients with sepsis over one year. Severe sepsis is defined as sepsis with hypotension, hypoperfusion and organ dysfunction. Sepsis is characterised by activation of the patient's immune system and enhancement of the creation of mediators that play an important role in the development of multiple organ system failure in patients with sepsis. The strategy for preventing acute renal failure in patients with sepsis includes early targeted therapy (in the first 6 hours), which consists of an early increase in blood volume circulating fluids (at least 20 ml/kg crystalloid in the first hour). Initial therapy should be achieved by central venous pressure of 8-12 mmHg, mean arterial blood pressure greater than 65 mmHg, urine output greater than 0.5 ml/kg/h and mixed venous blood saturation of oxygen greater than 70%. The ventilation strategy to protect the lungs and kidneys in patients on mechanical ventilation includes a tidal volume of 6 ml/kg and an end-inspiratory pressure plateau less than 30 cmH_2O. To remove a mediator from the serum of patients, high-volume haemofiltration and continuous haemodiafiltration with PMMA are used.*

Conclusion: *Early follow-up and early implementation of targeted therapies play a key role in preventing the development of acute heart and kidney damage.*

Key words: *sepsis, cardio-renal syndrome, dialysis therapy*

SAŽETAK

Uvod. *Sepsa je najčešći uzrok akutnog oštećenja bubrega u jedinicama intenzivnog lečenja.*

Cilj. *Rad je imao za cilj da analizira etiopatogenezu sepse i klinički značaj ranog otkrivanja i pravovremenog lečenja sepse kod bolesnika u jedinicama intenzivnog lečenja.*

Metodologija. *Analizirani su stručni radovi i kliničke studije koje se bave etiopatogenezom, dijagnostikovanjem i lečenjem sindroma sepse.*

Rezultati. *Jednogodišnja stopa porasta broja bolesnika sa sepsom iznosi 1.5%. Teška sepsa se definiše kao sepsa sa hipotenzijom, hipoperfuzijom i poremećajem funkcije organa. Sepsa se odlikuje aktivacijom imunskog sistema bolesnika i pojačanim stvaranjem pro- i antiinflamatornih medijatora. Pojačan i neregulisan odgovor imunskog sistema i pojačano stvaranje medijatora imaju značajnu ulogu u razvoju insuficijencije više sistema organa kod bolesnika sa sepsom. Strategija za prevenciju razvoja akutnog oštećenja bubrega kod bolesnika sa sepsom uključuje ranu ciljnu terapiju (u prvih 6 sati), koja se sastoji u ranoj pojačanoj nadoknadi zapremine krvi u cirkulaciji tečnostima (najmanje 20 ml/kg kristaloida u prvom satu). Početnom terapijom treba da se postigne centralni venski pritisak od 8-12 mmHg, srednji arterijski krvni pritisak veći od 65 mmHg, diureza veća od 0.5 ml/kg/h i zasićenost centralne venske krvi kiseonikom veća od 70%. Strategija ventilacije za zaštitu pluća i bubrega kod bolesnika na mehaničkoj ventilaciji uključuje tajdl volumen od 6 ml/kg i pritisak end-inspiratornog platoa manji od 30 cmH_2O. Za odstranjivanje medijatora iz seruma bolesnika koriste se visoko-volumenska hemofiltracija i kontinuirana hemodijafiltracija sa PMMA membranom.*

Zaključak. *Rano praćenje bolesnika i primena rane ciljne terapije imaju ključnu ulogu u sprečavanju razvoja akutnog oštećenja srca i bubrega.*

Ključne reči: *sepsa, kardio-renalni sindrom, dijalizna terapija*

UDK: 616.94 / Ser J Exp Clin Res 2013; 14 (4): 181-187
DOI: 10.5937/SJECR4363

Correspondence: Assist. Prof. Dejan Petrović, MD, PhD,
Clinic for Urology and Nephrology, Clinical Center Kragujevac, ZmajJovina 30, 34000 Kragujevac

INTRODUCTION

Cardiorenal syndrome is a pathophysiological disorder of the heart and kidneys, where acute or chronic dysfunction of one organ stimulates acute or chronic dysfunction in the other [1-6]. We distinguish five types of cardiorenal syndrome, of which Type 5 CRS causes severe sepsis and septic shock and leads to acute disorders of the heart and kidneys (septic cardiorenal syndrome) [1-6]. Due to the complexity and severity of acute kidney injury (AKI) (sepsis and multiple organ dysfunction syndrome), patients in intensive care units require a team approach, skilled staff and technical equippedness, continuous and enhanced cooperation between a nephrologist and intensivist anaesthesiologist and the use of a new treatment plan, which includes multiple organ support therapy [7]. Several of the main tasks of an intensivist physician in the ICU include detecting sepsis early, treating septic patients by achieving optimal blood volume status and preventing the development of cardiorenal syndrome [7].

Sepsis: definition, aetiopathogenesis and clinical significance

Sepsis is a serious clinical syndrome in medicine and a major health problem in both developed and underdeveloped countries. The number of septic patients has grown by 1.5% in the past year, and 934,000 patients in the USA developed sepsis in 2010. Regardless of technological advances (dialysis supportive therapy, respiratory supportive therapy), the mortality rate of patients with severe sepsis remains high at 30-50%, and the cost of one-year treatment of such patients exceeds 50 trillion USD [8].

Definition

Bacteraemia is the presence of bacteria in the patient's blood. A patient`s response to an infection is systemic inflammatory response syndrome (SIRS) and is defined as the presence of two or more related criteria: body temperature > 38^{0}C or < 36^{0}C, heart rate > 90 beats per minute, respiratory rate > 20 breaths per minute or arterial partial pressure of pCO_2 less than 32 mmHg, white blood cell count > 12 x 10^9/mL or < 4 x 10^9/mL or > 10% immature neutrophils (band forms) [9, 10]. Sepsis is defined as SIRS with a confirmed infection. Severe sepsis is defined as sepsis accompanied with hypotension, hypoperfusion, organ dysfunction (serum lactate > 2 mmol/L), altered mental status, capillary volume recharge ≥ 3 s, diuresis < 0.5 mL /kg/h, serum creatinine levels increase of ≥ 26.4 μmol/L, platelet count < 100 x 10^9/mL or disseminated intravascular coagulopathy, acute lung injury/acute respiratory distress syndrome and heart disorder (cardiac index < 2.2 L/min/m^2) [9, 10]. Septic shock is defined as severe sepsis with hypotension, with no improvement following intravenous infusion therapy (i.e., restoring fluid volume in circulation), evidence for hypoperfusion or organ dysfunction, mean arterial blood pressure < 60 mmHg (< 80 mmHg in previously hypertensive patients) after crystalloids infusion (0.9% saline) in the dose range of 40-60 mL/kg in the first hour or pulmonary capillary filling pressure in the range of 12-20 mmHg and the need for dopamine > 5 μg/kg/min or epinephrine < 0.25 μg/kg/min to maintain mean arterial blood pressure > 60 mmHg (> 80 mmHg in previously hypertensive patients). Refractory septic shock is defined as the need for dopamine > 15 μg/kg/min or epinephrine > 0.25 μg/kg/min to maintain mean arterial blood pressure > 60 mmHg (> 80 mmHg in previously hypertensive patients) [9, 10].

Etiopathogenesis

Sepsis is a serious medical condition characterised by the activation of the patient's immune system and enhanced production of pro- and anti-inflammatory mediators [11]. The initial patient response to infection (i.e., the interplay between host and microbial agent) is followed by an increased and unregulated immune system response, increased production of proinflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α), and haemodynamic instability accompanied by reduced renal blood flow [11]. The proinflammatory phase is then replaced by the compensatory anti-inflammatory response phase, which blocks the immune system and is characterised by the reduced proliferation and increased apoptosis of lymphocytes, decreased chemotaxis, reduced phagocytosis and increased production of interleukin-10 (IL-10) [11]. The highest concentration of serum mediators (presumption of mediator "peak levels") plays an important part in the pathogenesis of multiple organ dysfunction syndrome in patients with severe sepsis [12-15]. Extracorporeal blood cleansing techniques such as high-volume continuous veno-venous haemofiltration (HVHF) and continuous veno-venous haemodiafiltration using PMMA membrane (PMMA-CHDF) improve the patient's haemodynamic status, reduce the need for vasopressors, restore the balance of immune system response (i.e., the balance between pro- and anti-inflammatory mediators), reduce the concentration of cell apoptosis-inducing mediators and improve survival in patients with severe sepsis [12-15].

Type 5 Cardiorenal Syndrome: Mechanisms of Aetiopathogenesis

The relationship between heart and kidneys is important for regulating the volume of arterial blood , which provides blood flow and oxygen supply to the bodily tissues [16-18]. Endothelial dysfunction, arterial vasodilation, decreased myocardial function and reduced blood volume in the arterial circulation, which normally enables renal perfusion, play a major role in causing septic cardiorenal syndrome [16-18]. A reduction in arterial blood volume may occur due to reduced heart rate and/or arterial vasodilation [16-18]. In patients with severe sepsis and septic shock, decreased renal blood flow is caused by arterial vasodilata-

tion, due to increased production of nitrogen oxides (NO), and decreased myocardial function (increased myocardial concentrations of cytokines/myocardial inflammation and elevated levels of NO in the myocardium) [16-18]. Decreased renal blood flow (reduced blood volume in the arterial circulation, which normally enables renal perfusion) leads to ischaemia of the renal tubular epithelial cells and the development of acute kidney injury (ischaemic acute tubular necrosis) [16-18]. Acute kidney injury and fluid retention in the body have an adverse effect on myocardial function and thus trigger a vicious cycle of kidney and heart disorders [16-18].

Type 5 Cardiorenal syndrome: Diagnosis

Sepsis biomarkers

Leukocyte count and C-reactive protein levels are the "gold standard" for diagnosing infection. Normal concentrations of serum procalcitonin are < 0.05 ng/mL and increases in severe bacterial infections with systemic manifestations [19]. A serum procalcitonin level below 0.5 ng/mL indicates a possible local infection and inflammation, the absence of a significant inflammatory response and a low risk of progression to severe systemic infection (severe sepsis). Procalcitonin concentrations should be remeasured at 6- to 24-hour intervals due to the possibility of disease development [19]. With serum procalcitonin concentrations ranging from 0.5-2.0 ng/mL, systemic infection (sepsis) is possible, systemic inflammatory response is moderate, and the risk of a progression to severe systemic infection (severe sepsis) is moderate as well. Procalcitonin testing is recommended at 6-24 hours after the last measurement [19]. Serum procalcitonin concentrations ranging from 2-10 ng/mL indicate systemic infection, severe systemic inflammatory response and a high risk of progression to severe systemic infection (severe sepsis). Daily procalcitonin measurements are recommended [19]. Procalcitonin values ≥ 10 ng/mL indicate pronounced systemic inflammatory response, severe sepsis and septic shock, as well as possible multiple organ dysfunction syndrome and a high risk of death. Daily procalcitonin measurements are recommended [19].

Cardiac injury biomarkers

Determination of the natriuretic peptides BNP and NT-proBNP is needed to diagnose heart failure [20-24]. In patients suffering from chronic kidney disease, serum BNP and NT-proBNP concentrations may be increased, even if clinical manifestation of heart failure is absent due to reduced renal function through decreased renal clearance of natriuretic peptides, myocardial scarring and thickening of the left ventricular wall [20-24]. In patients whose endogenous creatinine clearance is ≥ 60 mL/min/1.73 m^2, BNP values greater than 100 pg/mL and NT-proBNP values greater than 300 pg/mL indicate heart failure. If endogenous creatinine clearance is < 60 mL/min/1.73 m^2, then BNP values greater than 200 pg/mL and NT-proBNP values greater than 1200 pg/mL indicate heart failure [20-24]. Cardiac troponins (cTnI/cTnT) are biomarkers used to demonstrate ischaemia-induced myocardial injury [24, 25]. An increase in serum troponin I of ≥ 20% from baseline indicates ischaemic damage to cardiac cells (cardiomyocytes), while values ≥ 2.0 ng/mL indicate the development of acute myocardial infarction [24, 25].

Renal injury biomarkers

In the last decade, a number of biomarkers for the early detection of acute kidney injure were discovered: interleukin-18, a kidney injury protein called KIM-1 (*kidney injury molecule-1*), NGAL (*neutrophil gelatinosa-associated lipocalin)* and cystatin C [23-25]. Lipocalin is used in clinical practice. NGAL values in urine in excess of 100 ng/mL two hours after initial event indicate a development of acute kidney damage [23-25]. Cystatin C is a cysteine protease blocker filtered in the glomeruli and completely reabsorbed by proximal tubular epithelial renal cells. An increase in cystatin C serum concentrations over 0.3 mg/L in the first 48 hours from the initial event, when compared to baseline, is indicative of the development of acute kidney injury. An increase in the cystatin C serum concentration occurs 1-2 days prior to an increase in serum creatinine, indicating that cystatin C is a more sensitive parameter for the detection of acute kidney injury compared to creatinine[23-25].

Monitoring of patients with severe sepsis and septic shock

Patients in intensive care units require monitoring of infection parameters (e.g., WBC, CRP, procalcitonin), haemodynamic status parameters (e.g., mean arterial blood pressure, central venous pressure [CVP] or internal jugular vein catheterisation, central venous oxygen saturation [$ScvO_2$], pulmonary capillary wedge pressure [PCWP] or pulmonary artery catheterisation), perfusion parameters and organ dysfunction (e.g., serum lactate concentrations, cardiac index [CI], left ventricular ejection fraction [EF], diuresis, serum creatinine concentration, platelet count, D-dimer, protein C, coagulation status [PT, aPTT, INR]) [26, 27].

Treatment of Type 5 Cardiorenal Syndrome

Strategic approach to prevention of acute renal injury in sepsis

Guidelines for the management of severe sepsis highlight the importance of early detection and timely administration of initial treatment within the first six hours of symptom onset to stop the septic cascade. Initial treatment should achieve a central venous pressure (CVP) of 8-12 mmHg, mean arterial blood pressure (MAP) greater than 65 mmHg, diuresis greater than 0.5 mL/kg/h and central venous oxygen saturation greater than 70% [28-32]. The treatment strategy for preventing the development of acute kidney injury includes early goal-directed therapy

(EGDT), which consists of increased early compensation of blood volume in fluid circulation (i.e., at least 20 mL/kg of crystalloids in the first hour of admission followed by 500 mL crystalloids every 60 minutes in first 6 hours until a central venous pressure of 8 to 12 mmHg is reached) with strict monitoring of patient response [28-32]. When adequate compensation of the circulating blood volume (3000 mL crystalloids) does not lead to increased blood pressure (MAP < 65 mmHg) and adequate tissue perfusion, a vasopressor treatment is used (dopamine or epinephrine vasopressor therapy) while inotropic therapy (dobutamine) is used when the cardiac index is reduced (CI < 2.2 L/min/m^2) [28-32]. The saturation of central venous blood reflects a balance in oxygen transport and oxygen consumption; thus, when saturation falls below 70%, even after CVP and MAP are normalised and haematocrit levels are less than 30%, a transfusion of packed red blood cells should be administered [27-32]. In tertiary health care institutions, EGDT is administered in the clinical practice by only 7% of physicians attending to ICU patients. Training of health care professionals in ICUs plays an important part in the use of early goal-directed therapy in septic patients [28-32].

Sepsis treatment

Intravenous antibiotic therapy should be initiated immediately within the first hour of determining sepsis symptoms and following the collection of peripheral blood samples. Effects of antibiotic therapy should be evaluated after 48-72 hours using microbiological and clinical data, and antibiotic drugs should be used in accordance with the antibiogram. Source of infection (e.g., abscess, infected necrotic tissue, gastrointestinal perforation) should be removed as soon as possible using the least aggressive surgical method in accordance with adequate patient care [28].

Additional therapies used in the treatment of severe sepsis and septic shock include corticosteroids and recombinant human activated protein C (rhAPC - drotrecogin alfa). Intravenous corticosteroids are recommended in patients with septic shock with relative adrenal insufficiency, as indicated by a cortisol increase of less than 9 μg/dL after the application of 250 μg adrenocorticotropic hormone (ACTH) [28]. Hydrocortisone at 50 mg IV q6h for 5 days is to be administered, and the dose should be gradually decreased to 50 mg IV q12h through 6-8 days, followed by an increase to 50 mg IV q24h through 9-11 days [28]. Recombinant human activated protein C (rhAPC) exerts anti-thrombotic, profibrinolytic, anti-inflammatory and cytoprotective effects [28, 32-34]. It is used in patients with sepsis and multiple organ dysfunction syndrome (MODS) or in patients suffering from septic shock with high risk of death (APACHE score > 25) when there is no absolute contraindication due to bleeding (i.e., active internal bleeding, cerebral bleeding in the last three months) at a dose of 24 μg/kg/h for 96 h as an IV infusion [28, 33-35].

Frequent glucose control as well asand continued glucose and insulin infusion to achieve a target blood glucose level of 6-9 mmol/L are recommended. Lactic acidosis treatment includes bicarbonate therapy when arterial blood pH decreases below 7.15. Stress ulcer prophylaxis is achieved by hydrogen receptor blockers. Unfractionated heparin and low molecular weight heparin (LMWH) are used for the prevention of deep vein thrombosis in adequate dose monitoring (anti-factor Xa activity) [28, 33].

In patients in intensive care units suffering from severe sepsis and septic shock, acute lung injury and acute kidney injury may occur simultaneously (i.e., multiple organ dysfunction syndrome). Acute lung injury (ALI) (arterial oxygen tension PaO_2 and fractional inspired oxygen FiO_2 ratio below 300), acute respiratory distress syndrome (ARDS) (PaO_2/FiO_2 ratio below 200) and mechanical ventilation contribute to the development of acute kidney injury (direct interconnectedness between lungs and kidneys) [36-38]. Positive pressure ventilation (PPV) (mechanical ventilation) lowers renal perfusion with its haemodynamic and non-haemodynamic effects. Positive pressure ventilation increases the pressure in the chest cavity and decreases venous return (preload reduced), resulting in decreased heart rate and cardiac output and, consequently, the activation of neurohormonal systems, such as the renin-angiotensin-aldosterone system, sympathetic nervous system and non-osmotic vasopressin system, release and ANP production. Finally, the activation of neurohormonal systems will reduce renal blood flow, decrease glomerular filtration rate (GFR), and increase sodium and water retention in the patient's body [36-38]. Patients in septic shock with acute respiratory failure should undergo protective lung ventilation using low respiratory volume (tidal volume of 6 mL/kg of ideal body weight), with end-inspiratory pressure plateau below 30 cmH_2O, while using the smallest positive end-expiratory pressure (positive end-expiratory pressure - PEEP) to achieve satisfactory oxygenation [36-38].

Methods of extracorporeal blood cleansing in septic patients

Sepsis is the leading cause of death in intensive care units. Treatment using extracorporeal blood cleansing techniques change a patient's immune response to the infection by non-selectively removing inflammatory mediators and toxins/bacterial products [39]. Endotoxins and other bacterial products (e.g., lipopolysaccharide [LPS], peptidoglycan, flagellin) stimulate increased production and release of cytokines into the serum of septic patients ("cytokine theory"). Increased concentrations of serum cytokines play an important part in the development of multiple organ dysfunction syndrome (MODS) (the assumption of "peak levels"). Removal of cytokines from a septic patient's serum significantly improves renal and patient outcomes. The two most important extracorporeal cleansing techniques for cytokine blood removal are high-volume continuous veno-venous haemofiltration (HVHF) and continuous veno-venous haemodiafiltration using a polymethylmethacrylate membrane haemofilter (PMMA-CDHF) [39].

Treatment initiation time for dialysis supportive therapy

In patients with AKI, it is necessary to assess the severity of injury and the presence of absolute indications for treatment with renal replacement therapy (RRT) (dialysis supportive therapy). Absolute indications for initiation of dialysis treatment include: serum urea concentration ≥ 36 mmol/L, complications of uraemia (uremic encephalopathy, uremic pericarditis), resistant hyperkalaemia ($K^+ > 6.5$ mmol/L with or without electrocardiographic changes), hypermagnesaemia ($Mg^{2+} \geq 4.0$ mmol/L and absence of deep tendon reflexes), severe metabolic acidosis (arterial blood pH ≤ 7.15) and volume overload (pulmonary oedema) resistant to diuretics in the presence of oliguric AKI [40]. In patients with severe AKI (RIFLE-F or AKIN III), initiation of dialysis supportive therapy is to be considered, and increased monitoring and treatment is recommended in those with mild/moderate AKI (RIFLE-R or I, AKIN I or II). Prior to making a decision to initiate RRT treatment in patients with mild or moderate renal injury, the treatment objectives, primary diagnosis, severity of the patient's clinical condition, renal functional reserve and the need to prevent development of complications should be taken into account. In patients with sepsis (high catabolism), there is a potential benefit of early initiation of dialysis treatment [40]. When making the decision to start dialysis treatment with supportive therapy, clinical conditions that adversely affect renal function in ICU patients, such as increased intra-abdominal pressure, mechanical positive pressure ventilation and use of nephrotoxins and radiocontrast agents, should be considered [40].

Selecting modes of dialysis supportive therapy

Patients with AKI are treated with numerous dialysis modalities, employed to remove uremic toxins and substances from the blood using mechanisms of diffusion (peritoneal dialysis, standard haemodialysis, slow low efficiency dialysis [SLEDD], continuous veno-venous haemodialysis), convection (intermittent haemofiltration, continuous veno-venous haemofiltration [CVVHF], continuous veno-venous high-volume haemofiltration [HVHF]), a combination of diffusion and convection (intermittent haemodiafiltration, slow low-efficiency diafiltration [SLEDDf], continuous veno-venous haemodiafiltration [CVVHDF]) and adsorption (continuous veno-venous haemodiafiltration using a *polymethylmethacrylate* membrane haemofilter [PMMA-CHDF]) [41-49].

Intermittent haemodialysis is performed in patients who are haemodynamically stable, with high levels of nitrogen and severe hyperkalaemia, and in patients with an increased risk of bleeding. Individual session doses for conventional intermittent (3 times per week) and enhanced intermittent (6 time per week) haemodialysis are single pool Kt/V index ≥ 1.2 [47, 48]. Intermittent haemodialysis has little impact on most of the major inflammatory cytokines. Hybrid modes of dialysis therapy, such as slow low-efficiency dialysis (SLEDD), provide excellent clearance of low molecular weight uraemic toxins, moderate clearance of medium molecular weight uremic toxins and good haemodynamic stability of patients [47, 48]. Individual session doses for conventional SLEDD (3 times per week for 6-12 hrs) and enhanced SLEDD (6 times per week for 6-12 hrs) are a single pool Kt/V index ≥ 1.2 (single pool Kt/V index = 1.2-1.4) [47, 48]. SSC (*Surviving Sepsis Campaign*) guidelines recommend that, in the absence of haemodynamic instability, intermittent and continuous dialysis treatment modalities should be equally considered [49]. Haemodynamically unstable patients (septic shock) in intensive care units suffering from AKI, with multiple organ dysfunction syndrome, elevated levels of serum cytokines (interleukin-6 concentrations ≥ 1000 pg/mL), increased catabolism and hypervolaemia require treatment modalities of continuous renal replacement therapy (CRRT) [41-49].

Most immune system mediators are substances of medium molecular weight (5-50 kD), soluble in water and removable using continuous renal replacement modalities. The standard dialysis dose in continuous veno-venous haemofiltration (CVVHF) and continuous veno-venous haemodiafiltration (CVVHDF) is 20-30 mL/kg/h, while in patients with sepsis and acute kidney injury the dose of continuous dialysis modality should be 35 mL/kg/h [41-45, 50, 51]. High-volume continuous veno-venous haemofiltration (HVHF) is used for the removal of pro- and anti-inflammatory mediators, due to the high convective transport, at > 35 mL/kg/h. It may be used continuously, with an ultrafiltration rate of 50-70 mL/kg/h (35-80 mL/kg/h) over 24 hours, or as pulse high-volume haemofiltration, with an ultrafiltration rate of 85-100 mL/kg/h (100-120 mL/kg/h) over 4-8 hours, followed by the standard dose afterwards [48-50]. High-volume haemofiltration significantly reduces the concentration of inflammatory mediators and restores the balance between inflammatory syndrome and compensatory anti-inflammatory systemic responses [41-45, 50, 51].

Continuous veno-venous haemodiafiltration with PMMA membrane (PMMA-CHDF) is administered within 24 hours of developing septic shock, and it removes cytokines via the adsorption process to the dialysis membrane matrix (i.e., cytokine adsorption to highly specific membranes/membranes with high cytokine removal capacity). It is effective in the treatment of clinical conditions associated with increased concentration of serum cytokines (interleukin-6 concentrations 6 ≥ 1000 pg/mL), such as septic shock, multiple organ dysfunction syndrome caused by sepsis, acute respiratory distress syndrome and severe acute pancreatitis [41-45, 50, 51]. The results of clinical trials (*EUPHAS Study*) show that PMMA-CHDF is more effective in cleansing blood of patients with severe sepsis and septic shock compared to PMX-DHP (*Polymyxin-B direct haemoperfusion*), which plays an important part in the removal of endotoxin (lipopolysaccharide) from the blood of septic patients [41-45, 50, 51].

Continuous modalities of dialysis supportive therapy should provide haemodynamic stability and homeostasis of the immune system. It provides an opportunity to use CRRT not only as supportive therapy, but also but also as a treatment to prevent injury progression and the development of multiple organ dysfunction syndrome in septic patients [50, 51].

Conclusion

The early identification of patients at increased risk for developing acute kidney injury and timely implementation of an appropriate treatment plan can prevent the development of acute kidney injury and reduce morbidity and mortality in septic patients. Haemodynamically unstable patients (septic shock) in intensive care units suffering from acute kidney injury, on mechanical respiration, with multiple organ dysfunction syndrome, elevated levels of serum cytokines, increased catabolism and hypervolaemia require treatment modalities of continuous renal replacement therapy. Well-controlled, prospective, randomised clinical trials should more precisely determine the place and role of various modalities of dialysis therapy in patients with sepsis and AKI in intensive care units.

Acknowledgments: The authors would like to express their deepest gratitude to the Serbian Ministry of Science and Technological Development for their Grant N⁰175014, which was used as one of the sources to financially support the study.

REFERENCES

1. Ronco C, House AA, Haapio M. Cardiorenal syndrome: refining the definition of a complex symbiosis gone wrong. Intensive Care Med 2008; 34(5): 957-62.
2. Ronco C, Haapio M, House AA, Anaveker N, Bellomo R. Cardiorenal Syndrome. J Am Coll Cardiol 2008; 52(19): 1527-39.
3. Ronco C, Chionh C-Y, Maapio M, Anavekar NS, House A, Bellomo R. The Cardiorenal Syndrome. Blood Purif 2009; 27(1): 114-26.
4. Ronco C, McCullough, Anker SD, Anand I, Aspromonte N, Bagshaw SM, et al. Cardio-renal syndromes: report from the consensus conference of the Acute Dialysis Quality Initiative. Eur Heart J 2010; **31**(6): 703-11.
5. Petrović D, Jagić N, Miloradović V, Nikolić A, Stojimirović B. Cardiorenal syndrome - definition, classification and basic principles of therapy. Ser J ExpClin Res 2010; 11(2): 67-71.
6. Petrović D, Milovanović D, Miloradović V, Nikolić A, Petrović M, Đurđević P, Poskurica M. Kardio-renalni sindrom tip 2: etiopatogeneza, dijagnostika i lečenje. Medicinski Časopis 2012; 46:(1): 30-4.
7. Ronco C. Introduction. In: Acute Blood Purification. Contrib Nephrol. Suzuki H, Hirasawa H (eds). Basel, Karger 2010; 166: 1-3.
8. Stoneking L, Denninghoff K, DeLuca L, Keim SM, Munger B. Sepsis Bundles and Compliance With Clinical Guidelines. J Int Care Med 2011; 26(3): 172-82.
9. Dollins MD. Part 2: Circulation. In: Critical Care Nephrology. Molitoris B, ed. London: Remedica, 2005: 21-42.
10. Hunter JD, Doddi M. Sepsis and the heart. Br J Anaesth 2010; 104(1): 3-11.
11. Zarjou A, Agarwal A. Sepsis and Acute Kidney Injury. J Am Soc Nephrol 2011; 22(6): 999-1006.
12. Ronco C, Brendolan A, Bellomo R, Ricci Z, Bonello M, Ratanarat R, et al. The Rationale for Extracorporeal Therapies in Sepsis. Advances in Sepsis 2004; 4(1): 2-10.
13. House AA, Ronco C. Extracorporeal Blood Purification in Sepsis and Sepsis-Related Acute Kidney Injury. Blood Purif 2008; 26(1): 30-5.
14. Venkatarman R, Subramanian S, Kellum JA. Clinical review: Extracorporeal blood purification in severe sepsis. Crit Care 2003; 7(2): 139-45.
15. Claudio R, Tetta C, Mariano F, Wratten ML, Bonello M, Bordoni V, et al. Interpreting the Mechanisms of Continuous Renal Replacement Therapy in Sepsis: The Peak Concentrations Hypothesis. Artif Organs 2003; 27(9): 792-80.
16. Chelazzi C, Villa G, De Gaudio AR. Cardiorenal Syndromes and Sepsis. Int J Nephrol 2011: ID 652967.
17. Schrier RW. Role of Diminished Renal Function in Cardiovascular Mortality. J Am Coll Cardiol 2006; 47(1): 1-8.
18. Schrier RW. Decreased Effective Blood Volume in Edematous Disorders: What Does This Mean? J Am SocNephrol 2007; 18(7): 2028-31.
19. Čanović P, Nešić Lj, Tomović M, Čanović D. Dijagnostički i prognostički značaj merenja prokalcitonina u toku septičkih stanja. Medicinski Časopis 2006; 40(3): 52-7.
20. Anwaruddin S, Lloyd-Jones DM, Baggish A, Chen A, Krauser D, Tung R, et al. Renal function, congestive heart failure, and amino-terminal pro-brain natriuretic peptide measurement: results from the ProBNP Investigation of Dyspnea in the Emergency Department (PRIDE) Study. J Am Coll Cardiol 2006; 47(1): 91-7.
21. David S, Kümpers P, Seidler V, Biertz F, Haller H, Fliser D. Diagnostic value of N-terminal pro-B-type natriuretic peptide (NT-proBNP) for left ventricular dysfunction in patients with chronic renal disease stage 5 on hemodialysis. Nephrol Dial Transplant 2008; 23(4): 1370-7.
22. Valle R, Aspromonte N. Use of Brain Natriuretic Peptide and Bioimpedance to Guide Therapy in Heart Failure Patients. In: Fluid Overload: Diagnosis and Management. Ronco C, Costanzo MR, Bellomo R, Maisel AS (eds). ContribNephrol. Basel, Karger, 2010, vol 164, pp 209-16.
23. Maisel AS, Katz N, Hillege HL, Shaw A, Zanco P, Bellomo R, et al and for the Acute Dialysis Quality Initiative (ADQI) consensus group. Biomarkers in kidney and Heart disease. Nephrol Dial Transplant 2011; 26(1): 62-74.
24. Petrović D, Jagić N, Miloradović V, Stojimirović B. Clinical importance of biochemical markers of cardiac damage in hemodialysis patients. Ser J ExpClin Res 2008; 9(1): 5-8.

25. Lassus JPE, Nieminen MS, Peuhkurinen K, Pulkki K, Siirila-Waris K, Sund R, et al. Markers of renal function and acute kidney injury in acute heart failure: definitions and impact on outcomes of the cardiorenal syndrome. Eur Heart J 2010; 31(22): 2791-8.
26. Liu KD. Critical Care Nephrology: Core Curriculum 2009. Am J Kidney Dis 2009; 53(5): 898-910.
27. Moranville MP, Mieure KD, Santayana EM. Evaluation and Management of Shock States: Hypovolemic, Distributive, and Cardiogenic Shock. J Pharm Pract 2011; 24(1): 44-60.
28. Jevđić J, Šurbatović M, Drakulić-Miletić S, Vukićević V. Primena novih terapijskih protokola u lečenju sepse i septičnog šoka u jedinicama intenzivnog lečenja kliničkog centra u Kragujevcu. Srp Arh Celok Lek 2008; 136(5-6): 248-52.
29. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, et al. Early Goal-Directed Therapy in the Treatment of Severe Sepsis and Septic Shock. N Engl J Med 2001; 345(19): 1368-77.
30. Otero RM, Nguyen HB, Haung DT, Gaieski DF, Goyal M, Gunnerson KJ, et al. Early Goal-Directed Therapy in Severe Sepsis and Septic Shock Revisited. Chest 2006; 130(5): 1579-95.
31. Petrović D. Akutno oštećenje bubrega: etiologija, dijagnostika i lečenje. Medicinska Istraživanja 2011; 45(3): 7-13.
32. Lukić S, Petrović D. Prevencija akutnog oštećenja bubrega u jedinicama intenzivnog lečenja. Med Čas 2012; 46(2): 100-4.
33. Rudis MI, Rowland KL. Current Concepts in Severe Sepsis and Septic Shock. J Pharm Pract 2005; 18(5): 351-62.
34. Gupta A, Williams MD, Macias W, Molitoris BA, Grinelli BW. Activated Protein C and Acute Kidney Injury: Selective Targeting of PAR-1. Curr Drug Targ 2009; 10(12): 1212-26.
35. Bernard GR, Vincet JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Eng J Med 2001; 344(10): 699-709.
36. Ronco C, Kellum JA, Bellomo R, House AA. Potential interventions in Sepsis-Related Acute kidney Injury. Clin J Am Soc Nephrol 2008; 3(2): 531-44.
37. Koyner JL, Murray PT. Mechanical Ventilation and the Kidney. Blood Purif 2010; 29(1): 52-68.
38. Cawley MJ. Mechanical Ventilation: Introduction for the Pharmacy Practitioner. J Pharm Pract 2011; 24(1): 7-16.
39. Rimmele T, Kellum JA. Clinical Rewiev: Blood purification for sepsis. Critical Care 2011; 15(1): 205.
40. Bagshaw SM, Cruz DN, Gibney RTN, Ronco C. A proposed algorithm for initiation of renal replacement therapy in adult critically ill patients. Crit Care 2009; 13(6): 317-25.
41. Nakada T, Matsuda K, Sadahiro T, Nakamura M, Abe R, Hirasawa H. Continuous Hemodiafiltration with PMMA Hemofilter in the Treatment of Patients with Septic Shock. Mol Med 2008; 15(5-6): 257-63.
42. Davenport A. Dialytic Treatment for Sepstic Patients with Acute Kidney Injury. Kidney Blood Press Res 2011; 34(4): 218-24.
43. Kawanishi H. Terminology and Classification of Blood Purification in Critical Care in Japan. Indications for Blood Purifications in Critical Care. In: Acute Blood Purification. Suzuki H, Hirisawa H (eds). ContribNephrol. Basel, Karger, 2010, vol. 166, pp 11-20.
44. Hirasawa H. Indications for Blood Purifications in Critical Care. In: Acute Blood Purification. Suzuki H, Hirisawa H (eds). Contrib Nephrol. Basel, Karger, 2010, vol. 166, pp 21-30.
45. Abe R, Oda S, Shinozaki K. Continuous Hemodiafiltration Using a Polymethyl Methacrylate Membrane Hemofilter for Severe Acute Pancreatitis. In: Acute Blood Purification. Contrib Nephrol. Suzuki H, Hirasawa H (eds). Basel, Karger 2010; 166: 54-63.
46. Prowle JR, Bellomo R. Continuous renal replacement therapy: recent advances and future research. Nat Rev Nephrol 2010; 6(9): 521-9.
47. John S, Eckardt K-U. Replacment Therapy in the Treatment of Acute Renal Failure - Intermitent and Continuous. Semin Dial 2006; 19(6): 455-64.
48. John S, Eckardt K-U. Renal Replacement Strategies in the ICU. Chest 2007; 132(4): 1379-88.
49. Ricci Z, Picca S, Guzzo I, Ronco C. Kidney diseases beyond nephrology: intensive care. Nephrol Dial Transplant 2011; 26(2): 448-54.
50. Joannidis M. Continuous Renal Replacement Therapy in Sepsis and Multisystem Organ Failure. Semin Dial 2009; 22(2): 160-4.
51. Prowle JR, Schneider and Bellomo R. Optimal dose of continuous renal replacement therapy in acute kidney injury. Crit Care 2011; 15(2): 207-14.

SUBMISSIVENESS TO HEALTH AUTHORITIES AS AN OBSTACLE TO PRACTICING EVIDENCE BASED MEDICINE

Ilickovic Ivana[1], Jankovic M Slobodan[2].
[1]Farmegra d.o.o, 4. Jula 60, Podgorica, Montenegro
[2]Faculty of Medical Sciences, University of Kragujevac, Serbia

Received / Primljen: 28.10.2013. Accepted / Prihvaćen: 26.12.2013.

Dear Editor,

In the Balkan countries, submissiveness to medical authorities is a widespread attitude among physicians, who consider ministries of health to be a primary source of initiative for the introduction of new knowledge and skills in medical practice [1]. To examine whether such an attitude might inhibit the practice of evidence-based medicine (EBM) by family physicians, we conducted a qualitative, semi-structured survey of a sample of family physicians in Podgorica, Montenegro. The topic of the survey interview was secondary prevention of related morbidity and mortality of patients who have experienced myocardial infarction (MI).

The interview schedule was developed through consultation with fellow clinical pharmacists and pharmacologists, and its face and content validity were checked means of using a pilot study. Eight physicians (age range: 45 – 57 years; 5 males and 3 females), out of a total of 57 family physicians working in 14 state-owned primary care facilities serving 150,000 inhabitants in Podgorica, were randomly sampled and interviewed. To analyse the taped interviews, the framework approach, validated through repeat analysis, was employed.

The findings suggest that, despite the explicitness and robustness of evidence for secondary prevention of related morbidity and mortality of patients who have experienced MI and the broad availability of open-access sources of such evidence, primary care physicians in Podgorica are unaware of either effective prophylactic therapy or the evidence-based information sources. In addition, we observed a discrepancy between information that was explicitly described by interview subjects as reliable and influential (for example, CME events or journals), despite its unavailability, and extensively -used sources that prescriptions were actually based on. Physicians mostly relied on non-evidence based sources of information, such as opinion leaders, colleagues, unsystematic experience, pharmaceutical companies and uncritical internet searches [2]. Their main goals were to become *"encouraged"*, *"affirmed"* and *"supported"*, which resulted in psychological gain but did not guarantee benefits for patients. The most trustworthy information sources were regarded as national experts recognised by the heath authorities, namely, well known "professors", regardless of whether they practiced EBM.

An interesting finding of our study was unsubstantiated enthusiasm for EBM among interviewed family physicians. Although they had a false conception of research evidence, lacked retrieval and appraisal skills, practiced therapeutic conservatism, and relied on "shortcuts" in interpreting research studies, they nevertheless highly valued the role of research evidence in clinical decision-making [2]. EBM appeared to them as an ideal that is not applicable in everyday work. They complained of many contextual and individual barriers: unavailability, inaccessibility and a lack of organised dissemination of unbiased, up-to-date information. In addition, they cited reluctance to change practice patterns and practical constraints. However, they did not perceive themselves as main sources of change but expected initiatives from outside sources, primarily the state and its responsible officers. They expected educational, organisational, and structural interventions, which will "tell them what to do".

Passivism, conservatism and submissiveness to health authorities were also observed in studies of primary care physicians' attitudes towards EBM conducted in other countries with authoritarian social structures [3, 4]. Instead of directly using numerous free EBM resources available on the Internet for self-education and implementing this knowledge into their practices, the majority of primary care physicians in such countries expect to be offered formal education organised and conducted by health authorities and their "experts" [3, 4, 5]. Although they have heard of EBM and view it positively, primary care physicians generally lack sufficient initiative to alter their routine behaviour and make significant changes.

Ser J Exp Clin Res 2013; 14 (4): 189-190
DOI: 10.5937/SJECR4764

Correspondence: Assist. Prof. Dejan Petrović, MD, PhD,
Clinic for Urology and Nephrology, Clinical Center Kragujevac, Zmaj Jovina 30, 34000 Kragujevac

REFERENCES

1. Jakovljevic MB. Resource allocation strategies in South-eastern European health policy. Eur J Health Econ 2013; 14(2): 153-9.
2. Đorđević ND, Janković SM. Characteristics of decision-making process during prescribing in general practice. Vojnosanitetski pregled 2006; 63(3): 279-285.
3. Al-Ansary LA, Khoja TA.The place of evidence-based medicine among primary health care physicians in Riyadh region, Saudi Arabia. Fam Pract 2002; 19(5): 537-42.
4. Al Omari M, Khader Y, Jadallah K, Dauod AS, Al-Shdifat AA, Khasawneh NM. Evidence-based medicine among hospital doctors in Jordan: awareness, attitude and practice. J Eval Clin Pract 2009; 15(6): 1137-41.
5. Sackett D, William MC, Rosenberg JA, Muir G, Haynes B, Richardson S. 1996 Evidence based medicine: what it is and what it isn't. BMJ 1996; 312: 71-72.

INSTRUCTION TO AUTHORS FOR MANUSCRIPT PREPARATION

Serbian Journal of Experimental and Clinical Research is a peer-reviewed, general biomedical journal. It publishes original basic and clinical research, clinical practice articles, critical reviews, case reports, evaluations of scientific methods, works dealing with ethical and social aspects of biomedicine as well as letters to the editor, reports of association activities, book reviews, news in biomedicine, and any other article and information concerned with practice and research in biomedicine, written in the English.

Original manuscripts will be accepted with the understanding that they are solely contributed to the Journal. The papers will be not accepted if they contain the material that has already been published or has been submitted or accepted for publication elsewhere, except of preliminary reports, such as an abstract, poster or press report presented at a professional or scientific meetings and not exceeding 400 words. Any previous publication in such form must be disclosed in a footnote. In rare exceptions a secondary publication will acceptable, but authors are required to contact Editor-in-chief before submission of such manuscript. the Journal is devoted to the Guidelines on Good Publication Practice as established by Committee on Publication Ethics-COPE (posted at www.publicationethics.org.uk).

Manuscripts are prepared in accordance with „Uniform Requirements for Manuscripts submitted to Biomedical Journals“ developed by the International Committee of Medical Journal Editors. Consult a current version of the instructions, which has been published in several journals (for example: Ann Intern Med 1997;126:36-47) and posted at www.icmje.org, and a recent issue of the Journal in preparing your manuscript. For articles of randomized controlled trials authors should refer to the „Consort statement“ (www.consort-statement.org). Manuscripts must be accompanied by a cover letter, signed by all authors, with a statement that the manuscript has been read and approved by them, and not published, submitted or accepted elsewhere. Manuscripts, which are accepted for publication in the Journal, become the property of the Journal, and may not be published anywhere else without written permission from the publisher.

Serbian Journal of Experimental and Clinical Research is owned and published by Medical Faculty University of Kragujevac. However, Editors have full academic freedom and authority for determining the content of the journal, according to their scientific, professional and ethical judgment. Editorial policy and decision making follow procedures which are endeavoring to ensure scientific credibility of published content, confidentiality and integrity of auth ors, reviewers, and review process, protection of patients' rights to privacy and disclosing of conflict of interests. For difficulties which might appear in the Journal content such as errors in published articles or scientific concerns about research findings, appropriate handling is provided. The requirements for the content, which appears on the Journal internet site or Supplements, are, in general, the same as for the master version. Advertising which appears in the Journal or its internet site is not allowed to influence editorial decisions.

Manuscripts can be submitted by using the following link:
http://scindeks-eur.ceon.rs/index.php/sjecr

MANUSCRIPT

Original and two anonymous copies of a manuscript, typed double-spaced throughout (including references, tables, figure legends and footnotes) on A4 (21 cm x 29,7 cm) paper with wide margins, should be submitted for consideration for publication in Serbian Journal of Experimental and Clinical Research. Use Times New Roman font, 12 pt. Manuscript should be sent also on an IBM compatible floppy disc (3.5"), written as Word file (version 2.0 or later), or via E-mail to the editor (see above for address) as file attachment. For papers that are accepted, Serbian Journal of Experimental and Clinical Research obligatory requires authors to provide an identical, electronic copy in appropriate textual and graphic format.

The manuscript of original, scinetific articles should be arranged as following: Title page, Abstract, Introduction, Patients and methods/Material and methods, Re-

sults, Discussion, Acknowledgements, References, Tables, Figure legends and Figures. The sections of other papers should be arranged according to the type of the article.

Each manuscript component (The Title page, etc.) should begins on a separate page. All pages should be numbered consecutively beginning with the title page.

All measurements, except blood pressure, should be reported in the System International (SI) units and, if necessary, in conventional units, too (in parentheses). Generic names should be used for drugs. Brand names may be inserted in parentheses.

Authors are advised to retain extra copies of the manuscript. Serbian Journal of Experimental and Clinical Research is not responsible for the loss of manuscripts in the mail.

TITLE PAGE

The Title page contains the title, full names of all the authors, names and full location of the department and institution where work was performed, abbreviations used, and the name of corresponding author.

The title of the article should be concise but informative, and include animal species if appropriate. A subtitle could be added if necessary.

A list of abbreviations used in the paper, if any, should be included. The abbreviations should be listed alphabetically, and followed by an explanation of what they stand for. In general, the use of abbreviations is discouraged unless they are essential for improving the readability of the text.

The name, telephone number, fax number, and exact postal address of the author to whom communications and reprints should be sent are typed et the end of the title page.

ABSTRACT

An abstract of less than 250 words should concisely state the objective, findings, and conclusions of the studies described in the manuscript. The abstract does not contain abbreviations, footnotes or references.

Below the abstract, 3 to 8 keywords or short phrases are provided for indexing purposes. The use of words from Medline thesaurus is recommended.

INTRODUCTION

The introduction is concise, and states the reason and specific purpose of the study.

PATIENTS AND METHODS/MATERIAL AND METHODS

The selection of patients or experimental animals, including controls, should be described. Patients' names and hospital numbers are not used.

Methods should be described in sufficient detail to permit evaluation and duplication of the work by other investigators.

When reporting experiments on human subjects, it should be indicated whether the procedures followed were in accordance with ethical standards of the Committee on human experimentation (or Ethics Committee) of the institution in which they were done and in accordance with the Helsinki Declaration. Hazardous procedures or chemicals, if used, should be described in details, including the safety precautions observed. When appropriate, a statement should be included verifying that the care of laboratory animals followed accepted standards.

Statistical methods used should be outlined.

RESULTS

Results should be clear and concise, and include a minimum number of tables and figures necessary for proper presentation.

DISCUSSION

An exhaustive review of literature is not necessary. The major findings should be discussed in relation to other published work. Attempts should be made to explain differences between the results of the present study and those of the others. The hypothesis and speculative statements should be clearly identified. The Discussion section should not be a restatement of results, and new results should not be introduced in the discussion.

ACKNOWLEDGMENTS

This section gives possibility to list all persons who contributed to the work or prepared the manuscript, but did not meet the criteria for authorship. Financial and material support, if existed, could be also emphasized in this section.

REFERENCES

References should be identified in the text by Arabic numerals in parentheses. They should be numbered consecutively, as they appeared in the text. Personal communications and unpublished observations should not be cited in the reference list, but may be mentioned in the text in parentheses. Abbreviations of journals should conform to those in Index Serbian Journal of Experimental and Clinical Research. The style and punctuation should conform to the Serbian Journal of Experimental and Clinical Research style requirements. The following are examples:

Article: (all authors are listed if there are six or fewer; otherwise only the first three are listed followed by "et al.")

12. Talley NJ, Zinsmeister AR, Schleck CD, Melton LJ. Dyspepsia and dyspeptic subgroups: a population-based study. Gastroenterology 1992; 102: 1259-68.

Book: 17. Sherlock S. Diseases of the liver and biliary system. 8th ed. Oxford: Blackwell Sc Publ, 1989.

Chapter or article in a book: 24. Trier JJ. Celiac sprue. In: Sleisenger MH, Fordtran JS, eds. Gastro-intestinal disease. 4th ed. Philadelphia: WB Saunders Co, 1989: 1134-52.

The authors are responsible for the exactness of reference data.

For other types of references, style and interpunction, the authors should refer to a recent issue of Serbian Journal of Experimental and Clinical Research or contact the editorial staff.

Non-English citation should be preferably translated to English language adding at the end in the brackets native langauage source, e.g. (in Serbian). Citation in old language recognised in medicine (eg. Latin, Greek) should be left in their own. For internet soruces add at the end in small bracckets ULR address and date of access, eg. (Accessed in Sep 2007 at www.medf.kg.ac.yu). If available, instead of ULR cite DOI code e.g. (doi: 10.1111/j.1442-2042 .2007.01834.x)

TABLES

Tables should be typed on separate sheets with table numbers (Arabic) and title above the table and explanatory notes, if any, below the table.

FIGURES AND FIGURE LEGENDS

All illustrations (photographs, graphs, diagrams) will be considered as figures, and numbered consecutively in Arabic numerals. The number of figures included should be the least required to convey the message of the paper, and no figure should duplicate the data presented in the tables or text. Figures should not have titles. Letters, numerals and symbols must be clear, in proportion to each other, and large enough to be readable when reduced for publication. Figures should be submitted as near to their printed size as possible. Figures are reproduced in one of the following width sizes: 8 cm, 12 cm or 17 cm, and with a maximal length of 20 cm. Legends for figures should be given on separate pages.

If magnification is significant (photomicrographs) it should be indicated by a calibration bar on the print, not by a magnification factor in the figure legend. The length of the bar should be indicated on the figure or in the figure legend.

Two complete sets of high quality unmounted glossy prints should be submitted in two separate envelopes, and shielded by an appropriate cardboard. The backs of single or grouped illustrations (plates) should bear the first authors last name, figure number, and an arrow indicating the top. This information should be penciled in lightly or placed on a typed self-adhesive label in order to prevent marking the front surface of the illustration.

Photographs of identifiable patients must be accompanied by written permission from the patient.

For figures published previously the original source should be acknowledged, and written permission from the copyright holder to reproduce it submitted.

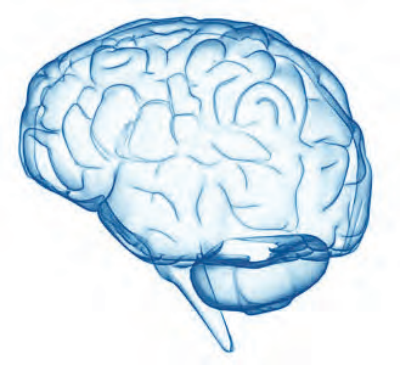
Color prints are available by request at the authors expense.

LETTERS TO THE EDITOR

Both letters concerning and those not concerning the articles that have been published in Serbian Journal of Experimental and Clinical Research will be considered for publication. They may contain one table or figure and up to five references.

PROOFS

All manuscripts will be carefully revised by the publisher desk editor. Only in case of extensive corrections will the manuscript be returned to the authors for final approval. In order to speed up publication no proof will be sent to the authors, but will be read by the editor and the desk editor.



CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

61

SERBIAN Journal of Experimental and Clinical Research
editor-in-chief Vladimir Jakovljević.
- Vol. 9, N° 1 (April 2008) -
- Kragujevac (Svetozara Markovića 69) :
Medical Faculty, 2008 - (Kragujevac : Medical Faculty). - 29 cm

Je nastavak: Medicus (Kragujevac) = ISSN 1450-7994
ISSN 1820-8665 = Serbian Journal of
Experimental and Clinical Research
COBISS.SR-ID 149695244

FACULTY OF MEDICAL SCIENCES
Svetozara Markovica 69, 34000 Kragujevac, SERBIA
P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
e-mail: sjecr@medf.kg.ac.rs

www.medf.kg.ac.rs