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REVIEW PAPER

RHEUMATOID ARTHRITIS: A NOVEL APPROACH IN DIAGNOSIS AND TREATMENT

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ABSTRACT

The rheumatoid arthritis is chronic disease with progressive course and deteriorations of joints as well as other organs. The pathogenesis of rheumatoid arthritis is characterized with chronic synovitis and inflammation. The main roles in development of rheumatoid arthritis have auto-reactive T cells and inflammatory cytokines, especially tumor necrosis factor a, interleukin 1 and interleukin 6. The management of rheumatoid arthritis has evolved significantly in the past twenty years, especially with introduction new diagnostic criteria by European League for Rheumatoid Arthritis which are very sensitive for early arthritis. The main goal of treating rheumatoid arthritis is to start with therapy in the phase of the disease when destruction of joints can still be prevented. Therapeutic strategies for rheumatoid arthritis involve wide palette of different drugs which can be divided into conventional and biological Disease Modifying Antirheumatic Drugs. The use of methotrexate in combination with biological drugs provide targeting not only structural changes in rheumatoid arthritis but also and immunological pathways in development of rheumatoid arthritis. These drugs synergistically provide clinical remission and low activity of rheumatoid arthritis in the majority of patients. The uses of biological drugs are limited due their high costs or safety profile. In order to reduce costs and toxicity in the treatment of rheumatoid arthritis, new treat-to-target concept is established. The new class of drugs which modulate signal pathways and activity of tyrosine kinase are under investigations in post marketing surveys in patients with rheumatoid arthritis as in efficacy as in safety issues.

***Keywords**: Rheumatoid arthritis, conventional Disease, Modifying Antirheumatic Drugs, biological Disease Modifying Antirheumatic Drugs.*

INTRODUCTION

Rheumatoid arthritis (RA) is a serious chronic disease which is characterized by persistent synovitis, systemic inflammation, and resence of autoantibodies (particularly to rheumatoid factor and pcitrullinated peptide) (1, 2). Epidemiological data indicate that RA is one of the most prevalent chronic diseases with inflammatory genesis (3). The primary targets in development of pathological processes in RA are joints but prevalence of extraarticular manifestations in RA patients is high too which lead to increased level of complications and comorbidities and reducing quality of life in RA patients (4-6). The introduction of biological drugs, especially those which target tumor necrosis factor-alpha have changed the management of RA and in the past decade initiated a novel approach in diagnosis and therapy of RA (1). In this review, we will evaluate the novel approach in diagnosis and therapy in patients with RA.

Epidemiology and clinical manifestations of rheumatoid arthritis

The prevalence of RA is 1% among general population, mostly involving people during their economically productive part of life (3). The prevalence of rheumatoid arthritis is 3 to 4 times higher among the women with rising tendency with aging (4-5). The onset of disease can be acute, but more often RA develops gradually with progressive course, which leads to structural changes of affected joints and surrounding tissues decreasing their functional ability and reducing quality of life of patients with RA (6). Mortality rate and morbidity due to RA are higher than in general population, since patients with RA have increased susceptibility to cardiovascular diseases, lymphomas and other extra-articular manifestations of RA (7). Extra-articular manifestations of RA are presented in 40% of patients with RA and they increase mortality for 3 to 4 times compared to patients without extra-articular complications.

Etiology and pathogenesis of RA

The etiology of RA is partly known (8). Genetic predisposition has an important role in pathogenesis of rheumatoid arthritis. Recent studies have shown that microbiological agents as Parvo viruses could lead to polyarthritis and development of RA, but in small percent of patients (2, 7%). The other etiological factors are obesity, smoking, heat shock proteins and presence of rheumatoid factor (RF) and/or antibodies against cycled citrullinated peptide (ACCP antibodies). (9, 10). RF is an anti-body (IgM and IgA) which binds to Fc portion of IgG and ACCP antibodies are autoantibodies which are directed to citrullinated peptides. (9).

The primary pathological process in RA mainly affects cells of synovial membrane and cartilage. In pathological circumstances which dominate in the pathogenesis of RA, cells of synovial membrane, fibroblast synovial cells and macrophages like synovial cells lose protecting role and transform into joint destroying cells which lead to conditions for development of increased production of pro-inflammatory cytokines (1, 4, 11, 12). The inflammation process on joints in RA developed due to increased influx of immune cells, especially macrophages, dendritic cells, lymphocytes, neutrophiles, and mastocytes which lead to hyperplasia of cells of synovial membrane (13).

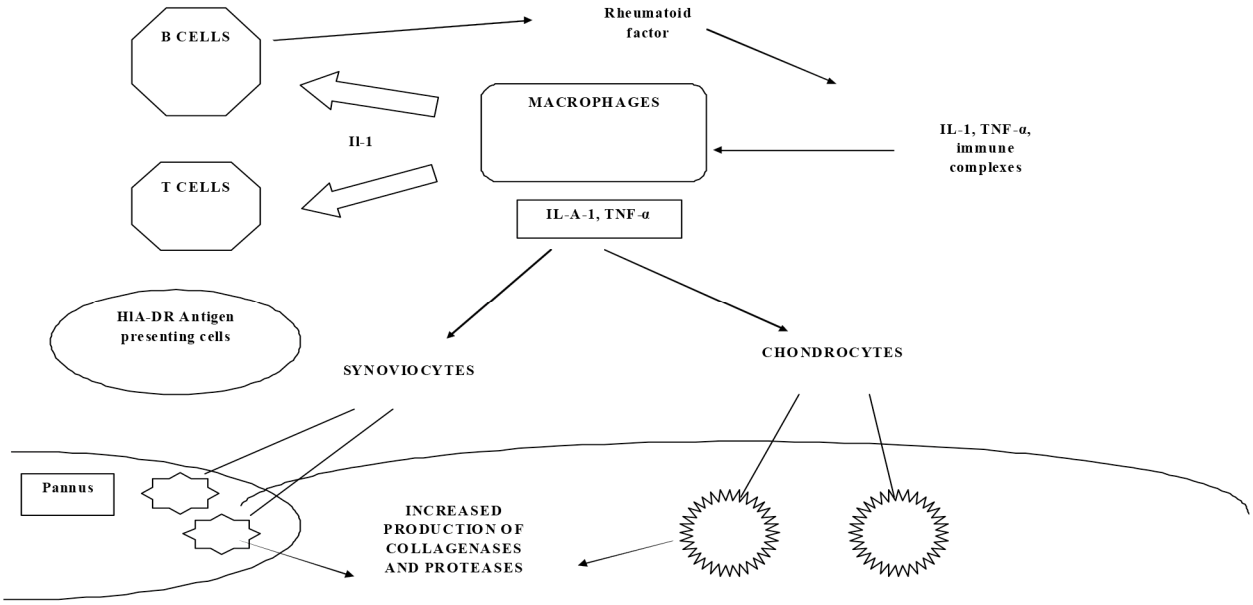
On the surface of inflamed joints, fibroblasts and mononuclear cells contribute to pannus formation, another characteristic of RA which has a significant proteolytic activity and implicates further damage of local tissue (2). Angiogenesis has a substantial role in the pathogenesis of RA too, especially in exacerbation of inflammation process. Cytokines: tumor necrosis factor-alpha (TNF-α) and interleukin 1, 6, and 8 (IL-1, IL-6, IL-8) can be powerful inducers or blockers of angiogenesis. TNF-α, IL-1, IL-6 may increase directly angiogenic activity or they can modulate vascular endothelial growth factor (VEGF)-dependent pathways. TNF-α induces the process of neovascularisation by itself and also trough capillary formulation in process via VEGF activity. IL-6 also enhances the angiogenesis process due to initiation of VEGF production. IL-8 is involved in process of induced activity of transformed synovial fibroblast which leads to induction of angiogenesis due VEGF pathways. In the presence of blockers of these cytokines the level of angiogenesis is decreased. Most of these mediators are targets for biological drugs used in the treatment of RA (1, 14).

The increased influxes of immune cells in the pathogenesis of RA are regulated by: TNF-α, IL-1 and IL-6 (13). TNF-α has a dominant role in profoundness of inflammation process on synovial membranes and in deterioration of affected joints in RA. The production of these cytokines is result of interaction between antigen presentating cells and CD4-T cells. Antigen presenting cells expose complexes of class II major histocompatibility compex molecules and peptides antigens which induce binding with receptors on T cells. The following step is activation of macrophages with secretion of proinflammatory cytokines (4).

The immune cells and their migration into affected joints in patients with RA can lead to impairment of bone and cartilage tissue around affected joints, not only by cytokine- mediated processes, but also due to effects of metalloproteinases (MMPs) and aggrecanases originating from bone matrix which disturb the balance between anabolic and catabolic processes in the bone tissue. MMPs, especially MMP-s 7 in injured cells cleaves ectodomain of syndecan-1 which induce releasing of chemokine named KC which than increase influx of inflammatory cells. (16)

Activated fibroblast with accumulated T-cells and B-cells, macrophages and monocytes enhance production of osteoclasts via promotion of receptor activator of nuclear factor κ B ligand (RANKL) T-cells and B-cells and via RANK receptor on macrophages, dendritic cells and proosteoclasts (17).

Figure l. The mechanism of developing synovitis in rheumatoid arthritis



These newly derived osteoclasts have crucial role in process of bone resorption which leads to progression of inflammatory process of RA in deeper structures of bone (16).

Clinical manifestations in RA

Since that in RA crucial processes on the synovial membrane are chronic inflammation and its complications, clinically in patients with RA dominate pain, stiffness, joint swelling, movement difficulty, but also systemic inflammatory changes such as fever, fatigue, anemia, as well as changes in laboratory parameters: elevated erythrocyte sedimentation rate, elevated reactive protein C (CRP), hypergammaglobulinemia and elevated levels of various auto antibodies (18). Clinical manifestations of RA are the result of intense inflammation process on joints and it is presented mainly as fluctuated swelling of joints primarily on hand and wrist joints, joints of the feet, or on the larger joints such as joints of the cervical spine, shoulders, knees and hips which result with different level of disability in patients with RA (2). In patients with RA special concern is needed due to extraarticular manifestations of this dis-ease, especially in patients with poor response on therapy. Extra-articular manifestations include multiorgan diseases as pulmonary, ocular, vascular, cardiac, neurological and cutaneous are. These extraarticular manifestations in-volve the presence of rheumatoid noduli on different organs or developing inflammation processes on different organs in patients with RA. The special concern in patients with RA are needed due to higher incidence of comorbidities on cardiovascular system (heart failure, myocardial infarction, stroke, hypertension), higher incendence of cancer (lymphoma, lymphoproliferative disease, lung cancer, skin cancer), infections and other diseases (depression, osteoporosis, psoriasis, etc) (2, 4).

Diagnostic criteria for rheumatoid arthritis

In 1978, American College of Rheumatology (ACR) established criteria for diagnosis of RA. According to these criteria during clinical assessment, four of the following criteria must be presented in patients: morning stiffness which lasts at least one hour, arthritis with edema in three or more joints confirmed by specialist, arthritis on joints of a hand (with edema more than in one joint), symmetric arthritis, rheumatoid skin noduli, positive value of laboratory tests for rheumatoid factor, radiologically confirmed typical findings for RA. All criteria, except the last two last, must last at least six weeks in moment of clinical assessment of RA (19-21).

In 2010, European League for Rheumatoid Arthritis (EULAR) has recommended an amendment of ACR criteria for RA, since these criteria lack in sensitivity for early phase of RA. According to this amendment, for diagnosis of RA, a new scale is established where score should be six or more than six, with included criteria such as involvement of the joints, the positive values of serological analysis, the presence of elevated levels of laboratory tests C reactive protein and erythrocyte sedimentation and duration of symptoms (21). These recommendations are especially related to newly diagnosed patients with RA with clinical presentation with synovitis and edema within one joint and in patients with synovitis where etiology is not determined (21).

Disease activity score (DAS) was designed in 1983, for purpose of improvement of former criteria for measuring activity of RA-index of RA. Nowadays DAS is a gold standard for estimating activity of RA, with values of low and high activity of RA. This score is the result of formula which obtains number of joints with edema (with examination of 28 joints: joints of shoulders, elbows, wrists, knees,

metacarpophalangeal joints and proximal interphalangeal joints), value of blood sedimentation and general health state of patient according to visual analogue scale (VAS) (22, 23). The range of DAS 28 is if value of disease activity score is higher from 5, 1, then RA has high activity, value of DAS 28 between 3, 2 and 5, 1 RA has moderate activity and RA has low activity if the value of DAS 28 is between 2, 6 and 3, 2. EULAR has developed criteria for estimating RA patients' response on therapy which are based on DAS 28 criteria. Patient's response to therapy can be considered good if change of value of DAS28 is significant and disease activity is low. According to these EULAR criteria, there are three pat-terns of response to therapy among patients with RA: good response, moderate response and no response to therapy. Decreasing of value of DAS 28 for 0, 6 indicates that patient with RA has no response to therapy, while decreasing of value of DAS 28 for 1, 2 and more indicates on moderate and good response. If value of DAS 28 is less than 2, 6, then patients with RA are in remission (24).

Rheumatoid arthritis decreases the ability of patients for management of everyday activity, which leads to a decrease of quality of these patients. Health Assessment Questionnaire involves functional ability of patients in several domains: inability, pain and discomfort, adverse reactions of drugs and economic sphere of treating RA. Each domain of HAQ is assigned with a grade, summarizing patient's answers clinicians obtain from HAQ score and value they got, which can vary from zero to three, where zero represent state without disability and three represents state of full disability (25-27).

Laboratory parameters in patients with rheumatoid arthritis can indicate presence and course of inflammation process and can be useful for assessment of development of RA and for monitoring of patients' response to therapy. Increased values of erythrocyte sedimentation rate, level of fibrinogen, C reactive protein and rheumatoid factor are repercussion of induced effects of TNF-α, IL-1, IL-6 partly on immune cells, partly on liver (28). The more significance antibodies for RA are those directed against cycled citrullinated peptides (ACCP antibodies). ACCP antibodies are more specific for patients with RA and their presence is better indicator for poor response on therapy and progressive joint deteriorations. The results of recent clinical studies indicate that in synovial tissue of ACCP antibodies positive RA patients dominate lymphocytes and in ACCP antibodies negative RA patients synovial membrane are changed due to fibrosis. Circulating ACCP antibodies can indicate on prerheumatoid arthritis, since it can be detected in patients with RA 10 years before diagnosis. The presence of ACCP antibodies indicate on increased joint deteriorations and on low remmision rate. The values of ACCP antibodies and RF decrease due to effects of therapy, but patients with RA rarely became ACCP antibodies negative comparing to RF whereas seropositive RF patients more frequently convert to seronegative RF patients (4, 10).

Therapeutic strategies in patients with rheumatoid arthritis

The main aim of therapy in rheumatoid arthritis is to prevent spreading of chronic inflammation process and to ensure protection of deteriorated joints from further dam-ages (15, 29).

The idea of early introduction of therapy in patients with RA was substantial for better management of RA, but the most crucial step in historical development of therapy for RA was introduction of biological drugs (30-33).

Modern concept of treatment of rheumatoid arthritis involves achieving a state of remission in patients with-out evidence of inflammation and joint damage. In broad terms, the goals of therapy can include reduction in dis-ease activity, reduction of pain, maintenance of functional status of the joints and preservation of working ability, but also the ability for daily activities of patients. Since the nature and course of molecular mechanisms responsible for symptoms of synovitis in chronic inflammation are different from the mechanism which is responsible for the structural deterioration of joints, therapy of rheumatoid arthritis should affect both pathophysiologal processes (15).

Disease-modifying antirheumatic drugs in therapy of rheumatoid arthritis

"Go low go slow" concept based on use of physical therapy, non pharmacological treatment and low doses of non steroidal anti-inflammatory drugs (NSAIL) was abandoned during the eighties of the last century. Despite the use of NSAIL in patients with RA which provides reduction in symptoms, recommendations for treatment early phase of RA indicate that Disease-modifying antirheumatic drugs (DMARD-s) that affect the course of the disease should be the first choice treatment (35-37). DMARD-s are administered mainly orally and in lower doses they provide anti-inflammatory effects, prevent further deteriorations of affected joints and their surrounding tissues so they can be used for management of RA for longer period. Disease modifying antirheumatic drugs include two major classes of drugs: synthetic and biological drugs. Further synthetic DMARD-s can be divided into two groups of drugs: conventional synthetic DMARD-s and targeted synthetic DMARD-s. The targeted synthetic drugs, like tofacitinib and baricitinib are janus kinase inhibitors which can modify the specific reaction in propagation of inflammation. Conventional synthetic DMARD-s were introduced in the treatment of RA trough positive experience but their mode of action in RA has still been unclear (38).

The earlier use of conventional synthetic DMARD-s provides better control of disease activity in patients with RA and improves effects of combination of conventional synthetic DMARD-s and biological drugs. Conventional synthetic DMARD-s applied in patients with RA decrease swelling of joints, reduce pain, lower the parameter values of acute

phase of inflammation and improve joint function (21, 39-45).

Conventional synthetic DMARD-s

Conventional synthetic DMARD-s include the broad spectrum of drugs: metotrexate, glucocorticoids, sulfasalazine, leflunomide, hydroxychloroquine, gold therapy.

EULAR criteria for management of RA with synthetic DMARD-s and biological drugs recommend that therapy of RA should be initiated with methotrexate with a low dose of glucocorticoides (46).

Methotrexate (MTX) is a gold standard in the therapy of RA which has been used in the last 25 years. The mechanism of action of MTX is directed on dihydrofolate reductase which MTX competitively and irreversibly inhibits with disabling conversion of dihydrofolate in tetrahydro-folate. By this step, MTX inhibits synthesis of DNA, RNA and proteins in gastrointestinal, medullar and neoplastic cells. The potential anti-inflammatory action of MTX can be explained by its blocking of thymidylade synthase which increases intra and extracellular adenosine activity. By these mechanisms, only part of anti-inflammatory action of MTX as its final effects on decreasing of proinflammatory mediators: TNF-α, IL-1, IL-6, metalloproteinases, prostaglandins and adhesion molecules can be explained (47). The dose regimen for MTX in patients with RA for oral or parenteral administration varies from 7.5 to 25 mg per week. The results of numerous studies have shown that the adequate use of MTX in patients with RA provides improvement as in preservation of functional ability of affected joints as well as in prevention of further structural damages (39, 47).

Despite these facts, the effects of MTX in patients with RA may fail due to toxicity or inefficacy. According to guidelines, use of MTX in patients with RA should be followed with associated administration of folic acid (5-15mg per week) or folinic acid (leucovorin in dose 27, 5 mg per week) due to decreasing adverse events (48). The most frequent adverse events are gastrointestinal ones with mild clinical presentations as dyspepsia, nausea, vomiting and abdominal pain. Rarely, severe clinical presentation as hepatic, pulmonary, haematologic, neurological, cutaneous and infectious adverse events can occur during MTX administration. Among hepatic adverse events in patients with MTX therapy, hepatic fibrosis and cirrhosis are most frequent. In order to prevent these adverse effects, special monitoring of liver enzymes (transaminases, gamma-glutamyl transferase, and alkaline phosphatase) is recommended and special concern is needed if these enzymes are higher two or more than two times in patients with MTX therapy as decreasing the dose of MTX and discontinuation of therapy are (47, 48). Chest X-ray should be performed at the beginning and during MTX therapy due to evaluated pulmonary toxicity induced by MTX (interstitial pneumonitis, pulmonary fibrosis, and non-cardiogenic pulmonary oedema). In the interest of prevention of hematological adverse events, patients with MTX therapy should undergo the tests of blood cell counts. Infectious diseases are more common in patients undergoing MTX therapy. Among neurological adverse events due to MTX toxicity headache, dizziness or impairments of speech, vision or cognition are more often. It is recommended that patients with RA treated with MTX should undergo frequent medical testing and supervisions, not only by rheumatologists but also by other specialists. The supplementation with folic acid is another preventive measure for reducing of MTX induced toxicity (48-52). MTX therapy is X category according to Food and Drug Administration and it is not recommended during pregnancy; the treatment with MTX should be determined 1 to 3 months be-fore conception (53).

MTX is indicated as monotherapy or in combination with other drugs in the treatment of RA. (45). The glucocorticoides are indicated as in the early phase of RA as in RA with developed extra-articular manifestations of RA. The dose regimen varies from use of small doses (< 7, 5 mg prednisone or equivalent per day) in the first six months of treatment, use of average doses (10-30 mg per day), use of large doses (>30mg per day) and use of pulse therapy with a dose higher than 250 mg of methylprednisolone per day via infusion (54, 55). The main disadvantages of use of glucocorticoides are adverse drug reactions of this group of drugs, which can be divided into two groups: prevent-able and non preventable (47). Preventable adverse drug reactions include wide range of impairments: heart failure, hypertension, peptic ulcer, diabetes mellitus, osteoporosis, myopathy, insomnia and mood disturbance. Considering that facts that pathological processes in RA affect bone too, osteoporosis induced by glucocorticoides remains the most endangering adverse drug reaction due to its decreasing effects on the process of bone formation and increasing effects on the bone resorption. Non-preventable adverse reactions involve infections, cataract, cutaneous modifications, accelerated atherosclerosis, and weight gain (54). For this reasons, crucial recommendations regarding the use of glucocorticoides in RA are that their use should be favorable in settings where their benefit exceeds risks: short term systemic use during relapse of RA, where their effects lead to rapid improvement and local use whenever it's possible (55-57).

Hydroxychloroquine and chloroquine are antimalarial drugs with antirheumatic activity which is directed to lysosomal membranes. These drugs change pH activity of lysosomes and stabilize the membranes of these cell structures which leads to suppression of immune response and activity of cells with dominant role in inflammation process: T cells and granulocytes and their migration. These drugs are mainly used in combinations as triple therapy with MTX and sulfasalazine, since their use as monotherapy has moderate efficacy in patients with RA. The use of these drugs is related to adverse reactions mostly with mild clinical presentations: nausea, anorexia, rash, photosensitivity, while those with severe clinical outcome (irreversible retinopathy) are rare (58, 59).

Sulfasalazine is pro drug, which is transformed by bacteria in colon into active forms of sulfapyridine and 5-amino salicylic acid. Anti-inflammatory activity of sulfasala-zine is related to reduction of the secretion of TNF-α, IL-1 and IL-6, decreasing production of immunoglobulin G and rheumatoid factor by B lymphocytes and to inhibition of T lymphocytes. The dose regimen of sulfasalazine in patients of RA is 2-3 g/day, as monotherapy as in combination with conventional or biological DMARD-s (50, 60-63). The side effects of sulfasalsalazine have mild clinical presentations: nausea, dyspepsia, anorexia, mild damage of liver, rash, itching, photosensitivity, anxiety, headaches and sleep disturbance. The multiform erythema and serious hematological adverse effects as lymphopenia, neutropenia and agranulocytosis are rare during sulfasalazine treatment in patients with RA (63). Treatment of RA during pregnancy with sulfasalazine is mostly safe, but due to its' concentrating in milk, special concern is needed during breastfeeding (64).

Leflunomide is another pro-drug which is converted after first hepatic passage in the submucosal intestinal into active form and that leads to inhibition of dihydroorotate dehydrogenase and decreasing synthesis of enzymes in-volved in pyrimidine synthesis and further to diminishing of activity of tyrosine kinase and nuclear factor (NF)-kB activation. The usual dose for leflunominde is 20 mg per day and it is effective in treatment of RA, as monotherapy as in association with other DMARD-s. Leflunomide is used as effective treatment both in early and late stage of RA leading to improvement, remission and prevention of further structural deterioration. The safety profile of leflunomide is similar of those of MTX (65, 66).

In the treatment of RA, the gold therapy can be used too, since they inhibit cytokine production and decrease the level of mediators of inflammation which leads to suppression of macrophages. But, despite its efficacy, the gold therapy is only used if patients haven't responded adequately to previous treatment, since the presence of its significant adverse reactions (34, 67).

Biological DMARD-s

Since the crucial role of immunopathogenic pathway in development of RA, biological therapy has demonstrated efficacy in prevention as well as in functional and structural deteriorations in patients with RA. Biological therapy in treatment of RA is directed to cytokines such TNF-α, Il-1, IL-6 and these groups of drugs are defined as cytokines inhibitors. The other group of biological drugs, non-cytokines agents are involved via their' mechanism of action in pathways of T-cell co-stimulation blockade, and B-cell depletion and on non-cytokine as CD-20 receptor on B cells (68-70).

The blockade of TNF-α is a potent mechanism of action which is directed to cytokine with a pivotal role in the pathogenesis of RA. These drugs can be administered intravenously (infliximab) or subcutaneously (adalimumab, etanercept, golimumab and certolizumab pegol). Among TNF blockers, the structure of etanercept matches with the structure of TNF receptor, while other drugs operate as monoclonal antibody or its part (40, 48).

Etanercept is a recombinant form of the soluble human

TNF receptor, which binds specifically to circulating TNF-α and prevents further proinflammatory role of TNF-α in RA. The use of etanercept in the treatment of RA is indicated in presence of a moderately severe or severe form of RA where patients have previously responded inadequately to conventional DMARD-s. The usual dose regimen is 50 mg per week (40, 71, 72).

Adalimumab is human IG-1 monoclonal anti TNF-α antibody, which targets specifically TNF, blocking further interactions with TNF receptor and interfering with reactions which are mediated by this mediator. Adalimumab is indicated as subcutaneous injection in dose of 40 mg every other week in patients with a moderately severe or severe form of RA who have previously an inadequate response to conventional DMARD-s (71, 72).

The other fully human monoclonal antibody which binds TNF-α is golimumab with usual dose regimen of 50 mg per month. Certolizumab is derived from human monoclonal anti TNF-α antibody and it contains only Fab

fragment which is covered with polyethylene glycol. Certolizumab is used every two weeks in dose of 200 mg (40, 48).

Infliximab is a chimeric monoclonal antibody which is consisted of parts of human IG1 and variable murine Fv regions. It is the only TNF-α blocker for intravenous ad-ministration with dose regimen of 3-10 mg / kg every 4-8 week (40, 48).

The safety profile of biological drugs encompasses increased level of infections, cancer, demyelinating disease, deterioration of heart disease, allergic reactions and production of anti drug antibodies and anti ds- DNA antibodies (73, 74).

Tocilizumab is biological drug with anti IL-6 effects. It is administered via intravenous infusion in dose of 8 mg/kg once a month. Tocilizumab is used as monotherapy or in combination with other cDMARD-s in patients who didn't respond to the pervious treatment with MTX or anti-TNF therapy. During therapy with tocilizumab, adverse reactions as infections, diverticulis, dyslipidemia, liver damage and neutropenia can occur (75, 76).

Abatacept is the first biological drug whose mechanism of action is involved into modulation of T lymphocyte activity. Abatacept binds to CD80/86 and CD 28 costimulatory factors and via these mechanisms, it decreases activity of T lymphocytes. Abatacept is administered via intravenous infusion in dose regimen of 500-1000mg (at weeks 0, 2, 4 and then once monthly) or as subcutaneous injection in dose of

125 mg per week. The most frequent adverse reactions are increased risk of infections, reduced positive response to vaccines and infusion related reactions. (76-79).

Rituximab is a chimeric monoclonal antibody whose mechanism of action is directed at CD 20. It is administered intravenously in dose of 1000 mg every 6 months who are unresponsive to TNF-blockers. The use of rituximab is indicated in patients with RA who are unresponsive to TNF-blockers. The safety profile of rituximab is more favorable than in other biological drugs especially in patients with RA who have concomitant diseases: multiple sclerosis, lymphoma or latent tuberculosis with contraindications of prophylaxis with isoniasid whereas other biological drugs should be avoided since they can provoke these diseases (40, 45, 80-82).

The use of biological therapy during pregnancy is disputed, results of recent studies indicate that use of TNF inhibitors does not correlate with conception or teratogenic risk, similar data have been published for tocilizumab and abatacept (83, 84).

Review of effectiveness of different therapeutic strategies in RA

The results of clinical studies which compare MTX and glucocorticoides and MTX and biological drugs in patients with RA haven't shown significant difference in outcomes. In patients with RA, who were treated with glucocorticoides in low doses in combination with MTX better structural protection of affected joints was provided than in the group with monotherapy with MTX (85). After six months of therapy, the dose of glucocorticoides should be gradually decreased and stopped when DMARD-s achieved full effects (85, 86). The clinical outcomes in patients with RA, in studies which compare MTX, sulfasalazine and leflunomide were similar, but MTX remains the core of therapy of RA especially because it optimizes effects of further therapy with biological drugs. In the numerous clinical studies triple therapy for RA (MTX, sulfasalazine and hydroxychloroquine) was compared to monotherapy with MTX, but greater efficacy of triple therapy remains "blured" since in that arm of study glucocorticoides were applied in higher doses. Further results of clinical randomised studies where lower dose of glucocorticoides was administered in triple-and in mono-therapy group have shown that no significant advantage was detected in the group with triple therapy, but only higher costs and more adverse effects (86, 87). According to ACR guidelines, combination of cDMARD-s is not recommended as early first line therapy in patients with RA due to limitations of numerous clinical studies which investigate use of these kind of therapeutic strategy in RA. But, in patients with RA with low risk of progressive disease and with poor response on MTX adding of other cDMARD-s or switching on other cDMARD-s may be optimal treatment (88, 89). EULAR provides guidelines for treating patients with high disease activity, high values of autoantibodies and RF and early joint damages on radiography; according to these recommendations patients with these characteristics should be switched to the biological therapy in combination with cDMARD-s and glucocortiocides (45).

The results of numerous clinical studies have shown benefits of biological treatment in patients with RA. MTX in combination with TNF inhibitors or with tocilizumab provide clinical remission in 30-60% of RA patients (30). The biological DMARD-s accomplishe more significant effects in patients with RA if they are in combination with MTX or other cDMARD-s, especially leflunomide. The results of recent studies indicate that tocilzumab should be biological drug of choice in settings where patients with RA due to ineffectiveness or intolerance of MTX. In patients with RA, tocilizumab has shown better outcomes in functional as well as in structural changes, compared to monotherapy with TNF inhibitors or monotherapy with MTX (90-92).

Review of the cost effectiveness analyses of different therapeutic strategies in RA

Due to their high costs, prescription of biological drugs is limited with special set of demands and only in patients who previously failed with two drugs from DMARD-s including MTX (92, 94). The results of cost effectiveness studies which compare different TNF- inhibitors are mainly given by socio economic conditions and health policy of country where study was performed. Kobelt et al. have shown that infliximab were cost effective for rheumatoid arthritis in economic settings of Sweden and Great Britain, from societal perspective, since in the infliximab group direct and indirect costs were reduced and incremental cost effectiveness ratios (ICER) were close to threshold. The similar findings were in study conducted by Bansback et al which was performed in Sweden. TNF inhibitors have favorable cost effectiveness ratio in case where threshold was estimated from 50 000 to 100 000 €/ QALY, and if threshold is 35000 €/QALY rituximab was found to be the most cost-effective alternative compared to other biologics among the patients with an insufficient response to TNF inhibitors (95). In our country, socio economic settings are different, the costs of the biological drugs are high as in other countries, while the costs of medical services are significantly lower than in other countries, which leads that biological drugs, tocilizumab is not cost effective for treatment of RA compared do cDMARD-s (92, 93).

Targeted synthetic DMARD-s

The size of bDMARD-s (90000-150000 Dalton) provides that their mechanism of action is directed only at cytokines and molecules which are part of cell membrane. Orally available cDMARD-s have lower molecular weight and interfere with structure positioned in cytoplasm and directly regulate intracellular signal pathways. The process of phosporylation of kinase proteins especially of Janus kinase mediates processes of cell proliferation, differentiation and adhesion which are crucial for development of RA. Janus kinase (JAK) family involves homodimer or heterodimer of Jak1, Jak2, Jak3 and tyrosine kinase 2 (Tyk 2) (96).

Tofacitinib is the first orally approved targeted synthetic DMARD which inhibit family of Jak kinase. Tofacitinib in combination with MTX, in dose of 5 mg twice a day, has shown similar efficacy as biological therapy in patients with RA. In contrast to most biological drugs, tofacitinib was superior to MTX in patients with RA. Tofacitinib has been approved only in USA and other countries, but not yet in the European Union. The side effects of tofacitinib involve nasopharyngitis, elevation of transaminase and level of creatine, increase of total cholesterol, neutropenia and anemia (96-98).

The inhibition of pan-JAK is a promising new mechanism which induces production of palette of new orally drugs: baricitinib, decernotinib, peficitinib and filgotinib with expectations that they would be valuable therapy with cDMARD-s and bDMARD-s not only in RA, but also in other autoimmune disease (96).

CONCLUSIONS

The primary goal of therapy of RA is to achieve clinical remission and to prevent further structural and functional deterioration of affected joints. Early diagnosis and induction of DMARD-s are crucial for maintaining remission and prevention of complications of RA. The new concept in treatment of RA includes treat-to-target principal which provoke that treatment should last while remaining course of disease. The core of this principal encompasses selection of drug with high efficacy and low rate of hazards, dose reduction of drugs in a remission phase and even therapy discontinuation especially for bDMARD-s in patients with RA (46).

The selection of therapeutic strategies for RA due to all these reasons may be a challenge for clinical practitioners and should be based on clinical guidelines and recommendations which summarize global evidences of the highest level, but also on individual characteristics of patients (29).

The targeted synthetic DMARD-s are new therapeutic strategy for RA but its well defined position among other available therapeutic strategies should be investigated in efficacy aspects as well as in safety aspects (40).

COMPETING INTERESTS

There are no conflicts of interest.

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ORIGINAL SCIENTIFIC ARTICLE

THE ROLE OF PHOSPHOCREATINE IN THE PERCONDITIONING AND POSTCONDITIONING OF ISOLATED RAT HEART

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ABSTRACT

The present study strives to assess the cardioprotective role of phosphocreatine as an agent for postconditioning and perconditioning of isolated rat heart.
Rat hearts (n=30) were perfused with a Langendorff apparatus and randomly assigned to three groups subjected to 20 minutes of global ischemia and 30 minutes of reperfusion: control group (untreated rat hearts), postconditioning group (hearts treated with 0.2 mmol/l of phosphocreatine during the first 5 minutes of reperfusion), and perconditioning group (hearts treated with 0.2 mmol/l of phosphocreatine during the first 5 minutes of ischemia). During the experimental protocol, cardiodynamic parameters were evaluated, while oxidative stress parameters such as superoxide anion radical, hydrogen peroxide, nitrites and index of lipid peroxidation were determined in coronary venous effluent.
Postconditioning and perconditioning with phosphocreatine improved contractile function, heart rate and coronary flow, while the examined oxidative stress parameters in coronary venous effluent were significantly reduced in groups of treated rat hearts.
The results of this study indicate that phosphocreatine has the potential as a therapeutic agent for perconditioning and postconditioning the heart in ischemia reperfusion injury.

Keywords: *Langendorff apparatus, creatin phosphate, cardioprotection, oxidative stress.*

INTRODUCTION

A major problem of the health system around the world is the high prevalence and poor prognosis of patients with heart failure. In order to start discovering new therapeutic strategies, it is crucial to fully elucidate all mechanisms involved in the development of cardiac dysfunction. Given that cardiomyocytes' energy needs are high, it is extremely important to have an adequate balance between energy produced and expended by continuous resynthesis in order to maintain an optimal contractile response of the heart muscle (1, 2). Chemical energy in the form of adenosine triphosphate (ATP) is created in mitochondria during the process of oxidative phosphorylation, and most of the substrate for energy production are fatty acids, while less than 10% is obtained from glucose, lactate, and ketone bodies (3, 4). The phosphagen system should provide the energy necessary for myocardial function through the interaction of creatine and ATP in the presence of creatine kinase. In addition to numerous studies, the precise role of high-energy phosphate metabolism in cardiovascular disease has not yet been fully elucidated. There is thought to be an association between phosphocreatine levels and the development and progression of heart disease, and this claim is supported by the results of studies conducted on both animal models and the human population (5).

During ischemia, the process of oxidative phosphorylation is disturbed, the production of ATP and the energy supply of the heart muscle are reduced. Damaged membrane potential on mitochondria and inability to undergo oxidative phosphorylation lead to deeper myocardial damage. However, available data suggest that significant cardioprotection can be achieved by accelerating the creatine kinase system in the heart (6), while the loss of creatine kinase system components can be extremely harmful in the conditions of ischemia/reperfusion (I/R). Given that, it is believed that phosphocreatine can reduce the release of heart enzymes and the part of the heart affected by a heart attack. On the other hand, exogenous administration of phosphocreatine can prevent the occurrence of potentially fatal arrhythmias after ischemia and reperfusion, which together with the aforementioned benefits can greatly improve heart recovery after ischemia (7).

Given that the research focuses on novel modalities of conditioning, it would be very interesting to examine the effects of phosphocreatine as a post- and perconditioning agent. By definition, perconditioning is the administration of a specific conditioning agent during ischemia, while postconditioning is the administration of an agent immediately after ischemia. According to the literature, the application of a protective agent during ischemia will lead to a reduction in the necrotic area and the infarct size of the affected tissue, however, the mechanisms leading to protection are different from the mechanisms involved in preconditioning and postconditioning (8).

Since there is a lack of data regarding the role of phosphocreatine in the therapy of ischemic/reperfusion injury, the aim of this study was to examine the role of phosphocreatine as an agent for postconditioning and perconditioning of isolated rat heart.

MATERIALS AND METHODS

This is an experimental study on *ex vivo* animal material (isolated rat heart). During the experimental work, the provisions of the prescribed acts (EU Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 86/609 / EES) and the principles of ethics were respected.

Animals and experimental protocol

In order to investigate the effects of phosphocreatine on I/R heart damage, healthy *Wistar albino* male rats, 8 weeks old, weighing 200-250 g, were used. Rats were raised in the vivarium of the Center for Clinical and Functional Research, Faculty of Medical Sciences, University of Kragujevac in standard laboratory conditions - air temperature 23 ± 1° C, relative humidity 50%, 12:12 hours cycle light:darkness (with at the beginning of the light period at 9:00 am) and with free (*ad libitum*) access to water and food.

Ex vivo examination of cardiac function was performed using the Langendorff model of retrograde perfusion of an isolated heart (Langendorff apparatus, Experimetria Ltd, 1062 Budapest, Hungary). Hearts of all animals (30 in total) were isolated and placed on cannula of Langendorff apparatus. After placing the heart on the device, stable cardiac work was confirmed by detecting unchanged values of coronary flow and heart function parameters after several consecutive measurements. All hearts were subjected to 20 minutes of global ischemia, followed with 30 minutes of reperfusion.

Isolated hearts were randomly divided into control (n = 10, hearts of rats with acute I/R injury) and two experimental (n = 20) groups depending on the period of application phosphocreatin; postconditioning group - phosphocreatine at a dose of 0.2 mmol/l was applied in the heart during the first 5 minutes of reperfusion, perconditioning group - phosphocreatine at the same dose was administered during first five minutes of reperfusion.

Examination of the effect of phosphocreatine on cardiodynamic parameters and coronary flow

Using the Langendorf apparatus and software followed cardiodynamic parameters were continuously monitored: dp/dt max - maximum rate of change of pressure in the left ventricle, which is expressed in mmHg/s; dp/dt min - minimum rate of pressure change in the left ventricle expressed in mmHg/s; SLVP - systolic pressure in the left ventricle expressed in mmHg; DLVP - diastolic pressure in the left ventricle expressed in mmHg; HR - heart rate expressed as beats per minute (bpm). Coronary flow (CF) was measured flowmetrically and expressed in mL of coronary venous effluent per minute. Values of cardiodynamic

parameters as well as coronary venous effluent were collected at: stabilization points (S), first (1), fifth (5), tenth (10), fifteenth (15), twentieth (20), twenty-fifth (25) and in the thirtieth (30) minute of reperfusion.

Examination of the effect of phosphocreatine on cardiac redox status

In order to examine the effects of phosphocreatine on cardiac production of prooxidans in coronary venous effluent samples, oxidative stress parameters were determined. All biochemical analyzes were performed spectrophotometrically (Specord S-600 Analytik Jena, UK).

The lipid peroxidation index was determined indirectly by measuring the products of the lipid peroxidation reaction with thiobarbituric acid, ie the levels of TBARS (Thiobarbituric Acid Reactive Substances). The spectrophotometric method is based on the determination of lipid peroxide levels based on the reaction of malonyldialdehyde with thiobarbituric acid (9).

Measurement of nitrite released levels in coronary venous effluent is a suitable method for indirect assessment of the functionality of the endothelial L-arginine, based on the use of Griess reagent (10).

The determination of the amount of superoxide anion radical (O_2^-) in coronary venous effluent and plasma is based on the reaction of O_2^- with nitro tetrazolium blue (NBT) to form nitroformasan blue (11).

The determination of H_2O_2 is based on the oxidation of phenol red by a hydrogen peroxide reaction catalyzed by the enzyme Horse Radish Peroxidase. With this method, it is possible to determine the formation and release of H_2O_2 during a time interval of 5-60 minutes (12).

Statistics

During the experimental protocol, the parameters of interest were monitored at the time of stabilization, as well as every five minutes during the thirty-minute reperfusion (points marked with 1, 5, 10, 15, 20, 25, 30). All obtained results are presented as mean ± standard deviation and are presented using graphs created in Microsoft Excel. In order to perform adequate comparison between groups and subgroups, all points of interest were also presented as percentage values in relation to the stabilization point, which was 100%.

RESULTS

The effects of different phosphocreatine conditioning modalities on cardiodynamic parameters and coronary flow of isolated heart of rats subjected to acute ischemic-reperfusion injury

Figure 1 shows the absolute values of cardiodynamic parameters (dp / dt max, dp / dt min, SLVP, DLVP, HR), as well as CF of the control and experimental groups recorded every five minutes during thirty-minute reperfusion period (points 1, 5, 10, 15, 20, 25, 30). Based on the obtained results, it is clear that the values of most of the monitored parameters were statistically significantly higher in experimental than in the control group. Only the value of DLVP in the control group was significantly higher in the first and the fifth minute of reperfusion compared to the experimental groups. In the group of hearts postconditioned with phosphocreatine, most of the monitored parameters were almost constant during the reperfusion period, except for SLVP where a decrease was observed. Similar results were obtained in perconditioning group except in HR where significant decrease during the first 5 minutes of reperfusion is observed, but during the remaining 25 minutes of reperusion the HR values gradually increased. To compare the effects of different models of phosphocreatine conditioning on cardiodynamic parameters and CF of an isolated rat heart, results were presented also as a percentage relative to stabilization (Figure 2). In perconditioning group most of the monitored parameters after thirty minutes of reperfusion have values of about 90% of the values recorded before ischemia, except for diastolic pressure (73.59%) at which a decrease was observed. More importantly, all values in the perconditioning group were significantly higher than the values obtained in the control group.

The effects of different phosphocreatine conditioning modalities on oxidative stress parameters of isolated heart of rats subjected to acute ischemic-reperfusion injury

Concentrations of prooxidants such as O_2^-, NO_2^-, H_2O_2 and TBARS were determined from coronary venous effluent collected at stabilization time (S), as well as every five minutes during thirty-minute reperfusion (points marked 1, 5, 10, 15, 20, 25, 30). However, three points of interest were considered for data analysis: the 1st, 5th, and 30th minute of reperfusion. Figure 3 shows the absolute values of these prooxidants during the ex vivo experiments. Based on these results it can be noticed that O_2^- and TBARS values were significantly higher, while NO_2^- was significantly lower in the control group, compared to experimental groups during the first minutes of reperfusion. On the other hand, at the end of reperfusion only TBARS remained higher in the control compared to the examined groups. Also, on this graph it is clear that the use of 0.2 mmol/l phosphocreatine after ischemia significantly increased the values of O_2^- and H_2O_2, compared to the group in which this agent was used during ischemia (perconditioning). Furthermore, Figure 4 shows the values in all points of interest expressed as a percentage through stabilization values of 100%. Both tested phosphocreatine conditioning maneuvers at a concentration of 0.2 mmol/l showed potential in reducing prooxidation parameters, especially O_2^-, H_2O_2, and NO_2^-. Namely, these graphs show that postconditioning with phosphocreatine led to significant fluctuations in O_2^- levels during reperfusion (67.08% in the first minute and 114.43% in the last minute of reperfusion), while the values of other measured prooxidants remained unchanged during the whole experimental protocol. On the other hand, use of phosphocreatine during the first five minutes of ischemia

significantly prevented fluctuations in monitored prooxidants, except for O_2^- levels which, after thirty minutes of reperfusion, were 27.47% higher than those measured at the time of stabilization.

Figure 1. Cardiodynamic parameters and coronary flow of the control (CTRL), postconditioning (Post) and perconditioning (Per) groups of rats, presented as absolute values (mean ± standard deviation) recorded every five minutes during the 30-minute reperfusion period. Interrupted line – control group, gray line – postconditioning group, black line – perconditioning group.

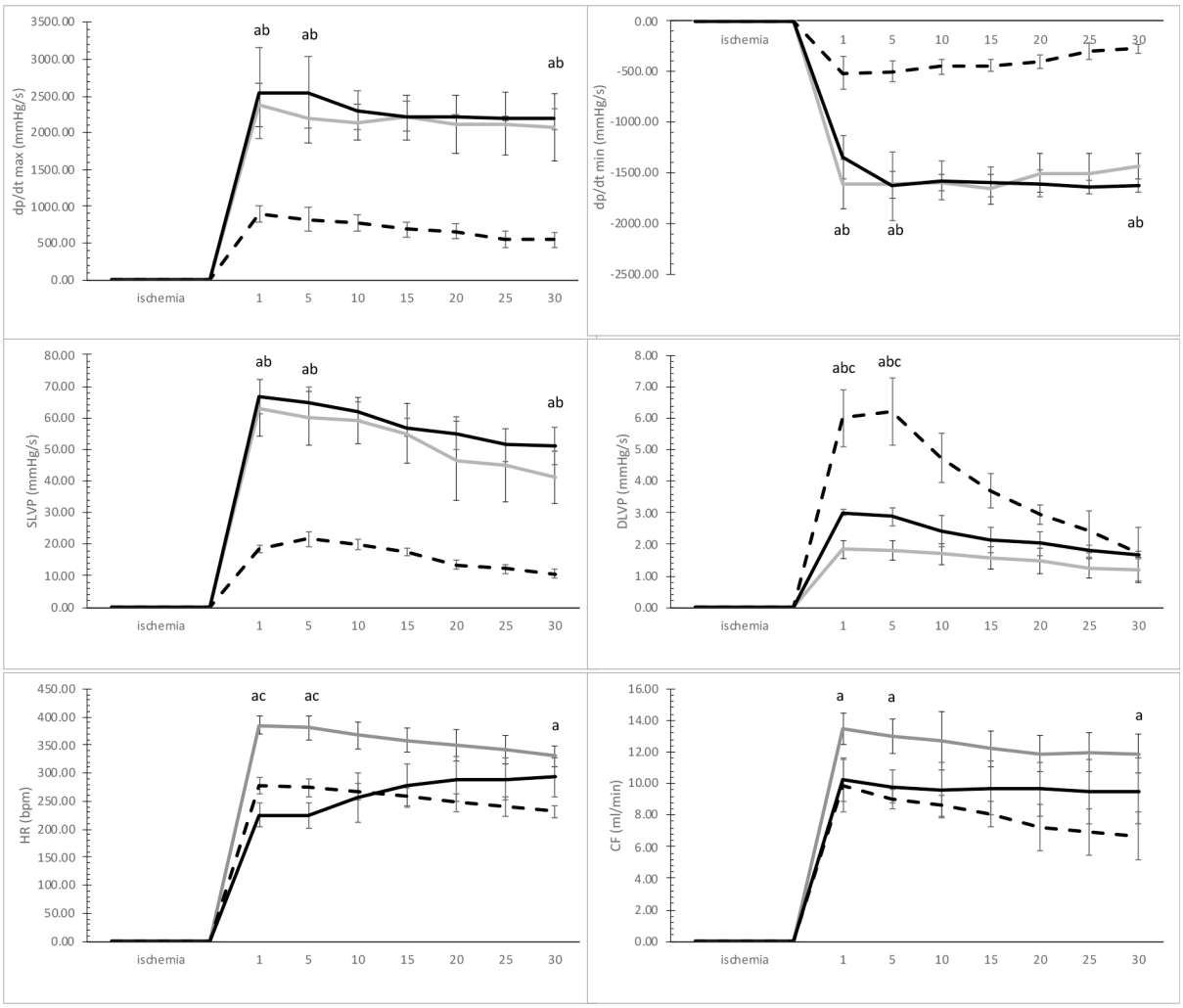


Figure 2. Values of cardiodynamic parameters and coronary flow of the control group and groups whose isolated hearts were subjected to different modalities of phosphocreatine conditioning expressed as a percentage of the stabilization point of 100%.

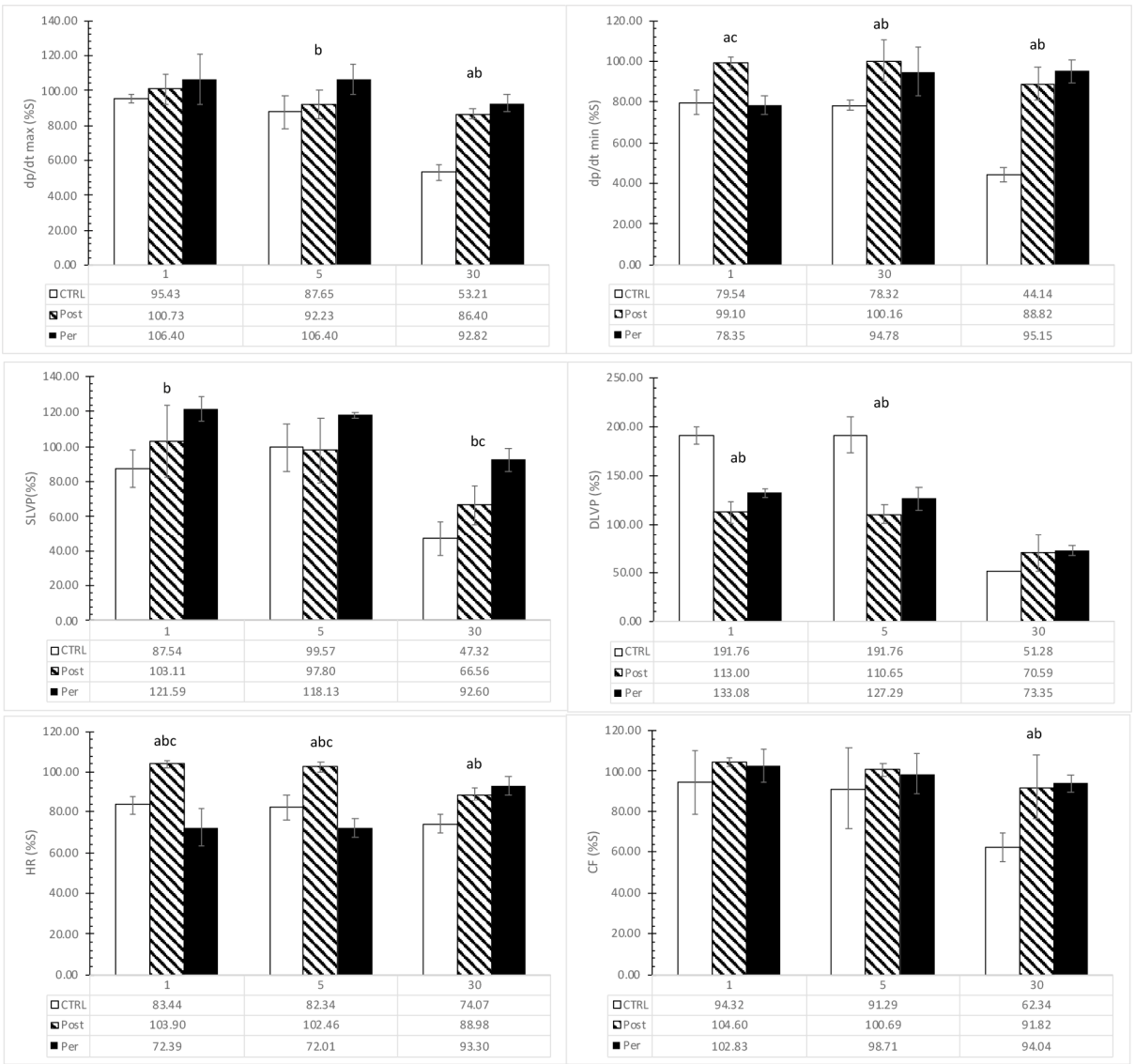


Figure 3. Oxidative stress parameters of the control (CTRL), postconditioning (Post) and perconditioning (Per) groups of rats, presented as absolute values (mean ± standard deviation) recorded every five minutes during the 30-minute reperfusion period. Interrupted line – control group, gray line – postconditioning group, black line – perconditioning group.

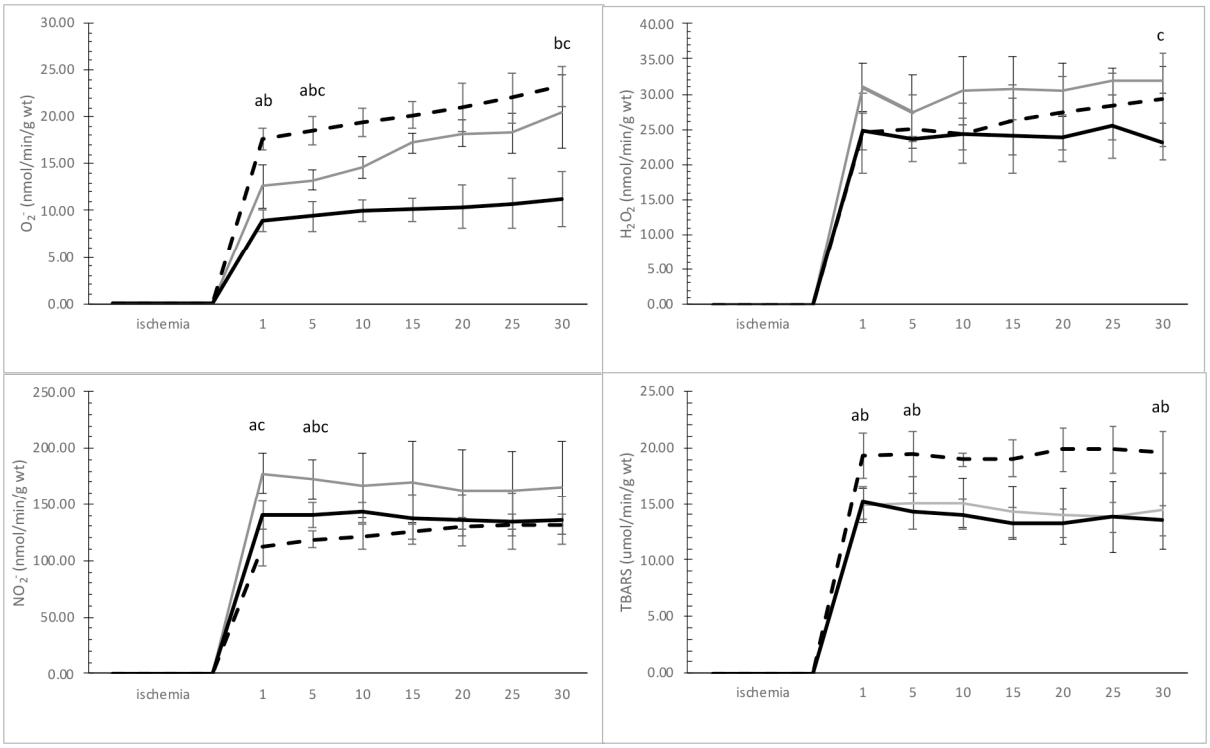
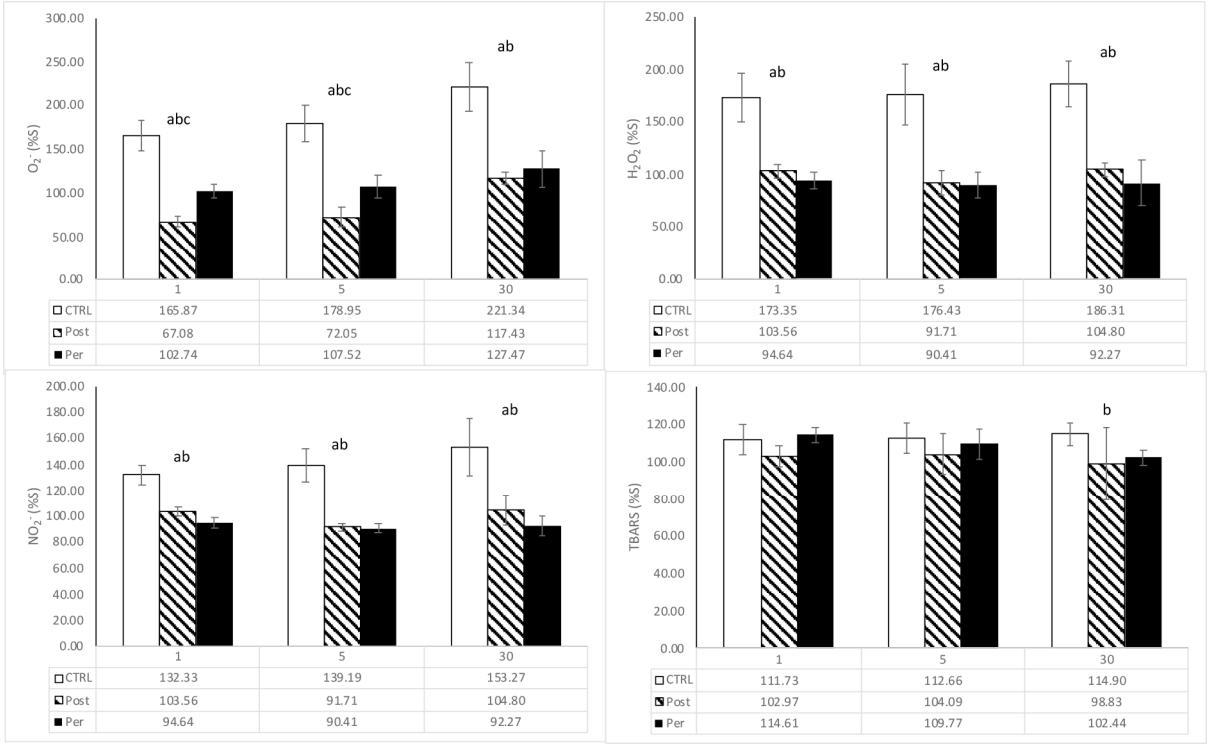


Figure 4. Values of oxidative stress parameters of the control group and groups whose isolated hearts were subjected to different modalities of phosphocreatine conditioning expressed as a percentage of the stabilization point of 100%.



DISSCUSSION

In the period of ischemia, anaerobic metabolism is of a crucial importance in energy delivery to the heart and preservation of myocardial viability, while rapid energy depletion worsens I/R injury (13-16). Therefore, we hypothesized that the use of phosphocreatine after, or during ischemia itself could be a crucial step in alleviating I/R-induced heart damage, especially because phosphocreatine is first consumed in ischemia.

Postconditioning attracts a lot of attention from scientists who have done a lot over the years to prove that the model used in preconditioning can be applied to postconditioning (17). Time stands out as a very important factor because only agents applied in early reperfusion have the potential to protect tissue while a more significant effect cannot be achieved 5-10 minutes after. These allegations are completely logical because the applied agents at this moment prevent the occurrence of further damage, but without affecting the already caused damage (18-20). Our results showed that phosphocreatine administered immediately after ischemia decreased fluctuations of cardiodynamic parameters during reperfusion. Similar results were obtained in a previous study, which examined the postconditioning effect of phosphocreatine (200 mg/kg through the femoral artery) on a model of ischemia induced by left anterior descending artery blockage for 30 minutes, followed by reperfusion for 120 minutes. The results showed that the use of this agent can reduce the serum level of creatine kinase and lactate dehydrogenase, which is an indicator of the protective role of phosphocreatine on damaged myocardium (7).

During the years, researchers have been trying to find out whether changes in the myocardium occur predominantly under the influence of ischemia or during the reperfusion. Since numerous pathophysiological processes occur in ischemia, it is hypothesized that better effects would be expected if management starts before or during ischemia (21, 22). Having in mind the pathological events that occurred in ischemia, we wanted to examine whether the exogenous use of phosphocreatine as a high-energy compound can prevent heart damage and whether its use during ischemia is more beneficial than in reperfusion, after ischemia. In our study, it was observed that acute application of phosphocreatine in ischemia shows a tendency to keep the values of cardiodynamic parameters unchanged during the entire reperfusion period. The changes observed were mild and reversible and did not affect cardiac output. Although both tested modalities of conditioning the isolated heart of rats with phosphocreatine at a concentration of 0.2 mmol/l showed that this agent preserves heart function during ischemic-reperfusion injury, it is undeniable that phosphocreatine showed better effect if used during ischemia, as a perconditioning agent.

The creatine kinase system acts as an energy buffer for the rapid regeneration of ATP when energy demand becomes greater than supply as occurs during ischemia (23). Our study showed that acute application of phosphocreatine, i.e. direct delivery of energy sources significantly contributes to the functional recovery of the myocardium in ischemic conditions. Since reperfusion leads to the return of phosphocreatine and gradual recovery of the heart, it is of great importance to restore phosphocreatine reserves and to establish ionic homeostasis. In this way, myocardial damage will be prevented (6). Phosphocreatine, due to its extreme polarity, cannot pass through the membrane by passive transport, so the potential to stabilize the membrane stands out as one of its key beneficial effects that is very important for I/R injury (24, 25).

It is also important to note that one of the crucial factors contributing to heart damage in reperfusion is oxidative stress (14). The danger of prooxidant accumulation arises not only from the potential to cause direct damage to cellular structures, but also from the ability to induce secondary ROS release by activating immune cells and disrupting mitochondrial function (26-32). It is also very important that cardiomyocyte death during I/R injury occurs due to changes in the extent of mitochondrial metabolism, which is also associated with excessive ROS production (29). For this reason, the question arises as to whether the use this agent during reperfusion can protect the myocardium and reduce the rate of damage. In this regard, we also wanted to examine the effects of phosphocreatine in a model of perconditioning and postconditioning on cardiac status by measuring oxidative stress parameters in coronary venous effluent.

Postconditioning with phosphocreatine in our study prevented an increase in the measured prooxidants, which significantly protects the heart from oxidative damage in I/R. Namely, under the influence of NADPH oxidase-NOX present in heart cells, a large amount of prooxidants forms. During the reperfusion process, neutrophils in which the NOX enzyme is present are activated. This enzyme catalyzes the reduction of O_2 with NADPH as an electron source, resulting in toxic reactive oxygen species $O_2^{\cdot-}$. The formation of ROS seriously contributes to heart damage, as well as loss of functional and structural characteristics of the heart. These findings have been confirmed in both animal and human I/R myocardial models and are consistent with our results in the control group of animals (14, 30). Also, perconditioning with phosphocreatine prevented an increase in most of the measured prooxidants in reperfusion and our results have shown that phosphocreatine has potential to improve redox signaling after ischemia.

CONCLUSION

Acute application of phosphocreatine during the period of ischemia or during period of reperfusion, leads to the maintenance of cardiodynamic parameters, which clearly shows the beneficial effect of this agent in conditioning models. Also, perconditioning and postconditioning with phosphocreatine prevents the rise of most of the measured proxidants during the reperfusion period. Given all the results obtained in this study as well as the previously mentioned studies indicating the potential of phosphocreatine to maintain ATP levels in

ischemia, this substance should be nominated as potentially cardioprotective and possibly with a better effect in perconditioning than preconditioning.

ETHICS APPROVAL

All research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU) and approved by the Ethics Committee for welfare of experimental animals, Faculty of Medical Sciences University of Kragujevac.

COMPETING INTERESTS

There are no conflicts of interest.

FUNDING

None.

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ORIGINAL SCIENTIFIC ARTICLE

FERTILITY AFTER THE OPERATION OF CRYPTORCHISM IN CHILDHOOD

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ABSTRACT

Cryptorchism is a congenital anomaly of male genitalia, and is defined as a disorder of lowering testicles into the scrotum. In our study, the quality of sperm and fertility of men who were operated from unilateral or bilateral cryptorchism in childhood were analyzed. According to the age in which they were operated, patients were classified into different time groups, subjected to clinical examination and sperm analysis. A normal sperm count was found: 36.9% of the total number of patients operated from unilateral to bilateral cryptorchism. The highest percentage of normal sperm counts was 73.97%: the data which was found in the group that was operated from one-sided cryptorchism to the end of the second year of life. Regardless of the age of the cryptorchism operation, it is possible to expect a disorder of spermatogenesis.

Keywords: *cryptorchism, sperm count, fertility, orchidopexy.*

INTRODUCTION

Cryptorchism is defined as the incomplete descent of one or both testicles from the abdominal cavity through the inguinal channel into the scrotum. It is a multifactorial etiological abnormality that affects 1-1.8% of male neonates (1). It can be considered to be associated with infertility and testicular cancer (2). In order to reduce these correlations, it is necessary to set and correct testicles in the scrotum (2, 3). [Supplementary Concept], Estrogen Replacement Therapy, Orchiopexy

It is not known when the ideal age of surgical treatment is and which the ideal therapy is (4). However, several studies have shown that as soon as possible, patients should start treatment because they have an improved spermatogenesis (3, 5).

Damage to the cells may be of secondary origin due to abnormal testicular position. These lesions are basically characterized by a progressive decrease in the number of cells and in the size of the tubular space with peritubular fibrosis and hyalinization, resulting in a reduction in the number of Leydig cells. These changes can affect the production of sex hormones that cause testicles atrophy with subsequent infertility (6, 7, 8). Therefore, the level of testicles and hormone levels is associated with maintaining spermatogenesis, and is considered indirect consequences for fertility (9, 10, 11). But, it is possible that the undescended testicle in the scrotum is indirectly related to atrophy (12, 13, 14, 15).

The surgery is recommended to be performed within 15-18 months, since cell loss is very rare and loss of germinal cells is associated with a subsequent risk of infertility (16, 17, 18).

MATERIALS AND METHODS

In our research, we analyzed a total of 84 men who had the operation of unilateral or bilateral cryptorchism at different times of their youth. Of the total of 84 patients, 46 were unilateral and 38 subjects had bilateral cryptorchism. We grouped them into three groups: a group of patients operated by the end of the second year of life (1), a group of patients operated from the second to the end of the seventh year (2), and a group operated seven years after (3). All patients gave three samples of spermograms, and all samples were made in the same laboratory.

In the period from February 2016 to the end of April 2017 the sperm analyses were carried out at the Institute for Health Care of the Medical Center in Krusevac. In all patients, three samples of sperm counts were taken, and a sample of the highest quality was analyzed. All samples were made in the same laboratory. Seed samples of patients tested for cryptorchism were taken by masturbation after 3 to 5 days of sexual abstinence and tested for 30 minutes at 37S. The concentration and mobility of the number of sperm counts are estimated according to the WHO recommendation. Samples are grouped into four groups: Normal finding, oligospermia (<20x106/ml), severe oligospermia (<5 x106/ml) and azoospermia.

Only a case of unilateral and bilateral cryptorchism is analyzed without analysis of family history (anomalies of the urogenital tract in the family, diseases and specific conditions of parents that can have an effect, for example, on mothers with diabetes, premature birth, weight of preoperative testis status such as atrophic testicles, use of hormonal gonadotropins, hernias, hypospadias, postoperative complications that may affect the number of spermatozoa and fertility estimation in men after operated cryptorchism). The test was conducted in accordance with the ethical principles of clinical trial on humans under the Helsinki Declaration and approved by the local ethics committee.

The obtained results are expressed as the mean±SD. In order to test the statistical significance of the results, we used Student's t-test and Pearson's correlation coefficient. Data processing is done using the Microsoft Office Excel 2003 software package in the Windows XP Professional Professional environment.

REZULTS

A normal finding of sperm count was found in 31 (36.9%) patients of a total of 84. The largest number of normal sperm count findings was found in patients operated from one-sided cryptorchism in the period up to the end of the second year of life. Oligospermia was found in 23 (28.5%) subjects, while heavy oligospermia was found in 14 (21.4%) patients operated by cryptorchism.

Azoospermia was found out in 11(13.09%) of a total of 84 subjects. In terms of age groups and cryptorchid side, a normal finding of sperm count is the most common in patients operated from one-sided cryptorchism until the end of the second year of life (73.9%) (Table 1).

Table 1. Normal sperm count

Age group	Nº cryptorchism			Normal sprmogram			% normal			Spermogram		
	U	B	∑	U	B	∑	U	B	∑	U	B	∑
0-2	23	16	39	17	7	24	73.9	43.7	61.5	36.9	18.4	28.5
2-7	14	14	28	3	2	5	21.4	14.2	17.8	6.52	5.26	5.9
< 7	9	8	17	2	0	2	22.2	0	11.7	4.34	0	2.38
Total	**46**	**38**	**84**	**22**	**9**	**31**	**47.8**	**32.6**	**36.9**	**47.8**	**23.6**	**36.9**

U-unilateral; **B-** bilateral; ∑-total

Table 2. Test variables in the first step (Step 1): year; one-sided-both sided

Step 1	B	S.E.	Wald	df	Sig.	Exp(B)
Year	.038	.012	10.208	1	.001	1.039
Unilateral		.542	4.923	1	.026	3.325
Bilateral	1.202					
Constant	2.578	.901	8.195	1	.004	.076

B= coefficients; **S.E.-** standard error coefficients;
Wald (the significance of the coefficient in the model); **Sig. (**p=**)**; **Exp (B)**= eB

Using the t-test, there is a statistically significant difference between the first and second groups (p=0.001), between the first and third groups (p=0.008), and between the first and fourth groups (p=0.014).

The highest percentage of normal sperm was 73.9% in an age group operated by one-sided cryptorchism until the end of the second year of life. In the case of surgeries with one-sided cryptorchism, we have twice more normal findings than in both cases (22 to 9).

The normal finding of sperm count is the most common in the age group operated by the end of the second year of life. It amounts to 43.77%, while in other age groups we have a very declining percentage of normal sperm finding in patients operated by bilateral cryptorchism (14.2% 0%).

There is a statistically significant difference (p=0.018) in the appearance of normal levels of spermatozoa in patients treated with single or double cryptorchism. Binary Logistic Regression examines the dependence of the findings (normal-abnormal) from the time when the operation of a single-sided or double cryptorchism was performed. Finding depends on age (p=0.001), and whether cryptorchism is one-sided or double-sided (p=0.026) (Table 2).

If the age is increased by one month, the risk that the finding does not occur normally increases by 3.9%. If cryptorchism is two-sided, the risk of finding outcomes increases by 3.32 times in relation to unilateral cryptorchism.

DISCUSSION

Infertility, due to its frequency, is not only the problem of marital partners, but of the entire society (19, 20). Looking at the age groups and the side of cryptorchism, the normal finding of spermograms is most common in patients with unilateral cryptorchism by the end of the second year of life (73.9%) (Table 1) (21, 22, 23).

On the 46 operations 22 (47.8%) of the subjects had a normal spermogram. On the 38 operations on both sides of cryptorchism 9 of the subjects (23.6%) had a normal spermogram. Cryptorchism occurs in about 4% of boys, which leads to a fall of testis from the abdominal cavity to the scrotum.

Infertility is due to damage of the epithelial epithelium caused by elevated temperature in the abdominal cavity. In cryptorchism, the disturbed function of the testicle can cause hormonal disorders. The level of hormone disorder is an indicator of damaged testes and can be used as a prognostic factor (24, 25, 26, 27).

The disorder of spermatogenesis is much more severe in patients with bilateral cryptorchidism compared to one-sided (24). The reason is the poor quality of sperm as well as permanent tubular damage in infertile men who have probably been operated in correlation with the time in which the surgical correction was performed (28).

Sperm parameters are often disrupted in patients with a history of cryptorchism (29). Boys older than 8 months have a low spermatogenic index, which indicates that surgery should be done before that time (22).

CONCLUSION

Cryptorchism leads to damage of spermatogenesis, as the loss of Leydig cells leads to sterility. The damage of spermatogenesis is more pronounced in patients with bilateral than in one-sided cryptorchism. Therefore, surgical treatment should be done earlier.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

COMPETING INTERESTS

There are no conflicts of interest.

FUNDING

None.

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ORIGINAL SCIENTIFIC ARTICLE

C REACTIVE PROTEIN AND PROCALCITONIN AS DIAGNOSTIC MARKERS IN CRITICALLY ILL PATIENTS WITH SUSPECTED SEPSIS

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ABSTRACT

The primary aim of this retrospective study was to estimate significance of determining C-reactive protein and procalcitonin for a diagnosis of sepsis in adult patients in early triage. Also, the aim of this study was to measure the sensitivity of the SIRS criteria, PCT and CRP levels and sepsis definitions to identify the most serious sepsis cases in the prehospital setting and at the Emergency Department (ED) triage. All patients were divided into two groups according to specific criteria for defining sepsis. First group (SIRS+ group) of patients were patients with clinically and/or laboratory confirmed sepsis (or systemic inflammatory response syndrome (SIRS) to bacterial infection with different localization). For confirmation of the SIRS we consider positive two or more clinical criteria (≥2 clinical criteria). The SIRS criteria use the clinical criteria of the Surviving Sepsis Campaign (SSC) for the SIRS, comprising at least two of the following criteria: HR > 90/min, RR > 20/min and temperature < 36° or ≥ 38.3°C and the next laboratory parameters such as leucocytosis > 15x109/L, leucopenia < 4x109/L, > 10% immature leucocytes. Second group of patients were patients with the SIRS negative criteria as a diagnostic tool (SIRS- group). We have founded that the CRP showed high sensitivity but no specificity in patients with sepsis, but on the other side, the PCT as a diagnostic marker showed a high sensitivity and high specificity in these patients. Also, the PCT is in positive correlation with the SIRS criteria, which could be of a clinical significance in early diagnosis of septic infections.

Keywords: *sepsis, C-reactive protein, procalcitonin, early diagnostic markers.*

INTRODUCTION

Patients hospitalized in the Intensive Care Units have a high risk of developing different complications. Infection is one of the most common and most serious complications. Infection is defined as "a pathologic process caused by the invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganisms" (1, 2).

In critically ill patients the intensity of infections is difficult to estimate especially in the initial stages of the disease. The diversity of a clinical picture of the underlying disease can often lead to an altered clinical presentation of the infection. In some cases, infection can be associated with an inadequate or inappropriate host response, and when this results in the development of organ dysfunction, the term "sepsis" is used (2, 3). Because of the complexity of reactions that are simultaneously triggered in the body and at various levels of homeostatic mechanisms of sepsis in patients, the intensity of the infection itself may vary from local infection to the development of sepsis with multiple organ failure (MOF) (4-7).

The incidence of sepsis is increasing at global level. Approximately 2% of hospitalized patients are under suspicion of sepsis. At the same time, the mortality rate remains high despite current progress in understanding pathophysiological mechanisms, diagnosis and therapy (8, 9). Sepsis and its various adverse sequelae, such as septic shock, the Acute Respiratory Distress Syndrome (ARDS), and Multiple Organ Dysfunction (MODS) continue to be among the most common causes of death in the non-coronary Intensive Care Unit (10).

For the past thirty years, a great deal of attention has been paid to the concept of sepsis as better as possible, to determine clinical and laboratory parameters for its rapid diagnosis and to improve the treatment of sepsis.

The importance of rapid identification of a patient with a suspicion on sepsis as well as the microorganisms of causative agents of severe infections is a crucial for the patient survival and a choice of antibiotic therapy (11). Guidelines for sepsis therapy are recommended by intravenous the use of wide-spectrum antibiotics within 1 h of the recognition of severe sepsis and septic shock. The delay in antibiotic therapy is associated with a significant increase in mortality (12).

In addition to the recommended clinical tests in the rapid identification of septic patients, with hemocultures that represent the "gold standard", numerous biomarkers from the blood and body fluids of the patient are used. An inflammatory markers, such as leukocyte (white blood cells - WBC) count in complete blood picture, C-reactive protein (CRP), and procalcitonin (PCT), have been applied in the diagnosis of the inflammation and infection.

White blood cells (WBC) or leucocytes produce, transport and distribute antibodies as a part of the human immune system response. Normal values of white blood cells are $4\text{-}10x10^9$ in adult. Number of leukocytes is an unspecified and insensitive indicator, but if it is elevated it can indicate the occurrence of a local or systemic infection (12, 13). Neutrophils and monocytes are also activated during sepsis. A poor prognosis is associated with lower expression of activation markers on monocytes and neutrophils, which indicates that poor outcome in these patients is due to a compensatory anti-inflammatory response (14).

In physiological conditions, the PCT concentrations are low (<0.15 ng/ml). Cytokines caused by the infection promote the production of extrathyroide by increasing the PCT level after 3-4 hours and reaching maximum values after 6 h. This level is maintained for the next 24-48 h. PCT is less than 0.5 ng/ml, indicating localized bacterial infection. Values of 0.5-2 ng/ml for the possible systemic infection. Values of 2-10 ng/ml and more for the safe systemic infection (15). PCT is a good marker of the bacterial infection in patients with systemic autoimmune diseases, even when they are being treated with corticosteroids and immunosuppressive agents. In such patients they rarely exceed the limit of 5 ng/ml (15).

The primary aim of this retrospective study was to estimate significance of determining C-reactive protein and procalcitonin for the diagnosis of sepsis in adult patients in early triage. Also, the aim of this study was to measure the sensitivity of the SIRS criteria, PCT and CRP levels and sepsis definitions to identify the most serious sepsis cases in the prehospital setting and at the Emergency Department (ED) triage.

PATIENTS AND METHODS

Study design and setting

This retrospective cross sectional clinical study included 55 patients which were admitted between May 2018 and August 2018 in the Health Center Valjevo in Serbia. This study was performed under the Good Clinical Practice guidelines and according to the Declaration of Helsinki.

Population and data sources

All patients with age above 18 and suspected or proven infection were included. Patients <18 years old, prisoners, pregnant women, patients in cardio-respiratory arrest, severe trauma victims, malignancies and epileptic seizure cases were excluded.

All patients were divided into two groups according to specific criteria for defining sepsis. First group (SIRS+ group) of patients were patients with clinically and/or laboratory confirmed sepsis (or systemic inflammatory response syndrome (SIRS) to bacterial infection with different localization). For confirmation of the SIRS we consider positive two or more clinical criteria (≥2 clinical criteria). The SIRS criteria use the clinical criteria of the Surviving Sepsis Campaign (SSC) for SIRS (16), comprising at least two of the following criteria: HR > 90/min, RR > 20/min and temperature

<36° or ≥ 38.3°C and the next laboratory parameters such as leucocytosis > $15x10^9$/L, leucopenia < $4x10^9$/L, > 10% immature leucocytes. Second group of patients were patients with the SIRS negative criteria as a diagnostic tool (SIRS-group).

During the period of three months, we observed the anamnestic and clinical data from medical history such as demographic characteristics (sex, age), comorbidities and previous diseases, reason of hospitalization, levels of procalcitonin and C reactive protein in serum samples, biological characteristics (laboratory values, microbiological data of blood and urine samples), therapy interventions (surgical intervention), as well as the SIRS criteria (positive or negative) and outcome for each patient.

Statistical analysis

Simple descriptive statistics were used to analyze population characteristics. We described data using percentages or medians with the Interquartile Range (IQR). To evaluate the differences between means we used Mann Whitney (Z test) and Spearman correlation (Rho coefficient) to evaluate associations between categorical and continual variables. Sensitivities, specifies (ROC curve), medians, averages and percentages were calculated using the SPSS 22.0, statistical software.

RESULTS

Demograhic characteristics of study population

In all, 34 (61.8%) patients were male, the mean age was 67.18±1.95 years (range: 31-90), and 29 (52.7%) were selected for surgical intervention (Table 1). Following the SIRS criteria, 42 (76.36%) patients were classified in the SIRS+ group and 13 (23.64%) in the SIRS- group (Table 1). Hemoculture test was positive in 24 cases in SIRS+ group and in 2 cases in SIRS negative group. Urine culture test was positive in 16 patients in SIRS+ positive group and in 2 patients in SIRS- group (Table 1). Also, negative outcome was present in 21 patients in SIRS+ group (Table 1).

Table 1. Study group characteristics

	All patients	**SIRS+ group**	**SIRS- group**
N (cases)	55 (100%)	42 (76.36%)	13 (23.64%)
Sex *	M 34 (61.8%) F 21 (38.2%)	M 24 (57.14 %) F 18 (45.86%)	M 10 (76.9%) F 3 (23.1%)
Mean age (years)	67.17±1.95	68.48±2.05	65.85±5.14
Surgical intervention	No 29 (52.7%) Yes 26 (47.3%)	No 26 (61.9%) Yes 16 (38.1%)	No 3 (23.07%) Yes 10 (76.93%)
Hemoculture test	No 29 (52.7%) Yes 26 (47.3%)	No 18 (42.85%) Yes 24 (57.15%)	No 11 (84.6%) Yes 2 (15.4%)
Urine Culture Test	No 37 (67.3%) Yes 18 (32.7%)	No 26 (61.9%) Yes 16 (38.1%)	No 11 (84.6%) Yes 2 (15.4%)
Outcomes (Mortality)	No 34 (61.8%) Yes 21 (38.2%)	No 21 (50%) Yes 21 (50%)	No 13 (100%) Yes 0 (0%)

According to the localization of infection, in all patients gastrointestinal tracts is the most common with 26 (47.27%) patients. In SIRS+ group is 17 patients (40.47%) and SIRS-9 (69%) patients the cause of gastrointestinal tract infections. Respiratory infection was present in 9 (16.36%) patients where 8 (19.36%) in SIRS+ group and 1 in SIRS-group (Table 2). Genito-urinary, Skin/Joint and Central nervous system represented by 3 (5.45%) patients. 1 patient in genito-urinary tract in SIRS- group. Other in SIRS+ genito-urinary 2 (5.45%), Skin/Joint 3 (7.14%) and Central nervous system 3 (7.14%) patients. Also, 2 patients (4.76%) unknown focus of infection in SIRS+ group 4.76%.

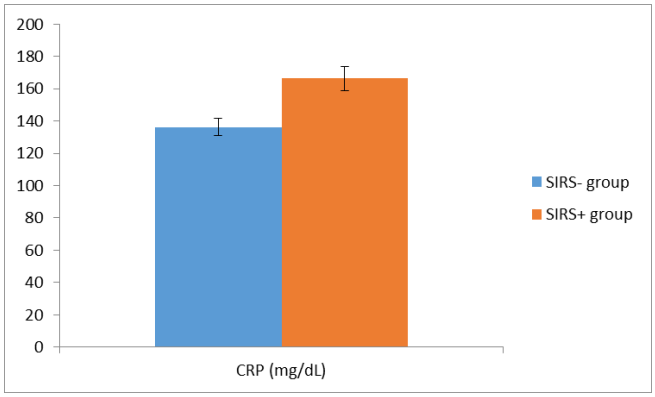
Table 2. Focus of infection in study population

Focus of infection	All patients	SIRS+ group	SIRS- group
N (cases)	55 (100%)	42 (76.36%)	13 (23.64%)
Respiratory (%)	9 (16.36%)	8 (19.04%)	1 (7.69%)
Genito-urinary (%)	3 (5.45%)	2 (4.76%)	1 (7.69%)
Gastrointestinal (%)	26 (47.27%)	17 (40.47%)	9 (69.23%)
Skin/joint (%)	3 (5.45%)	3 (7.14%)	0 (%)
Central nervous system (%)	3 (5.45%)	3 (7.14%)	0 (%)
Cardiovascular (%)	9 (16.36%)	7 (16.66%)	2 (15.38%)
Unknown (%)	2 (9.09%)	2 (4.76%)	0 (%)

Sensitivity of C reactive protein in SIRS+ and SIRS- groups

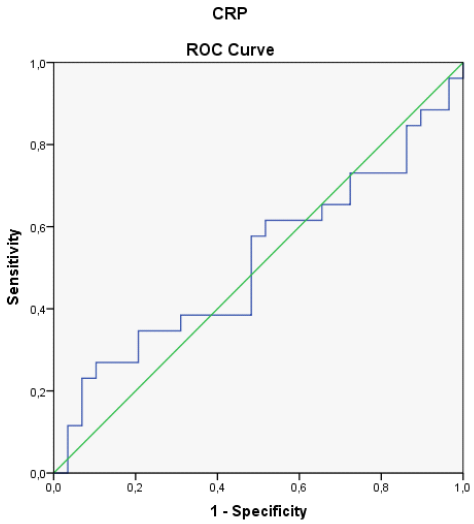
Mean value of the CRP in SIRS+ was 166.386 mg/dL and in SIRS- group was 136.21 mg/dl. Levels of the CRP in SIRS+ and SIRS- groups were very similar and without a statistically significant difference (p= 0.276) (Figure 1) but with slightly higher levels of this diagnostic marker in a group of patients with positive criteria for the systemic inflammatory response syndrome. Furthermore, we evaluated the sensitivity of the CRP in diagnosing sepsis by statistical methods (Figure 2). The area under ROC curve for C-reactive protein was 0.516. Cut-off value for C reactive protein was 1.685 mg/dl (sensitivity 96.2%, specificity 100.0%) (Figure 2).

Figure 1. Levels of CRP (mg/dl) in all groups of patients



Values are presented as mean±standard deviations. Asterisks (*) presents statistical significant differences (p<0.05) between means in SIRS+ and SIRS- groups confirmed by Mann Whitney test Z test.

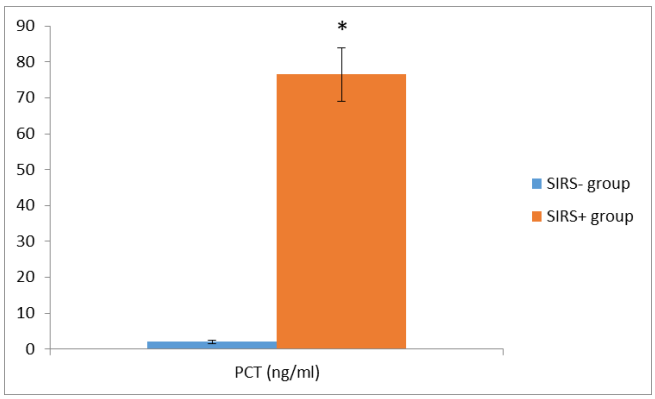
Figure 2. ROC curve for CRP



Sensitivity of procalcitonin in SIRS+ and SIRS- groups

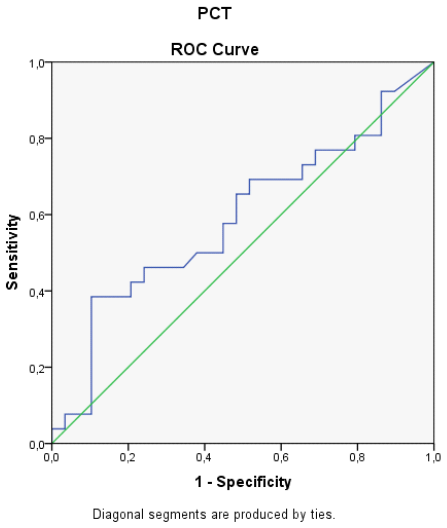
Mean value of the PCT in SIRS+ was 76.489 ng/mL and in SIRS- group was 2.085 ng/ml. Levels of the PCT in SIRS+ and SIRS- groups were different and with a statistical significant difference (p= 0.023) (Figure 3). Values of the PCT was significantly higher in group of patients with positive criteria for systemic inflammatory response syndrome. Also, we evaluated the sensitivity of the PCT in diagnosing of sepsis by statistical methods (Figure 4). The area under ROC curve for procalcitonin was 0.590. Cut-off value for PCT was 0.060 mg/dl (sensitivity 92.3%, specificity 82.7%) (Figure 4).

Figure 3. Levels of the PCT (ng/ml) in all groups of patients



Values are presented as mean±standard deviations. Asterisks (*) presents statistical significant differences ($p<0.05$) between means in SIRS+ and SIRS- groups confirmed by Mann Whitney test Z test.

Figure 4. ROC curve for the PCT



Correlation between CRP and PCT in study population

Procalcitonin and C reactive protein are in moderate positive correlation ($p=0.024$, $Rho=0.305$) in study group. Separately, the CRP was not in association with theSIRS criteria ($p=0.280$, $Rho=0.148$) while levels of the PCT were in moderate positive correlation with SIRS criteria ($p=0.021$, $Rho=0.310$) in study group (Table 3).

Table 3. Correlation between diagnostic parameters and SIRS tools in patients with and without sepsis. Results are presented as p values (p) and Spearman correlation coefficient (Rho).

Correlations

			PCT	CRP	SIRS_criteria
Spearman's rho	PCT	Correlation Coefficient	1,000	,305*	,310*
		Sig. (2-tailed)	.	,024	,021
		N	55	55	55
	CRP	Correlation Coefficient	,305*	1,000	,148
		Sig. (2-tailed)	,024	.	,280
		N	55	55	55
	SIRS_criteria	Correlation Coefficient	,310*	,148	1,000
		Sig. (2-tailed)	,021	,280	.
		N	55	55	55

*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

The primary aim of this retrospective study was to estimate significance of determining C-reactive protein and procalcitonin for the diagnosis of sepsis in adult patients in early triage. Also, the aim of this study was to measure the sensitivity of the SIRS criteria, PCT and CRP levels and sepsis definitions to identify the most serious sepsis cases in the prehospital setting and at the Emergency Department (ED) triage.

C-reactive protein (CRP) is an acute-phase reactant, synthesized by the liver and adipocytes mainly in response to IL-6. IL-6 is a cytokine that generates an initial response to injury or infection; its levels rise significantly during early sepsis, and therefore it is used to diagnose sepsis and predict the outcome of the patient's treatment (17). Synthesis of C-reactive protein begins in hepatocytes. After a latent period of about 6h, the serum level is doubled every 8h, and the highest concentration reaches 36-48h from the duration of the inflammatory process. When administered with an adequate

antibiotic therapy, the CRP decreases in the first two days to 50%. The normal C-reactive protein serum concentration is 0.8 mg/l. The level above this value indicates abnormalities and indicates a disease (12).

In our study, mean value of the CRP in SIRS + was 166.386 mg/dL and in SIRS- group was 136.21 mg/dl. Levels of the CRP in SIRS+ and SIRS- groups were very similar and without a statistically significant difference (p=0.276) (Figure 1) but with slightly higher levels of this diagnostic marker in group of patients with positive criteria for systemic inflammatory response syndrome. Furthermore, we evaluated the sensitivity of CRP in diagnosing of sepsis by statistical methods (Figure 2). The area under ROC curve for C-reactive protein was 0.516. Cut-off value for C reactive protein was 1.685 mg/dl (sensitivity 96.2%, specificity 100.0%) (Figure 2).

Meta analyses conducted by Shabuj et al, evaluated a role of the CRP as prognostic factors in neonatal sepsis. Meta-analysis showed that the CRP had a moderate accuracy (AUC=0.8535) for the diagnosis of NS. CRP is a helpful biomarker for diagnosis of NS. However, authors should combine the results with clinical symptoms and signs, laboratory and microbial results (18). On the other hand, Ticinesi et al measured the CRP in geriatric patients hospitalized for acute infection. C-reactive protein (CRP) is the most used biomarker of inflammation, and a substantial amount of the reference has demonstrated its importance and clinical usefulness in adult subjects. They concluded that the CRP dosage at hospital admission is helpful to detect acute infection, and particularly sepsis, in geriatric patients, and that CRP elevation may provide valuable short-term prognostic information. Also, at the current state of art, serial CRP measurements are instead not indicated to monitor disease course and plan hospital discharge in this setting (19).

Definitely, the clinical significance of serum CRP determination has not been completely clarified in older subjects with an acute infection, especially in the light of the age-related rearrangements in immunity and cytokine production.

Procalcitonin (PCT) is considered a relatively innovative and highly specific biomarker for the diagnosis of clinically relevant bacterial infections and sepsis; therefore it is increasingly recognized as an important diagnostic tool in clinical practice (20, 21). Procalcitonin is a prohormone calcitonine with secretory protein properties, which, in normal metabolic conditions, is only produced in C cells of the thyroid gland. After proteolytic digestion, only hormone activated calcitonin is secreted. For this reason, the blood of healthy people has the level of PCT very low or immeasurable (22). In patients with bacterial infection in the blood, high concentrations of intact PCT were detected. High circulation levels of PCT do not flow from the thyroid gland. An increased PCT concentration in patients with infection is secreted in the extratiroide tissue, they are the predominantly macrophage-monocytic system of various organs particularly lungs, liver and intestinal tract (23).

Furthermore, in our study mean value of the PCT in SIRS+ was 76.489 ng/mL and in SIRS- group was 2.085 ng/ml. Levels of the PCT in SIRS+ and SIRS- groups were different and with a statistically significant difference (p=0.023) (Figure 3). Values of the PCT was significantly higher in the group of patients with positive criteria for the systemic inflammatory response syndrome. Also, we evaluated the sensitivity of PCT in diagnosing of sepsis by statistical methods (Figure 4). The area under ROC curve for procalcitonin was 0.590. Cut-off value for PCT was 0.060 mg/dl (sensitivity 92.3%, specificity 82.7%) (Figure 4).

Prompt and accurate diagnosis of sepsis is of the high importance for clinicians. Procalcitonin (PCT) and C-reactive protein (CRP) have been proposed as markers for this purpose. Beqja-Lika et al examined the serum PCT levels in diagnosing of sepsis as an early diagnostic marker (24). Levels of the PCT and CRP were taken from 60 patients with sepsis criteria and 39 patients with the SIRS symptoms. Sensitivity, specificity and predictive values for the PCT and CRP were calculated. They found that PCT and CRP levels were increased in parallel with the severity of the clinical conditions of patients. The mean PCT level in patients with sepsis was 11.28 ng/ml versus 0.272 ng/ml in patients with the SIRS symptoms, with a sensitivity of 97.4% and a specificity of 96.6% for PCT >0.5 ng/ml. The mean CRP level in septic patients was 146.58 mg/l vs. 34.4 mg/l in patients with SIRS, with a sensitivity of 98.6% for sepsis and a specificity of 75% for CRP > 11mg/l. They concluded that the PCT and CRP values are useful markers to determine an early diagnosis and severity of an infection and the PCT was found to be a more accurate diagnostic parameter for differentiating SIRS from sepsis and may be helpful in the follow-up of critically ill patients (24).

Procalcitonin as a diagnosis and prognosis marker for sepsis: Many studies have demonstrated that serum PCT levels are increased in patients with sepsis, and the high levels of PCT correlate with the outcome of the disease. PCT can be used for differential diagnosis, prognosis, and follow-up of critically sick patients. However, it cannot be recommended as the single definitive test for sepsis diagnosis but rather it must be interpreted in context with information from clinical data (25, 26).

CONCLUSION

We can conclude that the CRP showed a high sensitivity but no specificity in patients with sepsis, but on the other side, the PCT as a diagnostic marker showed high sensitivity and high specificity in these patients. Also, the PCT is in positive correlation with SIRS criteria, which could be of a clinical significance in early diagnosis of septic infections. Further the cohort prospective clinical study is necessary to confirm our assumptions.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study

COMPETING INTERESTS

There are no conflicts of interest.

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ORIGINAL SCIENTIFIC ARTICLE

RISK FACTORS OF ESOPHAGEAL BLEEDING IN CHILDREN WITH VARIOUS ETIOLOGIES OF LIVER CIRRHOSIS – A SINGLE-CENTER REPORT FROM IRAN

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ABBREVIATIONS

AL – Alanine aminotransferase,
APRI – AST to Platelet Ratio Index,
AST – Aspartate aminotransferase,
CHF– Congenital Hepatic Fibrosis,
GSD – Glycogen Storage Disease,
INR – International normalized ratio,
MELD – Model For End-Stage Liver Disease,
PELD – Pediatric End-Stage Liver Disease,
PFIC – Progressive Familial Intrahepatic Cholestasis

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ABSTRACT

Esophageal bleeding is a common complication in patients with liver cirrhosis. In thee present study, our aim was to divulge major factors predicting esophageal bleeding in Iranian children with liver cirrhosis. This was a cross-sectional study including 101 children < 18 years old referred to the Pediatric Endoscopy Unit of Nemazee Teaching Hospital of Shiraz from 2014 until 2016. Children with esophageal varices were included. The patients were divided into two groups including those with and without history of esophageal bleeding. Statistical methods were performed in SPSS 16 software. There were 49 boys and 52 girls. The mean age was 7.74±5.26 years old. A history of esophageal bleeding was observed in 53 (52.4%). In univariate analyses, significant relationships were found between esophageal bleeding and varices size (P=0.001), Child-Pugh score (P=0.01), age of bleeding initiation (P<0.001), serum creatinine (P=0.01), and serum sodium (P=0.002). There was no statistically significant difference in the mean of PELD/MELD score among children with (12.34±12) and without (14.61±17.51) history of esophageal bleeding (P=0.5). Among various etiologies of cirrhosis, a significant association was observed between autoimmune hepatitis and the history of esophageal bleeding (P=0.01). Regarding the clinical importance of esophageal bleedings in children with liver cirrhosis, it is recommended to further divulge the risk factors predisposing to this event.

Keywords: *esophageal varices, liver cirrhosis, prognosis, risk factors, esophageal bleedin.*

INTRODUCTION

Liver cirrhosis is a late stage of chronic hepatic disorder. Cirrhosis is characterized with liver fibrosis and thedevelopment of a dysregulated nodular structure (1). Advanced liver fibrosis is seen in many liver diseases and it results from the accumulation of extracellular matrix components (e.g. collagen, glycoproteins, and proteoglycans) within hepatocytes (2). Cirrhosis can be associated with a wide range of complications including ascites, encephalopathy and esophageal bleeding (3, 4).

Esophageal bleeding is one of the main complications associated with high mortality and morbidity rates in children with cirrhosis (5, 6). This sequela is the outcome of portal hypertension and esophageal varices (7). The overall incidence of esophageal varices has been described in 6% of cirrhotic patients (8). Bleeding from esophageal varices has been noted in 5-19% of children (5, 6, 9). In patients with esophageal varices, the annual risk of esophageal bleeding has been reported in 4% increasing to 15% of patients with large varices (7). Also, the risk of bleeding has been reported in 10-75% of untreated patients (10). The 35-day mortality risk following gastrointestinal bleeding has been reported in 35% of children with esophageal varices (5).

Esophageal bleeding in children is treated according to the protocols available for adults including pharmaceutical, endoscopy and surgical interventions (11). Although beta-blockers and endoscopic band ligation have been noted as prophylactic managements for esophageal bleeding (6, 12), these methods have not always been effective and may even worsen the condition in some cases (13). Endoscopic band ligation has been suggested as a prophylactic intervention in children at high risk of esophageal bleeding (9). There is an incomplete understanding of risk factors predisposing to esophageal bleeding in children with liver cirrhosis. In this study, we described the risk factors, clinical and laboratory features, as well as endoscopic findings in cirrhotic children with esophageal bleeding.

PATIENTS AND METHODS

This was a cross-sectional study which included children <18 years old with liver cirrhosis referred to the Pediatric Endoscopy Unit of Nemazee Teaching Hospital affiliated with Shiraz University of Medical Sciences from 2014 until 2016. The children were evaluated for possible esophageal varices and those without esophageal varices in endoscopy examination were excluded from the study. Finally, 101 children with liver cirrhosis and esophageal varices were analyzed.

The patients were divided into two groups - those with and without history of esophageal bleeding.

Demographic data including age, sex, duration and etiology of liver cirrhosis, age of the first bleeding episode, history of encephalopathy and ascites and other paraclinical information (albumin, bilirubin, liver enzymes, creatinine, platelet, and sodium) were recorded. The PELD/MELD and Child-Pugh score were also determined.

Esophageal varices were categorized based on their sizes to small, medium, and large groups according to the classification described previously (14). In patients with multiple-size varices, the size of the largest one was considered. Furthermore, the presence of vessels on vessel, red spots, gastric varices and hypertensive gastropathy were sought in endoscopy.

Statistical methods were performed in SPSS 16 software. Nominal variables were described by frequencies and their relationships with each other were checked by Chi-square test. The distribution of quantitative variables was checked by Kolmogorov-Smirnov test. Mann-Whitney U test and independent sample student t-test were used as non-parametric and parametric tests for comparing mean values of quantitative variables between the study groups.

RESULTS

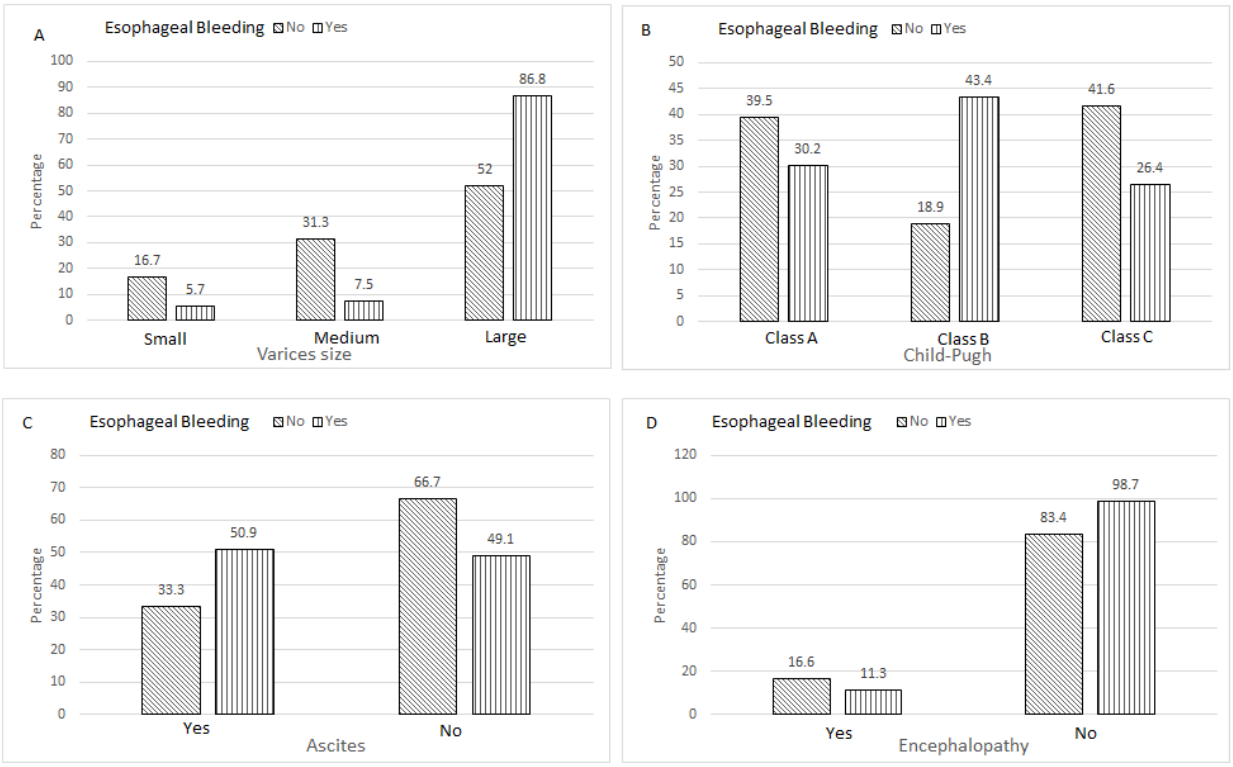
101 children <18 years old including 49 boys and 52 girls with liver cirrhosis and esophageal varices were included in this study. The mean age was 7.74±5.26 years old. Propranolol was administrated as prophylaxis for all patients. The band ligation was additionally used as prophylaxis for patients with medium and large size varices. Secondary therapy included sclerotherapy for children < 3 years old and ligation band for those >3 years old.

From 101 patients, 53 (52.4%) showed history of esophageal bleeding. Significant relationships were found between varices size (P=0.001) and Child-Pugh score (P=0.01) and esophageal bleeding (Figure 1).

There was no statistically significant difference in the mean PELD/MELD scores among children with (12.34±12) and without (14.61±17.51) history of esophageal bleeding (P=0.61). There were also significant differences between the two groups regarding the age of bleeding initiation (P<0.001), serum creatinine (P=0.01) and sodium (P=0.002) levels (Table 1).

No evidence of red spots or vessel on vessels was observed in endoscopy findings. However, two patients showed hypertensive gastropathy in endoscopy evaluation. Among various etiologies of cirrhosis, a significant association was observed between autoimmune hepatitis and the history of esophageal bleeding (P=0.01) (Table 2).

Figure1. Association of esophageal bleeding with main clinical parameters in 101 cirrhotic children (53 with and 48 without esophageal bleeding)



Significant differences were found in the distributions of varices size (P=0.001, A) and Child-Pugh classes (P=0.01, B) between children with and without esophageal bleeding. There were no significant differences in the distribution of either ascites (C) or encephalopathy (D) in children with or without esophageal bleedings.

Table 1. Association of esophageal bleeding with main laboratory parameters in 101 cirrhotic children

Laboratory parameters	**Esophageal bleeding**		**P**
	Yes N=53 n (%)	No N=48 n (%)	
MELD/PELD Score	12.34±12	14.61±17.51	0.5
Age (years)	7.5±5.14	7.46±5.26	0.97
Duration of hospitalization (days)	4.04±3.35	3.24±2.90	021
Bleeding age (years)	5.58±4.75	-	NA
Albumin (mg/dl)	4.25±4.75	3.7±0.74	0.53
Total Bilirubin (mg/dl)	5.97±7.58	7.55±10.92	0.47
INR	1.07±1.03	2.46±2.43	0.47
AST (IU/l)	157.54±316.81	164.72±193.78	0.33
ALT (IU/l)	107.34±202.62	118.81±187.48	0.70
Platelet (10^3/ul)	117.57±87.29	117±76.78	0.73
Creatinine (g/dl)	0.54±0.31	0.35±0.22	0.01
Na (mEq/l)	138.65±4.23	136±3.55	0.002
APRI	6.65±10.34	6.05±7.61	0.55

PELD; Pediatric End-Stage Liver Disease, MELD; Model For End-Stage Liver Disease, INR; International normalized ratio, APRI: AST to Platelet Ratio Index, AST; Aspartate aminotransferase, ALT; Alanine aminotransferase

Table 2. Association of esophageal bleeding with underlying diseases in 101 cirrhotic children

Underlying disease	Esophageal bleeding		P
	Yes N=53 n (%)	No N=48 n (%)	
Biliary atresia	12 (22.9)	14 (29.3)	**0.969**
Autoimmune hepatitis	7 (13.2)	2 (4.1)	**0.01**
Willson disease	1 (1.8)	2 (4.1)	**0.57**
PFIC	1 (1.8)	1 (2)	**1.00**
Neonatal hepatitis	1 (1.8)	2 (4.1)	**0.57**
CHF	4 (7.6)	2 (4.1)	**0.12**
Cryptogenic	3 (5.7)	1 (2)	**0.62**
Portal vein thrombosis	6 (11.4)	5 (10.7)	**0.13**
HPV	0 (0)	1 (2)	**0.43**
Galactosemia	1 (1.8)	0 (0)	**1.00**
Caroli disease	1 (1.8)	0 (0)	**1.00**
Gauncher disease	2 (3.7)	1 (2)	**0.50**
GSD	1 (1.8)	1 (2)	**1.00**
Unkwon	12 (22.9)	16 (33.6)	**0.58**
Lipodystrophy	1 (1.8)	0 (0)	**1.00**

PFIC; Progressive Familial Intrahepatic Cholestasis, CHF; Congenital Hepatic Fibrosis, GSD; Glycogen Storage Disease

DISCUSSION

Bleeding from esophageal varices is one of the important causes of morbidity and mortality in cirrhotic children. Therefore, it is critical to timely detect esophageal bleeding, divulge its risk factors, and appropriately manage its complications. Overall, 53 (52.4%) out of 101 evaluated cirrhotic children represented history of esophageal bleeding in our study. According to our findings, no significant relationships were found between the history of bleeding and encephalopathy, gastric varices, hypertensive gastropathy, and PELD/MELD scores. On the other hand, esophageal bleeding was significantly associated with Child-Pugh score, varices sizes, the age of bleeding initiation, serum sodium and creatinine levels and autoimmune hepatitis. Nonetheless, neither of these parameters remained an independent significant risk factor for esophageal bleeding in multivariant analyses.

Esophageal bleeding should be considered an alarming phenomenon to think of liver transplantation in cirrhotic patients (15). In fact, esophageal bleeding has been noted as an independent predictor of survival in patients with liver cirrhosis (16). In a recent report on children with chronic liver diseases of various etiologies, thrombocytopenia, hypoalbuminemia and splenomegaly were identified as risk factors of esophageal bleeding (17). In our study, however, platelet count and serum albumin were not significantly different comparing children with or without history of esophageal bleeding. Nevertheless, similar to our report, there were no associations between the risk of esophageal bleeding and serum bilirubin, liver enzymes and INR in the recent report (17). As well, no significant deviation was noted in the distribution of liver diseases etiologies in the recent report (17). Instead, we here found that autoimmune hepatitis may be a potential risk factor for esophageal bleeding in cirrhotic children. Further studies should yet be done to ascertain a definite association between autoimmune hepatitis and esophageal bleeding. Other significant risk factors for esophageal bleeding in cirrhotic patients have been noted as defects in the hemostatic pathway (18), viral load in viral-infection associated cirrhosis (19), the size of esophageal varices (20-23), Child-Pugh score (23), serum fibrinogen level (20), presence of gastric varices and hypertensive gastropathy (7, 9, 20-24), as well as the presence of red spots (20, 21, 23-26). In addition to these, we here noticed that serum sodium and creatinine levels may have a role as predictors of esophageal bleeding in cirrhotic children. Hypertensive gastropathy was noted in two of our patients, while no red spots were found in endoscopic evaluations. As mentioned, varices size and Child-Pugh score were associated with the risk of esophageal bleeding in our study as well. Although these were significant factors in univariate analysis, the results were not significant in multinomial regression analysis which may root in the relatively low power of the study. Splenomegaly and hypertension have also been noted as independent risk factors for esophageal bleeding. In present study; however, we did not assess these two factors in our patients. For appropriate and timely management of esophageal bleeding it is inevitable to develop risk stratifying parameters and reliable predicting factors. Liver stiffness has been a promising indicator of

esophageal varices and esophageal bleeding delivering sensitivity, specificity, positive predictive value, and negative predictive value of respectively 83%, 62%, 76.2% and 71.3% with the area under curve value of 0.780 at 31 kPa cut off for predicting significant esophageal bleeding (27).

The study limitations included not reporting splenomegaly and hypertension in the patients. In conclusion, regarding the clinical importance of esophageal bleeding in children with cirrhosis, it is recommended to divulge more details on the risk factors predisposing to this complication in future studies. Also, developing an updated universal guideline on the risk factors, diagnosis, classification, prognosis and treatment of esophageal varices in children is warranted.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study

COMPETING INTERESTS

There are no conflicts of interest.

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ORIGINAL SCIENTIFIC ARTICLE

ASSOCIATIONS OF VITAMIN D LEVEL AND GLUCOREGULATORY PARAMETERS IN TYPE 2 DIABETES MELLITUS

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ABSTRACT

Vitamin D is known to affect the functions of pancreatic beta cells, but the effects of vitamin D deficiency on glucoregulatory mechanisms are still inconclusive. The aim of this study was to link vitamin D levels with parameters of insulin resistance and insulin secretion. The study included 70 male and female participants, 40 newly diagnosed patients with type 2 diabetes mellitus (T2DM) and 30 healthy controls. All participants were tested for fasting glucose, hemoglobin A1c, fasting insulin, vitamin D levels, and the HOMA indexes were calculated using HOMA2 calculator. Fasting glucose levels, insulinemia, hemoglobin A1c levels and HOMA IR were all significantly higher in the diabetic group ($p<0.001$), while vitamin D levels and HOMA S index were significantly lower ($p<0.001$). HOMA-B values did not differ between the two groups ($p=0.31$). Vitamin D levels moderately correlated with HOMA S and HOMA B indexes ($r=0.466$, $p<0.001$; $r=0.394$, $p<0.001$, respectively), whereas a negative correlation was found between vitamin D levels and HOMA IR ($r=-0.285$; $p<0.001$). Multiple regression analysis showed that vitamin D levels significantly predicted the values of HOMA B index ($p=0.001$), but they had no predictive value on HOMA IR ($p=0.26$). In conclusion, the group of newly diagnosed patients with T2DM showed significantly lower vitamin D values compared to the healthy control group. The connection between vitamin D, glucose levels, hemoglobin A1c and insulin secretion index underlines the role of this vitamin in glucoregulation.

Keywords: *vitamin D, insulin resistance, insulin secretion, HOMA index, glu-cose metabolis.*

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INTRODUCTION

Some of the most common chronic metabolic disorders of present time are insulin resistance and vitamin D deficiency, and this is probably due to common risk factors, such as lack of physical activity, less sun exposure, bad dietary habits based on increased intake of concentrated carbohydrates and lower intake of dairy products and fibers, as well as chronic stress (1).

A published study has shown that individuals with vitamin D deficiency have reduced insulin secretion (2). Vitamin D receptors (VDR) are found in pancreatic β cells which also possess the 1-alpha hydroxylase enzyme essential for the synthesis of vitamin D, providing autocrine and paracrine biological effects (1,3,4). Furthermore, there is a study which suggests that vitamin D promotes increased synthesis of the mRNA for the preproinsulin of the pancreatic β cells in mice (1). The foregoing facts could indicate that vitamin D plays a significant role in physiological regulation of the endocrine pancreas. Additionally, it is believed that vitamin D may also affect the sensitivity of the peripheral insulin receptors due to the existence of VDRs in skeletal muscle, adipose tissue and hepatocytes.

Data considering the effects of vitamin D substitution on insulin resistance and secretion are still inconclusive. In some studies, vitamin D supplementation did not have any effect on insulin resistance in patients with prediabetes and type 2 diabetes mellitus patients (T2DM) (5,6). On the other hand, some studies conducted on T2DM patients and patients with impaired fasting glucose, showed that vitamin D substitution results in significant decrease of blood glucose levels, as well as improvement of calculated indexes of insulin resistance and/or insulin secretion (7-9).

Therefore, the aim of this study was to establish vitamin D status in patients with newly diagnosed T2DM, and to examine the link between vitamin D levels and the parameters of insulin resistance and secretion.

PATIENTS AND METHODS

This cross-sectional study was conducted in the Center of Laboratory Medicine in cooperation with the outpatient department of the Clinic for Endocrinology, Diabetes and Metabolic Disorders, Clinical Center of Vojvodina, during the summer and autumn of 2016. The study included 70 participants (57 male and 13 female). The mean age of the participants was 56.3 ± 5.1 for males and 55.8 ± 6.0 for females. Based on the patient history, clinical examination and laboratory analysis (fasting plasma glucose > 7 mmol/L and/or HbA1c > 48 mmol/mol), 40 participants were classified into a group of T2DM (10). All T2DM patients in this study were newly diagnosed and therapy-naïve. The control group consisted of 30 healthy (age and sex matched) subjects (individuals with normal fasting plasma glucose, insulin and glycated hemoglobin A1c levels).

Criteria for exclusion from the study were: kidney disease, psychiatric disorders and the presence of metabolic bone disease, as well as supplementation with vitamin D and/or calcium.

Laboratory assay

All laboratory analyses were performed before implementing any treatment of T2DM. After an eight-hour overnight fast and 15-minute rest in the laboratory after the arrival, blood samples were taken in order to determine fasting plasma glucose (FPG) and insulin (FPI) concentration, vitamin D level (25(OH)D) and glycated hemoglobin A1c (HbA1c). Glucose level was determined by an enzyme-specific, GOD-Perid method (reference range from 4.0 to 6.1 mmol/L) and the insulin was measured on an automated system ADVIA Centaur XP (reference value is 3.0 to 25.0 mIU/L). 25(OH)D was measured by direct chemiluminescent technology on platform ADVIA Centaur XP. Vitamin D status was defined according to blood levels of 25(OH)D as follows: vitamin D deficiency as 25(OH)D below 30 nmol/L, and vitamin D insufficiency as 25(OH)D of 30 to 50 nmol/L, while normal vitamin D status as 25(OH)D concentration greater than 50 nmol/L (11). HbA1c was determined by immunoturbidimetric method of inhibition of agglutination on the microparticles, on an automated system (Abbott Architect ci 4100). HOMA indexes were estimated using HOMA 2 calculator: HOMA index for estimation of insulin resistance (HOMA-IR), insulin sensitivity (HOMA-S) and secretory capacity of pancreatic β cells (HOMA-B) (12).

Data were presented using descriptive statistical methods for continuous variables as mean and standard deviation. In order to evaluate differences in parametric variables we used Student's T-test. Relations among variables were assessed using Pearson's correlation coefficient. Using linear correlation analysis, we estimated the relationship between all examined parameters and 25(OH)D. Multiple regression analysis was performed in order to estimate the independent relation between 25(OH)D levels and calculated parameters of insulin resistance/sensitivity as well as insulin secretion. A 2-tailed p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the Data Analysis Excel (Microsoft Corp., Redmond, WA) and MedCalc 12.1.4.0 statistical software (MedCalc Software, Mariakerke, Belgium).

RESULTS

Comparing T2DM patients with healthy controls, statistically significant difference was found for all tested parameters except for HOMA-B, ($p=0.31$). Compared with the control group, the T2DM group had significantly lower vitamin D levels ($p<0.001$) and HOMA-S ($p<0.001$) index, and significantly higher HOMA-IR index, BMI, FPG, FPI and HbA1c ($p<0.001$) (Table 1). In the T2DM group, 30 out of

40 participants were D vitamin insufficient (D vitamin below 50 nmol/l), while all healthy controls had vitamin D level above 50 nmol/l ($p<0.001$).

Using linear correlation analysis on both examined groups together (N=70), vitamin D levels moderately correlated with HOMA S and HOMA B indexes, whereas a negative correlation was found between vitamin D levels and HOMA IR. Negative correlations were also determined between 25(OH)D and FPG and HbA1c. However, we did not establish a correlation between 25(OH)D and FPI (Table 2).

Multiple regression analysis was used to assess the ability of the predictor model containing 25(OH)D and BMI to predict the values of HOMA IR, HOMA B% and HOMA S. As presented in Table 3, the model could successfully explain 48%, 41% and 13% of their respective variances. 25(OH)D was found to be a significant predictor of HOMA S and HOMA B, but not HOMA IR. As for BMI, it was a significant predictor of HOMA S and HOMA IR, but not HOMA B.

Table 1. Characteristics of glucoregulatory parameters of two examined groups

VARIABLE	T2DM group n=40	Control group n=30	P value
	X±SD	X±SD	
BMI (kg/m²)	29.94±5.77	22.05±1.89	p<0.001
FPG (mmol/L)	7.86±1.98	4.77±0.39	p<0.001
FPI (mIU/L)	17.24±7.07	7.40±4.64	p<0.001
HbA1c (mmol/mol)	47.9±17.72	31.1±4.01	p<0.001
HOMA-IR	2.82±1.12	0.96±0.58	p<0.001
HOMA-S (%)	42.07±18.98	135.86±70.88	p<0.001
HOMA-B (%)	88.15±40.19	98.45±28.74	p=0.31
25(OH)D (nmol/L)	45.17±20.65	70.7±16.33	p<0.001

Legend: BMI - body mass index; FPG - fasting plasma glucose; FPI - fasting plasma insulin; HOMA-IR - index for estimation of insulin resistance; HOMA-S - index for estimation of insulin sensitivity; HOMA-B - index for estimation of secretory capacity of pancreatic β cells; 25(OH)D - 25 hydroxyvitamin D; p - value

Table 2. Correlation between vitamin D levels and examined parameters

	25(OH)D N=70	
	r	P
BMI (kg/m²)	-0.261	p<0.001
FPG (mmol/L)	-0.600	p<0.001
FPI (mIU/L)	-0.201	p>0.05
HbA1c (mmol/mol)	-0.616	p<0.001
HOMA-IR	-0.285	p<0.001
HOMA-S (%)	0.466	p<0.001
HOMA-B% (%)	0.394	p<0.001

Legend: BMI-body mass index; FPG-fasting plasma glucose; FPI- fasting plasma insulin; HOMA-IR- index for estimation of insulin resistance; HOMA-S- index for estimation of insulin sensitivity; HOMA-B-index for estimation of secretory capacity of pancreatic β cells; 25(OH)D - 25 hydroxyvitamin D; r - correlation coefficient; p - value

Table 3. Multiple regression analysis

Dependent variable	Adjusted R^2	P value	Predictors		
N=70					
				β	p(β)
HOMA-IR	0.484	p<0.001	BMI	6.986	<0.001
			25(OH)D	-1.144	0.257
HOMA-S (%)	0.412	p<0.001	BMI	-4.693	<0.001
			25(OH)D	3.316	0.001
HOMA-B (%)	0.13	0.006	BMI	0.532	0.596
			25(OH)D	3.297	0.001

Legend: BMI - body mass index; FPG - fasting plasma glucose; FPI - fasting plasma insulin; HOMA-IR - index for estimation of insulin resistance; HOMA-S - index for estimation of insulin sensitivity; HOMA-B - index for estimation of secretory capacity of pancreatic β cells; 25(OH)D - 25 hydroxyvitamin D; Adjusted R^2 - the coefficient of determination that is consistent with the number of independent variables included in the model; β - standardized regression coefficient; p - p value of standardized regression coefficient

DISCUSSION

The main purpose of this study was to compare vitamin D levels in T2DM and healthy controls and to examine the relationship between vitamin D and insulin secretion and resistance indexes. The results showed that the T2DM group had significantly lower vitamin D levels compared with the control group. Also, 75% of patients with T2DM were vitamin D insufficient.

It has not been clarified yet if low vitamin D status is responsible for insulin resistance, or if it is just a consequence of the prediabetic state, including obesity, which exists together with insulin resistance. One of the major cohort studies concluded that reduced vitamin D levels lead to an increased risk of T2DM (13). Also, published results showed that people who have been substituting vitamin D or calcium had a lower risk of T2DM (14). The absence of the anti-inflammatory effect, which is attributed to reduced levels of vitamin D, can contribute to the development of insulin resistance, and therefore disrupt the glucoregulatory mechanisms, as well as the onset of T2DM (1). On the other hand, it is known that people with a higher BMI have lower levels of vitamin D due to the sequestration of liposoluble vitamin D in the larger adipose compartment, so this vitamin would be low in the serum (15). On the other hand, Drincic et al. (16) concluded that simple volumetric dilution in obese patients can be the explanation for the low vitamin D status in obesity.

Compared with the control group, the T2DM group had significantly higher HOMA-IR and insulin levels and lower HOMA-S values. The obtained results are expected and are in accordance with the previously known literature data. The pathophysiological basis of T2DM refers to development of insulin resistance, and therefore, significantly higher fasting insulin levels in our T2DM group represents an attempt to overcome the present insulin resistance (17). On the other hand, the T2DM group had a lower HOMA-B than the control group (88.15 ± 40.19% vs. 98.45 ± 28.14%), but without statistical significance. HOMA-B value depends on the level of insulin resistance, and the functional ability of pancreatic β cells. Thus, the reduction in insulin secretion can result from the preserved sensitivity of the peripheral tissues, and therefore a lower need for insulin secretion. On the other hand, after the hyperinsulinemic phase in T2DM, the insulin secretion becomes insufficient and reduced in relation to the level of current glycemia. Thus, HOMA-B values may be lower in healthy subjects, but also, as a consequence of the relative exhaustion of pancreatic β cells and a gradual decrease in insulin secretion, in T2DM patients. Also, knowing that HOMA-B is calculated using FPI and FPG, this index shows whether this insulin secretion is capable of maintaining fasting glucose in a normal range. We can see that in our control group HOMA-B is near the value of 100% which means that insulin secretion is optimal for that glucose level, whereas in the diabetic group, although fasting insulin is higher than in the control group, that insulin level is not capable of maintaining glucose level in euglycemic range, and that is the reason for lower HOMA B index in this group.

Results of this study also showed a statistically significant relationship between 25(OH)D and HOMA-B which is in accordance with previously published studies (18). Using multiple regression analysis, we have found that vitamin D is a predictor of insulin secretion estimated by HOMA-B index. It is known that the insulin secretion is glucose dependent due to the activation of GLUT-2 transporters, ATP synthesis, blockage of ATP-dependent potassium channels, as well as consequent depolarization of the cell membrane and influx of calcium ions. Cell influx of Ca^{2+} ions directly stimulates exocytosis and the release of insulin molecules into the

circulation. In addition to this mechanism, the second pathway of regulation of insulin secretion is also indicated, via cAMP (19,20). Besides the glucose-dependent, there are also glucose independent pathways of insulin secretion (for example the effects of incretin, amino-acids or glucagon on β-cells) (21,22). The presence of VDR receptors and 1α-hydroxylase in the pancreatic β cells suggests the possibility of a regulatory role of vitamin D on the intracellular concentration of Ca^{2+} ions, as well as the effect of vitamin D on the transcription of the preproinsulin gene (1,23). The second theory is based on the fact that insulin secretion can be independently induced by initiators (glucose, Ca^{2+}) as well as potentiators (glucagon, acetylcholine, β-adrenergic receptor agonists, cAMP) which enhance insulin secretion, but only in the presence of glucose as the most dominant initiator. According to this hypothesis, vitamin D can affect insulin secretion by acting on the efficacy of the initiator and/or potentiator, modulating the effects of the initiator of insulin secretion (23). Vitamin D deficiency causes a change in the efficiency of the glucose-dependent cAMP pathway and, consequently, its role in the regulation of insulin secretion (24). As a confirmation of this theory, numerous studies on animals have been published. They showed the influence of vitamin D on the cAMP signaling pathway, as well as changes in the activity of the cAMP-dependent protein kinase in vitamin D deficient animals (24-26). On the other hand, a clinical study which observed changes in the level of insulin secretion during parenteral administration of vitamin D concluded that an increase in insulin secretion was found only in people with elevated glycemia above the euglycemic range, whereas in subjects with normal glycemia, no increase occurred (27-29). Jeddi et al. had similar results on experimental animals (30).

According to our results, significant negative correlation was found between 25(OH)D and HOMA-IR which is in accordance with previously published data (18,29,31). However, multiple linear regression analysis has shown that the level of vitamin D influence on HOMA-IR is statistically insignificant. This finding is in line with the latest findings concerning the association between vitamin D and insulin resistance (1). Also, multiple linear regression showed that vitamin D is a significant predictor of HOMA-S, an index that represents the sensitivity of peripheral tissues to insulin. Both HOMA-IR and HOMA-S indexes are relatively close and point to the similar occurrences from a different perspective. However, the parameters used to calculate these indexes are different. In the HOMA-IR equation, only the basal values of glucose and insulin are used, whereas the estimation of HOMA-S also includes a deviation factor from normal values characteristic for healthy population (100%) (32). Different models of these indexes also point to different aspects of insulin effects on peripheral tissues (12). The effect which vitamin D potentially achieves in insulin sensitive tissues is still not well-known and explained in the literature. The local concentration of vitamin D and the production of hormone D in tissues, which directly affect the level of insulin resistance or sensitivity, cannot be fully identified with the measured level of vitamin D in systemic circulation.

CONCLUSION

Our study showed that newly diagnosed T2DM patients had significantly lower vitamin D levels compared with healthy controls. The relation between vitamin D levels and glucoregulatory parameters such as glucose levels, hemoglobin A1c and insulin secretion index (HOMA-B) underlines the glucoregulatory role of this vitamin, primarily due to its influence on the secretory function of pancreatic β cells.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

COMPETING INTERESTS

There are no conflicts of interest.

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ORIGINAL SCIENTIFIC ARTICLE

THE ANATOMY OF RENAL ARTERIES IN ADULTS

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ABSTRACT

Detailed extraparenhymal renal hilar dissection was performed on 110 fixed cadaveric kidneys (60 from male cadavers and 50 from female cadavers). We analyzed the number of renal arteries, angles between renal arteries and abdominal aorta, length and diameter of the renal arteries. Multiple renal arteries were present in 20.9% of cases, with a slightly higher incidence on the right side (21.8%: 20.0%). The angle between the aorta and the RRA varied from 30° to 100° with a mean of 64.1°, while the angle between the abdominal aorta and the LRA was 40° to 115°, with a mean of 67.3°. The external caliber of the RRA at the point of origin from the abdominal aorta was 5 mm to 9.1 mm, with a mean of 6.8 mm. The same caliber of the LRA was 3.7 to 9.6 mm with a mean of 7.0 mm. The average length of the renal artery from the point of origin from the abdominal aorta to the branching point was 36.2 mm for the right renal artery and 30.7 mm for the left renal artery. The average length of the renal artery from the point of origin from the abdominal aorta to the renal hilum was 65.1 mm for the right one and 54.7 mm for the left one. Knowledge of the number of renal arteries, their mode of entry into the kidney, the angles they build with the abdominal aorta, their diameter and length has practical applications in interventional radiology and surgery of the kidney and its environment.

Keywords: *renal artery, accessory artery, polar artery.*

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INTRODUCTION

Usually, each kidney is supplied by a single artery which is named the main renal artery. It has relatively constant position and path from the site of origin to the hilum of the kidney. Renal arteries are located in the vascular pedicle behind renal veins. They arise from the abdominal aorta below superior mesenteric artery, between the first and the third lumbar vertebra (1). Graves (1956) described renal vessels, defining normal segmental vessels, accessory artery and aberrant renal artery. He states that accessory arteries enter the kidney through the hilum and aberrants not through it, but usually through the upper and the lower pole of the kidney (2). In 70% of cases, there is a single renal artery (3). The right renal artery often leaves the aorta at a slightly higher level than the left one. It usually passes behind the inferior vena cava, but rarely, it could be found in front of it. Renal arteries are also the origin of inferior adrenal artery and small inferior branches that feed the renal pelvis and upper ureter. Tiny branches for the renal capsule and perinephric fat may also stem from the main renal artery.

The pronephros, mesonephros and metanephros are the basic stages in embryonic kidney development. The metanephros is formed at level of the bifurcation of the aorta. The mesonephros develops between the fifth week and the sixteenth week of the embryological development (4). Then it is supplied by temporary aortic branches, even up to thirty mesonephric arteries (5). With complete mesonephros degeneration only nine mesonephric arteries remain, from the tenth thoracic to the third lumbar segment (6). Between the sixth and the ninth week the metanephros ascends from the pelvis to the retroperitoneal space. During the ascent, the metanephros receives vascularization from the surrounding arteries, first from the iliac and then from the abdominal aorta. Lumbar mesonephros vessels form the rete arteriosum urogenitale which connects the vessels of the metanephros with the mesonephric arteries and the aorta abdominalis. Mesonephric arteries slowly disappear and only one artery remains, which is the future renal artery. If more than one artery remains at this stage of transition from mesonephros to metanephros then multiple renal arteries are formed (7).

Variations of the main renal artery and vein are common, due to existence of several mesonephric arteries during fetal life. These arteries develop from aorta between C6 and L3 vertebrae and are divided into cranial, middle and caudal arteries. Only one of the mesonephric arteries will later transform to main renal artery (8). If the fusion of the rete arteriosum urogenitale is not fully completed, two variations could occur: 1) short stem of renal artery and its early division and 2) multiple renal arteries. There are many names in the references for multiple renal arteries such as: accessory, aberrant, additional, supernumerary, supplementary arteries, etc. Glodny et al. (2009) divided the multiple renal arteries into additional renal arteries arising from the abdominal aorta, and accessory renal arteries arising from branches of the abdominal aorta. He found additional and accessory renal arteries with frequency of 14.6% up to 56% (9). Other study describes accessory renal arteries, as one with the hilar renal entrance that more often arise below the main renal artery. They cross ureter anteriorly and have the potential to cause obstruction. If an accessory artery is damaged, the part of kidney supplied by it is likely to become ischaemic. Aberrant renal arteries enter into the kidney extrahilary, through the capsule, usually through the lower or upper pole (10). If renal artery in a patient originates above the celiac trunk, it is consequence of the persistent mesonephric vessel (11). Bayramoglu et al. (2003) described bilateral accessory renal arteries stemming from abdominal aorta; the right one was accompanied by the vein (12). Merklin and Michels (1958) classify accessory arteries as follows: 1) accessory renal arteries originating from the aorta, 2) accessory renal arteries originating from the main renal artery, 3) accessory renal arteries originating from other sources. They use terms the main renal, aortic superior/inferior polar and renal inferior polar arteries (13). Poisel and Spangler (1969) described accessory, supplementary, supernumerary and aberrant renal arteries (14) and Stephens (1982) considered that these terms were not adequate because these were segmental and terminal blood vessels (15). Terms as hilar - for the aortic branch penetrating the hilum, extrahilar - for the branch of the renal artery with an extrahilar penetration, superior polar - for the aortic branch penetrating the superior pole and inferior polar - for the aortic or common iliac artery penetrating the inferior pole of the kidney, were also used in references (16). Satyapal et al. (2001) introduced the term additional renal artery for the one that, in addition to main renal artery, exits the aorta and ends in the kidney (17). Vilhova et al. (2001) used the term “accessory” for the segmental polar artery with hilar renal entrance, while the term “perforant” was reserved for segmental polar arteries that entered extrahilary (through the kidney capsule). In both case, those were aortic branches (18). Bordai et al. (2004) described three types of renal arteries: 1) the upper and lower polar arteries that enter through the kidney poles 2) the main renal artery as the largest hilar artery, and 3) the hilar supplementary artery that enters the hilum with the larger, main artery (19). The accessory and aberrant arteries usually originate from the abdominal aorta or iliac arteries and rarely from the thoracic aorta or branches of the abdominal aorta (20). Türkvatan A et al. (2009) divided the additional arteries into the hilar and polar depending on how they enter the kidney (8). Some authors distinguish between two types of additional renal arteries: 1) early division arteries and 2) extra renal arteries, which are classified as hilar (accessory) arteries and polar (aberrant) arteries (17, 21). In 2010, Daescu et al. divided the renal arteries into the hilar and polar (upper and lower polar arteries). They further divided the polar arteries into four groups: 1) solitary 2) pedicular, if the second one is accompanied by a polar vein and a nerve plexus 3) false supernumerary, if it replaces the segmental artery and 4) true supernumerary artery, if the respective segmental artery emerges from the renal artery (22).

In this study, we analyzed the number of renal arteries, their place of origin, length and diameter. We classified the multiples arteries into accessory, if they entered the kidney through the hilum and aberrant (polar) arteries, if they

entered the kidney through his capsule, usually through the upper or lower poles. The aim of the study was to examine the pattern of renal vascularization, describe its prospective anatomical variations and to point out the significance of a good knowledge of renal vascular anatomy, which is of great importance for planning and conducting certain urological and surgical procedures in the abdominal cavity.

MATERIAL AND METHODS

The study was performed in the Department of Anatomy and Department of Pathological anatomy of the Faculty of Medical Sciences of the University of Kragujevac, Serbia. Detailed extraparenhymal renal hilar dissection was performed on 110 fixed cadaveric kidneys (60 from male cadavers and 50 from female cadavers). The kidneys and surrounding tissue were removed within 24 h after death en bloc with adjacent part of the aorta and the vena cava. Cause of death was not related to urinary tract diseases. Any accessory or aberrant renal arteries if they were seen were also preserved. After excision, perirenal fat and surrounding tissue were removed and renal vessels, pelvis and ureter were prepared. The length of the renal artery was measured from the point of origin to the point of branching as well as from the point of origin to the hilum of the kidney. We also measured the external diameter of the renal arteries at the site of their origin. The measurements recorded in the text were made by the Vernier caliper with an accuracy of 0.1 mm. Angles between the lateral side of the abdominal aorta and the lower side of the renal artery were measured by using a protractor. All kidneys were recorded by drawings or photographs. The analysis included descriptive and analytical statistical methods. We used the student's T test in comparison to obtained sizes between the left and right kidneys, as well between male and female sex, in order to see if there was significant difference in the length, diameter and number of renal arteries. The study was performed in the Department of Anatomy and Department of Pathological anatomy of the Faculty of Medical Sciences of the University of Kragujevac, Serbia. Detailed extraparenhymal renal hilar dissection was performed on 110 fixed cadaveric kidneys (60 from male cadavers and 50 from female cadavers). The kidneys and surrounding tissue were removed within 24 h after death en bloc with adjacent part of the aorta and the vena cava. Cause of death was not related to urinary tract diseases. Any accessory or aberrant renal arteries if they were seen were also preserved. After excision, perirenal fat and surrounding tissue were removed and renal vessels, pelvis and ureter were prepared. The length of the renal artery was measured from the point of origin to the point of branching as well as from the point of origin to the hilum of the kidney. We also measured the external diameter of the renal arteries at the site of their origin. The measurements recorded in the text were made by the Vernier caliper with an accuracy of 0.1 mm. Angles between the lateral side of the abdominal aorta and the lower side of the renal artery were measured by using a protractor. All kidneys were recorded by drawings or photographs. The analysis included descriptive and analytical statistical methods. We used the student's T test in comparison to obtained sizes between the left and right kidneys, as well between male and female sex, in order to see if there was significant difference in the length, diameter and number of renal arteries.

RESULTS

The number of renal arteries

A single renal artery was present in 79.1% cases of 110 dissected kidneys: on the right side in 78.2% and on the left side in 80.0%. Multiple renal arteries were present in 20.9% of cases, with a slightly higher incidence on the right side (21.8%: 20.0%). We found two arteries in 18.2% and three in 3.6% of the observed right kidneys. The same arteries in the left kidney existed in 20.0% of cases: two arteries in 16.4%, three in 1.8% and four arteries in 1.8% of the kidneys (Table 1).

Table 1. The number and frequency of the renal arteries of the right and left kidneys in males and females

Material	Number	Gender	1 artery		2 arteries		3 arteries		4 arteries	
			right	Left	right	left	right	left	right	left
The dissected kidneys	60	**male**	22 73.3%	25 83.3%	7 23.3%	4 13.3%	1 3.3%	--- ---	--- ---	1 3.3%
			47 **78.3%**		11 **18.3%**		1 **1.7%**		1 **1.7%**	
	50	**female**	21 84%	19 76%	3 12%	5 20%	1 4%	1 4%	--- ---	--- ---

Material	Number	Gender	1 artery		2 arteries		3 arteries		4 arteries	
			right	Left	right	left	right	left	right	left
The dissected kidneys			40 **80%**		8 **16%**		2 **4%**		--- ---	
The dissected kidneys	110	**total**	43 78.2%	44 80%	10 18.2%	9 16.4%	2 3.6%	1 1.8%	-- --	1 1.8%
			87 **79.1%**		19 **17.3%**		3 **2.7%**		1 **0.9%**	

Angles between renal arteries and abdominal aorta

The angle between the aorta and the RRA varied from 30° to 100° with a mean of 64.1°, while that angle on the left side was from 40° to 115°, with a mean of 67.3°. The difference in the angle values between the right and the left side was a statistically significant, with smaller angle on the right side ($DF = 108$, $p < 0.01$).

The diameter of the renal arteries

The external caliber of the RRA at the point of origin from the abdominal aorta was 5 mm to 9.1 mm, with a mean of 6.8 mm. The same caliber of the LRA was 3.7 to 9.6 mm with a mean of 7.0 mm. At the branch point, the diameter of the RRA was 4.0 mm to 8.1 mm with a mean value of 5.5 mm, while on the left it was 2.4 mm to 8.0 mm with a mean of 5.8 mm. The difference in the diameter of renal arteries was not statistically significant between the left and right arteries in males ($t = 0.75$, $p > 0.05$) and females ($t = 0.75$, $p > 0.05$). Diameters of renal arteries are shown in Tables 2 and 3.

Table 2. Diameters of the renal arteries in mm

Gender	Site	Renal artery diameter- mm.					
		At the beginning of RA			At the branching point of the RA		
		min-max		SD	min-max		SD
Male	**RRA**	5.0 - 9.1	**7.02**	0.95	4.0 - 8.1	**5.71**	0.97
	LRA	3.7 - 9.6	**7.23**	1.22	2.4 - 8.0	**6.18**	1.13
Female	**RRA**	5.0 - 8.4	**6.63**	1.05	4.0 - 8.0	**5.40**	0.98
	LRA	5.2 - 9.0	**6.82**	1.01	4.0 - 7.0	**5.39**	0.94

Length of renal arteries

The length of the RRA from the point of origin to the branching point was 5 mm - 60 mm with an average value of 36.2 mm. The length of the RRA from the point of origin to the hilum of the kidney was 45mm - 88 mm, with an average value of 65.1 mm. The length of the LRA from the point of origin to the branching point was 3 mm - 50 mm with the average value of 30.7 mm. The length of the LRA from the point of the origin to the hilum of the kidney was 35 mm - 82 mm with average value of 54.7 mm. If the renal artery was divided up to 15 mm from the aorta, we considered it as early branching (early division). Early division of the renal artery was in 5.5% in the RRA and in 7.3% in the LRA. Early branching of the renal artery was considered as a consequence of the unfinished embryonic development of the kidney that is, the remains of mesonephrotic vessels. There is no statistically significant difference in the length of the left and right renal arteries from the point of origin to the point of branching ($t = 2.52$, $0.05 < p < 0.01$). The statistically significant difference was found in the length of renal arteries from the point of origin to the renal hilum, with the right renal artery longer than the left ($t = 5.179$, $p < 0.001$), as expected considering the sinistroposition of abdominal aorta. Lengths of the renal arteries are shown in Table 3.

Table 3. The length of renal arteries in mm

Renal artery	Number	The length of the renal arteries - mm	
		To the branching point	To the renal hilum
RRA	**55**	5 - 60 ± SD = 36.24 ± 12.15	45 - 88 ± SD = 65.01± 10.15
LRA	**55**	3 - 50 ± SD = 30.74 ± 10.75	35 - 82 ± SD = 54.69 ± 10.87

DISCUSSION

If there is another artery in addition to the main renal artery then it is a multiple renal artery. The percentages of multiple renal arteries described in references varied and depended on the author, type of study and the studied population. Tardo et al. (2017) discovered multiple renal arteries in 22% of subjects and 12.12% of kidneys. There was no significant difference between left- and right-sided kidneys (13.8% vs. 12.5%). Unilateral additional renal arteries were in 16.7% but bilateral in 3.4% of cases. Variations among males were more than females (27.2% vs. 15.2%) (23). Apisarnthanarak et al. (2012) found the supernumerary renal arteries in 18.5% and early branching in 12.8% at the right side and 27.7% and 22.4% respectively at the left side (24). In another large angiographic series, additional renal arteries were found in 18.2% of cases. One additional artery was found in 8.9% of cases, two in 5.0%, three in 1.6%, four in 0.35%, five in 0.2% and six in 0.1% cases. In 6.6% of cases there was bilateral symmetry in the number of the additional renal vein (bilateral double in 6.3%, bilateral triple in 0.2% and bilateral quadruple in 0.1% of cases (7, 25).

Palmieri et al. (2011) found a high percent of multiple renal arteries of 61.5% in the angiographic study conducted in Brazilian population (56% in the right and 67% in the left kidney) (26). Contrary, Hlaing et al. (2012) found only 4% of accessory arteries in an anatomical study in Malaysian population (27). In another angiographic study, multiple renal arteries were found in 31.3% of cases (two arteries in 22.2%, three in 7.5%, four in 1.4%, five in 0.2% of cases) and prehilar branching in 6.5% cases (1).

In our study multiple renal arteries were found in 20.9% of cases, at the right side in 21.8% and at the left side in 20.0% of cases. Multiple arteries were slightly more often in males than in females (21.7%: 20.0%). Our findings are in agreement with the studies conducted in other populations (Table 4).

Table 4. The frequency of multiple renal arteries (different studies)

Studies	MA %	2 arteries %	3 arteries %	4 arteries %	5 arteries %
Çınar C, Türkvatan A, 2016 (1)	31.3	22.2	7.5	1.4	0.2
Tardo DT et al., 2017 (23)	6.9	5.6	1.4	--	--
Zăhoi DE et al., 2015 (35)	23.2	19.6	3.6	--	--
Matusz P et al., 2011 (25)	18.2	8.9	5.0	1.6	0.35
Natsis K et al., 2014 (36)	17.3	13.0	4.3	--	--
Our study, 2019	20.9	17.3	2.7	0.9	--

The existence of accessory renal arteries is most often without clinical symptoms. Usually, these arteries are detected by random radiographic examinations. If the accessory artery vascularizes the lower pole of the kidney, it often passes in front of the ureteropelvic junction or ureter and performs external compression leading to hydronephrosis (15). The existence of accessory renal vessels (arteries or veins) leading to ureterohydronephrosis must be surgically resolved either by the open surgery or laparoscopy. Simphoroosh et al. (2005) presented the laparoscopic management of ureteropelvic junction obstruction by division of the aberrant vein and cephalad relocation of the crossing artery (28).

Life-threatening bleeding may result from accessory artery injury during percutaneous renal biopsy for diagnostic or other purposes (29). The existence of accessory renal arteries impairs renal transplantation and can lead to pyeloureteral necrosis of the graft due to damage of the artery that vascularizes it (30). The exact number of arteries and their arrangement is especially important in the kidney transplantation. These findings are necessary to avoid undesirable injuries to these arteries during the explantation of the kidney and to prepare for their microsurgical reconstructions.

Unlike the renal veins, the RRA is slightly longer than the LRA. Palmieri et al. (2011) found that the average length of the main renal artery to its first branch was 3.96 ± 0.13 cm on the right and 3.41 ± 0.11 cm on the left side (26). Mohiuddin et al. (2017), found a significant difference in the caliber and length of the right (diameter was 6.66 ± 0.39 mm; length was 44.69 ± 2.48 mm) and left renal arteries (diameter was 6.79 ± 0.36; length was 35.10 ± 2.86 mm) (31). We found no significant difference in caliber between the right and left renal arteries. A significant difference in the length of the right and left renal arteries was found when we measured the length from their origin to the hilum of the kidney. The clinical relevance of the knowledge of the size of renal arteries is reflected in the evaluation of renovascular hypertension. Tortuous shape and small caliber of accessory arteries can lead to arterial hypertension (17). Knowledge of the size of the renal arteries is important for interventional radiologists in procedures such as angioplasty and arterial catheterization (32). Small caliber of renal arteries can lead to arterial stenosis which can cause renal hypertension and ischemic nephropathy (33). Renal artery stenosis is treated by percutaneous stenting and requires knowledge of the caliber and length of the renal artery (34). The larger length of the renal artery allows greater mobility of the kidney during the intervention on it. The short renal artery (early branching) can cause difficulty during a nephrectomy and kidney transplantation. In our study, early branching of the renal artery (15 mm from the aorta) was found in 5.5% on the RRA and in 7.3% on the LRA. Knowing the length and caliber of the renal artery is important before the kidney transplantation. Successful transplantation requires a renal artery of sufficient length and diameter. The transplant kidney with the appropriate vascular loop should be selected to minimize microsurgical vascular reconstruction and to make transplantation more successful.

CONCLUSION

Multiple renal arteries were present in 20.9% of cases of our study, with a slightly higher incidence on the right side. Knowledge of the number of renal arteries, their origin, course, and mode of entry into the kidney is of great importance for the successful conduct of radiological and surgical procedures. Multiple renal arteries can be injured during surgery and lead to bleeding of a life-threatening patient. They complicate kidney surgery as well as interventions in its environment such as in the case of retroperitoneal lymphadenopathy, surgery of aneurysm of the abdominal aorta and others. Multiple kidney arteries make it difficult and, in some cases, prevent kidney transplantation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by Ethical comittee of the Clinical Centre Kragujevac, Serbia, No. 01-19/3542. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

COMPETING INTERESTS

There are no conflicts of interest.

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ORIGINAL SCIENTIFIC ARTICLE

ADMINISTRATION OF 4-HYDROXY-3,5-DI-TERTBUTYL CINNAMIC ACID RESTORES MITOCHONDRIAL FUNCTION IN RABBITS WITH CEREBRAL ISCHEMIA

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ABSTRACT

The aim of the study is to evaluate the effect of 4-hydroxy-3,5-di-tertbutyl cinnamic acid on the change in mitochondrial function under conditions of experimental cerebral ischemia in rabbits. The study was performed on 48 male rabbits, which were used for modeling permanent cerebral ischemia by occlusion of the common carotid arteries. The test compound was administered before modeling ischemia for 14 days and after the occurrence of reproducing ischemia, in a similar time interval. After that, neurological deficit and the parameters of mitochondrial respiration, the intensity of anaerobic processes, the latent opening time of the mitochondrial permeability transition pore, the value of the mitochondrial membrane potential and the concentration of caspase – 3 were determined. The administration of 100 mg/kg of 4-hydroxy-3,5-di-tertbutyl cinnamic acid into the animals reduced neurological deficit and restored the mitochondrial membrane potential. Prophylactic administration of 4-hydroxy-3,5-di-tertbutyl cinnamic acid, contributed to an increase in ATP-generating ability, the maximum level of respiration and respiratory capacity by 4.1 times ($p<0.01$), 4.8 times ($p<0.01$) and 4.3 times ($p<0.01$), respectively. With therapeutic administration, these indicators increased by 11 times ($p<0.01$), 12.2 times ($p<0.01$) and 8.6 times ($p<0.01$), respectively. Also, both the prophylactic and therapeutic use of 4-hydroxy-3,5-di-tret-butyl cinnamic acid normalized aerobic/anaerobic metabolism, as well as reduced the concentration of caspase-3. Based on the obtained data, significant cerebroprotective properties of 4-hydroxy-3,5-di-tertbutyl cinnamic acid can be assumed. Moreover, the potential mechanism of action of this compound may be mediated by the normalization of mitochondrial function.

Keywords: *cerebral ischemia, cinnamic acid derivatives, mitochondrial dys-function.*

INTRODUCTION

Stroke is the most common cerebrovascular accident, as well as one of the leading causes of death and primary disability in both economically developed and developing countries (1). The typology of stroke includes hemorrhagic and ischemic subtypes, and the latter accounts for about 70-80% of all cases of stroke (2). In recent years, significant progress has been made in understanding the pathophysiology of ischemic stroke, which has significantly optimized therapeutic approaches to the treatment of this condition (3). However, relevant statistical data, as well as prognostic calculations by specialists of the World Health Organization, show a progressive increase in mortality from cerebrovascular diseases in the acute phase. In low-income countries, stroke ranks fifth among the causes of death, at the same time with an increase in the well-being of the population, i.e. in middle-low, middle-high and high-income countries, ischemic brain damage already takes the second place in the structure of total mortality. Moreover, as a rule, ischemic stroke occurs in people of working age, which negatively affects the economic activity of a number of countries (3). Etiopathogenetic stroke is characterized by cerebral vessel occlusion with the formation of a necrotic zone - a zone of cerebral infarction ("ischemic shadow"), surrounded by tissue with reduced blood flow and metabolic activity ("ischemic penumbra"), which, if an unfavorable outcome occurs, can be involved in the formation of a brain tissue necrosis zone (5). In the "ischemic penumbra", there is an increase in inflammation, oxidative stress, glutamate excitotoxicity and intracellular calcium concentration, as well as a decrease in ATP synthesis and activation of apoptosis, and activation of anaerobic processes with hyperlactatemia (6). In the activation of the above processes, a special role is given to the mitochondria of neurons. Mitochondria are cellular organelles that mainly perform an energy-producing function (macroergic substrates in the form of ATP): however, the functional activity of mitochondria is also realized in the regulation of the cell redox state and apoptosis (7). Active forms of oxygen are by-products of normal mitochondrial metabolism, which play a significant negative role in the pathogenesis of ischemic stroke, initiating the processes of cellular and subcellular lipoperoxidation, as well as activating calcium influx, increasing its intracellular concentration to a toxic level (8). Mitochondrial-dependent apoptosis, as a rule, is activated upon reaching the minimum ATP level acceptable for normal cell functioning and includes caspase-dependent and caspase-independent cascade reactions (9). The effector system of caspase-dependent apoptosis is caspase-3, which acts as an enzyme, a DNA sequencer (10). Caspase-independent apoptosis is activated by decreasing mitochondrial membrane potential, resulting in opening of the mitochondrial permeability transition pore with cytochrome C releasing, activation of the apoptosis-inducing factor and formation of apoptosome (11). As a result of the described pathogenetic mechanisms, intensification of neuronal death in the "ischemic penumbra" and an increase in the zone of cerebral infarction are noted, which in turn contribute to the clinical symptoms of ischemic stroke: cognitive dysfunction, neurological deficit, sensorimotor disorders, paresthesia, and depression (12). In this regard, it can be assumed that a targeted effect on mitochondria can reduce the degree of brain damage in conditions of ischemic stroke. In previous studies, potential cerebroprotective properties of 4-hydroxy-3,5-di-tertbutyl cinnamic acid have been identified, which may also be associated with the restoration of mitochondrial function by stabilizing the activity of cytochrome C oxidase and ATP synthetase (mitochondrial complex IV and V respectively) (13). In this respect, the aim of this study was to evaluate the effect of 4-hydroxy-3,5-di-tertbutyl cinnamic acid on the change of functional activity of the brain mitochondria of rabbits in conditions of cerebral ischemia.

MATERIALS AND METHODS

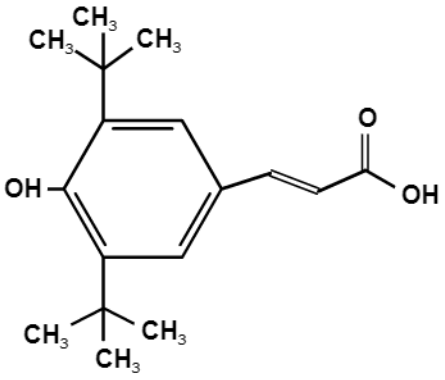
Experimental animals

As a biological model, this study used 48 male Soviet Chinchilla rabbits weighing 2300-2500 g. The animals were kept in standard living system laboratory conditions at the Pyatigorsk Medical and Pharmaceutical Institute with a natural change in the daily cycle (12 hours a day, 12 hours a night), relative humidity 60 ± 5 % and ambient temperature 20 ± 30 ° C. The rabbits were housed in individual cages with free access to food and water. The animals were removed from the experiment by cervical dislocation under chloral hydrate anesthesia (chloral hydrate 350 mg/kg, intraperitoneally). The keeping and manipulation of animals was in accordance with generally accepted standards of experimental ethics (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, adopted on September 22, 2010).

Test objects

In this study, the test object was 4-hydroxy-3,5-di-tertbutyl cinnamic acid (Fig. 1). The studied substance was obtained at the Department of Organic Chemistry of the Pyatigorsk Medical and Pharmaceutical Institute under the supervision of professor E.T. Oganesyan. The authenticity of the compound was confirmed by UV, IR and NMR spectroscopy. Ethylmethylhydroxypyridine succinate (Mexidol, FARMASOFT, Russia) at a dose of 100 mg/kg was used as a reference drug (14).

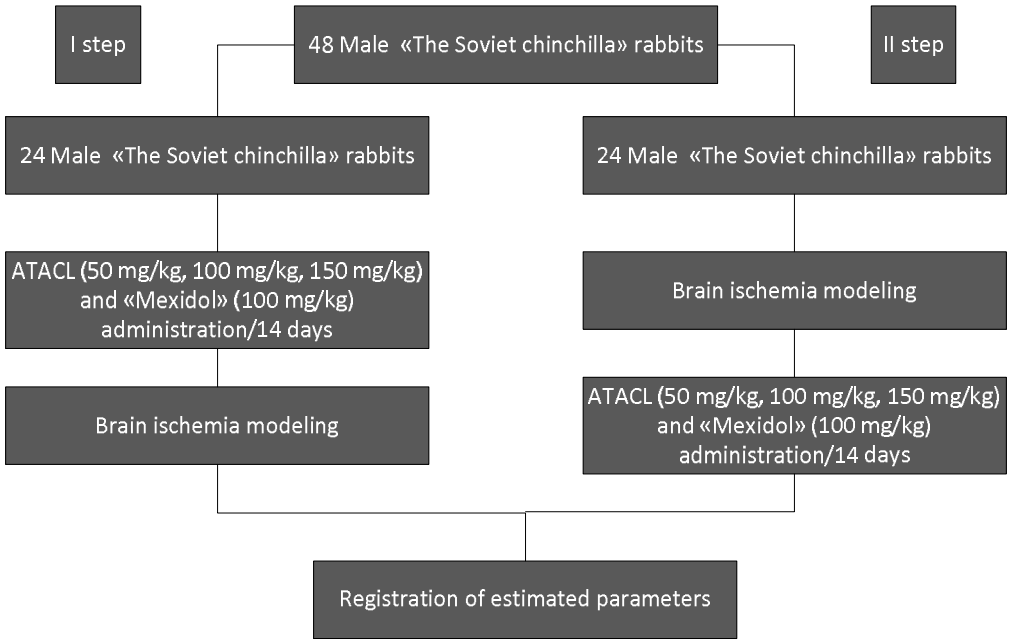
Figure 1. The structure of 4-hydroxy-3,5-di-tertbutyl cinnamic acid.



Study design

The study was approved by the local ethics committee (protocol No. 18 of June 25,2019). The study was realized in 2 steps. In the first step, the pharmacological efficacy of the test compound and the reference drug were evaluated with prophylactic administration. In the second step, the test object and reference drug were introduced after modeling cerebral ischemia in order to assess the therapeutic effect. In the course of the experiment, at each of the steps, six equal experimental groups of animals were formed (n=4 in each group). The first group included sham-operated animals (SO), to which all successive surgical procedures were applied except ligation of the common carotid arteries. The second group was the negative control group (NC); it included animals deprived of pharmacological support (this group of animals was administered isotonic sodium chloride solution). Groups No. 3-5 were given 4-hydroxy-3,5-di-tertbutyl cinnamic acid (laboratory code ATACL) at doses of 50 mg/kg, 100 mg/kg, and 150 mg/kg, respectively Animals of the sixth group were treated with the reference drug, Mexidol, at a dose of 100 mg/kg. In the first step of the study, the ATACL compound and the reference drug were administered *per os* for 14 days before the brain ischemia modeling. In the second stage of the experimental work, the ATACL substance and the reference drug were administered intragastrically after reproducing ischemic brain damage for 14 days. Animals were removed from the experiment by cervical dislocation with decapitation in the first step of the experiment, 24 hours after the reproduction of ischemia. In the second step of the experiment, the animals were removed after 14 days of administration of the test compound and the reference drug. The study design is presented in Fig. 2.

Figure 2. Study design



Cerebral ischemia model

Cerebral ischemia in rabbits was modeled by ligation of the common carotid arteries (subtotal permanent brain ischemia). In anesthetized animals (acepromazine 1 % solution, 0.05 ml/kg, intramuscularly + chloral hydrate 350 mg/kg, intraperitoneally + atropine sulfate 0.1 %, 0.1 ml, subcutaneously), the fell on the lower side of the neck was removed. Then an incision was made and soft tissues and muscles were dissected, after which the left and right common carotid arteries were visually detected. The arteries were isolated, cleaned of adjacent tissues and nerve fibers, after which a silk thread was inserted under the artery, which was used to bind the artery, while the termination of blood flow was visually noted above the ligation site. The tissue topography was then restored, and the wound was sutured and treated by antiseptic (Benzyldimethyl (3-myristoilamine) propyl) ammonium chloride monohydrate); 0.01 % solution).

Neurological deficit determination

The degree of development of neurological deficit was evaluated according to the *McGraw* scale (1976) according to the parameters presented in Table 1.

Table 1. McGraw neurological deficiency scale (1976)

Symptoms / parameters	***Neurological deficit score***
Inertness	0.5
Tremor	1

Symptoms / parameters	*Neurological deficit score*
One-side hemiptosis	1
Bi-side hemiptosis	1.5
Limb weakness	1.5
One-side ptosis	1.5
Bi-side ptosis	1.5
Circular motion	2.0
Limbs paresis (1-4)	2-5
Limb paralysis (1-4)	3-6
Coma	7.0
Death	10.0

The total score of 0.5-2.0 corresponded to a mild degree of neurological deficit; the score of 2.5-5.0 presented moderate severity; the score of 5.5-10 presented severe neurological deficit, while the degree of neurological deficit was determined by the sum of the relevant points (15).

Biomaterial sample preparation

Brain was used as biomaterial. The animals were decapitated under anesthesia and the organs were collected and divided into two parts, after which the first part of the biomaterial was homogenized in a Potter mechanical homogenizer in a selection medium (1 mmol EDTA, 215 mmol mannitol, 75 mmol sucrose, 0.1% BSA solution, 20 mmol HEPES, with a pH 7.2). The cell population was obtained by differential centrifugation, for which the obtained biogenic homogenate was centrifuged in the mode of 1400g by 3 min at 40 °C, after which the supernatant was transferred to 2 ml tubes. Next, the resulting supernatant was centrifuged at 13000g for 10 min and the supernatant (culture contains native mitochondria) was removed for respirometric analysis (16). The second part of the brain was homogenized in PBS with a pH 7.4 in a ratio of 1:7 and centrifuged in the mode of 10000 g for 5 min, then the resulting supernatant was taken for ELISA.

Respirometric analysis

Analysis of mitochondrial respiratory function was carried out by the method of respirometry using the AKPM1-01L laboratory respirometer system (Alfa Bassens, Russia). The mitochondrial respiratory function was assessed by the change in oxygen consumption in the medium against the introduction of mitochondrial respiratory uncouplers. In the last stage, there was a sequential addition of oligomycin -1 μg/ml, 4 - (trifluoromethoxy) phenyl) hydrazono) malononitrile (FCCP-1 μM), rotenone - 1 μM, and sodium azide - 20 mmol. Glucose was used as an oxidation substrate– at a dose of 15 mmol The overall assessment of mitochondrial function was determined by the level of oxygen consumption in the medium after sequential addition of oligomycin, FCCP and rotenone to the medium. The ATP-generating ability was determined by the difference in oxygen consumption after the addition of FCCP and oligomycin; the maximum level of respiration was determined according to the difference in oxygen consumption after the addition of FCCP and rotenone and the respiratory capacity was determined according to the difference in oxygen consumption after the addition of FCCP and the basal level of oxygen consumption. The activity of anaerobic processes was evaluated when glucose was used as an oxidation substrate during the registration of oxygen consumption under the conditions of sequential addition of glucose, oligomycin and sodium azide to the medium. The intensity of glycolysis was determined according to the difference in oxygen consumption after adding glucose and the basal level of oxygen consumption; glycolytic capacity was determined according to the difference in oxygen consumption after adding oligomycin and glucose; and glycolytic reserve was determined according to the difference in oxygen consumption after adding glucose and sodium azide. During the analysis, the biosample volume was 275 μl, and 25 μl of injected analyzers. Oxygen consumption was determined in ppm (17).

Study of mitochondrial pore opening

The effect of the ATACL compound and the reference drug on the opening of the mitochondrial pore was evaluated by the spectrophotometric method. The incubation medium contained: 0.5 ml of the analyzed supernatant, 200 mM KCl, and 0.5 ml of a 1 μm solution of cyclosporin A. The resulting mixture was adjusted to 2 ml with HEPES buffer solution with a pH of 7.4. The optical density of the mixture was recorded at λ= 540nm, then the resulting solution was incubated for 25 min at room temperature with constant stirring. At the same time, the latent time of opening of the mitochondrial pore in seconds was evaluated (by changing the optical density of the incubation medium) (17).

Study of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using the spectrophotometric method. The incubation medium contained 0.5 ml of the analyzed supernatant and 0.5 ml of a 9 μm solution of safranin O. The resulting mixture was adjusted to 2 ml with HEPES buffer solution with a pH of 7.4. The optical density of the mixture was recorded at λ=515 nm and λ=525 nm. The proton moving force (transmembrane electrochemical gradient, ΔΨ) was determined by the difference in the optical density: $\Delta\Psi=A_{515}-A_{525}$ (17).

ELISA study

In this study, the concentration of caspase-3 was determined by enzyme-linked immunosorbent assay in the supernatant of the brain of animals. The study was performed using a specific kit for ELISA manufactured by *Cloud clone corp.* (USA). The biosamples preparation and the course of the analysis were consistent with the recommendations of the kit manufacturer. The results were read using an *Infinite F50* microplate reader system (*Tecan*, Austria).

Statistical analysis

Statistical processing of the obtained data was carried out in the software package of statistical analysis STATISTICA 6.0 for Windows (StatSoft, USA). The results were expressed

as M (mean) ± SEM. Data was checked for normal distribution (Shapiro-Wilk test). Comparison of the groups of means was performed by the method of one-way analysis of variance (ANOVA) with post-processing of Newman-Keuls (normal distribution) or Kruskal-Wallis (abnormal distribution).

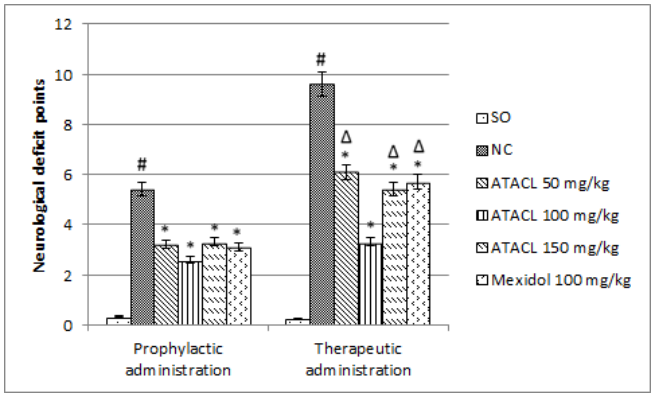
RESULTS

The effect of the ATACL compound and Mexidol on the change in neurological deficit in animals suffering from cerebral ischemia

In the first step of the study, in animals of the NC group in relation to the SO group, an increase in the degree of neurological deficit by 16.4 times ($p<0.01$) was evaluated. With the prophylactic administration of the ATACL compound at doses of 50 mg/kg, 100 mg/kg, 150 mg/kg, and Mexidol to animals after 24 hours of ischemia, a decrease of neurological deficit by 1.69 times compared with rabbits of the NC group by 1,69 ($p<0.05$), 2.08 ($p<0.05$), 1.63 ($p<0.05$) and 1.75 ($p<0.05$) times, respectively (Fig. 3) was noted.

In the second step of the experiment, an increase in neurological deficit by 38.4 times ($p<0.01$) was noted in animals of the NC group, after reproducing cerebral ischemia, in comparison with the SO group. At the same time, therapeutic administration (in the post ischemic period) of the ATACL compound at doses of 50 mg/kg, 100 mg/kg and 150 mg/kg contributed to a decrease in neurological deficit by 1.57 times ($p,<0.05$), 2.91 times ($p<0.05$) and 1.78 times ($p<0.05$), respectively. Moreover, the degree of neurological symptoms in animals treated with Mexidol after modeling cerebral ischemia was 1.68 times ($p <0.05$) less than that in the NC group (Fig. 4). In addition, in animals that were administered the ATACL compound at a dose of 100 mg/kg, neurological deficit decreased in relation to animals receiving the ATACL substance at doses of 50 mg/kg, 150 mg/kg and Mexidol by 84.8%($p<0.05$), 63.6% ($p,<0.05$) and 72.7% ($p<0.05$), respectively (Fig. 3).

Figure 3. Change in the degree of neurological deficit in animals that were treated with the ATACL compound and Mexidol in conditions of cerebral ischemia



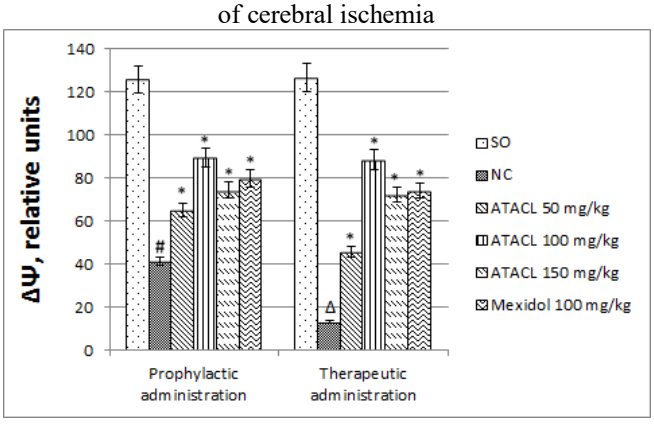
Note: # - statistically significant relative to the SO group of animals (Newman-Keuls test ($p<0.01$)); * - statistically significant relative to the NC group of animals (Newman-Keuls test ($p<0.05$)); Δ - statistically significant relative to the group of animals treated with the ATACL compound at a dose of 100 mg/kg (Newman-Keuls test ($p<0.01$)).

The effect of the ATACL compound and Mexidol on the change in the membrane potential of the brain mitochondria in animals under conditions of cerebral ischemia

In the first stage of the study, it was found that the value of the mitochondrial membrane potential (Fig. 5) decreased by 3.05 times ($p<0.05$) in the NC group of animals compared to the SO group, after 24 hours of ischemia. At the same time, in animals treated with the ATACL compound at doses of 50 mg/kg, 100 mg/kg and 150 mg/kg, the membrane potential of mitochondria increased in relation to the NC group by 58.2% ($p<0.05$), 2.17 times ($p<0.05$) and 80% ($p<0.05$), respectively. At the same time, against the background of prophylactic administration of Mexidol in rabbits, an increase in the electric potential of the mitochondrial membrane relative to the NC group of animals by 93.7% ($p<0.05$) was noted.

In the second step of the experimental work, under conditions of therapeutic administration of the ATACL compound and Mexidol (Fig. 4), it was found that the value of the mitochondrial membrane potential in animals of the NC group was decreased by 9.83 times ($p <0.01$) compared to the SO group. Moreover, against the background of the use of the compound ATACL at doses of 50 mg/kg, 100 mg/kg and 150 mg/kg, the value of the membrane potential of the brain mitochondria increased in relation to the NC group of animals by 3.54 times ($p<0.05$), 6.86 times ($p<0.05$) and 5.6 times ($p<0.05$), respectively. At the same time, therapeutic administration of Mexidol to animals contributed to an increase in the mitochondrial membrane potential in comparison with the NC group by 5.7 times ($p<0.05$).

Figure 4. Change in the membrane potential of brain mitochondria in animals with the administration of the compound ATACL and Mexidol under conditions of cerebral ischemia



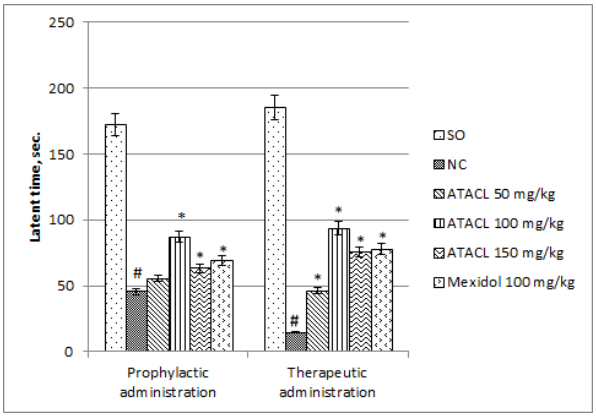
Note: statistically significant relative to the SO group of animals (Kruskal-Wallis test # - p<0.05; - p<0.01); * - statistically significant relative to the NC group of animals (Kruskal-Wallis test (p<0.05)).

The effect of the ATACL compound and Mexidol on the change in the degree of opening of the mitochondrial pore under cerebral ischemia

During the course of this study block, in animals of the NC group (step 1), in comparison with the SO group, a decrease of the latent time of opening of the mitochondrial pore (Fig. 5) by 3.77 times (p <0.05) was noted. The prophylactic administration of the ATACL compound at a dose of 50 mg/kg did not significantly affect the degree of opening of the mitochondrial pore, while the use of this compound at doses of 100 mg/kg and 150 mg/kg contributed to an increase (relative to the NC group) of the latent time of mitochondrial formation pores by 90.6% (p<0.05) and 39% (p<0.05), respectively. Against the background of prophylactic administration of Mexidol, the frequency of opening of the mitochondrial pore decreased in comparison with the NC group by 51.5% (p<0.05).

Under the conditions of therapeutic administration of the ATACL compound and Mexidol (step 2 of the experimental work), a decrease in the latent time of formation of the mitochondrial pore in the NC group of animals, with respect to the SO group, by 12.7 times (p <0.05) was noted. At the same time, in animals treated with the ATACL compound at doses of 50 mg/kg; 100 mg/kg and 150 mg/kg, the degree of opening of the mitochondrial pore was by 3.18 times (p<0.05), 6.42 times (p<0.05) and 5.18 times (p<0.05) less than the similar value of the NC group (Fig. 5). Against the background of the use of Mexidol, an increase in the latent time of opening of the mitochondrial pore with respect to the NC group of animals by 5.3 times (p <0.05) was noted. In addition, the latent time of mitochondrial pore formation in animals that were treated by ATACL compound at a dose of 100 mg/kg was 2 times (p <0.05) longer than that in rabbits treated with the ATACL substance at a dose of 50 mg/kg (Fig. 5).

Figure 5. Change in latent time of opening of the mitochondrial pore in animals with the administration of the ATACL compound and Mexidol under conditions of cerebral ischemia

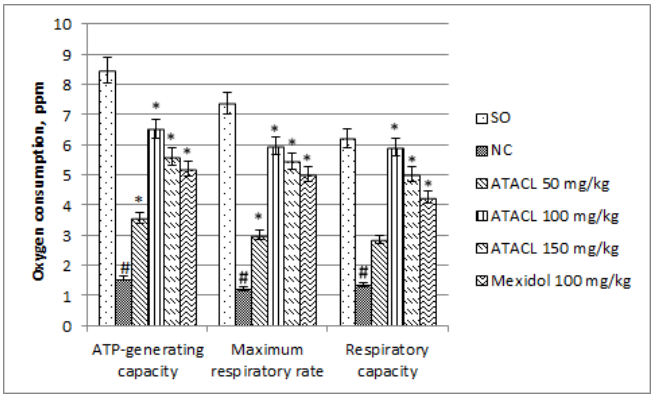


Note: # - statistically significant relative to the SO group of animals (Newman-Keuls test (p<0.05)); * - statistically significant relative to the NC group of animals (Newman-Keuls test (p<0.05)); Δ is statistically significant relative to the group of animals treated with the ATACL compound at a dose of 100 mg/kg (Newman-Keuls test (p<0.01)).

The effect of the ATACL compound and Mexidol on the change in the respirometric function of mitochondria in animals under cerebral ischemia

During the first block of the experimental study, a decrease in ATP-generating ability, maximum level of respiration and respiratory capacity in animals of the NC group, after 24-hour cerebral ischemia, relative to the SO group by 5.5 times (p<0.01), 6 times (p<0.01) and 4.6 times (p<0.01), respectively, was observed (Fig. 6). At the same time, the prophylactic use of Mexidol contributed to the increase in ATP-generating ability of animals by 3.3 times (p<0.01), the maximum level of respiration by 4.1 times (p<0.01) and respiratory capacity by 3.1 times (p<0.01), in relation to the NC group of animals. Against the background of administration (prior to the simulation of cerebral ischemia) to animals, the ATACL compound at a dose of 50 mg/kg compared with the NC group showed an increase in ATP-generating ability, maximum respiratory rate and respiratory capacity by 2.2 times (p<0.01), 2.4 times (<0.01) and 2 times (p<0.01), respectively. On the other hand, when using this compound at a dose of 100 mg/kg, the indicators evaluated at this stage of the experimental work increased by 4.1 times (p<0.01), 4.8 times (p<0.01) and 4.3 times (p<0.01), respectively, relative to the NC group of animals (Fig. 6). With the prophylactic use of the ATACL compound at a dose of 150 mg/kg in relation to the group of animals deprived of pharmacological support, an increase in ATP-generating ability by 3.5 times (p<0.01) and the maximum level of respiration by 4.4 times (p<0.01) and respiratory capacity by 3.7 times (p<0.01) was observed.

Figure 6. Change in the processes of mitochondrial respiration during the prophylactic administration of the ATACL compound and Mexidol under conditions of cerebral ischemia

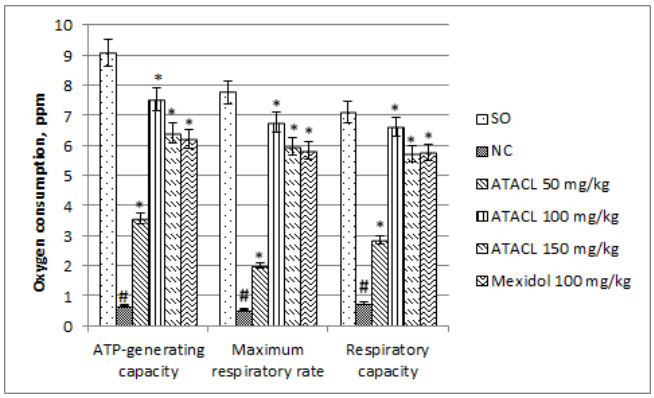


Note: # - statistically significant relative to the SO group of animals (Kruskal-Wallis test (p<0.01)); * - statistically

significant relative to the NC group of animals (Kruskal-Wallis test ($p<0.01$)).

During the second step of the study, there was a decrease in ATP-generating ability, maximum respiratory rate and respiratory capacity in animals of the NC group after 14 days of cerebral ischemia, in comparison with the SO group, by 13.3 times ($p<0.01$); 14.5 times ($p<0.01$) and 9.3 times ($p<0.01$), respectively, (Fig. 7). Against the background of therapeutic administration of Mexidol (Fig. 7) in relation to the NC group of animals, an increase in ATP-generating ability by 9.1 times ($p<0.01$), maximum respiratory rate by 10.8 times ($p<0.01$) and respiratory capacity 7.5 times ($p<0.01$) was observed. At the same time, the therapeutic administration of the ATACL compound at a dose of 50 mg/kg contributed to the restoration of the mitochondrial respirometric function, which was expressed as an increase (in comparison with the NC group of animals) of ATP-generating ability, maximum respiratory rate and respiratory capacity by 5.2 times ($p<0.01$); 3.7 times ($p<0.01$) and 3.5 times ($p<0.01$), respectively. When using the ATACL substance at a dose of 100 mg/kg in the post-ischemic period, an increase in ATP-generating ability, maximum respiratory rate and respiratory capacity in relation to the group of animals lacking pharmacological support was observed 11 times ($p<0.01$), 12.2 times ($p<0.01$) and 8.6 times ($p<0.01$), respectively. At the same time, the therapeutic administration of ATACL at a dose of 150 mg/kg contributed to an increase in ATP-generating ability relative to the NC of the animal group by 9.4 times ($p<0.01$), maximum respiratory rate by 11.1 times ($p<0.01$) and respiratory capacity by 7.5 times ($p<0.01$).

Figure 7. Change in the processes of mitochondrial respiration during therapeutic administration of the ATACL compound and Mexidol under conditions of cerebral ischemia

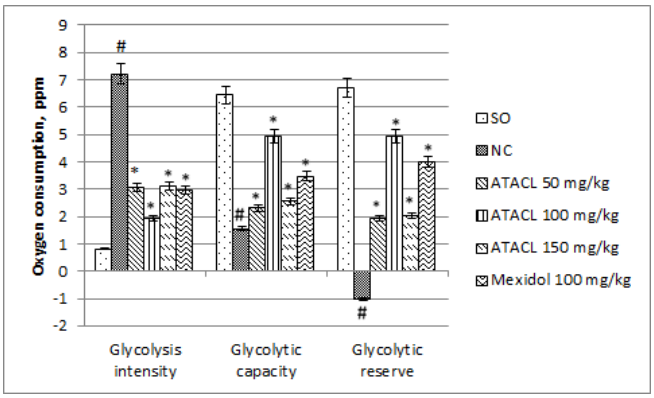


Note: # - statistically significant relative to the SO group of animals (Kruskal-Wallis test ($p<0.01$)); * - statistically significant relative to the NC group of animals (Kruskal-Wallis test ($p<0.01$))

The effect of the ATACL compound and Mexidol on changes in the activity of anaerobic processes in animal brain tissue under cerebral ischemia

During the first step of the study, in animals of the NC group compared to the SO group, there was an increase in glycolysis intensity by 8.8 times ($p<0.001$) and a decrease in glycolytic capacity by 4.1 times ($p<0.001$), while glycolytic reserve took a negative value (Fig. 8). Against the background of prophylactic administration of Mexidol, a restoration of the glycolytic reserve was noted, as well as an increase in glycolytic capacity by 2.2 times ($p<0.001$), accompanied by a decrease in the intensity of glycolysis compared to the NC group of animals by 2.4 times ($p<0.001$). When using the ATACL compound at a dose of 50 mg/kg, the glycolysis intensity decreased by 2.4 times ($p<0.001$) with respect to the NC group, while the glycolytic capacity increased by 1.5 times ($p<0.001$) against the background of the restoration of the glycolytic reserve. At the same time, the glycolysis intensity against the background of ATACL compound administration at doses of 100 mg/kg and 150 mg/kg decreased in comparison with the NC group by 3.7 times ($p<0.001$) and 2.3 times ($p<0.001$), respectively. The value of glycolytic capacity increased by 3.2 times ($p<0.001$) and 1.7 times ($p<0.001$), respectively. It should be noted that in animals prophylactically treated with the ATACL compound at doses of 100 mg/kg and 150 mg/kg, a restoration of the glycolytic reserve was noted (Fig. 8).

Figure 8. Change in the activity of anaerobic processes in animal brain tissue during the prophylactic administration of the ATACL compound and Mexidol under conditions of cerebral ischemia

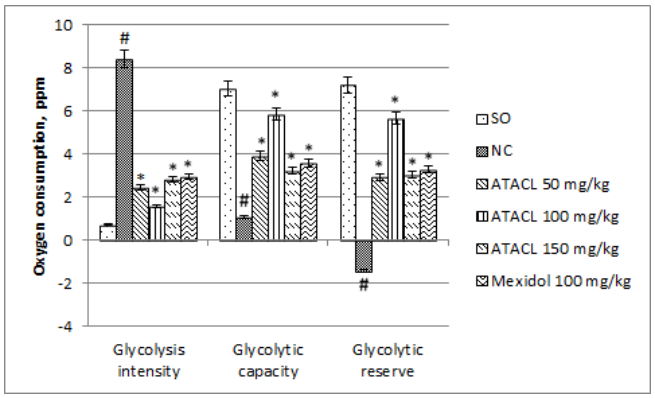


Note: # - statistically significant relative to the SO group of animals (Newman-Keuls test ($p<0.001$)); * - statistically significant relative to the NC group of animals (Newman-Keuls test ($p<0.001$)).

At the second step of the study, in animals of the NC group after 14 days of ischemia, in comparison with the SO group, an increase in the intensity of glycolysis by 11.7 times ($p<0.001$) was observed, accompanied by a decrease in glycolytic capacity by 6.5 times ($p<0.001$) with a negative value of the glycolytic reserve (Fig. 9). The therapeutic administration of Mexidol in relation to the NC group showed a

decrease in the intensity of glycolysis by 2.9 times ($p<0.001$) and an increase in glycolytic capacity by 3.4 times ($p<0.001$) when restoring the glycolytic reserve. At the same time, against the background of the ATACL compound use at a dose of 50 mg/kg, a decrease in the intensity of glycolysis and an increase in glycolytic capacity relative to animals deprived of pharmacological support by 3.4 times ($p<0.001$) and 3.7 times ($p<0.001$), respectively, were noted. In this case, the glycolysis intensity decreased by 5.4 times ($p<0.001$) and 3 times ($p<0.001$) when the animals were administered the ATACL compound at doses of 100 mg/kg and 150 mg/kg. On the other hand, the glycolytic capacity increased when the ATACL substance was administered at doses of 100 mg/kg and 150 mg/kg, compared with the NC group of animals, by 5.5 times ($p <0.001$) and 3 times ($p <0.001$), respectively. It should be noted that with the therapeutic use of the ATACL compound in the entire studied dose range, the restoration of the glycolytic reserve was observed (Fig. 9).

Figure 9. Changes in the activity of anaerobic processes in animal brain tissue during therapeutic administration of the ATACL compound and Mexidol under conditions of cerebral ischemia



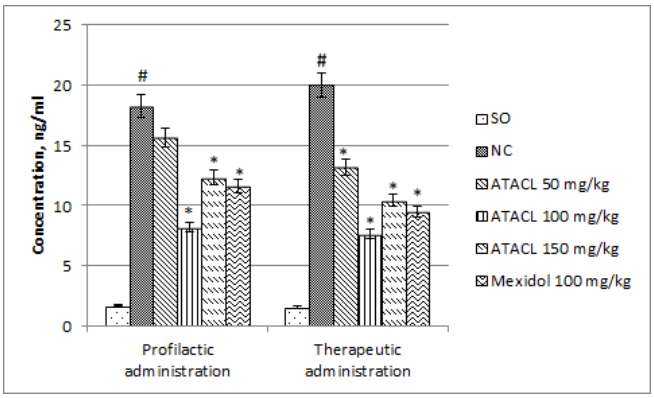
Note: # - statistically significant relative to the SO group of animals (Newman-Keuls test ($p<0.001$)); * - statistically significant relative to the NC group of animals (Newman-Keuls test ($p<0.001$)).

The effect of the ATACL compound and Mexidol on changing the concentration of caspase -3 in animal brain tissue under cerebral ischemia

During the first stage of the study, in animals of the NC group, in comparison with the SO group, an increase in the concentration of caspase-3 in the brain supernatant by 10.6 times ($p<0.001$) was noted. At the same time, prophylactic administration of Mexidol contributed to a decrease in the level of capsase-3 in relation to the NK group of animals by 57.4% ($p<0.05$). Also, the concentration of caspase-3 decreased with the administration of 100 mg/kg and 150 mg/kg ATACL compound to animals by 2.2 times ($p<0.05$) and 48.5% ($p<0.05$), while prophylactic use of this compound at a dose of 50 mg/kg did not significantly affect the change in caspase-3 level in the supernatant of animals brain (Fig. 10).

At the second stage of the study, in animals of the NC group, after 14 days of the ischemic period, the concentration of capsase-3 in the supernatant of the animals brain exceeded the similar indicator of the SO group by 12.8 times ($p<0.001$). Against the background of therapeutic administration of Mexidol in animals, a decrease in the level of caspase-3 by 2.1 ($p<0.05$) in comparison with the NC group was noted. At the same time, in animals therapeutically treated with ATACL at doses of 50 mg/kg, 100 mg/kg and 150 mg/kg, the concentration of caspase-3 in the supernatant of the brain was lower than that in the NC group of animals by 51.5% ($p <0.05$), by 2.6 times ($p<0.05$) and by 92.3% ($p<0.05$), respectively (Fig. 10).

Figure 10. Change in the concentration of caspase-3 in the animals' brain tissue with the administration of the ATACL compound and Mexidol under conditions of cerebral ischemia



Note: # - statistically significant relative to the SO group of animals (Newman-Keuls test ($p<0.001$)); * - statistically significant relative to the NC group of animals (Newman- Keuls test ($p<0.05$)).

DISCUSSION

The high medical, social and economic impact of ischemic cerebrovascular accident makes it relevant to search for optimal treatment and prevention strategies for this condition (19). Among the existing methods of treating ischemic stroke, thrombolytic therapy that aims at fast restoration of cerebral blood flow is generally recognized (20). However, despite the proven effectiveness of thrombolytic pharmacotherapy, this pharmacological approach has significant limitations for use (such as a small therapeutic window); In addition, there is a significant risk of reperfusion complications of thrombolytic therapy (21). It is important that measures used to prevent ischemic stroke are focused on reducing the impact of risk factors (hypreglycemia, atherosclerosis, coronary heart disease) on the human body, or limited to the use of dietary supplements of cerebrotropic action, which often have little evidence of use (22). In this regard, the development of new ways of preventing and treating brain ischemic damage becomes relevant. Literary sources provide data on the positive effect of compounds - derivatives of a cinnamic acid on the functional state of brain cells

under ischemic conditions. Thus, a study by Zhao et al, 2015 showed that the use of cinnamaldehyde helped to reduce neurological deficit and the cerebral infarction zone area, suppressed the activation of intracellular signal transduction molecules, and decreased the concentration of pro-inflammatory markers and leukocyte infiltration of the ischemic site in animals with permanent brain ischemia (23). Hemmati et.al., 2018 demonstrated the variable dose-dependent effect of cinnamic acid on the course of redox reactions and oxidative stress under conditions of streptozotocin-induced dementia. Under these experimental conditions, the use of cinnamic acid contributed to the restoration of the memorial trail in animals, normalization of the activity of antioxidant enzymes (superoxidedismutase and catalase) and a decrease in lipid peroxidation (24). Also, a study by Ren et.al.2017 showed that, in conditions of cerebral ischemia, the use of ferulic acid reduced the risk of reperfusion complications by suppressing apoptotic reactions and normalizing the antioxidant state of the cell (25). In this regard, it seems advisable to study cinnamic acid derivatives as cerebroprotective agents.

The study showed that the use of 4-hydroxy-3,5-di-tertbutyl cinnamic acid helped to restore the functional activity of the rabbits brain mitochondria under conditions of cerebral ischemia. In this case, the most pronounced pharmacological effect was noted in the use of 4-hydroxy-3,5-di-tertbutyl cinnamic acid at a dose of 100 mg/kg. Thus, the prophylactic and therapeutic administration of this ATACL dose to animals showed a decrease in neurological deficit and contributed to a restoration of the mitochondrial membrane potential, which was higher than that in the group of animals without pharmacological support. In addition, with the prophylactic (before the modeling of ischemia) administration of 4-hydroxy-3,5-di-tertbutyl cinnamic acid, an increase in ATP-generating ability, maximal respiratory rate and respiratory capacity, respectively, was noted. Under conditions of therapeutic administration, these parameters increased as well. Also, both the prophylactic and therapeutic use of 4-hydroxy-3,5-di-tertbutyl cinnamic acid contributed to the normalization of aerobic/anaerobic metabolism, and to a decrease in the concentration of the proapoptotic enzyme caspase-3.

In previous studies, the cerebroprotective properties of 4-hydroxy-3,5-di-tertbutyl cinnamic acid were established (13), but the detailed mechanism of action of this compound was not established. This study represent data on the positive effect of 4-hydroxy-3,5-di-tertbutyl cinnamic acid on bioenergetic processes occurring in the brain mitochondria under cerebral ischemia, and large laboratory animals that are more susceptible to ischemic effects were used as a biological model. Thus, the mechanism of the cerebrotropic action of 4-hydroxy-3,5-di-tertbutyl cinnamic acid can be mediated by the restoration of mitochondrial function. Literature also provides data on the positive effects of cinnamic acid derivatives on mitochondrial function. A study by Meeprom et.al., 2018 showed that the use of isoferulic acid reduces the degree of damage to mitochondria under conditions of methylglutoxal-induced apoptosis (26). The anti-apoptotic effect of cinnamic acid derivatives was also established by Wang et.al., 2018, where it was demonstrated that ligustrazine-cinnamon acid derivatives suppressed cytochrome C releasing and restored the activity of the anti-apoptotic complex Bcl-2 / Bax upon the induction of apoptosis of cobalt chloride (27). The positive effect of cinnamic acid was shown in the study by Anupama et.al., 2018, where the use of cinnamic acid contributed to the restoration of mitochondrial membrane potential and a decrease in the intensity of caspase-dependent apoptosis reactions (27). The suppression of apoptosis reactions under the influence of polyphenolic compounds, including derivatives of cinnamic acid, may be associated with the interaction of this class of compounds with components of the mitochondrial pore of transition permeability (mPTP). A study by Makoto N., et.al. 2019showed that polyphenolic compounds can directly interact with the N-terminal amino acid residues of VDAC, thereby changing the conformation of this structure and inhibiting the opening of mPTP. In addition, indirect inhibition of VDAC activity by polyphenolic compounds is possible, realized through a change in the activity of regulatory proteins - sirtuins, particulary sirtuin 1 (29). Cinnamic acid derivatives can also stimulate mitochondrial biogenesis, as confirmed by a study by Gannon et.al. 2015, in which trans-Cinnamaldehyde stimulated mitochondrial biogenesis through the activation of AMPK, PGC-1α, (PPARa) and PPARß / δ (29).

At the same time, 4-hydroxy-3,5-di-tertbutyl cinnamic acid compares with the favorably mitochondrial dysfunction correctors – triphenylphosphonium-based compounds, the synthesis and purification of which are laborious and not economically viable (31). At the same time, the synthesis of 4-hydroxy-3,5-di-tertbutyl cinnamic acid is easily performed and does not require significant economic investments (31), which makes this compound a promising and economically affordable cerebroprotective agent with high pharmacological activity aiming at restoring mitochondrial function under conditions of cerebral ischemia.

CONCLUSION

The study showed that both prophylactic and therapeutic use of 4-hydroxy-3,5-di-tertbutyl cinnamic acid under conditions of cerebral ischemia contributed to the normalization of mitochondrial bioenergetic processes and to a decrease in caspase-3 concentration, which ultimately contributed to a decrease in neurological symptoms in animals. Based on the obtained data, significant cerebroprotective properties of 4-hydroxy-3,5-di-tertbutyl cinnamic acid, realized through the restoration of mitochondrial function, can be assumed.

ETHICS APPROVAL

All research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU).

COMPETING INTERESTS

There are no conflicts of interest.

FUNDING

None.

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ORIGINAL SCIENTIFIC ARTICLE

THERAPEUTIC POTENTIAL OF „DERIVED- MULTIPLE ALLOGENEIC PROTEINS PARACRINE SIGNALING-D-MAPPS" IN THE TREATMENT OF DRY EYE DISEASE

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ABSTRACT

The primary aim of this retrospective study was to estimate significance of determining C-reactive protein and procalcitonin for a diagnosis of sepsis in adult patients in early triage. Also, the aim of this study was to measure the sensitivity of the SIRS criteria, PCT and CRP levels and sepsis definitions to identify the most serious sepsis cases in the prehospital setting and at the Emergency Department (ED) triage. All patients were divided into two groups according to specific criteria for defining sepsis. First group (SIRS+ group) of patients were patients with clinically and/or laboratory confirmed sepsis (or systemic inflammatory response syndrome (SIRS) to bacterial infection with different localization). For confirmation of the SIRS we consider positive two or more clinical criteria (≥2 clinical criteria). The SIRS criteria use the clinical criteria of the Surviving Sepsis Campaign (SSC) for the SIRS, comprising at least two of the following criteria: HR > 90/min, RR > 20/min and temperature < 36° or ≥ 38.3°C and the next laboratory parameters such as leucocytosis > 15x109/L, leucopenia < 4x109/L, > 10% immature leucocytes. Second group of patients were patients with the SIRS negative criteria as a diagnostic tool (SIRS- group). We have founded that the CRP showed high sensitivity but no specificity in patients with sepsis, but on the other side, the PCT as a diagnostic marker showed a high sensitivity and high specificity in these patients. Also, the PCT is in positive correlation with the SIRS criteria, which could be of a clinical significance in early diagnosis of septic infections.

Keywords: *sepsis, C-reactive protein, procalcitonin, early diagnostic markers.*

INTRODUCTION

Dry eye disease (DED) is a common, multifactorial disease of the lacrimal system and ocular surface [1]. Loss of homeostasis, instability and hyperosmolarity of the tears, chronic inflammation in the eye and consequent neurosensory dysfunction are usually manifested by visual disturbance, dryness, grittiness, scratchiness, soreness, irritation, burning, watering, and eye fatigue. Significantly reduced functional visual acuity impairs performance of vision-dependent daily activities (reading, writing, driving), greatly diminishing quality of life of DED patients [1]. Currently, there is no cure for dry eye, and the treatments are directed towards improving the symptoms in order to break the vicious circle of chronic inflammation [1, 2]. Since clinical manifestations of DED are observed in patients who suffer from chronic inflammatory and systemic autoimmune diseases (Sjögren's Syndrome, Rheumatoid arthritis, Systemic lupus erythematosus), it was postulated that detrimental immune response had a crucially important role in the development and progression of DED [2-6]. Therefore, molecular mechanisms responsible for the induction of inflammatory cascade in the eyes of DED patients have been evaluated by large number of research groups. Considering the important role of inflammation in DED development, the main treatment strategy has shifted from hydration and lubrication of dry ocular surface to the immunomodulation and immunoregulation-approach that should address the main pathologic processes responsible for disease progression [2-5]. However, it should be noted that long-term, systemic use of immunosuppressive drugs may result in the development of severe, secondary immunodeficiency, significantly increasing the risk for the development of infectious diseases and malignancy [6]. Therefore, new remedies for DED treatment should suppress detrimental immune response in the eye without affecting systemic inflammatory response [7].

Due to their capacity for production of immunosuppressive factors, mesenchymal stem cells (MSCs) and their secretome have been considered as potentially new agents in DED therapy since they may, after local application in the eye, regulate detrimental immune response without causing life-treating systemic immunosuppression [8]. Most recently, it was revealed that MSC-derived exosomes (MSC-Exos) were mainly responsible for beneficial effects of MSC-sourced secretome in alleviation of inflammatory eye diseases [9]. MSC-Exos are nano-sized (30-100nm) vesicles that carry nucleic acids, lipids and proteins (cytokines, chemokines) and are capable to modulate migratory and effector functions of immune cells (T lymphocytes, dendritic cells (DCs), macrophages) which have crucially important pathogenic role in DED development and progression. Since membranes of MSC-Exos are enriched in cholesterol, sphingomyelin, ceramide and lipid raft proteins, these nano-sized carriers of MSC-derived immunosuppressive factors may be taken by target cells through endocytosis or membrane fusion, regardless of biological barriers [9].

In line with these findings, we recently developed immunomodulatory ophthalmic solution "derived- Multiple Allogeneic Proteins Paracrine Signaling (d-MAPPS)" which activity is relied on immunosuppressive capacity of MSC-derived secretome [10]. d-MAPPS contains MSC-Exos, growth factors and immunosuppressive cytokines that are able to efficiently suppress generation of inflammatory phenotype in T cells and macrophages [10]. Herewith, we demonstrated that d-MAPPS protected human corneal epithelial cells from chemical injury and efficiently alleviated ocular discomfort and pain in DED patients.

MATERIAL AND METHODS

Preparation of d-MAPPS samples and eye drops

Sterile d-MAPPS is a bio-engineered biologic product obtained from amniotic fluid derived MSCs (AF-MSCs), previously collected from healthy human donors. Blood samples were given by the donor prior to or at the time of collection and were tested by laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) and were found negative using United States (U.S) Food and Drug Administration (FDA) licensed tests for detection of at minimum: Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus Types 1/2, Treponema Pallidum. AF samples were obtained with patient consent and kept at 4°C until processed. d-MAPPS samples were bio-engineered as AF-MSC-derived sterile product containing AF-MSC-Exos and AF-MSC-derived cytokines and growth factors, manufactured under current Good Manufacturing Practices (cGMP), regulated and reviewed by the FDA (10). Sterile d-MAPPS incorporate Regenerative Processing Plant's (RPP) proprietary patented sterilization process to provide for a safe, sterile product. d-MAPPS samples as well as d-MAPPS-based eye drops, used in this study, were manufactured under specific conditions in order to be applicable for bioavailability testing and for different therapeutic use.

Cells

Therapeutic potential of d-MAPPS in corneal protection was determined by using human corneal epithelial cells (HCEC). HCEC was purchased from Gibco (catalog no. C018-5C). The cells were cultured in keratinocyte serum free medium (SFM) (17005-042) at 37°C in a 5% CO_2 incubator. HCEC in 2nd passage was used throughout the experiment.

In vitro induction of corneal epithelial cell injury

HCEC were exposed to 1mL of benzalkonium chloride (BAK) at concentrations of 0.001% and 0.005% for 30 min. Control cells were treated with phosphate buffer saline [11]. HCEC were cultured for additional 48h either in the presence of d-MAPPS or medium.

In order to evaluate whether d-MAPPS improved viability of BAK-injured HCEC, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used.

HCEC were diluted with medium to $1x10^6$ cells/ml and were placed in individual wells in 96-multiplates. About 48h later, after the cell adherence, each well received 100µl of d-MAPPS or medium. Cells were incubated at 37°C in a 5% CO_2 incubator for 24h. After incubation, multiplates were centrifuged, the supernatant was removed, fresh medium and MTT solution (5 mg/ml in PBS) 20µl were added to each well and the plates were incubated for an additional 4h. The multiplates were centrifuged, cell-free supernatants were suctioned off, and DMSO (150µl) and glycine buffer were added to dissolve the crystals. The plates were shaken for 10 min. The optical density of each well was determined at 595nm using microplate multimode detector Zenyth 3100. [12].

Patients

There was a total of 131 DED patients recruited (27 male and 104 female) with a median age of 62 years (range 19-85). Patients received d-MAPPS eye drops and were followed-up for 12 months. The Principle of Good Clinical Practice and the Declaration of Helsinki were always adhered to. Patients were under continuous medical supervision by either their Ophthalmologist or Optometrist.

Clinical assessment of d-MAPPS based effects

Subjective symptoms were graded numerically using the VAS (visual analogue pain score). The scale ranged from 0 (absence of pain) to 10 (maximal pain). The subjects were asked to describe their discomfort or pain using the VAS. Standard Patient Evaluation of Eye Dryness Questionnaire (SPEED) is a questionnaire used for the evaluation of dry eye-related symptoms. The symptoms inquired by the SPEED questionnaire include dryness or grittiness or scratchiness, soreness or irritation, burning or watering, and eye fatigue reported and scored as sometimes-1, often-2, and constant-3, and whether these symptoms pose no problems-0, were tolerable-1, uncomfortable-2, bothersome-3, or intolerable-4 [13-14].

Statistics

Data were expressed as the mean ± standard error of the mean (SEM) for each group. Results were analyzed by Student's t test. Statistical analyses were performed using SPSS 25.0 for Windows software (SPSS Inc., Chicago, IL, USA). The difference was considered significant when $p < 0.05$.

RESULTS

d-MAPPS showed good tolerability on human corneal epithelial cell cultures

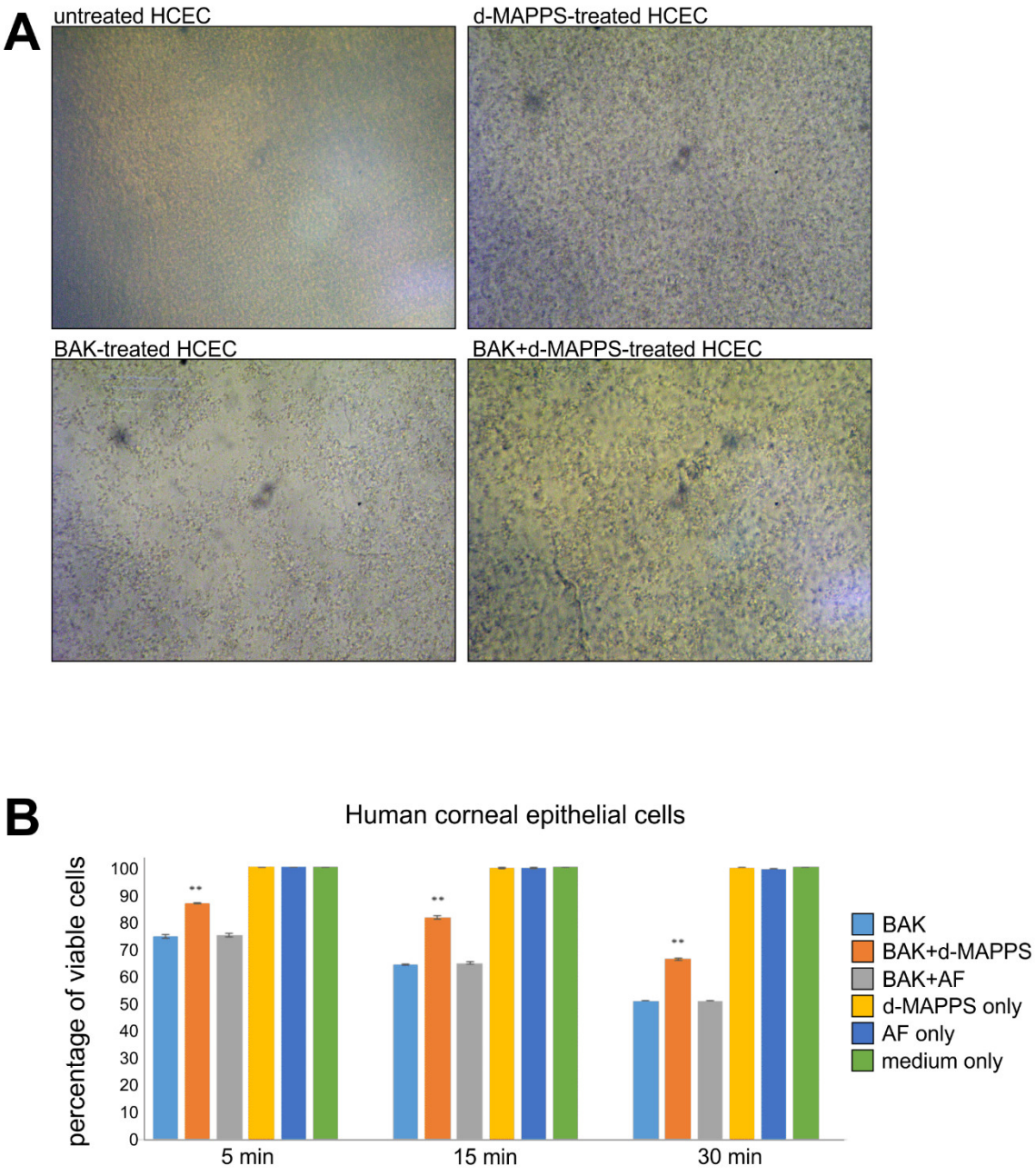
In order to determine whether d-MAPPS was well tolerated by HCEC, these cells were cultured in the presence of this MSC-derived product. As it is shown in Figure 1A, d-MAPPS-treatment was not toxic for HCEC. Morphology of HCEC was not altered by d-MAPPS and density of d-MAPPS treated HCEC was not significantly lower when compared to the cells that were grown under standard culture conditions. Loss of cell-to-cell contact was noticed in BAK-treated HCEC, while d-MAPPS treated HCEC grew in the same manner as under standard culture conditions. Importantly, morphology and density of BAK-injured HCEC was remarkably improved after d-MAPPS treatment (Figure 1A).

Importantly, viability of BAK-injured HCEC that were cultured in the presence of d-MAPPS was significantly higher when compared to BAK-injured HCEC that were grown under standard culture conditions or AF. After 5 min exposure to BAK, the percentage of viable HCEC had decreased by approximately 25%. After 15 min exposure to BAK, the viability of HCEC decreased for additional 10%, and at the 30 min time point, percentage of live cells significantly dropped down, being 48% lower than in the control, BAK-untreated group. Importantly, d-MAPPS treatment managed to significantly increase viability of BAK-injured HCEC at all time points (Figure 1B). The cell viability of BAK+d-MAPPS treated HCEC was 85% after 5 min exposure to BAK, 80% at the 10 min time point and percentage of viable HCEC was 65% after 30 min exposure to BAK (Figure 1B). d-MAPPS did not affect viability of BAK-untreated cells. There was no significant difference in the percentage of viable HCEC between BAK-untreated HCEC that grew under standard culture conditions and in the presence of d-MAPPS (Figure 1B).

d-MAPPS significantly attenuated VAS and SPEED scores in DED patients

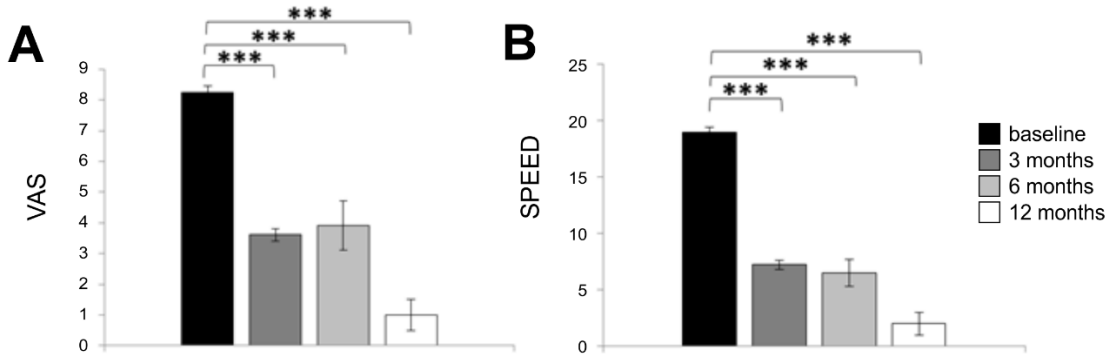
In line with the in vitro-observed results were findings obtained in clinical settings. Significantly reduced VAS (Figure 2A) and SPEED (Figure 2B) scores were noticed in d-MAPPS-treated DED patients, indicating that d-MAPPS eye drops managed to improve symptoms including pain, dryness, grittiness, scratchiness, soreness, irritation, burning, watering and eye fatigue (Figure 2). Importantly, d-MAPPS induced beneficial effects have been noticed during the entire observational period and significantly increased during the last 6 months of the follow-up. Significantly lower VAS and SPEED scores were documented 3 months after d-MAPPS treatment (Figure 2A-B, $p<0.001$), but the highest reduction in VAS and SPEED scores in DED patients were observed after 12 months of d-MAPPS-based therapy, indicating the long-lasting beneficial effects of d-MAPPS in alleviation of ocular symptoms in DED patients.

Figure 1. d-MAPPS showed good tolerability on human corneal epithelial cell cultures.



A) Morphology of human corneal epithelial cells (HCEC) was not altered by d-MAPPS. Density of d-MAPPS-treated HCEC was not significantly lower when compared to the cells that were grown under standard culture conditions. d-MAPPS significantly reduced benzalkonium chloride (BAK)-induced decrease in HCEC density. B) Results obtained by MTT assay showed that viability of BAK+d-MAPPS treated HCEC was significantly higher when compared to BAK-injured HCEC that were grown under standard culture conditions or amniotic fluid (AF). Results are shown as Mean+/-standard deviation (**$p<0.01$).

Figure 2. d-MAPPS eye drops significantly improved clinical symptoms in DED patients.



Significantly reduced (A) Visual Analogue pain (VAS) and
(B) Standard Patient Evaluation of Eye Dryness Questionnaire (SPEED) scores were noticed in 131 d-MAPPS treated DED patients, during the 12 month of follow-up (***p<0.001).

DISCUSSION

Herewith, we demonstrated therapeutic potential of newly designed MSC-derived product d-MAPPS eye drops in attenuation of DED. d-MAPPS was well tolerated by corneal epithelial cells and promoted their recovery from BAK-induced injury (Figure 1). Accordingly, d-MAPPS eye drops efficiently alleviated ocular symptoms in DED patients, indicating therapeutic potential of d-MAPPS in suppression of inflammatory diseases of the eye.

Several lines of evidence indicated that T cell-driven immune response has crucial role in the pathogenesis of DED [15-17]. Furthermore, inflammation can be considered as a cause and a consequence of DED [18]. Alterations in tear production and composition, particularly elevated osmolarity, promote inflammation on the ocular surface and lid margins, by activating c-Jun N-terminal kinase (JNK) and NF-kβ signaling pathways in epithelial cells which result in enhanced secretion of alarmins, pro-inflammatory cytokines and chemokines [15, 18]. Increased concentration of these mediators attracts circulating monocytes and lymphocytes into the lacrimal glands and ocular surface [18]. DCs and T cells within ocular surface and draining lymph nodes interact to defend the eye against the variety of microbial agents that populate or infect the cornea and ocular surface [15, 18]. Resident DCs capture bacterial antigens, present them to the naive CD4+ T cells in regional lymph nodes and through the secretion of IL-12 induce their differentiation in effector, IFN-γ-producing Th1 cells which, in turn, in IFN-γ-dependent manner promote polarization of resident macrophages in inflammatory M1 phenotype [15]. Th1 cell-derived IFN-γ promote apoptosis and squamous metaplasia of the ocular surface epithelia while M1 macrophage-derived matrix metalloproteinases (MMPs) and inflammatory mediators (tumor necrosis factor alpha (TNF-α) and nitric oxide) disrupt epithelial cell barriers [15, 18]. We recently demonstrated that d-MAPPS significantly attenuated concentration of IL-12 in the supernatants of activated human peripheral blood mononuclear cells (pbMNCs) and alleviate production of IFN-γ in activated lymphocytes [19]. d-MAPPS contains large number of immunoregulatory factors which are capable to suppress detrimental T cell-driven immune response [10]. Among them, GRO-γ is mainly responsible for the suppression of DCs:T cell cross-talk and for inhibition of DC-dependent generation of inflammatory Th1 cells [20]. Human MSCs secrete GRO-γ which was found in high concentration in d-MAPPS samples [20]. MSCs, in GRO-γ-dependent manner induce polarization of monocyte-derived DCs into myeloid derived suppressor cells. GRO-γ-treated DCs had a tolerogenic phenotype characterized by increased secretion of immunosuppressive IL-10, and reduced production of inflammatory cytokines IL-12 and IFN-γ [20]. In line with these findings, we assume that administration of GRO-γ-containing d-MAPPS eye drops, suppressed production of IL-12 in resident DCs, inhibited generation of Th1 lymphocytes and M1 macrophages and resulted in alleviation of eye inflammation in DED patients.

Inflammatory DCs in IL-1, IL-6 and IL-23-dependent manner induce differentiation of naïve T cells into effector Th17 cells that reduce tear production and promote progression of DED [18]. In similar manner as Th1 cells, effector Th17 cells promote corneal epithelial barrier disruption through the enhanced production of IL-17 [15]. Since d-MAPPS efficiently attenuated production of IL-17 in activated CD4+ T helper cells [19], we assume that d-MAPPS-induced suppression of Th17 cell-driven inflammation in the eye was, at least partially, responsible for beneficial effects of d-MAPPS eye drops in DED patients.

CD4+CD25+FoxP3+T regulatory cells (Tregs) have crucially important role in the suppression of eye inflammation in DED patients [21]. Reduced number of Tregs, usually accompanied with increased expansion of inflammatory CD4+ T cells (particularly Th17 cells) is observed in DED patients with aggravated diseases, while increased Tregs/Th17 cells is followed by tissue repair and regeneration [22-23]. Therefore, therapeutic agents that may induce generation and proliferation of Tregs have beneficial effects in attenuation of DED symptoms [23]. Among MSC-derived immunomodulatory factors, Indoleamine 2, 3-dioxygenase 1 (IDO1) was crucially responsible for the maintenance of immunosuppressive phenotype in resting Tregs [24-25]. During activation of resting Tregs, signals generated from activated T cell receptor, via mammalian target of rapamycin (mTOR) pathway, destabilize immunosuppressive phenotype of Tregs and cause their reprogramming into a pro-inflammatory helper-like phenotype ("ex-Tregs"), characterized by enhanced production of inflammatory cytokines, particularly IL-17 and IFN-γ [25]. As emphasized by us and others, IDO1 maintains population of Tregs in inflamed tissue by preventing transdifferentiation of Tregs in inflammatory Th1 and Th17 cells [26-27]. IDO1 activates general control nonderepressible 2 (GCN2) kinase in activated Tregs which inhibits mTOR signaling and prevents destabilization of immunosuppressive phenotype of Tregs enabling their expansion [25]. Having in mind that elevated IDO1 activity was measured in d-MAPPS samples [10], we believe that IDO-1-dependent expansion of Tregs could be, at least partially, responsible for beneficial effects of d-MAPPS eye drops in DED patients.

CONCLUSION

Due to their immunosuppressive and regenerative properties, d-MAPPS eye drops represent potentially new therapeutic agents that may efficiently alleviate eye inflammation and improve quality of life of DED patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

COMPETING INTERESTS

There are no conflicts of interest.

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REVIEW PAPER

THE PHOSPHODIESTERASE-5 INHIBITORS AND PROSTATE CANCER - WHAT WE RELY KNOW ABOUT IT?

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ABSTRACT

Phosphodiesterase-5 inhibitors (PDE5Is) represent a group of drugs that are registered for the treatment of erectiledysfunctions predominantly, but recently also for treatment of pulmonary hypertension and benign prostatic hypertrophy. However, more and more research deals with possible antitumor potential of PDE5Is in different types of cancers, including prostate cancer. Prostate cancer represents theone of the most common carcinoma in the male population, whose incidence is continuously increasing. Early detection combined with radical prostatectomy increases the survival rate, but also it is necessary to keep in mind the quality of lifeof patients undergoing prostatectomy in light of bladder control and erectile function. Authors of various clinical studies presented the results that often lead to totally opposing conclusions. For example, Chavez and colleagues have shown that use of PDE5Is in men with erectile dysfunction decreases the risk of developing prostate cancer, while, on the other hand, Michl and colleagues pointed out the adversely effect of PDE5Is on biochemical recurrence after bilateral nerve sparing radical prostatectomy. In that sense, the aim of this review was to present as many as possible of existing results dealing with of action of PDE5Is in the field of prostatic carcinoma. Taking into account all presented data, it can be concluded that effect of PDE5Is on formation, development andoutcome of treatment in patients with prostate carcinoma isvery intriguing question, whose response requires additionalboth experimental and clinical research.

Keywords: *Phosphodiesterase-5 inhibitors, Prostate carcinoma, Sildenafil, Tadalafil.*

INTRODUCTION

Phosphodiesterase-5 inhibitors (PDE5Is) were originally synthesized for the treatment of hypertension and angina pectoris, but they did not show efficacy in this therapeutic field. Sildenafil was firstly synthesized compound that belongs to this class of drugs, and therefore was the most intensively investigated. Namely, during experimental investigations it was shown that sildenafil induces penileerection, which was completely unexpected (1, 2). Consequently, clinical trials were conducted to investigate clinical benefit and safety for the treatment of erectile dysfunction (ED) with sildenafil (3) and sildenafil was approved foruse in ED and became the first official oral drug for ED treatment (4). After sildenafil, vardenafil and tadalafil wereapproved for treatment of ED (5, 6), and recently some newPDE5Is have been investigated, such as avanafil, lodenafil, udenafil and mirodenafil (7-10).

Besides the well documented and well described effects of PDE5Is in therapy of ED, there are grooving interests regarding actions of PDE5Is in other pathological andpathophysiological entities. There are a lot of data regarding cardioprotective effects of PDE5Is in patients with different forms of heart failure and in patients with diabetes mellitus (11, 12). It is shown that PDE5Is have protective effects due to other complications associated with diabetes mellitus such as neuropathy and diabetic nephropathy (13-16). In rat and mouse experimental models of stroke both sildenafil and tadalafil improved neurogenesis and neurological outcome (17-19), but there are still limited information considering the neuroprotective and neuro- restorative effects of PDE5Is human stroke patients (20). PDE5Is are also used for treatment of pulmonary arterial hypertension, since it has been shown that sildenafil causesvasodilatation of pulmonary arteries and improves the ex- change of gases in the lungs in patients with pulmonary hypertension (21).

Most intriguing effects of PDE5Is are probably related to their antitumor effects, increase of tumor sensitivity to anticancer drugs and protective effects to other organs and tissues during chemotherapy and radiotherapy. It was shown that PDE5Is induce apoptosis and reduction of cellgrowth in different human tumors, such as bile duct carcinoma, colorectal carcinoma, cervical cancer, and breast carcinoma (22, 23).

The objective of this paper is to review and summarizethe experimental and clinical findings considering the effects of PDE5Is as antitumor agents.

CYCLIC NUCLEOTIDE PHOSPHODIESTERASES - BASIC CHARCTERISTICS

Cyclic nucleotide phosphodiesterase enzymes (PDEs) represent a large family of enzymes that selectively catalyze the hydrolytic breakdown of the 3' cyclic phosphate bonds of adenosine and/or guanosine 3',5' cyclic mono- phosphate to produce 5'-AMP and 5'-GMP, respectively. (24). Cyclic adenosine 3',5' monophosphate (cAMP) and cyclic guanosine 3',5' monophosphate (cGMP) are second messengers that have crucial roles in regulation of numerous physiological processes and functions in biological systems, such as cell growth, proliferation and death, energy homeostasis, muscle contractility and relaxation, neuronalsignaling, immune and inflammatory responses, etc. The cAMP and cGMP concentrations in the cell, and thereforethe functions they regulate, are precisely determined by activity of adenylyl and guanylyl cyclases, which catalyze their synthesis, and PDEs, which catalyze their hydrolysis. Consequently, any disturbance in intracellular content of cAMP and cGMP and their signaling pathways lead to dis-ease or dysfunction, such as diabetes, pulmonary hyper- tension, heart failure, erectile dysfunction, etc. (25-29).

Incredible complexity of regulation of cAMP and cGMP in the cell, what more in different parts of the same cell, pointed out the possibility of expression of several different PDE classes, as well as the specificity of their localization and different modes of regulation (30). So far, eleven PDE isoenzyme families have been identified encoded by large and distinct gene superfamily, and it is estimated that there are more than a hundred different mRNA products from this gene superfamily due to alter- native sites for transcription initiation and various splicing mRNA precursor molecules. Most of PDE mRNA can be translated to proteins, but it cannot be said with certainty how many different PDE mRNAs are transcribed, whether they are all translated into proteins, and maybe most importantly does all of them have physiological role in the body and which it is. PDE isoenzyme families are classified according to their affinities to cAMP and cGMP, regulatory properties, sensitivity to specific inhibitors and activators, tissue localization (Table 1). Based on the substrate specificities PDEs can be sorted in three large groups: PDEs selective for cAMP hydrolysis (PDE4, PDE7, and PDE8), cGMP selective PDEs (PDE5, PDE6, and PDE9), and PDEs with dual specificity, which hydro- lyses both cAMP and cGMP (PDE1, PDE2, PDE3, PDE10 and PDE11) (31, 32).

Bearing in mind the fact that PDEs have crucial importance in regulation of cAMP and cGMP concentrations in the cells, and thus the downstream signaling pathways and biological responses, on one hand, and many specific isoforms of PDEs which are differently represented in cells,

tissues and organs, in which they have diverse physiological roles, on the other, PDE superfamily represents excellent therapeutic target (30, 33). Also, one of the main reasons that make PDEs a good therapeutic target refers to the pharmacological principle that change of degradation of any ligand (or second messenger) often has greater impact on change in concentration of that ligand than changes in rate of its synthesis (30).

Different isoforms of PDEs are located in specific part of the cell, thus enabling activation of individual signaling pathways due to cAMP or cGMP and propagation of specific, desirable signals. This compartmentalization of cAMP or cGMP signals is related to PDEs sequestrationin specific subcellular locations where they are embedded into different macromolecules and make connections withdifferent cellular structures (34).

Table 1. Overview of PDE isoforms

PDE isoform	Substrate	Tissue presence	Intracellular localization
PDE1A	cAMP/cGMP	Smooth muscles, heart, brain, lungs, sperm	Cytosolic (predominantly)
PDE1B	cAMP/cGMP	Smooth muscles, neurons, lymphocytes, macrophages	Cytosolic
PDE1C	cAMP/cGMP	Brain, smooth muscles, spermatids, olfactory epithelium	Cytosolic
PDE2A	cAMP/cGMP	Brain, adrenal medulla, heart, platelets, macrophages, endothelium	Membrane bound and cytosolic
PDE3A	cAMP	Heart, vascular smooth muscles, platelets, kidney	Membrane bound and cytosolic
PDE3B	cAMP	Vascular smooth muscles, liver, adipocytes, kidney, pancreatic β-cells, sperm, lymphocytes, macrophages	Membrane bound (predominantly)
PDE4A	cAMP	Brain, olfactory system, immune system, testis	Membrane bound (predominantly)
PDE4B	cAMP	Brain, immune system	Membrane bound (predominantly)
PDE4C	cAMP	Lungs, testis, some neurons	Cytosolic (predominantly)
PDE4D	cAMP	Brain, inflammatory cells	Cytosolic and particulate fractions
PDE5A	cGMP	Platelets, vascular smooth muscles, brain, lung, heart	Membrane bound/Cytosolic
PDE6A/ PDE6B	cGMP	Photoreceptors in retina (rods), pineal gland	Cytosolic
PDE6C	cGMP	Photoreceptors in retina (cones), pineal gland	Cytosolic
PDE7A	cAMP	Immune cells, heart, skeletal muscle, endothelium	Cytosolic
PDE7B	cAMP	Brain, heart, skeletal muscle, liver, pancreas, testis	Cytosolic
PDE8A	cAMP	Testis (predominantly), spleen, small intestine, ovary, colon, kidney	Cytosolic/ Particulate fractions
PDE8B	cAMP	Brain, thyroid gland	Cytosolic/ Particulate fractions
PDE9A	cGMP	Highly represented in the body	Cytosolic/Nucleus
PDE10A	cGMP	Brain, testis, heart, thyroid gland	Cytosolic/ Particulate fractions
PDE11A	cAMP/cGMP	Skeletal muscle, prostate, testis, thyroid gland, liver, salivary glands	Cytosolic

PHYSIOLOGY OF PHOSPHODIESTERASE-5

Phosphodiesterase-5 (PDE5) was firstly identified and characterized from platelets and lungs (35, 36), but they came into focus after discovering the role in regulation of smooth muscle contractility, and even more after discovery of sildenafil, as specific inhibitor of PDE5. PDE5 now ismost famous as molecular target for treatment of erectile dysfunction (ED), and recently for treatment of pulmonary hypertension. However, investigations regarding the PDE5and different PDE5Is indicate other roles of interest such as:

1) regulation of function of Purkinje cells in cerebellum (37, 38);
2) regulation of platelet function (39);
3) regulation of sodium homeostasis via renal sodium excretion (40);
4) regulation of neurogenesis and cognition (41, 42);
5) regulation of function of intestine cells (43, 44).

Only one gene encoding PDE5 was found for now, but there are three different variants of mRNA for PDE5A in humans and consequently three different polypeptides (PDE5A1, PDE5A2 and PDE5A3), which vary in their amino-terminal parts, but their first exons followed by common sequence of 823 amino acids are identical (30).

As well as other PDEs, PDE5 is a dimer, and the monomers are made up of an amio-terminal part which contains

phosphorilation domain, two allosteric cGMP binding sites and dimerization domain (or part of it), and carboxyl-terminal part containing catalytic domain (45, 46).

As mentioned above, the precise regulation of PDEs enzymatic activity have pivotal role in maintaining thecAMP and cGMP in adequate ranges for physiologicalcellular function. Few different mechanisms are included in regulation of PDE5 activity, and one of them is phosphorylation by protein kinase A (PKA) or protein kinaseG (PKG). Phosphorilation changes conformation of PDE5 and thus increases affinity for cGMP and catalytic activity for cGMP hydrolysis up to 50-70% (47, 48). It has been shown that treatment of platelets, cardiomyocytes and smooth muscle cells with nitric oxide (NO) induces immense increase in cGMP concentration, followed by rapiddecrease, produced by NO-induced protein kinase activity (49-51). Another mechanism implies the presence of two allosteric cGMP binding sites (GAF), and binding of cGMP at these allosteric sites induce increase in catalytic activity of PDE5, which some authors see as a new therapeutic opportunity (52). Actually, it is postulated that after binding of cGMP to GAF and increased enzymatic activity of PDE5, PKG induces phosphorilation, thereby stabilizing and prolonging the increased catalytic activity of PDE5. Possible mechanism for PDE5 deactivation implies dephosphorylation by catalytic activity of protein phosphatases, mainly protein phosphatase 1 (PP1), while cleavage of PDE5 into inactive form is performed by the action caspase-3 (53-56).

Bearing in mind that PDE5 is mostly represented in smooth muscles, it is practically present in almost all tis- sues and organs in the body, with different distribution of PDE5 isoforms (PDE5A1, PDE5A2 and PDE5A3), whereby the PDE5A2 is the most widespread (57). Using appropriate methods such as RT-PCR, *in situ* hybridization (ISH), Northern blotting, Western blotting or immunohistochemistry methods, PDE5 mRNA or PDE5 protein arefound in vascular smooth muscle, heart, lungs, platelets, brain, liver, pancreas, skeletal muscle, placenta, gastrointestinal tissues and reproductive system, with different distribution of PDE5A isoforms between the species, tissues and organs (58-62).

PHOSPHODIESTERASE-5 INHIBITORS

Phosphodestrase-5 AND mNO/cGMP signaling pathway in cancer

Results from a number of studies have pointed out the facts considering the presence of PDE5 in many types of carcinoma cells including colon adenocarcinoma, lung cancer, breast cancer, prostatic cancer and urinary bladder cancer,and also overexpression of PDE5 in breast malignant tumors, urinary bladder cancer, pancreas and prostatic cancer (63-70). Transformation of normal, healthy cell to malignantcell occurs as consequence of DNA damage and genetic alterations that appear due to procarcinogenic microenvironment. Chronic mild inflammation, for instance, increases NO production due to increased activity of inducible NO synthase (iNOS) which further enhances tumor growth, invasiveness and dissemination (71-73). Results from other investigations pointed out the other, anti-tumor nature of NOby inducing apoptosis and cytotoxicity (74, 75). This dual, paradoxical role of NO arises from the diversity of the signalpathways in which NO acts as a regulator, wherein the con-centration probably determines the promoting or inhibitoryeffects of NO in cancerogenesis (76, 77).

cGMP is synthesized by catalytic activity of solubleguanylyl cyclase (sGC) which is a receptor for NO. Namely,NO binds to ferrous heme of the β1 subunit of the sGC andcauses the rise in sGC activity and cGMP production (78).Consequently, cGMP level in the cells depends not onlyon enzymes involved in cGMP metabolism, but also on enzymes (endothelial, inducible and neuronal NOS) and pathways which regulate NO production and degradation (79). Similarly to NO, the roles of sGC and cGMP in tumor biology and cancerogenesis also remained paradoxical despite a large number of studies and investigations in re- cent decades, so the question considering the protective ordeleterious role of NO/sGS/cGMP signaling pathway still has no answer. Chang and coauthors recently indicated that activation of guanylyl cyclase and increased levels of cGMP have beneficial effects on inflammation-promoted colorectal neoplasia in mice (80). On the other hand Cesarini with colleagues highlighted the negative correlation between the increased expression of PDE5 and consequentdecreased levels of cGMP and tumor aggressiveness and clinical outcome in patients with glioblastoma multiforme (81). Scientific group gathered around Ferid Murad, No- bel Prize co-winner in Physiology or Medicine for NO signaling, assumed several possibilities for such effects:

1) beside role in physiological signaling, NO produced at high concentrations by iNOS, also exhibits cytotoxic and proapoptotic properties;
2) the components of NO/sGC/ cGMP (cGMP-dependent) pathway and NO oxidative pathway (cGMP-independent) vary between different cell types and tissues;
3) solid tumors are composed of parenchyma, which contains neoplastic cells, and stroma, whichincludes nonmalignant supporting tissues (connective tissue, blood vessels) with different behavior due to NO/ sGC/cGMP signaling (79, 82).

PHOSPHODIESTERASE-5 INHIBITORS AND PROSTATE CANCER

Prostate cancer is one of the most common solid malignant tumors in men population worldwide and thusrepresents a huge social and medical issue. In European Union prostate cancer is the most common malignancy in men with 365,000 new prostate cancer cases in 2015 (83). Early detection and radical surgical removal of the cancer (nerve-sparing radical prostatectomy) significantly increase the survival and quality of life due to recovery of bladder control and erectile function. Administration of daily doses of PDE5Is in patients after nerve-sparing radical prostatectomy (NSRP) due to localized prostate cancer showed increase in penile function

and positive effect on the recovery and maintenance of erectile function afterthe surgery (84). Despite routine use of PDE5Is in treatment of erectile dysfunction, as well as many researches dealing with problematics of different relations of PDE5Is and prostate cancer, many questions remained without answers and many results unclear.

Liu and coworkers dealt with role of PDE5/cGMP/PKG signal pathway in stemness and differentiation ofprostatic cancer stem cells (PCSC) (85). Namely, PCSC represent a small population of cancer cells with the ability for self-renewal, proliferation, invasive and meta- static growth. In their study these authors postulated that PDE5/cGMP/PKG signaling have crucial role in stemness remaining of PCSC. Furthermore, it is shown that activation of cGMP/PKG pathway by inhibition of PDE5 using vardenafil and tardalafil, results in activation of mammalian ste20-like protein kinase (MST – Hippo pathway) and consequent phosphorylation of transcriptional co- activator with PDZ-binding motif (TAZ), leading to its degradation and reduction of stemness in PCSC. Results from this investigation, conducted on *in vitro* on cell cultures and *in vivo* on xenografts, revealed interesting connection between PDE5/cGMP/PKG pathway and Hippo/ TAZ pathway in maintaining of PCSC stemness, as well as the possible reason for the usefulness of PDE5Is in prostate cancer therapy.

Das and coauthors, on the other hand, pointed out the sensitizing activity of sildenafil to prostate cancer cellson doxorubicin induced apoptosis through CD95 (86). Only co-treatment of prostate cancer cells with doxorubicin and sildenafil induced decrease in expression of FLIP (FLICE-like inhibitory protein), which represents one of the major regulators of CD95-mediated apoptosis. Furthermore, combined application of doxorubicin and sildenafil increased CD95 cell surface localization and decreased expression of Fas associated phosphatase-1 (FAP-1). Fas (APO-1/CD95) is death receptor which mediates in apoptosis of various types of cells, but many neoplastic cells are resistant to apoptosis induced by Fas. Increased expression of FLIP is brought into connection with increased tumor growth and immunologic escapeof tumors. On the other hand increased surface localization and activation of CD95 leads to the formation ofdeath-inducing signaling complex (DISC) and inductionof apoptosis, while FAP-1 disables the moving the CD95 (human Fas protein) to the membrane. Doxorubicin and sildenafil co-treatment also reduced the nuclear translocation of p65 and p50 and activation of NF-κB, thereby reducing FLIP expression, because it is shown that NF-κB up-regulates the expression of FLIP. Based on these results it was identified a new mechanism of inducing cell death in prostate cancer cells which implies the increased surface localization of CD95, decreased expression of FLIP and FAP-1, as well as inactivation of NF-κB affected by concomitant action of sildenafil and doxorubicin. Similar, beneficial effects of combined application of PDEIs with standard chemotherapy were noticed also in bladder and pancreatic tumor cells (69). Despite the clinical efficacy in combat with different types of malignancies, doxorubicin, as well as other anticancer drugs, exhibit severe side effects such as cardiotoxicity. The same group of authors in their previous study showed that PDE5 inhibitor tadalafil can attenuate cardiac dysfunction induced by doxorubicin, without simultaneous reduction of doxorubicin antitumor effect (87). Tadalafil during combined use with doxorubicin induced significant increasein manganese superoxide dismutase (MnSOD), cGMP levels and PKG activity in the heart, with preservation of ejection fraction. Assessment of the effects on cell-killing potential of doxorubicin in human osteosarcoma cancer cell lines and xenografts showed that tadalafil did not impede the anticancer activity of doxorubicin neither *in vitro* nor in the *in vivo*.

Results of the Ammirante and coauthors indicated that PDE5Is prevent myofibroblast activation and CXCL13 induction in castration-resistant prostate cancer (88). Cancer-associated fibroblasts (CAF) represent a various cell population that has many promoting roles in cancer progression, and myofibroblasts are part of CAF family. Chemoattractant C-X-C motif chemokine 13 (CXCL13)is chemoattractant for B-cells and mediates movement of B-cells into prostate cancer. Those lymphocytes that infiltrate cancer tissue produce a number of cytokines, suchas lymphotoxin, which contribute to the survival, development and dissemination of prostate tumor cells. In this investigation sildenafil prevented myofibroblast activationand decreased quantity of CXCL13 in castrated Myc-CaP (allografts of androgen-dependent mouse prostare cancer) tumor-bearing mice.

In research by Chavez and colleagues it have been indicated that men with erectile dysfunction treated with PDE-5Is tended to have less of a chance of being diagnosedwith prostate cancer (89). Authors have conducted retrospective study using electronic medical records of men suffering of erectile dysfunction during a period of 7 years.Participants in the study were selected based on similar risk factors for the development of prostate cancer, andof total number of 4974 men included into investigation 47.5% of them used PDE5Is, while the others did not use any drugs from this group. Results led to conclusion that use of PDE5Is is associated with lower values of prostate- specific antigen (PSA), higher incidence of benign prostatic hyperplasia (BPH) and, most importantly, lover risk of developing prostate cancer.

Following this investigation Jamnagerwalla and co- authors conducted a 4-year multicenter study in North American men investigated the association between the PDE5Is use and prostate cancer risk (90). The research involved 6,501 men 50-75 years old, with PSA within the physiological values for age and a single negative prostate biopsy. TRUS (ten-core transrectal ultrasound) guided prostate biopsies were performed at 2nd and 4th year of trial,regardless of the PSA value. Results from these authors showed that PDE5Is use was not associated with prostate cancer diagnosis, but there was an inverse between PDE5Isand prostate cancer diagnosis, although this was not statistically significant. Limitations of this study that were mentioned by the authors include a small number of respondents who used PDE5Is (5.6%), as

well as unavailable data considering the use and dosage of PDE5Is.

Jo and colleagues focused their interest on effects of PDE5Is on oncologic outcomes in patients with prostate cancer after radical prostatectomy (91). A total numberof 1082 patients who underwent radical prostatectomybetween January 2005 and December 2014 were divided according postoperative use of PDE5Is into three groups: non-PDE5I group, on-demand group, and penile rehabilitation group. The patients within the last two groups used several types of PDE5Is: sildenafil, tadalafil, vardenafil, avanafil, udenafil, and mirodenafil. Using appropriate statistical tests biochemical recurrence were assessed between groups that used PDE5Is and that did not, as wellas between group that used PDE5Is on demand and penilerehabilitation group. Based on data analysis authors concluded that PDE5Is treatment following radical prostatectomy have no impact on biochemical oncologic outcome and that PDE5Is use in patients after radical prostatectomyis clinically safe.

On the other hand, Michl and coworkers presented results that were completely opposite to the previously mentioned clinical and experimental investigations (92). Authors analyzed data of 4752 patients with prostate cancer, in whom bilateral nerve sparing radical prostatectomy was applied between January 2000 and December 2010, and assessed the risk of biochemical recurrence between the patients who used PDE5Is (23.4%) after the surgical treatment and patients who did not (76.6%). Five-year survival without biochemical recurrence in patients receiving PDE5Is was 84.7%, compared to 89.2% in group without PDE5Is treatment, and biochemical recurrencewas estimated due to PSA level (0.2 ng/ml or greater and increasing after). Based on the results conclusion weremade that use of PDE5Is following radical prostatectomy may adversely impact biochemical recurrence. For the explanation of the obtained results authors relied on previous researches where it have been demonstrated that PDEIs have proangiogenic and proneurogenic properties (93, 94). Although increased density of autonomic nerve fibers in tumor and surrounding tissue were brought into connection with poor clinical outcome, and angiogenesis is well known required factor for tumor development, conclusion that PDEIs represent independent risk factor for biochemical recurrence of prostate cancer due to enhancing role of PDEIs in nerogenesis and angiogenesis, remain in domain speculation (95, 96). On the contrary, recent study by El-Naa and coauthors showed that sildenafil potentiated antitumor activity of cisplatin by induction of apoptosis and inhibition of proliferation and angiogenesis in in Ehrlich solid-tumor-bearing mice (97).Few other authors also pointed out the limitations of the investigation conducted by Michl and coauthors such as lack of data due to the duration, dose, type and period of starting use of PDE5Is (98).

Regarding the results of the Michl and coauthors it was conducted investigation by Gallina and colleagues with similar but extended aims (99). Namely authors examined linkbetween usage of PDE5Is, therapy scheme of PDE5Is, number of taken PDE5-I pills, and biochemical recurrence (PSA $\geq$ 0.2 ng/ml) in 2579 patients with prostate carcinoma curedby bilateral nerve-sparing radical prostatectomy. Patients were classified in three groups due to PDE5Is usage withintwo years after the surgical procedure: on demand, rehabilitation schedule (daily use of PDE5Is for at least 3 months),and no use of PDE5Is. Using the multivariable Cox regression models it was confirmed that use of PDE5Is, either ondemand or on a rehabilitation schedule, was not associatedwith biochemical recurrence in patients treated by nerve- sparing radical prostatectomy due to localized prostate carcinoma. Furthermore, there were not significant differencesbetween patients taking PDE5Is pills on demand and thosewho were treated with a rehabilitation scheme, as well as due the number of PDE5-I pills taken by each patient.

CONCLUSIONS

Within this review are presented results from various investigations in which different experimental models were used regarding clarification role of PDE5Is in prostate cancer therapy, but still we cannot say with certainty where is the place of PDE5Is in cancer treatment protocols. It could be concluded that more results indicate a favorable role of PDE5Is in treatment of cancer, but this fact also remains in the domain of speculation, bearing in mind that the exact mechanisms of positive action of PDE5Is, as adjuvant anticancer drugs, are not fully understood. Additional experimental research is needed to clarify all potential mechanisms of action of PDE5Is in the field of cancer treatment, but also of crucial importance is to collect as many clinical data as possible. All these information together can allow us do fully understand all mechanisms of action of PDE5Is in tumor tissues and see should they be included in therapy or not, as well as should they be included in cure protocols of specific types of cancers.

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CASE REPORT

COVID-19 IN A PATIENT WITH X-LINKED AGAMMAGLOBULINEMIA: A CASE REPORT

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ABSTRACT

X-linked agammaglobulinemia (XLA), characterized by a profound deficiency of B lymphocytes, is caused by mutations in the gene encoding Bruton tyrosine kinase (Btk). . XLA patients have a susceptibility to viral infections. In this report, we present a 45-year-old man with known XLA, with about a 2-week history of fever, chills, diarrhea and vomiting. He was diagnosed with COVID-19 infection, which was confirmed by a real-time reverse-transcriptase-polymerase chain reaction. The antiviral drugs, antibiotics, and interferon-beta were administered to him. Unfortunately, the patient passed away after 5 days. During an epidemic of infectious diseases, the best strategy to overcome the potential challenges of treating XLA may be prevention. Early detection of biomarkers such as D-dimer and IL-6 might be more helpful for initiating more aggressive therapy and decreasing the duration of illness in these patients.

Keywords: *primary immunodeficiency disorders, X-linked agammaglobu-linemia, COVID-19.*

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INTRODUCTION

Primary immunodeficiency disorders (PIDs) are the group of immune deficiencies described by poor function in some elements of the immune system. These patients are at higher risk of several infections such as severe recurrent respiratory and gastrointestinal infections [1]. X-linked agammaglobulinemia (XLA) is one form of PIDs that is a result of gene defects in xq22 chromosomal location and a decrease in B-cell progenitor kinase (BTK) production [2]. It has been shown that males are affected by XLA much more commonly than females [3]. XLA is manifested by the partial or complete absence of gamma globulins including antibodies in the bloodstream which are vital for protecting against infections. The thymus gland is normal, but peripheral lymphoid tissues are typically absent. B cells and plasma cells are rare despite the normal numbers of pre–B cells in the bone marrow. They have significant problems with bacterial infections; they also have severe difficulty with persistent, disseminated echovirus infections, especially in the central nervous system.

XLA patients have a susceptibility to other viral infections, which can cause lethal infections [4]. Norovirus infection of the gut is another problematic viral infection in XLA cases [5]. Until now, the occurrence of coronavirus infection in patients with PIDs has not been studied. However, it is supposed that bacterial superinfections are more likely to occur following the coronavirus infection, which leads to a worse prognosis.

During the current novel coronavirus epidemic, one concerning problem about immunodeficient patients would be the higher risk of getting COVID-19 infection. Here, we present a patient with XLA who became infected with COVID-19.

CASE REPORT

A 45-year-old man with known XLA, that was diagnosed 6 years ago, had a 16-day history of fever, chills, diarrhea, vomiting and progressive dyspnea when he was admitted to our hospital. His medical history included XLA, cirrhosis and portal hypertension-induced esophageal varices, which led to band ligation 2 years earlier. His drug history was negligible except monthly intravenous immunoglobulin (IVIG) infusion therapy. High-resolution computed tomography (HRCT) was performed which was indicative of COVID-19 because the signs and symptoms were compatible with COVID-19 in the epidemic conditions of this infection. Patchy ground-glass opacity (GGO) consolidations were present in the lower lobes of both lungs accompanied by the reversed halo sign, which was indicative of viral pneumonia in general, and COVID-19 infection specifically (Figure1). The bronchiectatic changes were also observed in the posterior basal segment of the left lung which might be explained by his history of recurrent respiratory tract infections (RTIs) due to the underlying disease.

Despite receiving hydroxychloroquine and azithromycin combination therapy for 6 days in an outpatient setting, t his dyspnea got worse; thus he was admitted to hospital. At the admission, he had hypoxemia (the blood oxygen saturation of 82% while breathing the room air) with a body temperature of 38.7°C, blood pressure of 110/75 mm Hg, heart rate of 86 beats per minute, respiratory rate of 24 breaths per minute, and bilateral normal lung respiratory sounds (Table1).

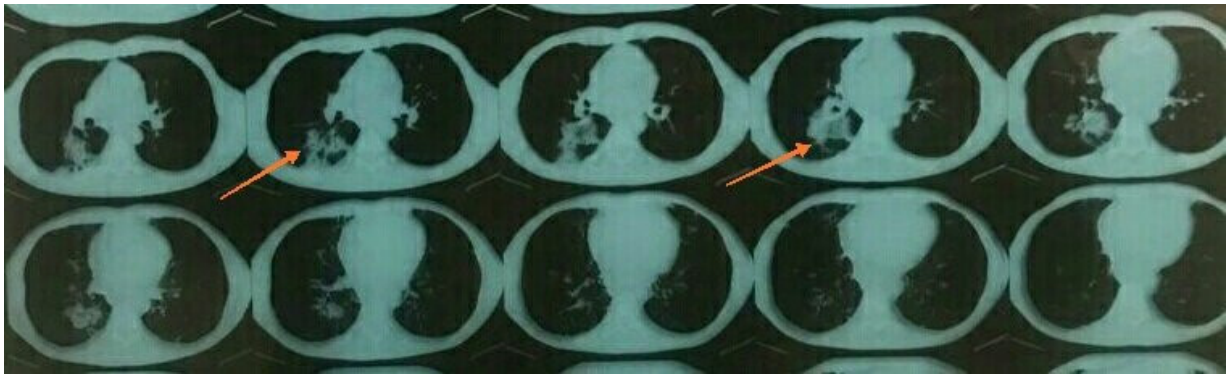
Table1. Examined parameters of patient infected with COVID-19

Parameter	Value
Body temperature	38.7°C
Blood pressure (mm Hg)	110/75
Heart rate (beats per minute)	86
Respiratory rate (breaths per minute)	24
White blood cell count, $\times 10^9$/L	3100
Lymphocyte count, $\times 10^9$/L	600
Platelet count, $\times 10^9$/L	122000
Haemoglobin (g/L)	11.6
Erythrocyte sedimentation (mm/h)	50
C-reactive protein (mg/L)	60
Procalcitonin (ng/mL)	0. 12
Interleukin-6 (pg/mL)	56
Pro B-type natriuretic peptide (pg/mL)	661
D-dimer (ng/mL)	368

Laboratory tests showed the white blood cell count (WBCs) per microliter (3.100×10^9/L) and 20% lymphocytes; hemoglobin, 11.6 gm/dL; platelet (PLT) , 122000 per microliter. The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and procalcitonin levels were 50 mm/h, 60 mg/L, and 0. 12 ng/mL, respectively.

Interleukin-6 (IL-6), pro B-type natriuretic peptide (pro-BNP) and D-dimer levels were 56 pg/mL, 661pg/mL, and 368 ng/mL, respectively. The liver and kidney function tests, electrolytes, and cardiac enzyme levels were within the normal range. He had positive RT-PCR results for COVID-19. Kaletra (lopinavir/ ritonavir) two 200 mg tablets twice daily, azithromycin 500 mg PO daily, meropenem 1 g IV q8hr, and vancomycin 1 gram q12hr were administered. He defervesced within 48 hours. Despite using the reservoir bags, his oxygen saturation (SaO_2) did not increase and his dyspnea continued. Therefore, interferon-beta was administered to him. Unfortunately, the patient passed away after 5 days.

Figure 1. CT scan performs GGOs in both lungs
accompanied by the reversed halo sign and bronchiectatic changes were observed in the posterior basal segment of the left lung.



DISCUSSION

X-linked agammaglobulinemia (XLA), characterized by a profound deficiency of B lymphocytes, is caused by mutations in the gene encoding Bruton tyrosine kinase (Btk). . This group of patients has a susceptibility to several types of infections [6].

Any types of immunosuppression such as humeral or cellular immune responses are risk factors for occurrence, severity, and poor prognosis of COVID-19. It is important to note, that a delayed diagnosis of infection in these patients due to the unspecific symptoms or radiographic patterns, and the poor innate immune defense in immunosuppressed individuals against respiratory infections, could generally lead to the low cure rate [7]. During an epidemic of infectious diseases, the best strategy to overcome the potential challenges of treating these patients may be prevention [8]. To achieve these goals, it is necessary for thesepatients to receive IVIG replacement monthly to defend more properly against the novel virus [9]. Also, the hospital infection control policies should be in place with the strictest precautions to isolate the patients with, or at risk of acquiring COVID-19 and the patients themselves should be advised to rigidly perform hand hygiene and other standard and respiratory precautions to prevent getting the infection.

Although, radiographic features in this patient were similar to immunocompetent patients ,there was no delay in the diagnosis. Moreover, high levels of the prognostic factors such as D-dimer and IL-6 would be associated with worse outcomes, more critical condition, and non-response to the standard therapy; perhaps, earlier checking of these biomarkers might be more helpful for initiating more aggressive therapy and decreasing the duration of illness in these cases. Some studies reported that IFNβ1 might account for a safe treatment against COVID-19 in the early stages of the disease. Also, in vitro studies propose that COVID-19 could be more sensitive to IFN-I than other coronaviruses [10, 11]. Probably, the use of interferon in our patient in addition to the antiretroviral therapy and antibiotics could improve his prognosis. But our patient was admitted in the early phase of this epidemic event of COVID-19 and in that period in beta formulations were not suggested in the studies and treatment protocols of COVID-19.

In conclusion, clinicians should pay attention to XLA patients who have a susceptibility to viral infections such as COVID-19 and earlier checking of some biomarkers might be more helpful for initiating the treatment and decreasing the duration of infection in these patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained prior to enrollment in the study.

COMPETING INTERESTS

There are no conflicts of interest.

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SYNCHRONOUS MANEC (MIXEDADENO-NEUROENDOCRINE CARCINOMA) OF THE COLON AND RENAL CELL CARCINOMA - CASE REPORT

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ABSTRACT

Synchronous multiple carcinomas represent two or more primary carcinoma that occur sumultaneously in the same patient. In order to fulfill the condition that tumors are synchronous, each tumor must be primary and not a metastasis of another tumor. The occurrence of renal carcinoma synchronously with colonic carcinoma is not so common. On the other hand, the pathohistological image in rare cases shows a mixed glandular and neuroendocrine component described in earlier works. In this paper, we present a patient who made a colonoscopy, a biopsy from a tumor change in the cecum due to malady, fainting, loss of appetite, and a positive test for faecal occult bleeding, and confirmed that it is an adenocarcinoma of the cecum. Multi slise computerized tomography of the abdomen also described a tumor change in the uretero-pelocalrix system of the left kidney region. The patient had no urinary tract disorders. The diagnosis of the synchronous tumor of the cecum and left kidney was set. A right hemicolectomy with latero-lateral ileo-transverse anastomosis, as well as left nephroureterectomy, was performed. What is particularly interesting in this case is that the pathohistological picture of the cecum carcinoma shows a rare form of tumor tone, mixed adeno-neuroendocrine carcinoma. In patients with diagnosed colorectal cancer, routine as well as additional preoperative diagnostic procedures should be performed to exclude the existence of kidney cancer, since, when synchronous with colorectal carcinoma occurs, renal carcinoma is mainly asymptomatic. In rare cases, the pathohistological picture may also show the neuroendocrine component of the tumor, which directs further therapy to the other direction.

Keywords: *synchronic carcinomas, cecum carcinoma, renal carcinoma, MANEC tumors.*

INTRODUCTION

Synchronous multiple cancers represent two or more primary cancers that occur simultaneously in the same patient. To fulfill the requirement that the tumors are synchronous, each tumor must be primary, not the tumor metastasis (1).The incidence of primary malignancies in the kidneys and colon is difficult to determine. The term "synchronous" means identity, but some authors even extend the time interval to detect another malignancy of up to 90 days (2). Mixed exocrine-neuroendocrinal tumors are rarely detected. According to the recent classification of the World Health Organization (VHO), neoplasms with exocrine and neuroendocrine components are called "mixed adenoneuroendocrine carcinomas" (MANECs) (3). Some authors say that MANEC is a neoplasm with dual adenocarcinoma and neuroendocrine differentiation, with each component representing at least 30% of the tumor (4). Also, this is a very rare tumor, and most are presented as a case report. In English literature, only seven cases in cecum and about 35 cases in the stomach were reported (5).

In this paper, we present the case of a patient with a synchronous renal and colonic cancer with MANEC's pathohistological characteristics.

CASE REPORT

The male patient, aged 77, had problems in the form of fatigue, fainting, loss of appetite. Blood tests indicated anemia, and analysis of the chair on faecal occult bleeding was positive. A colonoscopy was performed, whereby the tumor change in the length of several centimeters was described on the step-ascendent part of the column, which involves the whole circumference of the column and narrows its lumen. A biopsy was taken and the finding confirmed that it was an adenocarcinoma of the cecum. A multi-slice computerized tomography of the abdomen was developed, which, in addition to the already proven carcinoma of the cecum (Figure 1), also describes a tumor change in the uretero-pelocalrix system of the left kidney (Figure 2).

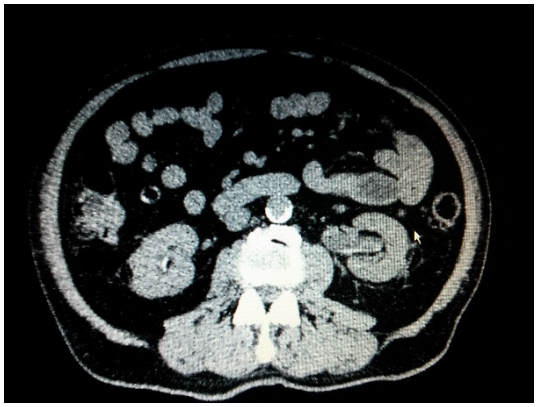

Figure 1. CT image carcinoma of the cecum

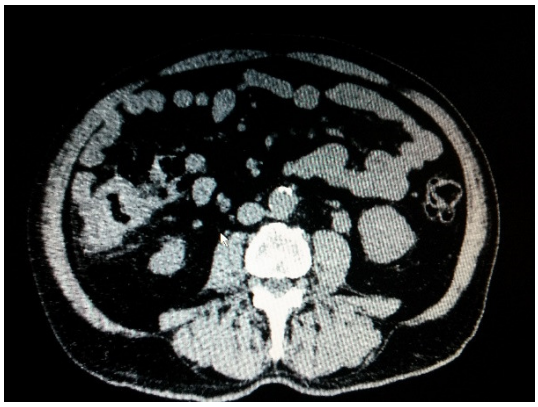

Figure 2. CT image of left kidney cancer

A decision was made on the operative treatment of both tumors during the same operation ((Figures 3 and 4), and a right hemicolectomy with latero-lateral ileo-transverso anastomosis, as well as left nephroureterectomy, was performed.

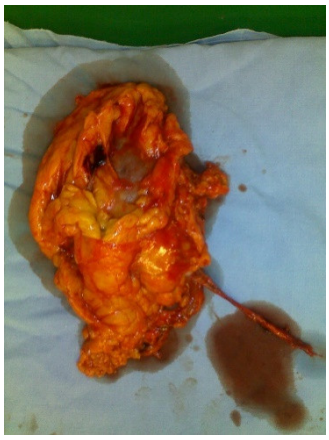

Figure 3. Left kidney

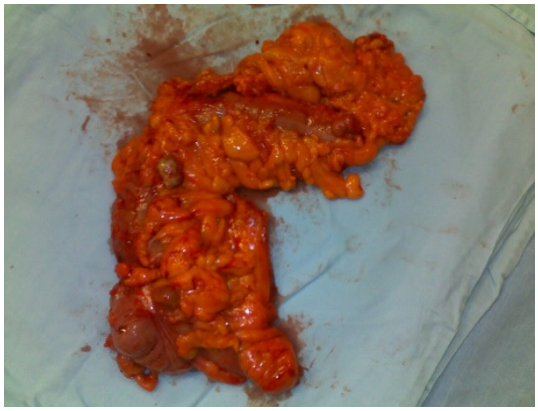

Figure 4. Right colon

The post-operative course was carried out neatly, and the patient was discharged home 10 days after surgery.

A definitive histopathological finding revealed the following:

- Reserved portion of the colon with pericolic fatty tissue, in the length of 300 mm, the diameter of the lumen 12 and 25 mm. Upon opening at 75 mm from the distal edge of the resection, there is an ulceroinfiltration change in the length of 50 mm, which involves 2/3 circuits and obstructs 1/3 lumen. On the intersection of the tumor changes the infiltrise wall to the bottom of the serous. In the rest of the mucous membranes, the morphology is common. The length of the neck is 50 mm, the diameter from 6 to 11 mm, at the intersection of wall thickness up to 4 mm. In the pericolic fat tissue, 9 lymph nodes have been found, between 2 and 9 mm diameter. Mixed adenocarcinoma and neuroendocrine carcinoma (MANEC) cecum, collision mode of combination.Adenocarcinoma invasivum coeci, high grade, pT3N0Mx.NET coeci, G2-intermedijal gradus, pT3N0Mx. (Figure 5,6)

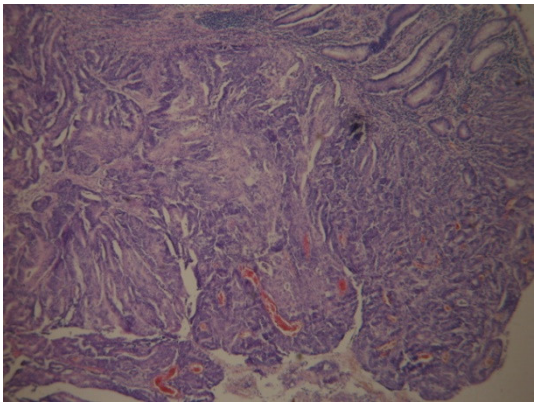

Figure 5. Colon carcinoma HEx100

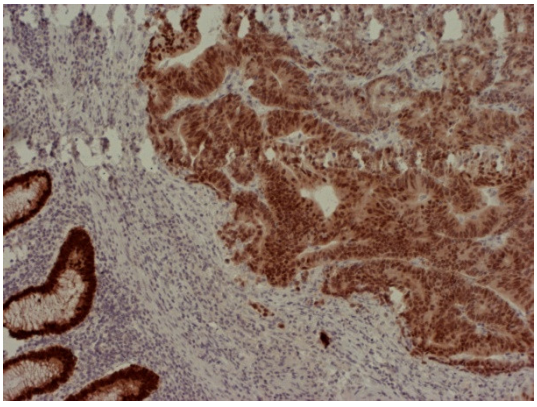

Figure 6. Colon carcinoma IHH LSABx2oo CDX2

- Kidney with perirenal fat tissue, total weight 780 g, diameter 91x67x26, with ureter length 100 mm, diameter from 6 to 9 mm, easily removable capsules. The surface is a lobular appearance, coarse-grained, yellow-brown color, with irregular leafy recesses, on the intersection of easily expanded renal sinus and kidney pelvis. There is a dual ureter, a hypoplastic appearance, a second ureter with a 30 mm long spatial enlargement that is filled with tumor mass, whitish colors, soft consistency that completely obstructs. Carcinoma papillare transitiocellular, histological grade I, nuclear grade II. Tumor infiltrate muscular layer, without elements of invasion of vascular structures. Pathological stage: pT2NxMx. (fig.7-10)

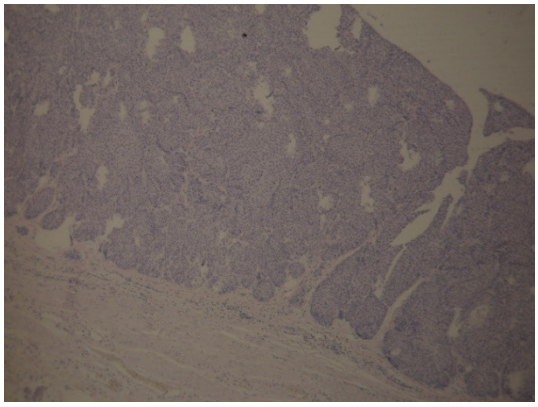

Figure 7. Ureter carcinoma HEx100

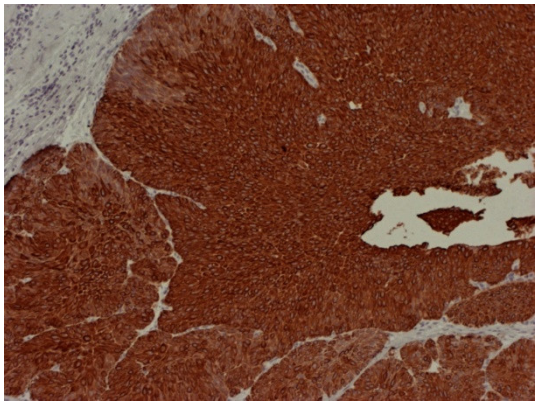

Figure 8. Ureter carcinoma IHH LSABx200 CK19

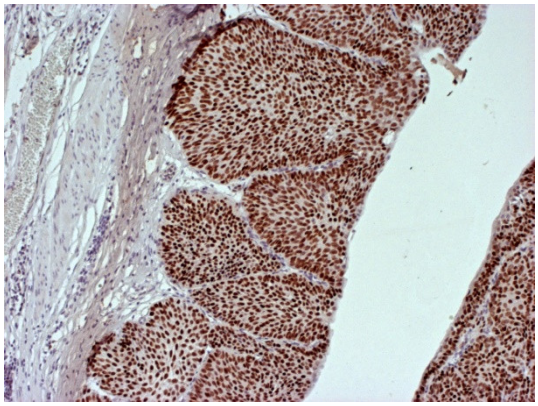

Figure 9. Ureter carcinoma IHH LSABx200 p63

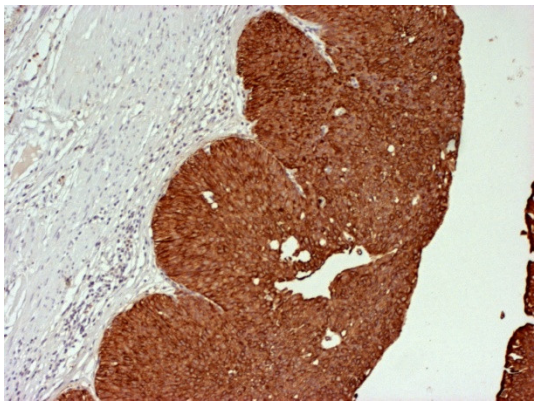

Figure 10. Ureter carcinoma
IHH LSABx200 CK7

DISCUSSION

Multiple primary carcinomas are present in about 16% of all diagnostic carcinoma (6). In relation to this percentage, most patients have two forms of the arm, but there are also patients with three or more carcinoma. The incidence of synchronous colon and kidney tumors is 0.1%. Synchronous asymptomatic carcinoma of cardiovascular diseases are found in 0.03-0.5% of patients with colorectal carcinoma (7). Not many studies have been done so far on this topic. One of these studies describes six cases of synchronous bowel and renal carcinoma between 1200 cancer patients with the current six-year period presenting a frequency of 0.5% (8). A synchronous tumor is defined as a tumor that has been reported at the same time or up to six months (although some authors define this period for 90 days) from the first tumors, if more than six months have passed since the onset of the first tumor, then tumors are called metacrons. In our patient, both tumors were diagnosed at the same time, so synchronous tumor criteria were released (9). It is known that carcinoma of the kidney can occur simultaneously with other primary carcinoma, and gastrointestinal carcinoma is most commonly carcinoma that has been reported synchronously with renal carcinoma (10).

In general, taking into account the synchronous primary carcinoma, usually occurs with the gastrointestinal and urogenital tract carcinoma. In our institution, this is the first description of the case of a synchronous tumor of the colon and kidneys. They are present in the patient, mostly over 74 years, and the main males are male. Our patient is male, aged 77 years. It is interesting to note that patients with urological cancer diagnoses have a higher risk of developing the following colorectal cancer than patients without cancer of urological diseases. Similarly, patients with colorectal cancer increased the risk of urologic cancer, especially in urethral carcinoma (11). The discovery of Renaissance cancer was purely accidental with a CT scan for setting up a tumor of the colon. As with other series of synchronous colon and renal carcinoma, the RCC was symptomatic, and the finding was an incident (12). A number of published works in the literature now describe only synchronic resections and colon on the same sides of the stomach.

When talking about surgical treatment as the initial and leading therapy of these diseases, the simultaneous resection of both malignancies was done in a single surgical session. The usual approach is open laparotomy, more convenient for the surgeons involved. However, some authors have shown that simultaneous laparoscopic resection of both malignancies is feasible with very low morbidity and zero mortality. In the literature, only three cases of synchronous laparoscopic resection of colorectal and renal cell carcinoma (13-15) were found.

In addition to the fact that there is a rare occurrence of synchronic cancer of the colon and kidneys, this case is especially interesting because of the pathohistological picture of the caecum carcinoma.

Exocrine-neuroendocrinal tumors of the gastrointestinal tract were first published in 1924 (16). Neoplasms are now referred to as "mixed adenoneuroendocrine carcinomas" (MANECs) according to the latest classification of the VHO classification of neoplasms of the gastrointestinal tract (17). These two components of the tumor can be in a different relationship and their treatment depends on the component that has prevailed in their relationship. Manecs containing a well-differentiated neuroendocrine tumor component and an adenocarcinoma component should be treated as adenocarcinoma. MANECs containing poorly differentiated neuroendocrine carcinoma (NEC) should be treated as NEC (18). For patients with MANEC lesions containing a higher proportion of the adenocarcinoma component, they could be expected to have a better prognosis than patients with a lower relationship. Chemotherapy protocols are not yet clearly defined because of the small number of patients with MANEC. Therefore, there is no one best mode of chemotherapy for patients with MANEC. The therapy is reduced to a case-by-case basis. New studies are necessary in terms of predicting the outcome of tumors with such pathohistological images.

CONCLUSION

In patients with diagnosed colorectal cancer, routine as well as additional preoperative diagnostic procedures should be performed to exclude the existence of kidney cancer, since, when synchronous, carcinoma of the kidney is mainly asymptomatic. On the other hand, people with urinary tract malignancies have a greater chance of malignancies of the gastrointestinal tract. The pathohistological image is of key importance first in determining the mixed forms of neoplasia, but also determining which component is more dominant, which in many ways is characterized by a clinical picture, a method of treatment, and in the outline and outcome of the disease.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained prior to enrollment in the study.

COMPETING INTERESTS

There are no conflicts of interest.

FUNDING

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