Summary

Vitamin D is the sunshine vitamin that is not only important for children’s and adults’ skeletal health but is also important for their overall health and wellbeing. Vitamin D deficiency has been defined as a 25-hydroxyvitamin D < 20 ng/mL and vitamin D insufficiency as a 25-hydroxyvitamin D of 21–29 ng/mL. The major source of vitamin D is sensible sun exposure since very few foods naturally contain vitamin D. Vitamin D deficiency is associated with increased risk for many acute and chronic diseases including infectious diseases, autoimmune diseases, cardiovascular disease, type 2 diabetes, neurocognitive dysfunction and muscle weakness. To achieve a blood level of 25-hydroxyvitamin D >30 ng/mL children require 600–1 000 IU and adults 1 500–2 000 IU of vitamin D daily.

Keywords: vitamin D, cancer, sunlight, 25-hydroxyvitamin D, infectious disease, osteoporosis, rickets

Historical Perspective

Some of the earliest life forms that have existed in the Atlantic Ocean for more than 500 million years and depended on sunlight for their energy source also produced vitamin D (1). As the industrial revolution swept across Europe in the mid-1600s it brought with it the scourge of rickets (2). In 1822 Sniaudecki realized the importance of sunlight for the prevention and cure of rickets. However, it was inconceivable how exposure of the skin to sunlight could have any impact on the bone disease. In 1889 Palm recommended sunbathing to prevent rickets, but his recommendation was also ignored. Finally, in 1919 Huldschinsky reported that children exposed to a mercury arc lamp could be cured of their bone deforming disease. Two years later, Hess and Unger reported that exposure to sunlight was effective in inducing radiologic improvement in rachitic lesions in young children (2–4). These and other observations resulted in the United States and governments in Europe recommending sensible sunbathing of children to prevent rickets (2).

Steenbock (5) introduced the concept of irradiating milk and other foods with ultraviolet radiation to impart antirachitic activity. The United States and Europe embraced this fortification not only for milk, but also many other products including custard and

Keywords: vitamin D, cancer, sunlight, 25-hydroxyvitamin D, infectious disease, osteoporosis, rickets

Kratak sadržaj

Vitamin D je „sunčani“ vitamin koji je važan ne samo za zdrave kosti dece i odraslih osoba već i za njihovo opšte zdravlje i dobro stanje. Nedostatak vitamina D definiše se kao nivo 25-hidroksivitamina D < 20 ng/mL a manjak vitamina D kao nivo 25-hidroksivitamina D 21–29 ng/mL. Glavni izvor vitamina D jeste izlaganje suncu u razumnoj meri, pošto je mali broj namirnica koje prirodno sadrže vitamin D. Vitamin D deficiency je asocijovane sa povećanjem rizika za mnoge acute i chronic bolesti uključujući infektivne bolesti, autoimune bolesti, kardiovaskularne bolesti, dijabetes tipa 2, neurokognitivnu disfonksiju i mišićnu slabost. Da bi se dostigao nivo 25-hidroksivitamina D u krvlje >30 ng/mL deci je potrebno 600–1 000 IU a odrasli osoba 1 500–2 000 IU vitamina D dnevno.

Ključne reči: vitamin D, kancer, sunčeva svetlost, 25-hidroksivitamin D, infektivne bolesti, osteoporoza, rafitis
even beer was fortified with vitamin D. However, in the 1950s several children were born with facial deformities and hypercalcemia and it was concluded they were vitamin D intoxicated due to overfortification of milk with vitamin D. This resulted in Europe banning the fortification not only of milk but also all other products with vitamin D (6). It is now recognized that the likely cause was a rare genetic disorder, Williams syndrome, that is associated with elfin faces and hypercalcemia. Only recently Sweden and Finland have reintroduced fortifying milk with vitamin D as a preventative measure for rickets.

Sources of Vitamin D

The major source of vitamin D is sensible sun exposure (2, 3, 7). An adult in a bathing suit exposed to an amount of sunlight that causes a slight pinkness to the skin (one minimal erythemal dose) is equivalent to ingesting approximately 20,000 IUs of vitamin D (8, 9). Very few foods naturally contain vitamin D. These include wild caught salmon, herring and other oily fish, cod liver oil and sun exposed mushrooms (Table I). Foods fortified with vitamin D usually contain 100 IUs per serving. Both vitamin D2 and vitamin D3 when given in physiologic doses are equally effective in maintaining serum levels of 25-hydroxyvitamin D (8, 10–12).

Vitamin D Deficiency Pandemic

Vitamin D deficiency is common worldwide. In the United States approximately 32% of children and adults were found to be vitamin D deficient (15). Vitamin D deficiency is common in Europe, China, Canada, Korea, and Japan among other countries in temperate climates (7, 14–17). However, vitamin D deficiency is as common in equatorial countries including UAE, Saudi Arabia, South Africa, Thailand, Brazil and India (7, 17–23). The problem is magnified if vitamin D insufficiency (defined as a 25-hydroxyvitamin D of 21–29 ng/mL) is also included. It has been estimated that upwards of 50% of children and adults are at risk for vitamin D deficiency or insufficiency worldwide (7, 8, 17). Even in the United States where vitamin D fortification of milk and other dairy products as well as some orange juices are common it has been estimated that 50% of children aged 1–5 years and 70% of children aged 6–11 years are vitamin D deficient or insufficient (24). Pregnant and lactating women are also at very high risk (25). Pregnant women who received 600 IUs of vitamin D during their pregnancy at the time they gave birth 76% of them and 81% of their infants were vitamin D deficient (26).

Definition of Vitamin D Deficiency and Insufficiency

The Institute of Medicine defined vitamin D deficiency as a 25-hydroxyvitamin D < 20 ng/mL and based it on their evaluation of vitamin D’s effect only on bone health (27). Endocrine Society defined vitamin D deficiency as a 25-hydroxyvitamin D < 20 ng/mL but concluded that to maximize bone health the blood level should be above 30 ng/mL (28). Therefore, they defined vitamin D insufficiency as a 25-hydroxyvitamin D of 21–29 ng/mL. This was based in part on several studies demonstrating that PTH plateaus between 30 and 40 ng/mL (29–31). Most importantly, a study of 675 presumed healthy German adults aged 20–70 years who met untimely deaths often due to a motor vehicle accident provided evidence for what blood level of 25-hydroxyvitamin D was essential for bone health (32). An evaluation of their bones for evidence of vitamin D deficiency osteomalacia was related to their blood level of 25-hydroxyvitamin D. The investigators reported that upwards of 36% had evidence of osteomalacia. There was also evidence for osteoidosis, i.e. osteoid buried within the mineralized bone, in approximately 35% of the healthy adults. The investigators concluded that to guarantee no evidence of osteomalacia a blood level of 25-hydroxyvitamin D should be >30 ng/mL (32).

Consequences of Vitamin D Deficiency

Vitamin D deficiency results in an increase in PTH levels which induces the formation of osteoclasts (33) which results in the destruction of the matrix and mineral that leads to bone loss in both children and adults (2, 7). The secondary hyperparathyroidism causes a lowering of the blood phosphate level resulting in an inadequate calcium phosphate product causing a mineralization defect of the newly laid down matrix. In children this results in rickets and in adults causes the painful bone disease osteomalacia (2, 7, 34).

For more than 100 years there have been a variety of observations relating living at higher latitudes with increased risk for dying of cancer (7, 8). In the 1990s several reports appeared suggesting that living at higher latitudes which was associated with lower blood levels of 25-hydroxyvitamin D was the cause for the observations (35–37).

Others have reported that higher intakes of vitamin D and exposure to more sunlight at lower latitudes was associated with a reduced risk for type 1 diabetes, multiple sclerosis, rheumatoid arthritis, Crohn’s disease, hypertension, cardiovascular disease, Alzheimer’s disease, depression, schizophrenia and cancer and cardiovascular mortality (7, 8, 38–57) (Figure 1). Healthy adults who had a blood level of 25-hydroxyvitamin D ~ 38 ng/mL reduced their risk of upper respiratory tract infections by more
Table I Sources of Vitamin D$_2$ and Vitamin D$_3$ (with permission, copyright Holick 2007).

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>VITAMIN D CONTENT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural Sources</strong></td>
<td></td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>~400–1,000 IU/tsp vitamin D$_3$</td>
</tr>
<tr>
<td>Salmon, fresh wild caught</td>
<td>~600–1,000 IU/3.5 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Salmon, fresh farmed</td>
<td>~100–250 IU/3.5 oz vitamin D$_3$, vitamin D$_2$</td>
</tr>
<tr>
<td>Salmon, canned</td>
<td>~300–600 IU/3.5 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Sardines, canned</td>
<td>~300 IU/3.5 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Mackerel, canned</td>
<td>~250 IU/3.5 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Tuna, canned</td>
<td>236 IU/3.5 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Shiitake mushrooms, fresh</td>
<td>~100 IU/3.5 oz vitamin D$_2$</td>
</tr>
<tr>
<td>Shiitake mushrooms, sun dried</td>
<td>~1,600 IU/3.5 oz vitamin D$_2$</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>~20 IU/yolk vitamin D$_3$ or D$_2$</td>
</tr>
<tr>
<td>Sunlight/UVB radiation</td>
<td>~20,000 IU equivalent to exposure to 1 minimal erythemal dose (MED) in a bathing suit. Thus, exposure of arms and legs to 0.5 MED is equivalent to ingesting ~ 3,000 IU vitamin D$_3$.</td>
</tr>
<tr>
<td><strong>Fortified Foods</strong></td>
<td></td>
</tr>
<tr>
<td>Fortified milk</td>
<td>100 IU/8 oz usually vitamin D$_3$</td>
</tr>
<tr>
<td>Fortified orange juice</td>
<td>100 IU/8 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Infant formulas</td>
<td>100 IU/8 oz vitamin D$_3$</td>
</tr>
<tr>
<td>Fortified yogurts</td>
<td>100 IU/8 oz usually vitamin D$_3$</td>
</tr>
<tr>
<td>Fortified butter</td>
<td>56 IU/3.5 oz usually vitamin D$_3$</td>
</tr>
<tr>
<td>Fortified margarine</td>
<td>429/3.5 oz usually vitamin D$_3$</td>
</tr>
<tr>
<td>Fortified cheeses</td>
<td>100 IU/3 oz usually vitamin D$_3$</td>
</tr>
<tr>
<td>Fortified breakfast cereals</td>
<td>~100 IU/serving usually vitamin D$_3$</td>
</tr>
<tr>
<td><strong>Pharmaceutical Sources in the United States</strong></td>
<td></td>
</tr>
<tr>
<td>Vitamin D$_2$ (Ergocalciferol)</td>
<td>50,000 IU/capsule</td>
</tr>
<tr>
<td>Drisdol (vitamin D$_2$) liquid</td>
<td>8000 IU/cc</td>
</tr>
<tr>
<td><strong>Supplemental Sources</strong></td>
<td></td>
</tr>
<tr>
<td>Multivitamin</td>
<td>400, 500, 1000 IU vitamin D$_3$ or vitamin D$_2$</td>
</tr>
<tr>
<td>Vitamin D$_3$</td>
<td>400, 800, 1000, 2000, 5,000, 10,000, and 50,000 IU</td>
</tr>
</tbody>
</table>

* Designated calciferol which usually means vitamin D$_2$. 
than 40% (58). Japanese school children who received 1200 IUs of vitamin D daily for 4 months during the winter reduced their risk of developing influenza A infection by 42% (59).

Mechanisms for the Pleotropic Effects of Vitamin D

Essentially, every tissue and cell in the body including the brain, gonads, skin, vascular smooth muscle and immune cells have a vitamin D receptor (VDR) (7, 8, 60). In addition, not only are the kidneys able to produce 1,25-dihydroxyvitamin D but also a wide variety of other cells including prostate, skin, brain, macrophages, colon and breast have the capacity to produce 1,25-dihydroxyvitamin D (7, 8, 60–62). It is believed that the local production of 1,25-dihydroxyvitamin D by these tissues and cells is for the purpose of regulating a wide variety of metabolic processes as well as controlling cellular growth and preventing malignancy (7, 8, 60–68). The production of 1,25-dihydroxyvitamin D by macrophages is important for helping macrophages fight infections including TB by increasing the expression of cathelicidin, a defensin protein that helps kill bacteria and other infectious agents (69) (Figure 2).

1,25-dihydroxyvitamin D increases the expression of transcription factors that can downregulate cellular growth including p21 and p27 (7, 63). It has been estimated that as many as 2000 genes may be directly or indirectly regulated by 1,25-dihydroxyvitamin D (7, 70). In addition, 1,25-dihydroxyvitamin D can also affect the epigenetics of the cell adding an additional method for influencing cellular activity.

Treatment and Prevention of Vitamin D Deficiency

The Institute of Medicine made its recommendations using a population model and recommended for most children and adults up to the age of 70 years that 600 IUs of vitamin D daily will prevent vitamin D deficiency (27) (Table II). The Endocrine Society made its recommendations for the treatment and prevention of vitamin D deficiency and insufficiency and recommended that children of one year and over receive 600–1000 IUs of vitamin D daily and adults 1500–2000 IUs of vitamin D daily (28) (Table II). There are a variety of patients including those with obesity, malabsorption syndromes and on medications that would enhance the destruction of vitamin D and its metabolites who require at least 2–3 times more vitamin D. Patients with sarcoidosis should be
carefully monitored because of their hypersensitivity to vitamin D because of the granulomas producing 1,25-dihydroxyvitamin D that can cause hypercalciuria and hypercalcemia (7, 28).

One strategy to treat vitamin D deficiency is to give 50,000 IUs of vitamin D once a week or its equivalent about 6000 IUs of vitamin D daily for 2 months (71). To prevent recurrence 50,000 IUs vitamin D once every 2 weeks or an equivalent of 3000 IUs of vitamin D daily is suggested. This has been effective for up to 6 years without any untoward toxicity. Vitamin D toxicity is usually not seen in adults until they ingest more than 10,000 IUs of vitamin D daily for at least several months (7, 71) (Figure 3).

**Conclusion**

Vitamin D deficiency and insufficiency is a global health problem for children and adults. Health
**Table II** Vitamin D dietary reference intakes.

<table>
<thead>
<tr>
<th>Life Stage Group</th>
<th>IOM Recommendations</th>
<th>Committee Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AI</td>
<td>EAR</td>
</tr>
<tr>
<td>Infants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 6 mo</td>
<td>400 IU (10 µg)</td>
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</tr>
<tr>
<td>6 to 12 mo</td>
<td>400 IU (10 µg)</td>
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</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–3 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>4–8 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9–13 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>14–18 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
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<tr>
<td>19–30 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
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<tr>
<td>31–50 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>51–70 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>&gt; 70 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9–13 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
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<tr>
<td>14–18 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
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<tr>
<td>19–30 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
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<tr>
<td>31–50 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>51–70 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>&gt; 70 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14–18 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>19–30 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>31–50 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>Lactation*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14–18 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>19–30 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
<tr>
<td>31–50 y</td>
<td>---</td>
<td>400 IU (10 µg)</td>
</tr>
</tbody>
</table>

* Mother’s requirement 4,000–6,000 (mother’s intake for infant’s requirement if infant is not receiving 400 IU/d)

Note: AI = Adequate Intake; EAR = Estimated Average Requirement; IU = International Units; RDA = Recommended Dietary Allowance; UL = Tolerable Upper Intake Level.
Figure 3 A. Mean serum 25-hydroxyvitamin D \([25(OH)D]\) levels in all patients: Includes patients treated with 50,000 IU vitamin D\(_2\) every 2 weeks (maintenance therapy; \(N=81\)), including those patients with vitamin D insufficiency who were initially treated with 8 weeks of 50,000 IU vitamin D\(_2\) weekly prior to maintenance therapy (\(N=39\)). Error bars represent standard error of the mean, mean result over 5 years shown. Time 0 is initiation of treatment, results shown as mean values averaged for 6-month intervals. When mean 25\((OH)D\) in each 6-month group was compared to mean initial 25\((OH)D\), \(p<0.001\) up until month 43; \(p<0.001\) when all remaining values after month 43 were compared to mean initial 25\((OH)D\).

B. Mean serum 25\((OH)D\) levels in patients receiving maintenance therapy only: Levels for 37 patients who were vitamin D insufficient (25\((OH)D\) levels < 30 ng/mL) and 5 patients who were vitamin D sufficient (25\((OH)D\) levels ≥ 30 ng/mL) who were treated with maintenance therapy of 50,000 IU vitamin D\(_2\) every two weeks. Error bars represent standard error of the mean, mean result over 5 years shown. Time 0 is initiation of treatment, results shown as mean values averaged for 6-month intervals. When mean 25\((OH)D\) in each 6-month group were compared to mean initial 25\((OH)D\), \(p<0.001\) up until month 43; \(p<0.001\) when all remaining values after month 43 were compared to mean initial 25\((OH)D\).

C. Serum calcium levels: Results for all 81 patients who were treated with 50,000 IU of vitamin D\(_2\). Error bars represent standard error of the mean. Time 0 is initiation of treatment, results shown as mean values averaged for 6-month intervals. Normal serum calcium: 8.5–10.2 mg/dL. Reproduced with permission.
There is no need to be screening every one for their vitamin D status but only those who are at risk (Table III). Although there is great concern about sun exposure and risk for skin cancer, it should be realized that sensible sun exposure especially of the arms, legs, abdomen and back to suberythemal doses of sunlight a few times a week will not significantly increase risk for non-melanoma skin cancer (72). The concern, of course, is melanoma, which is the most deadly form of skin cancer. However, often it is not appreciated that most melanomas occur on the least sun exposed areas and occupational sun exposure decreases risk for this deadly disease (73). Even Australia, the skin cancer capital of the world, recognizes that upwards of 40% of their children and adults are vitamin D deficient and now recommends sensible sun exposure as a means of preventing this disease of neglect.

Sources of support: This work is supported in part by the UV Foundation and the Mushroom Council.

### Table III: Conditions When 25(OH)D measurement is indicated.

<table>
<thead>
<tr>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rickets</td>
</tr>
<tr>
<td>Osteomalacia</td>
</tr>
<tr>
<td>Osteoporosis</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>Hepatic failure</td>
</tr>
<tr>
<td>Malabsorption syndromes</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>Bariatric surgery</td>
</tr>
<tr>
<td>Radiation enteritis</td>
</tr>
<tr>
<td>Hyperparathyroidism</td>
</tr>
<tr>
<td>Medications</td>
</tr>
<tr>
<td>Antiseizure medications</td>
</tr>
<tr>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>AIDS medications</td>
</tr>
<tr>
<td>Older adults with history of falls</td>
</tr>
<tr>
<td>Older adults with history of non-traumatic fractures</td>
</tr>
<tr>
<td>Obese children and adults (BMI&gt;30)</td>
</tr>
<tr>
<td>Granulomatous disorders</td>
</tr>
<tr>
<td>Sarcoidosis</td>
</tr>
<tr>
<td>TB</td>
</tr>
<tr>
<td>Histoplasmosis</td>
</tr>
<tr>
<td>Cocciidiomycosis</td>
</tr>
<tr>
<td>Berylliosis</td>
</tr>
<tr>
<td>Some lymphomas</td>
</tr>
</tbody>
</table>

References


Introduction

World Health Organization (WHO) has recognized chronic noncommunicable diseases, particularly cardiovascular diseases, diabetes, chronic respiratory diseases and cancer, as the most important public health problems in the developed part of the world. According to some epidemiological studies chronic noncommunicable diseases are becoming a great problem also in developing countries. Interestingly, the WHO did not include chronic kidney disease (CKD) among the most important chronic diseases despite the fact that between 5 and 10% of adult persons in the developed world suffer from chronic kidney disease (1). There are also some epidemiological data that the prevalence of CKD is of the same magnitude in developing countries. From the nephrological point of view there is no doubt that CKD is also an important chronic noncommunicable disease. On the other hand, a growing body of evidence suggests that vitamin D deficiency or insufficiency is a pandemic with more than half of the world’s population currently at risk. During the past ten years several observation studies have supported the concept that vitamin D is involved in the pathogenesis of cardiovascular and renal disease. At least, there is an epidemiological connection between vitamin D disturbance, i.e. deficiency and insufficiency and the prevalence of the majority of chronic noncommunicable diseases.

In this review, we will briefly discuss the vitamin D metabolism, the epidemiological data on vitamin D insufficiency and renal and cardiovascular disease, and the possible biological mechanism, i.e. the role of vitamin D in renal and cardiovascular disease. At the end we will discuss whether vitamin D has any potential as a drug in the prevention and treatment of renal and cardiovascular disease.
Vitamin D Metabolism

At the beginning of the last century the fat-soluble antirachitic substance in fish liver oil was discovered. In 1922, the treatment of rickets through exposure to UV light was introduced in clinical practice. Nine years later ergocalciferol, i.e. vitamin D, was discovered and five years after that 7-dehydrocholesterol. More than 40 years later, 25-hydroxyvitamin D, 1,25 dihydroxycholecalciferol and the vitamin D receptors were discovered (3–5).

There has been increasing interest in vitamin D in the past decade. In addition to its well-known role in maintaining an adequate level of serum calcium, phosphorus, parathyroid hormone and normal bone metabolism, there is evidence that vitamin D has a biological effect beyond mineral metabolism (4).

Vitamin D is a secosteroid that is made in the skin by the action of sunlight, or much less frequently ingested through diet. During ultraviolet B radiation, 7-dehydrocholesterol (provitamin D) is converted to vitamin D, which is converted into vitamin D. Vitamin D is very rarely found in food. In some countries foods like milk or bread products are fortified with vitamin D, but this is not an important source of vitamin D. In the liver, vitamin D is converted by the enzyme cytochrome 450 into calcidiol. This conversion is under low metabolic control. Calcidiol is biologically inert but it is used to determine vitamin D status because it has a long half-life, is easily measured, and there is a good correlation between the level of calcidiol and some diseases. Despite some controversy, vitamin D insufficiency might be defined as a calcidiol level less than 25 nmol/L, deficiency between 25 to 75 nmol/L and an optimal level more than 75 nmol/L. There are many reasons for vitamin D deficiency or insufficiency: skin pigmentation, aging, obesity, lack of sun exposure, chronic disease, particularly chronic kidney disease, latitude of residence etc. (3–6).

In the kidney, calcidiol is metabolized by the enzyme 1α-hydroxylase (CYP27B1) to calcitriol, an active metabolite of vitamin D. The production of calcitriol is very tightly controlled by calcium and phosphorus levels, by parathyroid hormone and fibroblast growth factor 23. Another enzyme in the kidney, 24-hydroxylase (CYP24), catabolizes calcidiol and calcitriol into biologically inactive calcitrionic acid (3, 4).

Calcitriol acts by activating the vitamin D receptor (VDR), which binds together with transcription factor RXR in specific regions of DNA (VDREs, vitamin D response elements) (8). Vitamin D receptors are widely distributed. In addition to tissue and organs involved in mineral and bone metabolism, VDRs are found in vascular smooth muscle, endothelium, the heart, brain, skin, pancreas, macrophages etc. Moreover, some cells like macrophages or vascular cells express 1α-hydroxylase, i.e. the possibility of converting calcidiol into calcitriol. This extrarenal calcitriol is not tightly controlled and the calcitriol produced in these cells has a local, autocrine or paracrine effect. The distribution of VDRs and the local production of calcitriol demonstrate that vitamin D is a pluripotent hormone involved in more than calcium homeostasis and bone metabolism. Today, there is a large amount of data suggesting that vitamin D deficiency or insufficiency is involved in the pathogenesis of bone disease, malignancies, metabolic and immunological diseases, cardiovascular disease and hypertension and progression of renal disease (3, 4).

Vitamin D and Renal Disease

The kidney is the major site of synthesis of calcitriol, the natural activator of VDR, and has an important function in mineral homeostasis (6, 8). Many studies showed a high prevalence of vitamin D deficiency or insufficiency in CKD patients. Recent experimental, observational and clinical studies have shown that dysregulation of vitamin D metabolism in CKD contributes not only to mineral metabolism disturbance but also to progression of renal disease and a high incidence of cardiovascular disease. There are several mechanisms for vitamin D deficiency in CKD, particularly in proteinuric renal disease (diabetic nephropathy and many glomerulopathies). The first one is the degree of proteinuria. Calcitriol is bound to vitamin D binding protein. Due to similar molecular weight as albumin, it is filtered at glomeruli and lost into the urine. The second mechanism is impaired reabsorption of calcitriol by megalin-mediated endocytotic activity. The third mechanism is elevated activity of the catabolic enzyme CYP24A1, involved in calcitriol and calcitriol degradation. At the same time, the activity of enzyme CYP27B1 (1α-hydroxylase) is very reduced i.e. there is reduced conversion of calcidiol to calcitriol. The fourth mechanism is increased activity of fibroblast growth factor 23, a major phosphatonin that suppresses calcitriol production (6, 8).

Vitamin D deficiency in CKD patients is associated with increased all-cause and cardiovascular mortality. For example, data from the Third National Health and Nutrition Examination Survey (NHANES III) in the United States revealed a correlation between low serum calcitriol and the risk of all-cause mortality in the general population and in patients with CKD not yet on dialysis (9). Data from Germany, i.e. the Ludwigshafener Risk and Cardiovascular (LURIC) Health Study confirmed that vitamin D deficiency is associated with all-cause and cardiovascular mortality in CKD patients (10). There is a lot of data indicating that
Vitamin D deficiency is associated with renal disease progression. Albuminuria is a major risk factor for renal disease progression. The abovementioned NHANES III study revealed a correlation between low levels of vitamin D and increased prevalence of albuminuria (9).

Today, we have enough experimental and clinical data showing that vitamin D could have antiproteinuric, i.e. renoprotective activity. The renoprotective activity of vitamin D could be mediated by the activation of VDR and through several mechanisms. First, by suppression of the renin-angiotensin system (RAS). This intrarenal system is a major mediator of renal damage. Li et al. (11) demonstrated that vitamin D, i.e. calcitriol, is a potent inhibitor of renin synthesis. They showed that renin expression and plasma angiotensin II production are increased in VDR receptor-null mice. In wild mice, i.e. mice with intact VDR receptors, the inhibition of calcitriol synthesis also led to increase in renin expression, whereas calcitriol injection led to renin suppression, i.e. vitamin D is a negative regulator of the RAS. The second mechanism is by suppression of NF-kB factor, an important factor involved in inflammation, proliferation and fibrogenesis. Vitamin D could be involved in the regulation of Wnt/β-catenin, an important factor in podocyte injury (12). Also, there are experimental data that vitamin D may regulate genes in the synthesis of the proteins involved in the formation of the slit diaphragm in the glomerulus. Several clinical studies have demonstrated the vitamin D renoprotective activity, particularly antiproteinuric activity. Alborzi P. et al. (13) have conducted a small pilot double-blind, placebo controlled trial to evaluate the effect of paricalcitol (vitamin D analog) on markers that are linked to the progression of CKD. In patients treated with paricalcitol significant reduction of albuminuria was observed. Similar results were obtained in CKD patients with proteinuria greater than 400 mg/24 h (14). In 31 patients treated with 1 mg paricalcitol significant reduction of proteinuria was observed in comparison with a placebo group. In a large randomized placebo controlled study, the VITAL study, involving 281 patients with type 2 diabetes and albuminuria and already on renin-angiotensin inhibitor therapy, significant reduction of albuminuria was seen in patients treated with 2 μg of paricalcitol. The reduction of albuminuria was recorded also in patients who were on 1 μg of paricalcitol, but this was not as significant. No incidence of hypercalcemia or any other adverse events was a very important observation (15). These and other clinical studies confirmed the antiproteinuric effect of vitamin D.

**Vitamin D and Cardiovascular Disease**

More than thirty years ago, the hypothesis that the increased CVD incidence in winter may be a consequence of vitamin D deficiency or insufficiency was postulated. This hypothesis together with the discovery of the VDR in the rat heart stimulated research of vitamin D and cardiovascular disease (16).

In the last twenty years, several cross-sectional studies and only a few prospective studies have been conducted in an attempt to correlate vitamin D levels with cardiovascular disease (16–18). One of the largest studies was the Third National Health and Nutrition Examination Survey (NHANES III). It is a representative study of the non-institutionalized US population. More than 12,000 patients were included in this cross-sectional observation study between 1988 and 1994. A significant inverse correlation between self-reported angina, myocardial infarction, heart failure, blood pressure and vitamin D level, i.e. calcidol was observed (19). More recently, combined data from the NHANES III and the NHANES 2001–2006 (more than 270,000 participants) showed that lower vitamin D levels are associated with increased heart rate and blood pressure; in other words, low vitamin D status may increase cardiac work. It is well known that high blood pressure is the most important cardiac risk factor (20). Several studies have reported an inverse association between vitamin D level and high blood pressure. In the NHANES 2001–2006 study a low level of calcidol and a high level of PTH were independently associated with high blood pressure. In fact, among more than 5,000 participants not taking any antihypertensive medication, systolic and diastolic blood pressure decreased linearly across quintiles of serum calcidol and increased linearly across quintiles of serum PTH. Even more similar results were observed for prehypertension (systolic blood pressure 120–140 mmHg and diastolic 80–90 mmHg) (21). Recently, Burgaz et al. (22) published a meta-analysis of blood calcidol concentration and hypertension. In the analysis 18 studies (14 cross-sectional, 4 prospective) were included, with a total of 78,028 participants. The pooled odds ratio of hypertension was 0.73 [95% confidence interval (CI) 0.63–0.84] for the highest versus the lowest category of blood calcidol level. In a dose response meta-analysis, the odds ratio for a 40 nmol/L increment in blood calcidol level was 0.84 (95% CI 0.78–0.9). Without a doubt, the conclusion from this meta-analysis is that calcidol level is inversely associated with hypertension (22).

**Biological Links between Vitamin D and Cardiovascular Disease**

Vitamin D may exert various direct effects on heart and blood vessels through VDR activation or by locally produced calcitriol with autocrine and paracrine function. Very briefly, there are a lot of experimental studies suggesting that vitamin D downregulates the genes involved in myocardial hypertrophy (12, 23). Also, the effect of vitamin D on the heart could be by regulation of cardiac extracellular matrix

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turnover or by modulation of heart contractility. Probably the most important activity is the suppression of RAS. The effect of vitamin D on blood vessels could be protection against atherosclerosis, endothelial dysfunction and calcification (23, 24). From the clinical point of view, vitamin D could have an effect on various cardiovascular risk factors: suppression of PTH, reduction of inflammation, prevention of diabetes mellitus, beneficial effect on dyslipidemia and on the progression of CKD, an important cardiovascular risk factor. Probably the most important effect of vitamin D in terms of cardiovascular protection is the well-known negative relationship between calcidol or calcitriol levels and RAAS activity, i.e. the inhibition of this system (23).

Vitamin D as a Cardiovascular and Antihypertensive Drug

Unfortunately, at this time not enough studies have been conducted to investigate the effect of calcitriol or calcitriol as a cardioantihypertensive agent (25). In a small trial Pfeiffer et al. (27) demonstrated greater systolic blood pressure reduction in the vitamin D plus calcium group versus only the calcium group (p=0.02). Calcitriol as a single i.v. dose significantly decreased systolic and diastolic pressure 2 h after administration in a small group of dialysis patients. Such changes were not observed in patients with essential hypertension or healthy volunteers (26, 27). In a pilot feasibility study Judd SE et al. (28) have demonstrated that blood pressure could be reduced with calcitriol. Nine hypertensive subjects were randomized to receive standard antihypertensive therapy in addition to placebo, vitamin D or calcitriol. Only seven subjects completed the study. Subjects on calcitriol therapy had a significant decrease in systolic blood pressure compared to the placebo. Interestingly, one week after the discontinuation of calcitriol therapy, the systolic blood pressure returned to pretreatment levels.

| Table I  Vitamin D and renal and cardiovascular disease: strength of evidence. |
|----------------|----------------|----------------|----------------|
| Disease        | Experimental  | Observational | Interventional |
| Hypertension   | +++           | ++            | +             |
| ISHD           | +             | +++           | 0             |
| PVD            | +             | ++            | 0             |
| CHD            | ++            | ++            | 0             |
| Dyslipidemia   | ++            | ++            | 0             |
| Renal disease  | +++           | +++           | +             |

ISHD, ischemic heart disease; PVD, peripheral vascular disease; CHD, congestive heart disease.
0, absence of evidence; +, limited evidence of association; ++, moderate evidence of association; ++++, strong evidence of association.

Unfortunately, at this moment there are no data regarding the therapeutic efficacy of vitamin D and its analogs as primary or complementary therapy in cardiovascular disease.

Conclusion

Clinical and epidemiological studies support a possible relationship between vitamin D and renal and cardiovascular disease (Table I). There are some plausible biological mechanisms. Treatment of patients with renal and cardiovascular disease is still a challenge for physicians (24). Patients with hypertension, cardiovascular, renal disease and vitamin D deficiency could benefit from vitamin D supplementation or calcitriol treatment, particularly patients with chronic kidney disease (23). Undoubtedly, we need more large prospective studies.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.
References


INHIBITION OF CHOLESTEROL BIOSYNTHESIS IN HYPERCHOLESTEROLEMIA – IS IT THE RIGHT CHOICE?

INHIBICIJA BIOSINTEZE HOLESTEROLA U HIPERHOLESTEROLEMJI – DA LI JE PRAVI IZBOR?

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Summary: Cholesterol biosynthesis is a complex pathway comprising more than 20 biochemical reactions. Although the final product created in the pathway is cholesterol, the intermediate products, such as ubiquinone and dolichol, also provide vital metabolic functions. Statins are HMG-CoA reductase inhibitors that stop the production of cholesterol by directly inhibiting the mevalonate production. Mevalonate is a precursor of two additional vital molecules, squalene and ubiquinone (coenzyme Q10). We hypothesized that inhibiting the cholesterol biosynthesis with statins for an extended duration may potentiate the oxidative stress, neurodegenerative disease and cancer. Our recommendation was to measure muscle enzymes, antioxidant capacity, and ubiquinone to monitor patients receiving the statins for prolonged periods of time.

Keywords: cholesterol, ubiquinone, statins

Cholesterol biosynthesis comprises more than 20 enzymatically catalyzed reactions and occurs in four main stages. In the first stage, acetyl-CoA molecules form a six-carbon molecule called mevalonate. In the second stage, mevalonate is converted to activated isoprene units, which are subsequently (stage 3) polymerized to form squalene. In the fourth and final stage, cyclization of squalene forms the steroid nucleus and an additional series of changes leads to formation of cholesterol (1, 2).

The first-stage reaction is clinically relevant, as it the target of certain anti-hyperlipidemia drugs. In the first step, two acetyl-CoA molecules condense to form acetoacetyl-CoA. Subsequently, a third acetyl-CoA condenses with the acetoacetyl-CoA to yield 3-hydroxyl 3-methylglutaryl CoA (HMG-CoA). HMG-CoA is then converted to mevalonate by HMG-CoA reductase. Conversion of HMG-CoA to mevalonate is the
rate-limiting step in the cholesterol biosynthesis, and the primary statin target.

In the second stage, mevalonate is converted to isopentenyl pyrophosphate (IPP). IPP units condense to form geranyl pyrophosphate (GPP) and then farnesyl pyrophosphate (FPP). In addition to cholesterol biosynthesis, the isoprene products perform many vital functions discussed in more details herein.

Despite its numerous beneficial structural and metabolic functions, elevated cholesterol levels are a significant risk factor of coronary artery disease. Thus, inhibiting the cholesterol biosynthesis is widely accepted as a method for treating the hypercholesterolemia, and numerous drugs have been developed to inhibit the enzymes at different steps during cholesterol biosynthesis.

**Inhibition of Cholesterol Biosynthesis by Statins**

Statins comprise a group of drugs (simvastatin, lovastatin, mevastatin, atorvastatin, pravastatin) that inhibit cholesterol biosynthesis. These drugs selectively inhibit the rate-limiting enzyme of the cholesterol biosynthesis, HGM-CoA reductase, thereby, lowering the blood cholesterol levels. However, statins do not inhibit the enzyme completely. They inhibit roughly 45% to 95% of HGM-CoA reductase activity depending on the dose and the type of statins used (3). HGM-CoA reductase exerts its effects during the first stage of cholesterol biosynthesis, and thus inhibition of HGM-CoA reductase inhibits all of the metabolically active intermediate molecules produced during the stages 2–4 of the cholesterol biosynthesis pathway.

**Hypothesis**

We hypothesized that HGM-CoA reductase is not the most appropriate inhibitory target for lowering the blood cholesterol levels, particularly for an extended duration. Doing so in a large population which meets the criteria for hypercholesterolemia may trigger serious diseases in many otherwise healthy individuals.

Cholesterol synthesis is a multi-step reaction, requiring 20 different enzymes to complete the pathway. All of these enzymes catalyze intermediate reactions and the resulting products accomplish many other metabolic functions. Two of these intermediate molecules of particular importance are IPP and FPP.

IPP is the precursor of isoprenoids, ubiquinone, and dolichol. Ubiquinone is an essential component of the electron transport chain, which is located within the inner mitochondrial membrane and is responsible for 95% of all human ATP synthesis reactions. Ubiquinone is small and hydrophobic, and thus mobile and freely diffusible within the lipid bilayer of the inner mitochondrial membrane. Ubiquinones accept electrons from FMN$^H_2$, which is produced by NADH dehydrogenase (complex I), and from FADH$^H_2$, which is produced by succinate dehydrogenase (complex II) and acyl CoA dehydrogenase. These steps are absolutely required for subsequent electron transfer to complex III. Ubiquinones carry both electrons and protons and play a central role in coupling the electron flow to proton movement, which is essential for oxidative phosphorylation. A decreased ubiquinone level results in decreased electron transport and subsequently decreased ATP production. Although ubiquinones are concentrated in the inner mitochondrial membrane, they are also widely distributed in other cellular membranes (4) and perform several cell metabolism functions other than ATP production, including the cell signalling and gene expression (5).

Oxidative stress causes DNA and protein damage, and plays an important role in the development of cancer, neurodegenerative diseases, cardiovascular disorders, and aging (6, 7). According to the oxidative stress theory, atherosclerosis is the result of the oxidative modification of low-density lipoproteins (LDL) by reactive oxygen species (ROS) in the arterial wall. ROS induce the oxidation of LDL, whose uptake by macrophages is easier than uptake of non-oxidized LDL. Oxidative stress, together with the weakened antioxidative defense system, induces the vascular dysfunction and promotes atherosclerosis. Ubiquinone inhibits atherosclerosis development as described by the oxidative theory (8), and is considered the anti-risk factor of atherosclerosis.

It has been shown that statin therapy decreases circulating and muscle ubiquinone levels (8, 9). Decreased levels of ubiquinone may be responsible for such statin side effects as myotoxicity and rhabdomyolysis (10). Although it is not still clear, some study results indicate that decreased levels of ubiquinone during statin therapy might be associated with the subclinical cardiomyopathy and that this situation is reversible with the ubiquinone supplementation (11).

Oxidative stress and mitochondrial dysfunction have been implicated in many neurodegenerative disorders including the Alzheimer’s disease, amyotrophic lateral sclerosis, and Parkinson’s disease. For example, studies suggest that, in Parkinson’s disease, there is a deficiency of the complex I activity in the mitochondrial electron transport chain. Interestingly, ubiquinone appears to be a promising agent in the treatment of Parkinson’s disease. Additionally, in animal models, the experimental studies suggest that ubiquinone protects against many neurodegenerative diseases (12–14).

In addition to ubiquinone, dolichol metabolism and prenylation play important roles in the impact of statins on human metabolism. Dolichol metabolism affects glycoprotein and glycolipid synthesis, which subsequently enables vital cell cycle functions. Glycoproteins and glycolipids comprise a group of structural and functional proteins of major metabolic
importance. Glycoproteins are found in the blood, extracellular matrix, outer surfaces of cell membranes, and organelles such as lysosomes and Golgi complexes. Glycolipids are located in cell membranes, and their polar heads are composed of carbohydrate molecules, which are activated by the attachment of dolichol. Inhibition of dolichol biosynthesis results in the abnormal glycosylation, which in turn has negative effect on many requisite cellular functions and structures. Specifically, it has been shown that the inhibition of dolichol synthesis or function blocks the cell cycle in the late G1 phase and if the exposure is prolonged, it induces apoptosis (15–18).

Prenylation is a common mechanism by which the proteins are anchored to the inner cellular membrane surfaces. The attached unit is either a farnesyl or a geranylgeranyl group. Protein prenylation is an important function of the isoprene derivatives, and statins can block prenylation (19). There is strong evidence that statin-induced blocking of the protein prenylation also blocks the cell growth (20).

Statins are approved only for treatment of lipid disorders; however, it is suggested that they may be useful in treating other conditions, such as osteoporosis and Alzheimer’s disease (21, 22). Clinical studies have yet to evaluate this assumption. The potential of statins as an anti-cancer treatment has been investigated extensively (20). Despite their benefits in treating the hypercholesterolemia and being studied extensively, statin safety remains unclear. It has been suggested that some statins reduce the risk of developing cancer (23); however, this association has not been validated by other studies (24). Recently, Vinogradova and coworkers (25) conducted a large population-based case control study on 88,125 cases and 362,254 matched controls and found reduced risk of hematological malignancies. More importantly, they found that the prolonged use of statins (≥ 4 years) was associated with the significantly increased risk of colorectal, bladder, and lung cancers. These cancer types are common in the age group of patients taking anti-hypercholesterolemia drugs. The association between the statins and cancer risk is an important point that warrants further intensive investigation.

When evaluating the metabolic functions of all cholesterol biosynthesis intermediates in hypercholesterolemia patients, physicians may conclude that the inhibition of HMG-CoA reductase with statins may not be the right choice. Rather, physicians may choose to target another inhibitory point in the cholesterol biosynthesis pathway. However, this decision is not straightforward because there are different known target points and associated drugs. For example, triparanol inhibits the final step of cholesterol biosynthesis, the conversion of desmosterol to cholesterol. When triparanol was used to treat hypercholesterolemia patients, it caused an accumulation of desmosterol in tissues, resulting in the development of alopecia, atherosclerosis and cataracts. Triparanol was used in 1960s and its main side effect is known as »Triparanol Disaster« (2).

In conclusion, cholesterol biosynthesis is a complex pathway with many vital intermediate downstream products that serve as potential anti-hypercholesterolemia drug targets. These intermediates are as important to human body function as cholesterol itself. The purpose of the cholesterol biosynthetic pathway is not exclusively to synthesize cholesterol, and mevalonate synthesis does not always result in cholesterol production. Alternatively, the body may utilize mevalonate for synthesizing the ubiquinone, dolichol and other molecules, as well as cholesterol. These molecules are defense against oxidative stress, cancer, neurodegenerative diseases and atherosclerosis. Statin usage over time inhibits cholesterol production, but also compromises the defense system, rendering the patients vulnerable to these diseases. Statins are overprescribed in populations not at risk of cardiovascular disease, and in these patients, the risk of statin use may far outweigh the benefits. Additional studies are required to identify new targets for development of anti-hypercholesterolemia drugs

Conflict of interest statement
The authors stated that there are no conflicts of interest regarding the publication of this article.


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CARDIOTOXICITY OF PALLADIUM COMPOUNDS
KARDIOTOKSIČNOST JEDINJENJA PALADIJUMA

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Summary: Previous studies have shown that palladium has toxic effects on the kidney and liver, leads to deterioration of the general condition of animals, and could cause allergy in animals and humans. Considering the limited data about the influence of palladium on the cardiovascular system, the aim of our study was to evaluate the effects of palladium on the heart from available published data, and to compare the toxicity of inorganic and organic palladium compounds. Relevant studies for our review were identified from PubMed and Scopus databases. The search terms included »palladium«, »palladium compound«, »cardiotoxicity«, »toxicity«, »heart«, »myocardium«, »oxidative stress« and »myocardial enzyme«, as well as combinations of these terms. There were only two published studies with the primary purpose to investigate the effect of palladium on the cardiovascular system, while others registered the side-effects of palladium compounds on the heart. Palladium could cause arrhythmias, a drop in blood pressure, decrease of the heart rate, as well as death of experimental animals. Based on the presented data it seems that palladium does not express significant cardiac toxicity when it is bound in an organic compound. Further investigation of the effects of palladium on the heart is necessary for a clear picture of the nature and extent of its cardiac toxicity.

Key words: palladium, palladium compound, toxicity, cardiotoxicity, oxidative damage

Introduction

Palladium (Pd) is a lustrous, silvery-white, heavy metal belonging to the platinum group of elements (PGMs). The presence of palladium and other PGMs in the Earth’s crust is small (<1 μg/kg) (1). It is obtained as a by-product of nickel, copper and other base metals refining. Numerous unsuccessful attempts at using different palladium compounds for treating tuberculosis (2), gout, obesity (3) and dermatological diseases (2) were made in the past.
Nowadays, Pd\textsuperscript{103} is used in oncology, in brachytherapy for prostate cancer (4–6) and ocular melanoma (7–9). The latest research indicated that some palladium complexes were effective in the treatment of non-small-cell lung cancer (10), breast cancer (11, 12) and ovarian cancer (13).

Little is known about the toxicity of palladium and its compounds. Previous studies conducted on rats and rabbits have shown that palladium has toxic effects on the kidney (14, 15), liver (16, 17), produces deterioration of the general condition of experimental animals (2, 18) and could cause allergy in animals (14, 19–21) and humans (22–26). Clinical signs of acute poisoning include death, tonic and clonic convulsions, ataxia, tiptoe gait, reduced intake of food and water, weakness and abdominal distension in animals (2, 18). Also, PdCl\textsubscript{2} caused testicular necrosis and destruction of all spermatozoa in mice (27) while its teratogenicity was not proven (28). On the other hand, studies of acute and chronic toxicity in rats and rabbits did not show specific histopathological or other toxic effects of palladium on the heart (29–31).

Considering the limited data about the influence of palladium on the cardiovascular system, the aim of our study was to evaluate the effects of palladium on the heart from available published data, and to compare the toxicity of inorganic and organic palladium compounds.

Material and Methods

Relevant studies for our review were identified from PubMed and Scopus databases (since 1975). The search terms included «palladium», «palladium compound», «cardiotoxicity», «toxicity», «heart», «myocardium», «oxidative stress» and «myocardial enzymes», as well as combinations of these terms. We found 331 published papers which contained these terms. References from relevant original scientific research studies and literature reviews written in English and non-English languages were included. Unpublished and unsystematic studies were omitted.

Results

The number of studies which investigated the impact of palladium on the cardiovascular system is relatively small (Table I). Moreover, there are only two studies which were primarily designed to investigate this effect (32, 33), while two others only observed the side-effects of palladium compounds on the heart. In the first study, after intravenous administration of palladium to unanesthetized rats in the form of inorganic compounds (Pd(NO\textsubscript{3})\textsubscript{2}, PdCl\textsubscript{2}, (NH\textsubscript{4})\textsubscript{2}PdCl\textsubscript{4}, K\textsubscript{2}PdCl\textsubscript{4} and PdSO\textsubscript{4}), arrhythmias and decrease in blood pressure were observed, and some of the animals died (32). In addition, Pd(NO\textsubscript{3})\textsubscript{2}, PdCl\textsubscript{2} and PdSO\textsubscript{4} were three times more toxic than the bivalent compounds ((NH\textsubscript{4})\textsubscript{2}PdCl\textsubscript{4} and K\textsubscript{2}PdCl\textsubscript{4}). In the second study, palladium (PdCl\textsubscript{2}) caused a drop in diastolic (DLVP) and mean blood pressure (MBP) and decrease in the heart rate (HR) (33). On the contrary, the organic compound of palladium (trans-dichlorobis(triethanolamine-N)palladium(II) complex (trans-[PdCl\textsubscript{2}(TEA)\textsubscript{2}])) produced a limited decrease in the heart rate (33). In accordance with this finding, it appears that palladium does not express significant cardiac toxicity when it is bound in an organic compound. There are two more studies which observed rapid death of animals when PdCl\textsubscript{2} was given intravenously (2, 18). The mechanism of this toxic effect was not described, and the authors offered only speculations about the possible palladium-induced damage and disturbance of the heart.

On the other hand, some findings suggested that palladium \(\alpha\)-lipoic acid (an organic form of palladium which is an ingredient of the food supplement \(\text{POLY-MVA}\)) has a protective effect on the heart, and might be useful in the prevention of cardiovascular and neurodegenerative diseases associated with aging (34, 35). In fact, the palladium complex significantly increased the activity of the main antioxidative mitochondrial enzymes in the myocardial cells of aged rats: the Krebs cycle enzymes (ICDH, \(\alpha\)-KGDH, SDH and MDH), mitochondrial complexes I, III, and IV (34), catalase (CAT) and glutathione peroxidase (GPx); the level of reduced glutathione (GSH) (35) was also increased. Moreover, the antioxidant potential of the palladium \(\alpha\)-lipoic acid complex was five times higher than that of alpha-lipoic acid itself (35).

The antioxidative effect of an organic palladium compound has been shown in another study, where trans-[PdCl\textsubscript{2}(TEA)\textsubscript{2}] decreased the index of TBARS (thiobarbituric acid reactive substances) (36). On the contrary, PdCl\textsubscript{2} did not affect significantly either NO, H\textsubscript{2}O\textsubscript{2}, O\textsubscript{2}– or TBARS. These findings further suggest that organic palladium compounds are less toxic than the inorganic ones.

Discussion

Studies of the distribution of palladium compounds in the tissues of rats, rabbits and dogs after intravenous or intratracheal administration indicated the highest percentage of retention in the kidneys and liver (8–21% of applied dosage), lymphatic nodes, adrenal glands, lungs and bones (37–40). Also, the retention period in some of the organs was up to 104 days long (37, 38). LD\textsubscript{50} of inorganic palladium compounds for rats, mice and rabbits ranged from 3 mg/kg to 4900 mg/kg (16, 38, 41). Oral administration of Pd showed the lowest toxicity compared to others due to the lowest bioavailability. The most toxic compound was palladium(II) chloride (PdCl\textsubscript{2}), while
Table I  Toxic effects of palladium on the cardiovascular system.

<table>
<thead>
<tr>
<th>Pd form</th>
<th>Experimental model/animal</th>
<th>Route</th>
<th>Dose(^a)</th>
<th>Clinical signs and effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PdSO(_4)</td>
<td>unanesthetized rat (n=41)</td>
<td>Intravenous: bolus 0.5 ml</td>
<td>0.4 ± 0.2</td>
<td>Cardiac arrhythmias with concomitant decrease in blood pressure; abnormal ECG patterns, death of the animal.</td>
<td>Wiester MJ (1975)</td>
</tr>
<tr>
<td>Pd(NO(_3))(_2)</td>
<td>unanesthetized rat (n=9)</td>
<td>Intravenous: bolus 0.5 ml</td>
<td>0.4 ± 0.2</td>
<td>Cardiac arrhythmias with concomitant decrease in blood pressure; abnormal ECG patterns, death of the animal.</td>
<td>Wiester MJ (1975)</td>
</tr>
<tr>
<td>(NH(_4))(_2)PdCl(_4)</td>
<td>unanesthetized rat (n=21)</td>
<td>Intravenous: bolus 0.5 ml</td>
<td>1.2 ± 0.3</td>
<td>Cardiac arrhythmias with concomitant decrease in blood pressure; abnormal ECG patterns, death of the animal.</td>
<td>Wiester MJ (1975)</td>
</tr>
<tr>
<td>K(_2)PdCl(_4)</td>
<td>unanesthetized rat (n=8)</td>
<td>Intravenous: bolus 0.5 ml</td>
<td>1.2 ± 0.3</td>
<td>Cardiac arrhythmias with concomitant decrease in blood pressure; abnormal ECG patterns, death of the animal.</td>
<td>Wiester MJ (1975)</td>
</tr>
<tr>
<td>PdCl(_2)</td>
<td>unanesthetized rat (n=6)</td>
<td>Intravenous: bolus 0.5 ml</td>
<td>0.4 ± 0.2</td>
<td>Cardiac arrhythmias with concomitant decrease in blood pressure; abnormal ECG patterns, death of the animal.</td>
<td>Wiester MJ (1975)</td>
</tr>
<tr>
<td>PdCl(_2)</td>
<td>isolated rat heart (n=6)</td>
<td>Langendorff perfusion technique</td>
<td>5.6 × 10(^{-8}) – 5.6 × 10(^{-4})</td>
<td>There was no effect on oxidative status (NO, TBARS, O(_2^\cdot), H(_2)O(_2)) on the isolated rat heart.</td>
<td>Živković V. et al. (2011)</td>
</tr>
<tr>
<td>PdCl(_2)</td>
<td>isolated rat heart (n=6)</td>
<td>Langendorff perfusion technique</td>
<td>5.6 × 10(^{-8}) – 5.6 × 10(^{-4})</td>
<td>Decrease in DLVP, MBP and HR, without effects on dP/dt max and SLVP.</td>
<td>Perić T. et al. (2012)</td>
</tr>
</tbody>
</table>

| Palladium α-lipoic acid complex (POLY-MVA) | rat (n=6) | Oral | 0.05\(^c\) | Administration of POLY-MVA significantly improved the antioxidant status in the heart mitochondria of aged rats. The Krebs cycle enzymes activities (ICDH, α-KGDH, SDH and MDH) and activities of complexes I, III, and IV significantly increased, compared to the aged control group. | Sudheesh NP et al. (2009) |

| Palladium α-lipoic acid complex (POLY-MVA) | rat (n=6) | Oral | 0.05\(^c\) | Administration of POLY-MVA significantly improved the antioxidant status in the heart mitochondria of aged rats (except Mn SOD activity). The activities of CAT, GPx and GSH increased in the POLY-MVA treated group, compared to the aged control group. | Sudheesh NP et al. (2010) |

| trans-[PdCl\(_2\)(TEA)\(_2\)] | isolated rat heart (n=6) | Langendorff perfusion technique | 2.1 × 10\(^{-8}\) – 2.1 × 10\(^{-4}\) | Decrease of TBARS, without effects on NO, O\(_2^\cdot\) and H\(_2\)O\(_2\). | Živković V. et al. (2011) |

| trans-[PdCl\(_2\)(TEA)\(_2\)] | isolated rat heart (n=6) | Langendorff perfusion technique | 2.1 × 10\(^{-8}\) – 2.1 × 10\(^{-4}\) | Decrease of HR, without effects on dP/dt max, SLVP, DLVP and MBP. | Perić T. et al. (2012) |

\(^a\)The mean dose of palladium, which caused mild effects, in mg/kg body weight, unless otherwise specified.
\(^b\)Increasing concentrations of the heart perfusion for compound in M/l.
\(^c\)The concentration of applied compound in ml/kg (which is equivalent to 0.38 mg α-lipoic acid complex/kg).
the least toxic compound was palladium(II) oxide (PdO) (42).

The mechanism of palladium toxicity is not understood yet. Some of the results suggest an association with changes of the membrane potential in myocardial cells (32). Recent research has shown that Pd(II) complexes have an affinity for binding with numerous ion channel proteins and enzymes, leading to disturbances in membrane potential and arrhythmias, decreased entry of calcium in cells, and decreased myocardial contractility (33). It seems that palladium interferes with thiol (SH) groups of membrane Na+/K+ ATPase (43, 44), Ca2+ Mg2+ dependent ATPase of the sarcoplasmic reticulum (45–47), and of some other important enzymes. Inhibition of these proteins could cause a disturbance of cardiac functioning.

Previous studies of palladium have indicated that its inorganic compounds in most cases have pro-oxidative effects (48, 49), emphasizing reactions with superoxide anion (O2−) and H2O2. Reactive oxygen species-mediated DNA damage (50) and inhibition of DNA (51–54) and RNA synthesis (55) are important toxic effects of the palladium ion. Moreover, inhibition of the main energetic enzyme in the cell, creatine kinase (CK) (29, 30), might reduce the amount of free energy in myocardial cells, which decreases the activity of Ca2+ ATPase and limits the calcium-binding capacity (56). It appears that palladium inhibits enzymes by binding with SH groups (29), or by substituting Fe2+ (57). Also, available data indicate that palladium inhibits some other cell enzymes: lactate dehydrogenase (LDH) (58), aldolase, carbonic anhydrase (30), etc. These cytotoxic effects of the palladium ion could explain the depressive effect of palladium compounds on the heart.

Based on the presented data it is not certain whether organic palladium compounds are less toxic than the inorganic ones. Further investigation of the effects of palladium on the heart is necessary to get a clear picture of the nature and extent of its cardiac toxicity.

Conflict of interest statement
The authors stated that there are no conflicts of interest regarding the publication of this article.

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THE ASSOCIATION OF OBESITY AND LIVER ENZYME ACTIVITIES IN A STUDENT POPULATION AT INCREASED RISK FOR CARDIOVASCULAR DISEASE

VEZA IZMEĐU GOJAZNOSTI I AKTIVNOSTI JETRENIH ENZIMA U STUDENTSKOJ POPULACIJI SA POVEĆANIM RIZIKOM ZA NASTANAK KARDIOVASKULARNIH BOLESTI

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Summary

Background: It has been reported that obesity is associated with metabolic syndrome, insulin resistance, cardiovascular risk but also with nonalcoholic fatty liver disease (NAFLD). The prevalence of obesity in children and adolescents is increasing rapidly all over the world. The aim of this study was to analyze the value of liver enzymes: AST, ALT and γGT in a group of obese students in order to establish their correlation to anthropometric parameters such as: BMI (body mass index), WC (waist circumference), HC (hip circumference), and WHR (waist-to-hip ratio) compared to non-obese students who comprised the control group (CG).

Methods: In this study, 238 students from the University of Novi Sad of both sexes (126 men and 112 women) with a mean age of 22.32 ± 1.85 years were included. According to the body mass index (BMI) lower and higher than 25 kg/m² and waist circumference (WC) lower and higher than 94 cm (80 cm for females) the whole group of 238 students was divided into 2 subgroups: the obese group at increased risk for CVD (Group 1) and the group at lower risk for CVD (Group 2). AST, ALT and γGT activities were determined in fasting blood samples.

Results: Statistical processing data revealed significantly higher values of AST, ALT and γGT in the group of students with BMI>25 kg/m², WC>94 cm for males and WC>80 cm for females, HC>108 cm for males and HC>111 cm for females, and WHR>0.90 for males and WHR>0.80 for females (P<0.001). Significant association was established...
between anthropometric parameters and liver enzyme levels (P<0.0001).

Conclusions: Obese students with higher BMI, WC, HC and WHR values have higher liver enzyme activities and a higher chance to develop NAFLD in the future.

Keywords: obesity, liver enzymes, nonalcoholic fatty liver disease, cardiovascular risk

**Introduction**

Recently, obesity and related cardiovascular disease (CVD) have become a major physical, social and economic burden globally. The prevalence of obesity in children and adolescents is increasing rapidly both in high-income and middle and low-income countries. There are about 155 million overweight children and adolescents worldwide, of which about 30 to 45 million are obese (1, 2).

The association of obesity with chronic diseases in adults, in particular cardiovascular disease is well known (2). One of the most important cardiovascular diseases associated with obesity is hypertension. Risk estimates from population studies suggest that more than 75% of hypertension cases can be directly attributed to obesity (3).

Increasing weight also has a strong correlation with elevated triglycerides and low-density lipoprotein (LDL), total cholesterol levels as well as low levels of high-density lipoprotein (HDL). Some studies found that overweight was a significant risk factor for development of diabetes in women (4). The association of obesity with insulin resistance the metabolic syndrome and cardiovascular risk has also been reported, and is not only related to the degree of obesity but also to the body fat distribution. Individuals with a higher degree of central adiposity develop this syndrome more frequently than those with a peripheral body fat distribution (5–8).

Nonalcoholic fatty liver disease (NAFLD) is another consequence of obesity. NAFLD includes different types of liver pathologies and outcomes, from simple steatosis to nonalcoholic steatohepatitis (NASH) (9). NAFLD usually develops in children who are obese (10). While abnormalities in liver enzymes in pediatric NAFLD are moderate (11), a correlation between the degree of obesity and the severity of hepatic steatosis at ultrasonography has been reported (10, 12). Among adults, NAFLD markers such as alanine aminotransferase (ALT) might predict the metabolic syndrome independently. A study of Oliveira et al. (13) in children and adolescents found a strong association of elevated ALT values with the metabolic syndrome and obesity.

The aim of our study was to analyse the values of liver enzymes (AST, ALT and GT) in obese and non-obese students in order to establish their correlation to anthropometric parameters such as: body mass index – BMI, waist circumference – WC, hip circumference – HC, and waist-to-hip ratio – WHR, in relation to increased risk for NAFLD in the group of students at increased risk for CVD.

**Materials and Methods**

In a cross-sectional study conducted at the Student Health Protection Institute, University of Novi Sad, a total of 238 students aged 18 to 29 (22.32 ± 1.85) were examined (126 males and 112 females). They went through a regular medical checkup from April 2009 to April 2011. From the total number of students, 164 were obese or overweight (BMI>25 kg/m²) and had a WC>94 cm for men and WC>80 cm for women, and they comprised the group with increased risk for CVD (Group 1). The remaining 74 students who had BMI<25 kg/m² and WC<94 cm (80 cm for females) comprised the control group (Group 2). Each student filled in a questionnaire about their habits, including physical activity, smoking, alcohol intake, family history of CVD, etc. The blood samples for analysis were taken after a 12–14 hours overnight fast. In the blood samples taken from the subjects, the following parameters were analysed: glucose, lipo- and apoprotein levels, AST, ALT and γGT activities. All laboratory tests were done immediately. All subjects gave their informed consent for participation in this study, approved by the local Ethics Committee.

Statistical analysis was performed by the MedCalc v.9.4.2.0 statistical package using the Student's t-test, Mann-Whitney U test, Chi-Square, and Fisher's exact test. Results were presented as mean ± SD and median and interquartile range. Spearman's rank and Pearson's correlation test was used to define correlations of the individual parameters between and within the tested groups. All statistical tests were two-tailed. P values ≤0.05 were considered statistically significant. Logistic regression analysis was used to assess the association between the anthropometric parameters and the liver enzyme levels.

**Results**

Statistical processing data revealed significantly higher values of ALT (P<0.0001), AST (P<0.0001)
and GT (P<0.0001) in the group of students with a BMI>25 kg/m² (Group 1) compared to nonobese students (Table I). There was no difference in glucose values between the tested groups (P>0.05). Higher waist circumference (WC) was found in 137 (83.5%) students in Group 1 and only 1 student (1.4%) in Group 2 (P<0.01). The WC values were significantly higher in Group 1 (90–104 cm) compared to Group 2 (68–79 cm) (P<0.000), as well as HC values (P<0.000) and the waist-to-hip ratio (P<0.000). The average BMI value in Group 1 was 28.85±3.86 kg/m², and that was significantly higher compared to the BMI value in Group 2 (20.52±2.07 kg/m²) (P<0.000).

A family history of CVD was found in 135 students (82.3%) from the group at increased risk for CVD (Group 1), and also in 48 students (64.9%) from the control group (Group 2) (P<0.01). A total of 134 students from Group 1 (81.7%) had a normal arterial blood pressure (BP, up to 120/80 mmHg), 10 students had a slightly higher BP (prehypertension; BP up to 130/90 mmHg) (6.1%) and 20 students had hypertension (140/90 mmHg) (12.2%). Compared to Group 2, there was no significant difference in BP frequency between the studied subgroups of students (χ²=3.87; P>0.05).

Significantly higher values of AST and ALT (P=0.005) but not γGT (P=0.158) were found in the males of Group 1 compared to females in the same group. A significant and positive correlation was found between BMI and AST in Group 1 (r=0.305; P<0.01), BMI and ALT (r=0.295; P<0.01), as well as between WC and AST (r=0.360; P=0.001) and WC and ALT (r=0.414; P<0.01). A significant and moderate correlation was found between WHR and ALT (r=0.345; P<0.001), WHR and AST (r=0.230; P=0.004) and WHR and γGT (r=0.210; P=0.008).

A positive and significant correlation was found between ALT and BMI in both male (r=0.441; P=0.000) and female subgroups (r=0.236; P=0.011), as well as between γGT and BMI (male/r=0.355; P=0.000 and female/r=0.281; P=0.002), while AST correlated positively with BMI only in the male subgroup (r=0.440; P=0.000). Liver enzymes correlated positively with WHR>0.90 in the male subgroup (ALT/r=0.275; P=0.005, AST/r=0.235;
Using logistic regression analysis, a weak but significant association between liver enzymes and the tested anthropometric parameter values higher than their cutoff values was found (Table II). BMI>25 kg/m² was weakly but significantly associated with ALT (OR: 1.09, 95% CI 1.05–1.13, P<0.000), AST (OR: 1.19, 95% CI 1.07–1.18, P<0.000) and γGT (OR: 1.14, 95% CI 1.09–1.20, P<0.000); WC>80 cm (for females) was associated only with γGT (OR: 1.05, 95% CI 1.0–1.09, P=0.011); while WC>94 cm (for males) was associated with ALT (OR: 1.04, 95% CI 1.01–1.06, P=0.0004), AST (OR: 1.05, 95% CI 0.998–1.095, P=0.031) and γGT (OR: 1.08, 95% CI 1.03–1.14, P=0.0001). WHR>0.90 (for males) was significantly associated with ALT (OR: 1.04, 95% CI 1.015–1.06, P<0.000), AST (OR: 1.09, 95% CI 1.4–1.15, P<0.000) and γGT (OR: 1.03, 95% CI 1.0–1.06, P=0.025); while WHR>0.80 (for females) was significantly associated with ALT (OR: 1.04, 95% CI 1.02–1.08, P<0.0036) and AST (OR: 1.11, 95% CI 1.04–1.2, P=0.0013). HC>108 cm (for males) was associated with ALT (OR: 1.06, 95% CI 1.0–1.11, P=0.0017), AST (OR: 1.13, 95% CI 1.03–1.23, P=0.0011) and γGT (OR: 1.12, 95% CI 1.05–1.20, P=0.0003), and HC>111 cm (for females) was significantly associated with ALT (OR: 1.03, 95% CI 1.0–1.06, P=0.035) and γGT (OR: 1.04, 95% CI 1.0–1.08, P=0.019).

Discussion

Based on the obtained results it can be concluded that students with higher BMI, WC, HC and WHR also had higher levels of the liver enzymes ALT, AST and γGT. The liver enzymes correlated positively with the tested anthropometric parameters in Group 1 and in the subgroups of males and females of Group 1. This study has documented that the values of BMI>25 kg/m² are significantly associated with all three tested liver enzymes, as well as a WC>94 cm (for males), while a WC>80 cm (for females) is associated only with γGT. Significant association was obtained also between WHR>0.90 and all three tested enzymes, while WHR>0.80 was associated with ALT and AST. HC was associated with ALT, AST and γGT in the male subjects, while HC>111 cm was associated with ALT and γGT in the female subjects. According to these results it can be concluded that female students with a BMI>25 kg/m², WC>80 cm, WHR>0.80 and HC>111 cm have a higher chance of developing NAFLD in the future, as well as male students with a WC>94 cm, WHR>0.90, HC>108 cm and BMI>25 kg/m², compared to students whose anthropometric parameters are below these cutoff values.

Overweight and obese children and adolescents are more likely to have elevated ALT and AST levels than normal-weight students. WC is a simple and effective indicator which can be used to screen central adiposity (14) as well as the cardiovascular risk profile (15–18). Oliveira et al. (13) demonstrated that for each 5 cm increase in WC and every 1-point increase in the BMI z-score, there was a 1.3-fold greater chance of having increased ALT levels.

Different studies have shown that a number of metabolic syndrome components, obesity and insulin resistance are strong predictors of increased ALT activity in NAFLD in children and adolescents (19, 20). Central obesity, raised triglycerides, reduced HDL-cholesterol, and elevated fasting glucose are the metabolic syndrome components that contribute to increased ALT and AST activities. Visceral adiposity, and in particular upper abdominal visceral adiposity is correlated with cardiometabolic risk factors in humans (21). Several hypotheses regarding the role of visceral fat in metabolic disease were suggested, including the secretion of proinflammatory molecules capable of inducing insulin resistance in other organs and a high rate of lipolysis in the visceral fat depot that increases the delivery of free fatty acids to the liver to induce hepatic insulin resistance (21). A major contributor to fatty liver is enhanced de novo synthesis of fatty acids (lipogenesis) which leads to hepatic steatosis. Kelishadi et al. (9) suggested that, in future interventional studies, in addition to liver enzymes, high-sensitive CRP, γGT, uric acid, vitamin D, adiponectin, ghrelin level, insulin level, insulin resistance and the oral glucose tolerance test could be the integral part of the evaluation of patients with NAFLD to further clarify the relationships among different biologic factors and the metabolic syndrome.

Some studies have shown that obesity and insulin resistance have also been associated with other risk factors such as elevated blood pressure (22, 23). The Coronary Artery Risk Development in Young Adults (CARDIA) study of 4576 young adults reported a weight-independent association between fasting insulin concentration and hypertension (24). Obesity, insulin resistance and increased circulating insulin concentration over time, at some point, lead to loss of blood glucose control, resulting in dietary glucose intolerance. It is known that obese individuals may develop different degrees of insulin resistance, and not all individuals develop glucose intolerance. The factors that make some individuals more likely to progress to type 2 diabetes mellitus are not well understood at the present time. It is not currently known what level of weight loss is necessary for adolescents to achieve improved glucose handling.
and diastolic hypertension have been found in obese adults. The early impact of prehypertension and hypertension parameters in adolescents and young adults was examined in the Strong Heart Study (26). Compared to normotensive individuals, both prehypertensive and hypertensive individuals were more likely to be obese. Seventy-nine percent of the hypertensive and 69% of the prehypertensive individuals had central adiposity as determined by waist circumference. Baker et al. (27) investigated the association between BMI in childhood (7–13 years) and coronary heart disease in adulthood (25 years and older) in a huge cohort of men and women in whom childhood BMI data were available. The risk of either a nonfatal or fatal cardiovascular event was positively associated with BMI at 7–13 years old for boys and 10–13 years old for girls. Children with higher BMI were at increased risk for coronary heart disease in adulthood and the associations were linear for each age.

There is increasing evidence indicating that obesity in children and adolescents is associated with short- and long-term cardiovascular risks. The long-term health issues are serious and carry a heavy burden both clinically and financially. Therefore, the prevention of obesity in children and adolescents is one of the major tasks for early management of obesity which can abate or reverse almost all of the cardiovascular consequences of obesity.

**Conflict of interest statement**

The authors stated that there are no conflicts of interest regarding the publication of this article.

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RESPONSE OF RAT ERYTHROCYTE OXIDATIVE STRESS MARKERS TO REPETITIVE HYPERBARIC OXYGEN EXPOSURES UP TO 40 DAILY SESSIONS

ODGOVOR MARKERA OKSIDATIVNOG STRESA U ERITROCITIMA PACOVA NA VIŠESTRUKO IZLAGANJE HIPERBARIČNOM KISEONIKU DO 40 SEANSI DNEVNO

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Summary
Background: Studies with single-session hyperbaric oxygen exposures have shown that HBO-induced oxidative stress is proportional to exposure pressure and duration. Since the efficacy of hyperbaric oxygen mainly depends on repetitive exposures, this study aimed to investigate the oxidative effect of hyperbaric oxygen administered for 5 to 40 sessions.

Methods: Sixty rats were divided into one control and 6 study groups. Study groups were exposed to 5, 10, 15, 20, 30, and 40 daily consecutive 2.8 atm/90 min hyperbaric oxygen sessions. Animals were sacrificed 24 h after the last hyperbaric oxygen administration. Malondialdehyde and carbonylated protein levels as well as superoxide dismutase activities were determined in isolated rat erythrocytes.

Results: Carbonylated protein levels increased significantly after just 5 hyperbaric oxygen exposures; reached a peak level with 10 exposures; were still significantly higher than controls after 15 sessions; and decreased to normal limits after 20 exposures. Malondialdehyde levels were found to be significantly increased in the 10 to 30, but not in the 5 and 40-session groups. Superoxide dismutase activity showed elevated levels only in the 5 and 10 times hyperbaric oxygen-exposed groups.

Conclusions: The suppressed oxidative stress level after 40 exposures suggests an effective endogenous antioxidant defense in repetitive HBO administrations.

Keywords: adaptive response, antioxidant defense, free radicals, oxidation products, reactive oxygen species

Kratak sadržaj
Uvod: Studije o jednostrukom izlaganju hiperbaričnom kiseoniku pokazale su da je oksidativni stres izazvan izlaganjem hiperbaričnom kiseoniku srazmeran pritisku i trajanju izlaganja. Pošto efikasnost izlaganja hiperbaričnom kiseoniku uglavnom potiče od višestrukog izlaganja, cilj ove studije bio je da se istraži oksidativni efekat izlaganja hiperbaričnom kiseoniku u trajanju od 5 do 40 seansi.

Metode: Sedeset pacova podeželjeno je na jednu kontrolnu i 6 eksperimentalnih grupa. Eksperimentalne grupe podvrgnjene su uzastopnim seansima udisanja hiperbaričnog kiseonika (2,8 atm/90 min) po 5, 10, 15, 20, 30 i 40 puta dnevno. Zivotinje su žrtvovane 24 časa posle poslednjeg seansa. U izolovanim eritrocitima pacova određeni su nivoi malondialdehida i karboniliranog proteina kao i aktivnosti superoksid-dizmutazes.

Rezultati: Nivoi karboniliranog proteina značajno su porasli posle samo 5 izlaganja hiperbaričnom kiseoniku; dostigli vrhunac nakon 10 izlaganja; ostali značajno povišeni u odnosu na kontrolu posle 15 seansi; i vratili se u normalne okvire posle 20 izlaganja. Nivoi malondialdehida bili su značajno povišeni u grupama sa 10 do 30 izlaganja, ali ne i sa 5 i 40 seansi. Aktivnost superoksid-dizmutazes pokazala je povišene niveo samo u grupama sa 5 i 10 izlaganja hiperbaričnom kiseoniku.

Zaključak: Sniženi nivo oksidativnog stresa nakon 40 izlaganja ukazuje na efikasnu endogenu antioksidantnu odbranu kod višestruke primene hiperbaričnog kiseonika.

Ključne reči: adaptivni odgovor, antioksidantna odbrana, slobodni radikali, proizvodi oksidacije, reaktive kiseonice vrste

List of abbreviations: HBO, hyperbaric oxygen; MDA, malondialdehyde; NBT, nitroblue tetrazolium; PCC, protein carbonyl content; SOD, superoxide dismutase
Introduction

It is widely known that (hyperbaric) hyperoxia can lead to an excessive production of reactive molecules that can trigger oxidation/peroxidation cascades of several functional and structural biomolecules (1). On the other hand, reactive molecules are now known not only to lead to cellular injury but also to act as signaling agents. In this way they play a role in a number of physiological functions in living organisms (2). Hyperbaric oxygen (HBO) treatment, a therapeutic modality depending on the inhalation of 100% oxygen under a pressure exceeding that of the atmospheric pressure, has been used for decades as a life saving application in critical cases including carbon monoxide poisoning, air/gas embolism and decompression illness as well as acute traumatic wounds, crush injuries, burns, gas gangrene and compartment syndrome (3). However, ‘oxygen toxicity’ was an important scientific issue in the former medical literature and it was often pointed out as a potential risk of HBO administrations (4).

From another point of view, more recent reports suggest that the enhanced level of reactive molecules, e.g., the superoxide radical and hydrogen peroxide, may play an important role for the beneficial actions of HBO therapy (5). Nowadays, it is not certain, apart from the well-explained oxygen supplying/enriching and bubble reducing effects, whether both oxygen and nitrogen derived reactive species may take part in the therapeutic mechanisms of HBO (6–8). Through the last decade, our laboratory has been concentrated on defining the oxidative action of HBO within therapeutically applied limits. In a series of experimental studies conducted in rats, we found that HBO-induced oxidative stress is directly proportional to the exposure pressure (9, 10) and duration (11, 12). Another experimental set revealed an important finding that the enhanced levels of oxidation products declined to their baseline values at one hour later following a single HBO exposure (13, 14). Finally, in our most recent studies, different from the abovementioned one-session HBO exposure procedures, after exposure to daily HBO sessions for up to 8 weeks, clear rises in lipid and protein oxidation products along with the antioxidant enzyme superoxide dismutase (SOD) were detected in the rats’ lung (15), but not in their brain tissue (16).

The present work was established as a complementary issue to previous studies in order to elucidate the HBO-induced oxidative interactions in longer administration periods one step forward. Erythrocytes, the oxygen-carrying cells and one of the main targets of hyperoxia-related oxidative action (9, 11, 14), were chosen for this purpose.

Material and Methods

Study design

The Experimentation Ethics Committee of our institution approved the experimental procedures of the study (issue 08/75K). Sixty adult male Sprague-Dawley rats bred at the Gulhane Military Medical Academy Research and Progress Center were used for the study. Rats were 12 weeks old and weighed 200–250 g at the beginning of the experiment. Housing was at 22–24 °C with light from 08.00 a.m. to 08.00 p.m. including free access to water. All animals were fed with a standard commercial rat chow during the experiment.

Rats were divided into 6 study groups (n=8 for each) which were exposed to HBO for 1, 2, 3, 4, 6 and 8 weeks. HBO administrations were set as 5 daily consecutive exposures followed by 2-day intervals. All animals in the study groups were sacrificed 24 h after their final HBO treatment. Separate control groups consisting of 6–8 animals for each time point were forbidden by our institutional Ethics Committee. In order to evaluate the possible effects of aging, 2 control animals were sacrificed at the same time with the 6 study groups; thus, the control group of the study consisted of 12 (6 × 2) animals. A detailed schedule of the study design is given in Table I.

Table I Detailed experimental schedule.

<table>
<thead>
<tr>
<th>Day 1 to 5</th>
<th>Day 6 (Sacrificing)</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>5 HBO sessions (48 animals)</td>
<td>8 study + 2 control rats</td>
</tr>
<tr>
<td>Week 2</td>
<td>5 HBO sessions (40 animals)</td>
<td>8 study + 2 control rats</td>
</tr>
<tr>
<td>Week 3</td>
<td>5 HBO sessions (32 animals)</td>
<td>8 study + 2 control rats</td>
</tr>
<tr>
<td>Week 4</td>
<td>5 HBO sessions (24 animals)</td>
<td>8 study + 2 control rats</td>
</tr>
<tr>
<td>Week 5</td>
<td>5 HBO sessions (16 animals)</td>
<td>No sacrificing</td>
</tr>
<tr>
<td>Week 6</td>
<td>5 HBO sessions (16 animals)</td>
<td>8 study + 2 control rats</td>
</tr>
<tr>
<td>Week 7</td>
<td>5 HBO sessions (8 animals)</td>
<td>No sacrificing</td>
</tr>
<tr>
<td>Week 8</td>
<td>5 HBO sessions (8 animals)</td>
<td>8 study + 2 control rats</td>
</tr>
</tbody>
</table>

Note that all HBO administrations and animal sacrifice were performed at 10.00 a.m.
HBO Exposure

An animal hyperbaric chamber (made in Etişehir Military Equipment Factory; Ankara, Turkey) was used for HBO exposure. The HBO sessions were set as 2.8 atm pressure for 90 min in all study groups. Compression and decompression of the chamber were completed gradually in 5–10 min; continuous 100% O₂ ventilation at a rate of 3–4 L/min was maintained throughout the 90-min exposure periods in the chamber. All administrations were started at the same hour in the morning (10.00 a.m.) to avoid the possible effects of circadian rhythm (17).

Tissue preparation

Animals were kept for one day after the last HBO session to exclude interference of the acute actions of HBO exposure. They were then anesthetized via an intraperitoneal injection of ketamine (85 mg/kg) plus xylazine (12.5 mg/kg). Their chests were opened and 4–6 mL blood specimens were obtained from the inferior vena cava. The rats were sacrificed by bleeding and hypovolemic shock under anesthesia.

Blood samples were separated into plasma and erythrocytes by centrifugation (for 10 min) at +4 °C (Hermle Z323K: Gosheim, Germany). Erythrocyte samples were washed three times with cold physiological saline and then hemolyzed by adding a 4-fold volume of distilled water. The final hemolysates were divided into three parts, put into eppendorf tubes and stored at −80 °C until assay.

Biochemical analysis

In the erythrocyte hemolysates, lipid peroxidation levels were measured using the thiobarbituric acid reaction by the method of Ohkawa et al. (18). This method was used to obtain a spectrophotometric (Helios epsilon, USA) measurement of the color produced during the reaction to thiobarbituric acid with malondialdehyde (MDA) at 535 nm. The calculated intra-assay coefficient of variation (CV%) and inter-assay CV% for MDA were 4.4% and 5.5%, respectively.

The carbonylated protein content (PCC) was determined with the method described by Levine et al. (19). MDA and PCC levels were expressed as millimoles per gram protein. The intra- and inter-assay CV% for PCC measurements were calculated as 4.8% and 6.1%, respectively.

The activity of the antioxidant enzyme SOD was assayed using the nitroblue tetrazolium (NBT) method of Sun et al. (20). Briefly, NBT was reduced to blue formazan by the superoxide anion radical, which has strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The estimated SOD activities were expressed as units per gram protein. Intra- and inter-assay CV% values for SOD activities were estimated to be 3.6% and 4.1%, respectively.

Finally, in order to normalize the measured data, the protein content of the hemolysates was measured according to the method of Lowry et al. (21) with bovine serum albumin as the standard.

Statistical analyses

Normality analyses were performed by using the Shapiro-Wilk test and the entire data set of the study was found to be normally distributed. Thus, parametric statistics were used for the evaluation of the results. Since the One Way Analysis of Variance (ANOVA) indicated intergroup significance, post hoc Bonferroni test was performed for group to group comparisons. P values less than 0.05 were considered as significant. All analyses were performed using the SPSS software (Version 15.0; SPSS, Chicago, IL, USA).

Results

According to general observations such as symptoms for barotraumas, hyperoxic convulsions, weight gain or loss, no unexpected effect due to HBO exposure was observed throughout the study. All animals survived until the scheduled time of sacrifice. The outcome is presented in the box-plot graphics (Figures 1–3) enabling one to see the median, minimum, maximum and quartile values at a glance.

Lipid peroxidation

Erythrocyte MDA levels tended to increase with the first measure point after 5 HBO sessions. However, statistical significance was noted initially after 10 HBO exposures and continued up to the end of the 6th week (30 HBO administrations). Twenty-four hours after the 40th HBO session, MDA levels were found to have declined to nearly control values. The values after 20 and 30 HBO administrations were also recorded as being significantly higher than the 5-session group’s MDA levels. Detailed P values are given with the graphical demonstration of the erythrocyte MDA levels in Figure 1.

Protein oxidation

The erythrocytes’ PCC values were found to be significantly higher than the controls just with the 5- and 10-session HBO exposure groups. After 15 HBO administrations a decline in erythrocyte PCC values was recorded but the degree was still significantly
Figure 1 Erythrocyte MDA levels were found to be significantly higher compared with *control, and **5-session HBO groups.

Figure 2 Carbonylated protein amounts of erythrocytes were significantly higher than *all groups except the 10- and 15-session HBO groups, **all groups apart from the 5-session HBO group, ***the control group, and ****the 40-session HBO group.

Figure 3 The SOD enzyme activities were increased significantly in the 5- and 10-session HBO groups as indicated by *vs. control values, **vs. the 20-session HBO group, ***vs. the 30-session HBO group, and ****vs. the 40-session HBO group.
higher than the control group as well as than the 40-
session HBO group. With 20 and more HBO sessions 
carboxylated protein levels were recorded within sim-
ilar ranges to controls. More detailed group to group 
comparisons and exact P values for PCC levels are 
shown in Figure 2.

Superoxide dismutase enzyme activity

The antioxidant enzyme SOD immediately 
responded via significant elevation of enzymatic activ-
ity to HBO exposure in the groups sacrificed earlier, 
i.e. the 5- and 10-session HBO groups. With longer 
HBO exposure periods, SOD activities tended to de-
cline; thus, the recorded SOD activities of the 5- and 
10-session HBO groups were also significantly higher 
than in the most of the other groups (Figure 3).

Discussion

In this study we investigated the potential oxida-
tive effect of repetitive HBO treatments for up to 40 
sessions on rat erythrocytes. The levels of lipid perox-
idation and protein oxidation were recorded in order 
to reflect the oxidative status, and meanwhile the 
activity of the antioxidant enzyme SOD was detected. 
Key findings of the study were: (i) a rise in both of the 
measured oxidation products, i.e. MDA and PCC, in 
the earlier stages with 5 to 15 HBO exposures, which 
were simultaneously accompanied by elevated SOD 
activities; (ii) gradual increment of MDA but not PCC 
and SOD activity levels up to 30 HBO sessions; and 
finally (iii) normalized values for all 3 measured para-
meters with no difference from control levels after 40 
HBO administrations.

Former studies clearly demonstrated that single 
HBO exposures cause oxidative reactions in cultured 
cell lines (22), experimental animals (9) and healthy 
human volunteers (23). Interestingly, this oxidative 
effect was shown to discontinue with following HBO 
exposures (24) along with an HBO-triggered adaptive 
protective mechanism (25). On the other hand, in a 
more recent study, the isolated lymphocytes obtained 
from combat divers, who were repetitively exposed to 
100% oxygen breathing under pressure due to their 
jobs, represented an enhanced sensitivity to oxidative 
damage (26). Thus, more research is needed in this 
field in order to elucidate the oxidative interactions 
with clinically relevant multiple-HBO exposures.

Studies focused on oxidative actions of repetitive 
HBO exposure were generally performed with patients undergoing HBO therapy for different rea-
sons (27). It is difficult to distinguish whether the oxidative stress levels measured in these studies 
depend on HBO or the pathology for which the patient was treated. Other studies performed on 
healthy volunteers were mostly ceased after the first 
or an additional second HBO exposure (23). How-
ever, the use of HBO in clinical conditions depends 
mainly on at least 10 treatments and may exceed 30 
sessions in cases of refractory pathologies (28). Indeed, exposing healthy human beings unnecessar-
ily to HBO for more than 10 times will be ethically dis-
putable and animal studies remain important.

In our previous work, an increasing oxidative 
effect of repetitive HBO exposure was demonstrated 
in the rat’s lung tissue (15). Briefly, with 20 daily 
exposures to HBO, significantly increased MDA and 
PCC levels were detected in the lung tissues and 
remained as high values after 30 and 40 exposures. 
The good news was that increased activities of antiox-
idant enzymes accompanied the rise of these oxida-
tion products. On the other hand, the brain tissue 
specimens of rats did not reflect any significant 
change for oxidant and antioxidant indices in the 
same experimental set (16). With regard to hyperox-
ial-induced oxidative injury, the lung tissue is the first 
target, since it is the entering site of oxygen; then, 
oxogen passes into the blood and the oxygen-carrier 
cells, erythrocytes, represent an ideal secondary tar-
got for detecting hyperoxic oxidative interactions (9). 
Therefore, in the present work, we focused on the 
erthrocytes in order to investigate possible similari-
ties and/or differences with the abovementioned lung 
and brain studies (15, 16). As a result, both MDA and 
PCC levels were detected to increase significantly at 
earlier time-points than previously seen in the lung 
(15). The activity of SOD also increased with a simi-
lar course to the oxidation products.

Among the many biological targets of oxidative 
stress, lipids are the most involved class of biomole-
cules (29–31). Lipid oxidation gives rise to a number 
of secondary products and MDA is the principal and 
most studied product of polyunsaturated fatty acid 
peroxidation (32). In our current study, erythrocyte 
MDA levels significantly increased at the time-point of 
10 HBO administrations and remained high for up to 
30 sessions (Figure 1) reflecting the presence of ox-
idative stress within this time interval. Then, after 40 
HBO exposures, a decline to insignificant values was 
detected indicating sufficient endogenous repair 
action of the organism.

Protein carbonylation is a type of protein oxida-
tion and a well-established marker for oxidative stress 
(33). In the present study, erythrocytes’ PCC levels 
increased significantly at the earliest stage with 5 
HBO exposures and this continued after 10 and 15 
sessions (Figure 2). This finding is interesting since, 
in the lung tissue, the first significant increase of PCC 
levels was recorded with 20 HBO administrations and 
continued up to 40 exposures (15). Again, similar to 
the final result for MDA values, the near-to-control 
PCC levels of the 20-, 50- and 40-times HBO
exposed groups provide evidence for a successful defense or regulatory mechanism.

The hyperoxic state during HBO treatments causes primarily an increased production of the superoxide radical (26) and, as a response, upregulation of the antioxidant enzyme SOD was reported for several times (34). Almost all of our previous one-session HBO exposure studies also resulted in increased SOD activities in rat erythrocytes (9, 11, 14). Similarly, we recorded significant elevation of SOD activities in the present work, but only in the early 5 and 10 times HBO administered groups. Then, SOD activities tended to decline and resulted in totally normalized values at the end-stage of the study (Figure 3).

Taken together, at the last measure point after 40 HBO administrations, all three measured parameters, i.e. MDA, PCC and SOD, were found to be within their normal ranges. Due to the short half-life of the enzyme SOD (35), but the relatively longer half-life of the oxidation end products such as MDA (36), the earlier decline of SOD in the present experimental set is an expected result. Hence, although MDA and PCC levels remained significantly higher for a longer period than SOD, their final normalized values supported the previous suggestions on adaptive protection against oxidative stress with repetitive HBO treatments (25). The sole measurement of SOD as a marker for the antioxidant systems has to be emphasized as a limitation of the present study. Normally, we planned to investigate the members of the glutathione system, but during the biochemical analyses unforeseen problems occurred and hindered further widening of our parameter spectrum.

In conclusion, when compared with the previous lung study (15), the main similarity is the relatively synchronous elevation of the oxidant and antioxidant system markers. The main difference, however, is the timing for these increased values. The harmony between the measured oxidation products and the antioxidant enzymes can be interpreted as evidence for a controlled level of oxidative stress recruiting reactive molecules into signaling pathways instead of harming biomolecules. The earlier increases of oxidation products in erythrocytes, or, from another standpoint, their postponed increases in the lung tissue may stand for different sensitivity ranges of diverse body cells. Antioxidant enzyme activities were not found to be depressed and no sign of exhaustion of the antioxidant system appeared. Thus, the overall results prove the safety of long-term HBO treatments.

More detailed studies, including detections of the previously suggested molecules heme oxygenase-1 (25) and hypoxia inducible factor-α (37), warrant more precise knowledge in order to elucidate the underlying mechanisms of HBO-related oxidant/antioxidant interactions.

Acknowledgements: The skillful help of our medical technical assistant Serap Obut is highly appreciated. Also thanks to Mrs. Pinar Demirkaya, English Instructor, for her assistance in improving the use of regular English throughout the text.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

References


THE EFFECTS OF ERYTHROPOIETIN ON BACTERIAL TRANSLOCATION AND INFLAMMATORY RESPONSE IN AN EXPERIMENTAL INTESTINAL OBSTRUCTION MODEL IN RATS

UTICAJ ERITROPOETINA NA BAKTERIJSKU TRANSLOKACIJU I INFLAMATORMI ODGOVOR U EKSPERIMENTALNOM MODELU INTESTINALNE OPSTRUKCIJE KOD PACOVA

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Summary

Background: Intestinal obstruction results in distortion of balance of antiinflammatory cytokines and release of oxidants, and also leads to bacterial translocation, sepsis and multiple organ failure. Asymmetric dimethylarginine is related to multiple organ failure as a new prognostic marker. Erythropoietin reduces the inflammatory response by decreasing the levels of proinflammatory cytokines and cytokine-induced apoptosis. In this study, we aimed to investigate the effectiveness of erythropoietin in reducing the severity of bacterial translocation and inflammatory response after intestinal obstruction and the relation between asymmetric dimethylarginine and inflammatory markers.

Methods: Forty Wistar albino rats (200–250 g) were divided into 4 groups as follows: Group 1 (Sham), only ileocaecal junction dissection; Group 2 (Erythropoietin), ileocaecal junction dissection and 3000 IU/kg erythropoietin subcutaneously; Group 3 (Intestinal Obstruction), complete ileal ligation; Group 4 (Intestinal Obstruction + Erythropoietin), complete ileal ligation and 3000 IU/kg erythropoietin subcutaneously. After 24 hours, the rats were sacrificed by taking blood from the heart for biochemical analyses. Peritoneal swab culture, liver, mesenteric lymph nodes, spleen and ileum were collected for microbiological and histopathological examinations.

Results: Erythropoietin reduced the secretion of inflammatory cytokines, oxidative damage and bacterial translocation, prevented the formation of inflammatory changes in the intestine, liver, spleen and mesenteric lymph nodes,
Introduction

Intestinal obstruction (IO) is a major problem for surgeons with the ratio of approximately 20% of emergency surgical diseases. Despite the development of treatment strategies, the mortality of mechanical IO is reported as still 5–20% (1). Beside the most important task of digestion and absorption, the small intestine creates a functional and mechanical barrier for the antigens, toxins and microorganisms (2). Under normal conditions, the small bowel contains very few bacteria, however the microbiological ecological balance is disrupted after IO (1). Bacterial growth as a result of disrupted ecological balance, immune dysfunction induced by deterioration of the balance of pro- and anti-inflammatory cytokines and release of oxidants after mucosal barrier dysfunction accelerate the development of bacterial translocation (3). Increased intestinal permeability leads to the development of systemic inflammatory response, infection, sepsis and multiple organ failure (MOF) (2) by increasing the translocation of bacteria and their products to the peritoneal space, mesenteric lymph nodes (MLNs), liver, spleen and systemic circulation which are normally sterile (4).

Interleukin-6 (IL-6), which is one of the pro-inflammatory cytokines appearing after the oxidative and inflammatory stress, is an important parameter in determining the level of inflammatory damage (5). Other important markers of inflammatory response are Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1Beta (IL-1β) (6). In addition, C-reactive protein (CRP) is both an acute-phase reactant and an important marker of systemic inflammatory response (7).

Recently, asymmetric dimethylarginine (ADMA) has been reported to be associated with MOF, liver failure and the severity and incidence of intensive care unit mortality in a concentration-dependent manner, and is also reported to be directly related to MOF or a new important indicator (5).

Erythropoietin (EPO) reduces the inflammatory response by decreasing the levels of proinflammatory cytokines and cytokine-induced apoptosis along with trophic effects on the bowel. In addition, recent data have suggested that EPO supports angiogenesis, reduces oxidative stress and accelerates wound healing (2).

Conclusions: Asymmetric dimethylarginine levels did not differ between the groups. Erythropoietin may be useful to preserve from intestinal injury and related sepsis in patients with intestinal obstruction. Asymmetric dimethylarginine is not a suitable prognostic marker.

Keywords: erythropoietin, intestinal obstruction, bacterial translocation, asymmetric dimethylarginine

In this study our aim was to investigate the effectiveness of EPO, microbiologically, biochemically and histopathologically, in reducing the severity of bacterial translocation and inflammatory response emerging as a result of mechanical IO, and we also aimed to investigate the correlation between inflammatory markers and ADMA, as an inflammatory marker.

Materials and Methods

Chemical

Erythropoietin was purchased from Sigma (E5627–Erythropoietin human recombinant, expressed in Chinese hamster ovary cells, lyophilized powder, cell culture tested, ~ 100,000 units/mg protein) and dissolved in phosphate buffer saline.

Animals

Forty Wistar albino rats, each weighing 200–250 g, were included in the study at the Dicle University Health Sciences Application and Research Center. The experimental manipulations and surgical operations in this study were approved by the Committee of Experimental Animals of Dicle University. All experimental procedures complied with the guide for the Care and Use of Laboratory Animals. Rats were housed in cages and allowed free access to standard rat chow and water before the experiments under standard conditions in an air-conditioned room with 12 h light and dark cycles, at constant temperature (22 ± 2 °C). The animals were fasted overnight the day before surgery, but had access to water.

Forty Wistar albino rats were divided into four groups (n=10): Group 1 (Sham, S), only ileocaecal junction dissection was performed; Group 2 (Erythropoietin, EPO), ileocaecal junction dissection was performed and 3000 IU/kg EPO was given subcutaneously; Group 3 (Intestinal Obstruction, IO), ileocaecal junction dissection with ileal ligation; Group 4 (Intestinal Obstruction + Erythropoietin, IO + EPO), ileocaecal junction dissection with ileal ligation and 3000 IU/kg EPO was given subcutaneously.

Surgical Procedure

Rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar®, Parke Davis, Eczacibasi,
Istanbul, Turkey) and 10 mg/kg xylazine (Rompun®, Bayer AG, Leverkusen, Germany) via intramuscular injection for all surgical procedures. For laparotomy, a midline incision was performed under sterile conditions and the ileocaecal junction was dissected. After 2 mL saline were given into the peritoneal area, the abdominal wall was closed in one layer in the groups S and EPO. In the groups IO and IO+EPO, after laparotomy and midline incision, the ileocaecal junction placed in the middle and distal ileum was ligated with 3–0 silk suture at 1 cm proximal to the cecum, obstructing the passage but not inhibiting the circulation of the vessels. Then 2 mL saline were given into the peritoneal area and the abdominal wall was closed in one layer (7).

After a period of 24 hours (3), the rats were anesthetized and sacrificed by taking blood from the heart for biochemical analyses. Under sterile conditions, a thoracoabdominal midline incision was performed immediately. After opening the abdomen, peritoneal swab culture was taken for microbiological analyses and a 1 mL blood sample was taken from the inferior vena cava. Liver, MLNs, spleen and ileum samples were collected for microbiological and histopathological examinations. Serum was obtained from the centrifugation of the blood and stored at –80 °C until analyses. The tissues for histopathological evaluation were put into plastic containers with 10% formaldehyde solution after washing with saline for removing the foreign tissue residues and blood.

Biochemical analyses

Total oxidant activity (TOA), total antioxidant capacity (TAC), paraoxonase (PONX), TNF-α, IL-6, IL-1β, CRP and ADMA analyses were performed in the blood samples.

TOA of supernatant fractions was determined using a novel automated measurement method, developed by Erel (8). Oxidants present in the sample oxidize the ferrous ion-α-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylene orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of μmol H₂O₂ Equiv/L.

TAC of supernatant fractions was determined using also a novel automated measurement method developed by Erel (9). In this method, the hydroxyl radical is produced, which is the most potent biological radical. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequentially produced radicals, such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, the antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The results are expressed as mmol Trolox Eq/L.

Serum PONX levels were measured spectrophotometrically by a modified Eckerson method (9). Initial rates of paraoxon hydrolysis (O,O-diethyl-O-p-nitrophenyl phosphate; Sigma Chemical Co. London, UK) were determined by measuring liberated p-nitrophenol at 405 nm at 37 °C. The results are expressed as U/L (10).

TNF-α, IL-6 and IL-1β (Diasource; Nivelles, Belgium), ADMA (Immundiagnostik; Bensheim, Germany) and CRP (DRG; NJ, USA) levels were measured using commercially available ELISA kits.

Microbiological assay

Blood samples were obtained from the heart and cultured aerobically and anaerobically using the BacT™ Peds battles (Becton-Dickinson Diagnostic Inc., Sparks, MD, USA). Identification was realized by the BD-Phoenix 100 TM system. Peritoneal swab and positive cultures were plated out on blood agar, eosin methylene blue (EMB) agar, chocolate agar and Sabouraud-dextrose agar. At the same time, MLNs, spleen and liver were removed and placed in sterile glass bottles containing sterile brain-heart infusion media. The bottles were re-weighed and tissue homogenates were prepared in 2 mL brain-heart infusion using a sterile mortar and pestle. A portion (0.1 mL) of each homogenate was cultured on blood agar, EMB agar, and chocolate agar and Sabouraud-dextrose agar. At the same time, MLNs, spleen and liver were removed and placed in sterile glass bottles containing sterile brain-heart infusion media. The bottles were re-weighed and tissue homogenates were prepared in 2 mL brain-heart infusion using a sterile mortar and pestle. A portion (0.1 mL) of each homogenate was cultured on blood agar, EMB agar, and chocolate agar and Sabouraud-dextrose agar. All agar plates were examined after 24 h and 48 h of incubation at 37 °C. The incidence of bacterial translocation was calculated by determining the number of rats with positive bacterial culture divided by the total number of rats studied.

Histopathological assessment

Ileal segment, MLNs and liver tissues were put into the 10% formalin solution in paraffin blocks and prepared by slicing 4-μm sections. Tissues stained with hematoxylin-eosin and standard protocols were applied.

Ileal segment, MLNs and liver samples were examined for the grade of inflammatory cell infiltrate, and the ileal segments were also examined for the ileal mucosal injury score by an expert pathologist, using light microscopy (Nikon ECLIPSE 80i). As concordant to the literature, the changes were graded as follows; Grade 0, no changes; Grade 1, mild changes; Grade 2, moderate changes; Grade 3, severe changes (1, 4). In addition, all tissue samples were examined under light microscopy by staining Giemsa for the evaluation of bacterial translocation.
### Table I Biochemical results of the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S (n=10)</th>
<th>EPO (n=10)</th>
<th>IO (n=10)</th>
<th>IO+EPO (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PONX (U/L)</td>
<td>35.54 ± 8.52</td>
<td>71.11 ± 13.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.68 ± 4.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.91 ± 6.78&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TAS (mmol Trolox Eq/L)</td>
<td>0.72 ± 0.06</td>
<td>1.50 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.71 ± 0.09</td>
<td>1.16 ± 0.35&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TOS (μmol H₂O₂ Equiv/L)</td>
<td>12.14 ± 1.21</td>
<td>16.93 ± 6.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.52 ± 10.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.44 ± 7.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADMA (μmol/L)</td>
<td>2.35 ± 0.80</td>
<td>3.26 ± 1.29</td>
<td>2.34 ± 0.95</td>
<td>2.52 ± 0.93</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.93 ± 0.86</td>
<td>1.84 ± 1.23</td>
<td>7.59 ± 1.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.41 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>31.25 ± 8.45</td>
<td>26.87 ± 5.86</td>
<td>65.83 ± 20.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.63 ± 7.90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0.47 ± 0.11</td>
<td>0.23 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.62 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79 ± 0.64&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>30.46 ± 4.64</td>
<td>28.27 ± 4.14</td>
<td>165.26 ± 41.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.50 ± 15.68&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data were given as Mean ± SD. <sup>a</sup> Significantly different when compared with S group (p ≤ 0.001). <sup>b</sup> Significantly different when compared with IO group (p ≤ 0.001). <sup>c</sup> Significantly different when compared with IO group (p=0.005).

### Table II Histopathological grading of the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S (n=10)</th>
<th>EPO (n=10)</th>
<th>IO (n=10)</th>
<th>IO+EPO (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver inflammation score</td>
<td>0.11 ± 0.31</td>
<td>0.11 ± 0.31</td>
<td>1.34 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLN inflammation score</td>
<td>1.5 ± 0.53</td>
<td>1.7 ± 0.67</td>
<td>2.5 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8 ± 0.63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum inflammation score</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
<td>2.59 ± 0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.66 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileal mucosal damage score</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>1.29 ± 0.66</td>
<td>0.66 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data were given as Mean ± SD. <sup>a</sup> Significantly different when compared with S group (p<0.05). <sup>b</sup> Significantly different when compared with IO group (p<0.05).

### Statistical analysis

Statistical analysis was performed by SPSS for Windows 11.5 (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean±SD (standard deviation) values for biochemical values. Groups were compared by using the nonparametric Kruskal-Wallis test. Mann-Whitney U test was used for binary comparisons. A P value of less than 0.05 was considered significant.

### Results

All animals survived throughout the experimental procedures. Biochemical results are summarized in Table I. IO is significantly associated with oxidative stress. Serum PONX, TAC and TOA levels were different among the groups. PONX activity was lower in the IO group than the S group and higher in the IO+EPO group than the IO group. However, TAC levels did not differ significantly in the IO group; EPO treatment supported TAC levels in the EPO and IO+EPO groups. TOA levels were increased in the IO group compared to the S group, and the treatment with EPO significantly prevented the increase of TOA levels in the IO+EPO group.

Also, the inflammatory cytokines (TNF-α, IL-6, IL-1β) and CRP were increased after IO. In the IO+EPO group, all these cytokines were significantly decreased when compared with the IO group. There was no significant difference in ADMA levels between the groups.

The histopathological grading of the liver, MLNs and ileum is summarized in Table II. There was no difference between the S and EPO group scores. Inflam-
Information scores of the liver (p<0.001), MLNs (p=0.03) and ileum (p<0.001) were higher in the IO group than the S group. In addition, the ileal mucosal damage score was higher in the IO group (p<0.001). The inflammation scores of the liver (p=0.003), MLNs (p=0.035) and ileum (p=0.003) were lower in the IO+EPO group when compared with the IO group, and also the ileal mucosal damage score (p=0.043) was lower in the IO+EPO group (Figure 1 and 2).

Figure 1 The effects of EPO on ileal inflammation and mucosal injury after IO evaluated by histological examination. A: In group Sham, minimal mucosal inflammation (H&E stain, x100). B: In group IO, subtotal villous atrophy and mild epithelial degenerative changes in the intestinal mucosa with severe inflammation and edema (H&E stain, x200). C: In group IO+EPO, mild to moderate inflammation and edema in the mucosa with minimal epithelial degenerative changes (H&E stain, x200).

Figure 2 The effects of EPO on liver inflammation after IO evaluated by histological examination. A: In group Sham, mild edema in the liver parenchima (H&E stain, x100). B: In group IO, moderate portal inflammation and edema in the liver (H&E stain, x200). C: In group IO+EPO, mild parenchimal inflammation of the liver (H&E stain, x200).

Table III Microbiological culture results of the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S (n=10)</th>
<th>EPO (n=10)</th>
<th>IO (n=10)</th>
<th>IO+EPO (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture (c/d)</td>
<td>0/10</td>
<td>1/10</td>
<td>8/10a</td>
<td>1/10b</td>
</tr>
<tr>
<td>Liver culture (c/d)</td>
<td>0/10</td>
<td>1/10</td>
<td>9/10a</td>
<td>3/10b</td>
</tr>
<tr>
<td>Spleen culture (c/d)</td>
<td>1/10</td>
<td>1/10</td>
<td>8/10a</td>
<td>2/10b</td>
</tr>
<tr>
<td>Peritoneal culture (c/d)</td>
<td>3/10</td>
<td>2/10</td>
<td>9/10a</td>
<td>2/10b</td>
</tr>
</tbody>
</table>

Data were given as Mean ± SD. a Significantly different when compared with S group, (p<0.05); b significantly different when compared with IO group (p<0.05).

The culture results are summarized in Table III as the number of rats with positive bacterial culture divided by the total number of rats. There was no difference between the groups S and EPO. Blood (p=0.002), liver (p<0.001), spleen (p=0.007), MLNs (p=0.007), and peritoneal (p=0.023) cultures were significantly positive in the IO group when compared with the S group. However, in the IO+EPO group positive cultures were decreased, suggesting the EPO
EPO has cytoprotective effects on many cells and tissues beyond its hematopoietic activity, and these have been studied widely. Exogenous administration of EPO in animal models attenuates ischemic brain and spinal cord injury (17), acute kidney injury (18) and ischemia reperfusion-induced lung injury (19). EPO mediates reconditioning (ischemic tolerance) and specifically limits the destructive role of TNF-α and other proinflammatory cytokines in the heart, brain, kidney, and other tissues. The therapeutic effects of EPO for tissue protection are very wide in experimental models, showing effectiveness when administered before, during, or after an insult and raising optimism for high clinical potential (20). In this study, EPO was given to rats after the IO. Although there are various studies for understanding the signaling pathways responsible for EPO’s tissue-protective actions that are similar to those employed for erythrocyte maturation, much work remains to be carried out because EPO has now emerged as a multifunctional tissue-protective cytokine (21–23).

Previous studies have reported the effects of EPO against I/R injury in the small intestine (24). Also, EPO decreased the serum levels of TNF-α and IL-6 at 6 hours after I/R injury, which are the major proinflammatory cytokines upregulated in I/R injury (25). Hu et al. (26) have reported that EPO treatment significantly reduced the gene expression of major proinflammatory cytokines (TNF-α, IL-6 and IL-1β), while Hojman et al. (27) revealed the increasing of proinflammatory cytokines after the administration of EPO in a human model of acute systemic low-grade inflammation. In addition, Villa et al. (28) revealed that the antiinflammatory effect of EPO was related with the direct effect on inflammatory cells rather than the inhibition of cytokines secretion. In this study, TNF-α, IL-6 and IL-1β levels were lower in the EPO treatment group. For that reason, we believe that EPO treatment attenuates the proinflammatory cytokine levels due to the reduction of proinflammatory cytokines secretion.

Plasma CRP is an acute-phase protein and increases markedly with acute invasive infections. CRP concentrations are correlated with the severity of inflammation and tissue injury (21). Cevikel et al. (7) have reported that CRP levels increase with the severity of bacterial translocation in acute IO. Similarly, our study demonstrated significant increases in CRP levels in the IO group 24 h after the onset of obstruction. The proinflammatory cytokines and mediators of the acute phase reactions are correlated with the surgical intervention, and the surgery-based nature of this model makes it difficult to differentiate the effects of surgical trauma from IO (22). Therefore, CRP levels in our sham-operated control group were higher than those in the EPO control group. El-Awady et al. (3) have suggested that the CRP is a reliable test of bacterial translocation during IO, and it is a predictor of vascular compromise and bacterial translocation se-

**Discussion**

The present study, using a rat model, has demonstrated that protective effects in the small intestine and remote organs after IO were obtained after the treatment with EPO. In IO, with the disruption of ecological balance and involvement of the immune system, the bowel becomes a source for the reservoir of systemic infection and MOF via bacterial translocation. As a result of the increased intestinal permeability, microorganisms may be translocated to the systemic circulation, MLNs and liver (11, 12). Terminal obstruction of the ileum has been shown to create more bacterial translocation (13). So, in this study, we obstructed the terminal ileum, and both histopathological and microbiological assessments showed the results of IO and bacterial translocation.

IO was significantly associated with bacterial overgrowth, oxidative stress and inflammatory response. IO increased oxidative stress in a manner similar with the one in the study reported by El-Awady et al. (3). There were significant inflammatory changes in the liver and ileal mucosal tissues after small bowel obstruction in the IO group when compared with the S group. These changes were attenuated significantly with the EPO treatment in the liver, spleen and ileum mucosal tissues except MLNs. EPO is a strong antioxidant (6), and it has been reported that EPO increased the activity of antioxidant enzymes and decreased lipid peroxidation (14, 15). Furthermore, Bakan et al. (16) suggested that there was a direct relationship between the levels of antioxidant enzymes and lipid peroxidation. In this study, EPO treatment increased TAC and PONX levels and decreased TOA levels. These findings demonstrated that EPO reduced the oxidative injury, and this was confirmed with histopathology.

Bacteria can be detected in the MLNs within 6 hours of IO, while bacteria spread to the liver, spleen and blood after 24 hours of IO (11). Cevikel et al. (7) demonstrated the occurrence of bacterial translocation in the samples of MLNs, liver, and blood in 67% of animals after IO. We determined, after 24 hours of IO, that the samples of MLNs, liver and peritoneum had positive culture results in 90% and spleen had positive culture in 80% of rats in the IO group. Positive hemocultures were observed in 80% of rats in the IO group. However, no evidence of bacterial translocation was observed in animals in either control group, S and EPO. This difference in bacterial translocation rates between the experimental and control groups was statistically significant (P<0.05).

EPO has cytoprotective effects on many cells and tissues beyond its hematopoietic activity, and these have been studied widely. Exogenous administration of EPO in animal models attenuates ischemic brain and spinal cord injury (17), acute kidney injury (18) and ischemia reperfusion-induced lung injury (19). EPO mediates reconditioning (ischemic tolerance) and specifically limits the destructive role of TNF-α and other proinflammatory cytokines in the heart, brain, kidney, and other tissues. The therapeutic effects of EPO for tissue protection are very wide in experimental models, showing effectiveness when administered before, during, or after an insult and raising optimism for high clinical potential (20). In this study, EPO was given to rats after the IO. Although there are various studies for understanding the signaling pathways responsible for EPO’s tissue-protective actions that are similar to those employed for erythrocyte maturation, much work remains to be carried out because EPO has now emerged as a multifunctional tissue-protective cytokine (21–23).

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verity. In our study, the CRP levels were decreased due to the effects of EPO on the inflammatory response.

Plasma ADMA concentration has been reported to be a strong and independent risk factor for intensive care unit mortality, and hepatic dysfunction is the most prominent determinant of ADMA concentration in critically ill patients (23). It is reported to be directly related to MOF and correlated with IL-6. Also, ADMA concentration is dependent on the kidney and liver functions (5). Bacterial translocation, caused by IO, creates a source for systemic infection and MOF (29). So, we decided that ADMA may be a novel prognostic marker in the monitoring of patients with IO and therefore the correlation between ADMA concentration and IO was investigated. But, there was no significant difference between the groups, and this can be attributed to the normal liver and kidney functions. Based on these results, ADMA is not a useful parameter in the monitoring of patients with IO.

The administration of EPO reduces intestinal mucosal injury, oxidative damage, bacterial translocation and secretion of inflammatory cytokines in the ileum after IO. This effect of EPO may be useful for preserving intestinal injury and related sepsis in patients with IO. ADMA, however, is not suitable as a prognostic marker for monitoring intestinal damage and related inflammatory response in IO.

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Conflict of interest statement
The authors stated that there are no conflicts of interest regarding the publication of this article.

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EFFECTS OF ERYTHROPOIETIN ON THE SERUM AND LIVER TISSUE LEVELS OF COPPER AND ZINC IN RATS WITH OBDUCTIVE JAUNDICE

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Summary

Background: Erythropoietin is an anti-apoptotic, anti-inflammatory, angiogenic cytokine and has protective properties against oxidative stress. In this study we investigated the effects of erythropoietin on the levels (serum and liver tissue) of copper and zinc in cholesstatic rats.

Methods: Thirty-two Wistar albino rats used in the study were divided into four groups – Group I: Sham; Group II: Erythropoietin; Group III: Obstructive Jaundice; Group IV: Obstructive Jaundice+Erythropoietin. After the first operation, rats were followed up for seven days and then operated for the second time. Rats were sacrificed by intracardiac blood taking, and the liver tissue samples were obtained immediately.

Results: Erythropoietin reduces copper, and increases zinc levels in serum and liver tissues after obstructive jaundice (p<0.05). Furthermore, it has been shown that the levels of alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transferase, alkaline phosphatase and total bilirubin/direct bilirubin were significantly lower in Obstructive Jaundice+Erythropoietin group than Obstructive Jaundice group.

Conclusions: Erythropoietin affects the changes in copper and zinc levels, thus decreasing the liver damage biochemically in rats with obstructive jaundice. However, further investigations are needed to discover how erythropoietin therapy might reduce target organ damage in cholesstatic liver cases by affecting copper and zinc levels.

Keywords: obstructive jaundice, erythropoietin, liver damage, copper, zinc

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Original paper
Originalni naučni rad

EFEKTI ERITROPOETINA NA NIVOE BAKRA I CINKA U SERUMU I TKIVIMA JETRE KOD PACOVA SA OPSTRUKTIVOM ŽUTICOM

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Kratak sadržaj

Uvod: Eritropoetin je citokin sa antiapoptotskim, antiinflamatornim i angiogenetskim svojstvima koji deluje protektivno u odnosu na oksidativni stres. U ovoj studiji istraživali smo efekte eritropoetina na nivo (u serumu i tkivima jetre) bakra i cinka kod pacova sa holestazom.


Rezultati: Eritropoetin snižava nivoe bakra, a podiže nivoe cinka u serumu i tkivima jetre posle opstruktnih `utica (p<0,05). Pored toga, pokazano je da su nivoi alanin aminotransferaze, aspartat aminotransferaze, gama-glutamyl transferaze, alcalna fosfataza i total bilirubin/direktnog bilirubina bili značajno niži u Grupi 4 nego u grupi sa opstruktnom žuticom.

Zaključak: Eritropoetin utiče na promene u nivovima bakra i cinka i na taj način biohemski umanjuje oštećenja jetre kod pacova sa opstruktnom žuticom. Međutim, potrebna su dalja istraživanja kako bi se otkrio na koji način terapija eritropoetinom, kroz uticaj na nivo bakra i cinka, može umanjiti oštećenja cijelih organa u slučajevima holestaze jetre.

Ključne reči: opstruktna žutica, eritropoetin, oštećenja jetre, bakar, cink
Introduction

Obstructive jaundice (OJ) is a frequent situation in surgery clinics resulting in higher mortality and morbidity. OJ is a clinical condition which may be caused by bile stones, cessation of bile flow due to tumors and strictures, and accumulation of bile acids in the liver (1). Its etiopathogenesis is not clear yet. The release of free oxygen radicals, due to bile acid damage, results in interference with Kupffer cells and neutrophils, and this clinical picture may not be limited to the liver, but its systemic effects could cause damage of various organs (2). OJ causes dilatation in hepatocytes and bile ducts, and due to high concentrations of hydrophobic bile acids it causes oxidative damage and inflammation (3). Mediators liberated as a result of oxidative damage, by increasing the quantity of free radicals, cause activation of the coagulation cascade, impairment of microcirculation and clinical pictures which may lead to multiple organ failure (4). In diseases such as primary biliary cirrhosis, alcoholic cirrhosis and OJ, it has been shown that zinc (Zn) levels decrease in the liver, while copper (Cu) and mangenese levels increase (5). Zn is one of the basic trace elements which plays a catalytic and structural role in many enzymes (6). Besides its anti-inflammatory and anti-apoptotic effects, Zn has important antioxidant properties and affects the processes such as growth and development, cancer formation and aging (7). While Zn is necessary for liver function, liver is important for zinc hemostasis (8). In experimental animal models, although not understood exactly, it has been seen that zinc has hepato-protective properties in terms of acute and chronic liver damage (8). Furthermore, it has been shown that zinc supplementation leads to reduction in blood ammonia (9). Cu is a trace element which takes its place as a co-factor in a number of enzymatic reactions (amine oxidase, Cu-dependent superoxide dismutase, cytochrome oxidase and tyrosinase) (10). In primary biliary cirrhosis, alcoholic cirrhosis and other cholestatic syndromes, accumulation of excessive Cu in the liver is reported (7). Erythropoietin (EPO) has an effect in reducing inflammatory response via reducing the levels of pro-inflammatory cytokines and apoptosis induced by cytokines (11). Recent data show that EPO supports angiogenesis, reduces oxidative stress and accelerates wound healing (12, 13). Our aim in this study is to investigate the effects of EPO on the levels of Cu and Zn which are both basic trace elements, and its effects on the liver tissue damage in rats with OJ.

Materials and Methods

Chemical

Erythropoietin was purchased from Sigma (E5627–Erythropoietin human recombinant, expressed in Chinese hamster ovary cells, lyophilized powder, cell culture tested, ∼ 100,000 units/mg protein) and dissolved in phosphate buffer saline.

Animals

Thirty-two female Wistar albino rats, each weighing 200–250 g, were included into the study at the Dicle University Health Sciences Application and Research Center. The study was conducted in accordance with the rules of the National Institute of Health Guide for the Care and Use of Laboratory Animals, following approval from the Ethics Committee. Rats were housed under standard conditions in an air-conditioned room with 12 h light and dark cycles, at a constant temperature (22 ± 2 °C). The rats were housed in cages, and allowed free access to standard rat chow and water before the experiments. The animals were fasted overnight the day before surgery, but had access to water.

Experimental design

Thirty-two Wistar albino rats were divided into four groups (n=8):

- Group 1 (Sham, S); only the common hepatic duct was dissected and followed up for 7 days,
- Group 2 (Erythropoietin, EPO); the common hepatic duct was dissected, and EPO was given at a dose of 500 IU/kg daily and followed up for 7 days,
- Group 3 (Obstructive Jaundice, OJ); the common hepatic duct was dissected and ligated; followed up for 7 days,
- Group 4 (Obstructive Jaundice + Erythropoietin, OJ+EPO); the common hepatic duct was dissected and ligated, EPO was given at a dose of 500 IU/kg daily and followed for 7 days.

Surgical procedure

Anesthesia was obtained by giving 50 mg/kg Ketamine hydrochloride (Ketalar®, Parke Davis, Pfizer, Istanbul, Turkey) and 10 mg/kg Xylazine (Rompun®; Bayer AG, Leverkusen, Germany) to the rats via intramuscular injection. For skin antisepsis, povidone iodine was applied and middle line incision was preferred. After laparotomy, the common bile duct was ligated with 4/0 silk and the incision was closed as a double layer after 4 mL physiologic solution was given to the peritoneal area. After 7 days, rats were given standard rat chow and water. The rats were anesthetised again by administering 50 mg/kg Ketamine hydrochloride (Ketalar®, Parke Davis, Pfizer, Istanbul, Turkey) and 10 mg/kg Xylazine (Rompun®; Bayer AG, Leverkusen, Germany) i.m., and then sacrificed by taking intracardiac blood. Liver tissue samples were taken out for analysis by the thoraco-abdominal incision.
Biochemical analyses

In the blood samples, alanine transaminase (ALT) (IU/L), aspartate transaminase (AST) (IU/L), alkaline phosphatase (ALP) (IU/L), gamma glutamyl transferase (GGT) (IU/L), total bilirubin (TB) (mmol/L), direct bilirubin (DB) (mmol/L), Zn (ppm) and Cu (ppm) analyses were performed. Also, Zn (μg/protein) and Cu (μg/protein) measurements were performed in the liver tissue.

AST, ALT, ALP, GGT, TB and DB were measured in serum by a spectrophotometric method using an Architect® c16000 autoanalyzer (Abbott Laboratories, Abbott Park, IL, USA).

Weighed tissue samples were taken into heat-resistant glass tubes and 2.5 mL 65% nitric acid was added and incubated at room temperature for 1 h, then incubated at 100–120 °C for 2 h. After cooling at room temperature, 0.5 mL 65% perchloric acid was added and incubated at 150–180 °C for 2 h. After cooling, a vehicle solution was added to obtain the final 5 mL solution for measurement (12). Zn and Cu were determined by a Shimadzu 6401S atomic absorption/emission spectrometer. The acetylene flow rate and the burner height were adjusted in order to obtain the maximum absorbance signal with a slit of 0.5 nm, at a wavelength of 213.9 nm for Zn and 324.8 nm for Cu. The radiation sources were hollow cathode lamps (Shimadzu, Japan). Operating conditions were those recommended by the manufacturer (Operation Manual-Atomic Absorption Spectrophotometer AA-6800, SHIMADZU, 2000).

Statistical analysis

Statistical analysis was performed using SPSS for Windows 11.5 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation for biochemical values. Groups were compared by using the nonparametric Kruskal-Wallis test. Mann-Whitney U test was used for binary comparisons. P value of less than 0.05 was considered as significant.

Results

All animals survived throughout the experimental procedures. Liver functions and bilirubin levels of all the groups are listed in Table I, and the serum and liver tissue levels of Cu and Zn in Table II. Comparison of the groups in terms of Cu and Zn levels in the serum and liver tissue is shown in Table II.

In Group III, the serum values of TB, DB, ALT, AST, GGT and ALP were found significantly increased

Table I BALT, AST, ALP, GGT, TB, DB levels in serum.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S (n=8)</th>
<th>EPO (n=8)</th>
<th>OJ (n=8)</th>
<th>OJ+EPO (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, IU/L</td>
<td>48.3±18.2</td>
<td>41.8±15.7</td>
<td>105.0±9.7</td>
<td>77.1±15.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST, IU/L</td>
<td>271.8±80.5</td>
<td>222.7±64.8</td>
<td>510.7±108.5</td>
<td>389.7±88.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALP, IU/L</td>
<td>52.3±10.9</td>
<td>51.37±11.0</td>
<td>113.1±18.1</td>
<td>89.3±11.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GGT, IU/L</td>
<td>33.8±9.7</td>
<td>33.37±9.5</td>
<td>120.5±11.5</td>
<td>103.7±5.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TB</td>
<td>0.3±0.04</td>
<td>0.3±0.1</td>
<td>10.8±2.6</td>
<td>6.4±1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DB</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>4.2±1.1</td>
<td>2.1±0.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

§ Significantly different when compared to Sham group (p<0.001), ¥ significantly different when compared to Sham group (p<0.01), ‡ significantly different when compared to Sham group (p<0.05), ¶ significantly different when compared to EPO group (p<0.001), ~ significantly different when compared to OJ group (p<0.001), * significantly different when compared to OJ group (p<0.01), ‡‡ significantly different when compared to OJ group (p<0.05).

Table II Cu (μg/protein) and Zn (μg/protein) levels in serum and liver tissue.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S (n=8)</th>
<th>EPO (n=8)</th>
<th>OJ (n=8)</th>
<th>OJ+EPO (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Cu</td>
<td>1.13±0.14</td>
<td>1.1±0.2</td>
<td>3.85±0.43</td>
<td>2.68±0.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Zn</td>
<td>0.75±0.14</td>
<td>1.2±0.3</td>
<td>0.54±0.13</td>
<td>0.71±0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver Cu</td>
<td>2.99±0.71</td>
<td>2.6±0.8</td>
<td>4.49±0.86</td>
<td>3.37±0.94</td>
<td>0.003</td>
</tr>
<tr>
<td>Liver Zn</td>
<td>24.72±4.97</td>
<td>26.7±5.0</td>
<td>16.23±2.40</td>
<td>21.47±3.97</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

‡ Significantly different when compared to Sham group (p<0.001), ¥ significantly different when compared to Sham group (p<0.01), ‡ significantly different when compared to EPO group (p<0.001), ¶ significantly different when compared to EPO group (p<0.01), ‡‡ significantly different when compared to EPO group (p<0.05), * significantly different when compared to OJ group (p<0.01), ‡‡‡ significantly different when compared to OJ group (p<0.05).
when compared to the other groups (p<0.05). Zn levels in serum were higher in Group II (p<0.001) compared to Group I, while the other parameters did not show any significant differences (p>0.05) in these two groups. When Group II was compared to Group III, all the parameters were significantly different (p<0.001). Comparison of Group II and Group IV showed no significant difference in terms of Cu levels in liver tissue (p>0.05), while Zn levels were found to be significantly different (p=0.028). All the other serum parameters of these two groups were significantly different (p<0.05). Cu and Zn levels in the liver tissue of Group III and Group IV were significantly different (p=0.010 and p=0.003, respectively), and all the serum parameters of these two groups were also significantly different (p<0.05).

Discussion

Diseases which result in liver damage may change the levels of trace elements like Fe, Zn, Cu and Mn in the liver tissue, and these changes play a key role in the liver fibrosis process. D’Uscio et al. (14) found that treatment with EPO increased the vascular expression of SOD1 (superoxide dismutase). Although modulatory effects of EPO on Cu and Zn levels in certain liver and kidney diseases have been reported in some studies, its effects in OJ have not been clarified (3, 15). In general, the increase of Cu and decrease of Zn levels in the liver tissue and serum may be the characteristics of chronic diseases, such as severe cholestasis, biliary atresia and cirrhosis (16, 17). In cirrhotic patients, low levels of Zn and high levels of Cu are associated with the severity of liver fibrosis (5). In our study it was also established that ligation of the main bile ducts of rats leads to an increase in Cu levels and a decrease in Zn levels in serum and liver tissue. In ischemia-reperfusion models of experimental animal studies increased Cu and reduced Zn levels have been shown to be associated with oxidative stress (18). Devipriya et al. (13) reported increased Cu levels and decreased Zn levels in alcohol given rats. In cholestasis associated with impaired bile flow, Cu accumulates in the liver and Cu metabolism is impaired. The finding of increased levels of Cu in OJ rats in our study was supported by the results of Devipriya et al. (13). Excess cumulation of Cu in rats triggers oxidative damage of the kidney and liver tissue DNAs and ultimately contributes to many degenerative disorders (19). In this study we observed that OJ group had higher levels of serum and liver Cu, respectively (p<0.001 and p=0.003), than other groups. Also, ALT and AST levels were significantly increased in OJ group compared to Sham and EPO groups (p<0.001). These results support the finding that the accumulation of Cu in serum and liver leads to organ damage. In a study by Rodriguez et al. (5) a control group was compared to patients with alcoholic cirrhosis and it was found out that the liver Zn concentration is lower in cirrhotic patients. Furthermore, it was shown that Zn treatment prevented liver damage induced by ethanol, in both acute and chronic exposure to alcohol (20). We also established that Zn levels were significantly reduced in OJ and OJ+EPO groups compared to the non-obstructed groups. When rats in OJ and OJ+EPO groups were compared, in OJ+EPO group Zn levels in serum and liver tissue were higher (p=0.038 and p=0.003, respectively), and ALT (p<0.001), AST (p=0.05), TB and DB (p=0.02), GGT (p=0.03), and ALP (p=0.007) levels were lower in serum samples. These results support the view that EPO treatment may increase the levels of Zn in serum and liver tissue, and reduce liver damage.

EPO is an anti-apoptotic, anti-inflammatory, angiogenetic cytokine that has protective properties against oxidative stress (21, 22). Nishiyama et al. (23) reported that EPO improves ventricular function after myocardial infarction, by increasing angiogenesis and reducing apoptosis. Johnson et al. (24) noted that EPO shows renoprotective effects by reducing acute renal damage. Liu et al. (25) showed that EPO implementation is cardioprotective due to suppression of the inflammatory response in myocardial ischemia-reperfusion damage. When given at early stages, EPO has been reported to have positive effects on liver ischemia-reperfusion damage by reducing oxidative stress and kaspase-3 activation (26). In our study an increase in serum Zn levels was observed (p<0.001) compared to Sham group, but there were no significant differences in other parameters. When OJ and OJ+EPO groups were compared, Cu levels in serum and liver tissue were lower (p<0.01), and Zn levels in serum and liver tissue were significantly higher in OJ+EPO group. Furthermore, in OJ+EPO group, GGT, ALP, TB, DB, ALT and AST (p<0.05) levels were lower compared with OJ group.

Conclusion

It is a well-known fact that the levels of Cu are increased, and the levels of Zn are decreased in the liver tissue of cirrhotic patients, and the severity of liver fibrosis is associated with these levels. It has been shown in this study that erythropoietin has regulatory effects on the serum and liver tissue levels of Cu and Zn, by decreasing Cu levels and increasing Zn levels. These results suggest that EPO can be used as an effective chemoprotective agent in OJ cases for regulating Cu and Zn levels. However, further investigation is needed to support our findings, and to explain the mechanism of how EPO treatment reduces target organ damage in cholestatic liver damage by affecting Cu and Zn levels.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.
References


THE EFFECTS OF CENTRAL ANGIOTENSIN II AND ITS SPECIFIC BLOCKERS ON NOCICEP TION. POSSIBLE INTERACTIONS WITH OXIDATIVE STRESS STATUS

EFEKTI CENTRALNOG ANGIOTENZINA II I NJEGOVIH SPECIFI^KNIH BLOKATORA NA NOCICEPCIJU. MOGU^CE INTERAKCIJE SA STATUSOM OKSIDATIVNOG STRESA

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Summary: It has already been demonstrated that a complete brain renin–angiotensin system (RAS) exists distinctly separate from the peripheral system and is implicated in complex functions such as memory, emotional responses and pain. Regarding the implications of angiotensin II (the main bioactive peptide of RAS) in pain, although there are many studies in this area of research,most of the results are controversial. Also, it seems that oxidative stress follows angiotensin II infusion, but the role of AT1 vs. AT2 receptors is not well established. In this context, we were interested in studying the effects of central RAS on nociception, through the intracerebroventricular administration of losartan and PD-123177 (antagonists for the AT1/AT2 receptors), as well as an ACE inhibitor (captopril) and also angiotensin II in rats, which were subsequently tested using the hot-plate task, a well known behavioral test for pain perception. We present here the analgesic effect of angiotensin II administration, as shown by increased latency-time in the hot-plate, as well as a nociceptive effect of angiotensin II blockers like AT1 and AT2 specific antagonists (losartan and PD-123177) and an ACE inhibitor (captopril), as their administration resulted in decreased latency-time. Moreover, we demonstrated a significant correlation between the results of the nociceptive and oxidative stress parameters.

Kratak sadr^aj: Do sada je ve^ pokazano da kompletan mo^dani renin–angiotenzin sistem (RAS) postoji zasebno od perifernog sistema i da je uklju^en u slo^ene funkcije kao ^to su memorija, emocionalni odgovori i bol. ^To se ti^e implikacija angiotenzina II (glavnog bioaktivnog peptida RAS) u bolu, mada u ovoj oblasti istra^ivanja postoje mnoge studije, rezultati su ve^inom opre^ni. Tako^e, izgleda da infuziju angiotenzina II prati oksidativni stres, ali uloga receptora AT1 i AT2 jo^ nije razja^njena. U tom kontekstu hteli smo da prou^imo efekte centralnog RAS na nocicepciju preko intracerebroventrikularne administracije lozartana i PD-123177 (antagonista za receptore AT1/AT2) kao i jednog ACE inhibitora (kaptopril) i angiotenzina II kod pacova, koji su potom testirani metodom grejne plo^e, poznatim behevioralnim testom za percepciju bola. Predstavljamo analgeti^ki efekat administracije angiotenzina II pokazan kroz pove^anu latentnciju na grejnoj plo^i, kao i nociceptivni efekat blokatora angiotenzina II kao ^to su antagonisti specifi^ni za AT1 i AT2 (lozartan i PD-123177) i ACE inhibitora (kaptopril), po^to je rezultat njihove administracije bila smanjena latentncija. ^To vi^e, uocili smo zna^ajnu korlaciju izmedju rezultata nociceptivnog behevioralnog testa i nivoa nekih od glavnih markera oksidativnog stresa. Na taj na^in obeze^eni su novi rezultati i novi namet za daljnje istra^ivanje u ovom podru^ju.

Introduction

The discovery that all components of the renin–angiotensin system (RAS) are present in the brain led investigators to postulate the existence of a local brain RAS (1). In this way, it has already been demonstrated that a complete brain RAS exists that is distinctly separate from the peripheral system and comprises all necessary precursors and enzymes required for the formation and metabolism of the biologically active forms of angiotensins (2).
behavioral task and the levels of some main oxidative stress markers. This provides additional evidence for an analgesic effect of Ang II administration, as well as for a nociceptive effect of Ang II blockers. Moreover, a significant correlation between the noception and angiotensin II-induced oxidative stress is presented.

**Keywords:** angiotensin II, pain, oxidative stress

Also, it is now generally accepted that the brain RAS with its bioactive peptides, which mainly include angiotensin II (Ang II), is involved not only in cardiovascular functions and body fluid homeostasis (3), but also in the regulation of some superior functions involving the regulation of cognitive functions (learning and memory processes) (4, 5), emotional responses (6, 7) and also nociception (8, 9).

These effects are modulated by specific angiotensin receptors. Numerous studies have led to the identification of two pharmacologically specific angiotensin receptors type 1 (AT 1) and type 2 (AT 2), which are well represented in various brain areas (10). Our group also previously demonstrated that the administration of losartan and PD-123177, which are selective antagonists for the AT 1 and AT 2, results in anxiolytic effects in rats (7). Also, similar effects were reported as the result of angiotensin-converting enzyme (ACE) inhibitors like captopril (7), which is also commonly used as an antihypertensive drug (11).

Regarding the implications of Ang II in pain, although there are many studies in this area of research, most of the results are conflicting. In this way, while some reports stated that Ang II administration resulted in diminishing morphine-induced analgesia (12) and also that specific blockers like spirapril and losartan exert antinociceptive effects (13), other authors demonstrated opposite effects with Ang exhibiting analgesic effects (14) and its blockers (enalapril or losartan) generating increased pain sensitivity (15). Moreover, combined effects were also reported sometimes within the same experiment, as both increased and decreased nociception was observed as a result of various Ang II blockers administration (16).

Additionally, it seems that oxidative stress follows Ang II infusion, but the role of AT 1 vs. AT 2 receptors is not very well established (17). Also, it has been shown that the administration of Ang II facilitates the formation of some free radicals like superoxide (O$_{2}^-$) (18), while losartan seems to exert antioxidant effects (19). There are also controversies regarding the effects of angiotensin II on oxidative stress, considering that in some experiments losartan significantly decreased angiotensin II-induced oxidative stress, while PD-123319 did not (20).

In this context, in the present paper we were interested in studying the effects of the central RAS on noception, through the intracerebroventricular (icv) administration of losartan and PD-123177 (agonists for the AT 1/AT 2 receptors), as well as an ACE inhibitor (captopril) and also Ang II in rats, which were subsequently tested in the hot-plate task, one of the most well-known behavioral tests for pain perception. Additionally, we were interested to know if there is a possible correlation between the effects of Ang II on noception and some oxidative stress markers which were determined from the temporal lobe of rats, considering that this is one of the brain areas most susceptible to oxidative stress (21).

**Material and Methods**

**Animals**

Sixty male Wistar rats weighing 200–250 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22 °C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water *ad libitum*. Rats were treated in accordance with the guidelines on animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC).

**Neurosurgery**

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg/kg b.w., i.p., Sigma) anesthesia. Rats were mounted in the stereotaxic apparatus 11° below horizontal zero plane.

Losartan, PD-123177, captopril and Ang II were i.c.v. administered (0.1 µg/kg b.w., Sigma) by freehand through a plastic (silastic) cannula (0.9 mm outer diameter), implanted stereotaxically in the left cerebral ventricle at the following coordinates: 0.5 mm posterior to bregma; 1.3 mm lateral to the midline; 4.3 mm ventral to the surface of the cortex (22). The cannula was positioned with acrylic dental cement and secured by one stainless steel screw. After surgery the rats were isolated in a separate cage and protected with streptomycin 300 mg/kg bw. The sham-operated rats were injected with saline. The location of the i.c.v. cannulas in lesioned rats was verified by injecting a dye (trypan blue) through each cannula at the end of the experiment. Brains were
removed and cut with a scalpel and the spread of the dye within the ventricles was examined. All cannulas were found to be in the right position. Pain testing was started after 7 consecutive days of treatment.

**Hot-plate**

The investigation of pain sensibility was performed using a hot-plate (Hugo Basile). A plastic cylinder is used to confine the rat to the heated surface of the plate which is maintained at 55 °C using a thermostate. The reaction time (the latency time) to two different types of behavior was monitored: licking the paw and jumping (23).

**Tissue collection**

After the behavioral tests, all rats were anesthetized, rapidly decapitated, and the whole brain was removed. The temporal lobes were collected. Each temporal tissue sample was weighed and homogenized with a Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in bidistilled water (1 g tissue/10 mL bidistilled water). Samples were centrifuged 15 min at 3000 rpm. Following centrifugation, the supernatant was separated and pipetted into tubes.

**Biochemical estimations**

Regarding the biochemical assessments, we decided to classically determine the main antioxidant enzymes (first line of defense in the way of free radicals) and a lipid peroxidation marker.

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 substrate (a water soluble tetrazolium dye) and xanthine oxidase using a SOD Assay Kit (Fluka, product number: 19160) according to the manufacturer’s instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Glutathione peroxidase (GPX) activity was measured using the GPX cellular activity assay kit CGP-1 (Sigma Chemicals). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity.

Malondialdehyde (MDA) levels were determined by the thiobarbituric acid reactive substances (TBARs) assay. Two hundred microliters of temporal lobe homogenate (supernatant) was added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (pH 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100 °C for 20 minutes. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm. The signal was read against an MDA standard curve, and the results were expressed as nmol/mg protein (24–26).

Total protein was measured using the Bradford dye-binding method, with bovine serum albumin as standard (27).

**Statistics**

The animal’s behavior in the hot-plate task was statistically analyzed using one-way analysis of variance (one-way ANOVA). The results are expressed as mean ± SEM. Post hoc analyses were performed using Tukey’s honestly significant difference test in order to compare losartan, PD-123177, captopril and angiotensin II groups. F values for which P<0.05 were regarded as statistically significant. Pearson’s correlation coefficient and regression analysis were used to evaluate the connection between the latency-time in the hot-plate and the central oxidative stress markers.

**Results**

Regarding the effects of RAS components in nociception, as studied in the hot-plate, we report here a significant decrease of the latency time to jump/licking paws in the case of both antagonists administered: losartan (F(1.22)=95, p<0.0001) and PD-123177 (F(1.22)=41, p<0.0001), as well as in the case of the angiotensin-converting enzyme inhibitor captopril (F(1.22)=18, p=0.003), as compared to the control group (Figure 1).

![Figure 1](https://example.com/figure1.png)

**Figure 1** Effect of angiotensin II on latency time in the hot-plate. The values are mean ± S.E.M. (n=12 animals per group). ***p<0.0001 vs. control group, **p=0.005 vs. control group.
Figure 2 Correlations between latency time in the hot-plate and SOD (A), GPX (B) and MDA (C).
Additionally, we observed a significant increase (F(1,22) = 40, p < 0.0001) of the latency time in the Ang II administrated group, as compared to control rats (Figure 1).

Also, post hoc analysis revealed significant differences between losartan and Ang II (p < 0.0001), PD-123177 and Ang II (p < 0.0001), as well as between captopril and Ang II (p < 0.0001) groups. Still, no significant differences were found between losartan and PD-123177 (p = 0.1), losartan and captopril (p = 0.06) and PD-123177 vs. captopril (p = 0.08) groups.

Moreover, we found significant correlations between the results of the behavioral task, represented by the latency time in the hot-plate, and the levels of some oxidative stress markers, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA). In this way, the Pearson’s correlation coefficient and regression analysis showed the following correlations: latency time vs. SOD (n = 60, r = -0.401, p = 0.001) (Figure 2A), latency time vs. GPX (n = 60, r = -0.343, p = 0.007) (Figure 2B) and latency time vs. MDA (n = 60, r = 0.590, p < 0.0001) (Figure 2C).

Discussion

In the present work we demonstrated an analgesic effect of Ang II administration, as shown by increased latency time in the hot-plate, as well as a nociceptive effect of Ang II blockers like AT 1 and AT 2 specific antagonists (losartan and PD-123177) and an ACE inhibitor (captopril), as their administration resulted in decreased latency time in the hot-plate. Moreover, we present here a significant correlation between the results of the nociceptive behavioral task and the levels of some main oxidative stress markers.

As mentioned, previous studies regarding the effects of Ang in nociception resulted in contrasting results with reports stating both analgesic and increased pain sensibility effects (12–16). Similar facts were also reported regarding the various blockers of Ang II, both at the receptor levels, as well as on the ACE blocking level (12–16). Furthermore, there are experiments where the administration of various Ang II blockers, such as saralasin, sarmesin, losartan or PD123319, generated both increased and decreased nociception, depending on the dose (16). Besides the dose used, it seems that the conflicting result described above can also be explained by the different strains of animals used, as well as various behavioral tasks selected for the study of nociception.

These aspects could be very important considering that most of the previous studies regarding various neurotransmitters implicated in the modulation of pain were especially focused on the opioid peptides. However, considering the integrative aspects which characterize the modulation of nociceptive processes, additional studies regarding other neurochemical systems such as the bioactive peptides of RAS (Ang II) are very significant.

Moreover, in the present work we demonstrated a significant correlation between the nociception expressed as the latency time in the hot-plate and the specific activity of the main antioxidant enzymes (SOD and GPX) and a lipid peroxidation marker (MDA). Actually, there are some authors who previously demonstrated that oxidative stress contributes to persistent pain (28–30). In this way, removal of excessive ROS by free radical scavengers, such as phenyl N-tert-butyl nitrotrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), produced significant analgesic effects in both neuropathic pain (31-33) and inflammatory pain (34).

Furthermore, the number of neurons showing mitochondrial ROS production was significantly increased in the lumbar spinal dorsal horn in spinal nerve ligated neuropathic rats (35). Also, increased levels of extracellular hydrogen peroxide were observed in the spinal trigeminal nucleus after formalin injection into the lip of the rat, and this increase coincided with pain behaviors (36).

Additionally, it was demonstrated that SOD, which converts free-radical superoxide to hydrogen peroxide (37), was very effective in reducing inflammation indicators and hyperalgesia after carrageenan injection into the rat paw (38).

Still, while it is becoming clear that ROS are involved in persistent pain, the mechanisms by which they contribute to pain are still unknown.

We demonstrated here the antinociceptive effects of Ang II administration, as shown by the increased latency time in the hot-plate, as well as a nociception-induced effect of Ang II blockers like losartan, PD-123177 and captopril. Further, we present a significant correlation between the results of the nociceptive behavioral task and the levels of some main oxidative stress markers such as SOD, GPX and MDA.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.
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