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Original Article

Protective effects of vitamin B12 on testicular torsion/detorsion injury in rat

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ABSTRACT

Torsion of the testis is a urologic emergency in males, which causes the interruption of the arterial blood flow of the testis; it can lead to ischemia in the testicular tissue that an important cause of male infertility. The goal of this work was to evaluate the effects of Vit B12 on testis torsion injury after torsion/detorsion. Thirty- five male Wistar rats were randomly divided into five groups containing 8 rats per group including sham, ischemia, ischemia + Vit B12 (4 mg/kg i.p), ischemia / reperfusion and ischemia / reperfusion + Vit B12 (4 mg/kg i.p). Three hours of torsion and 3 hr. of detorsion were created in the both testes. After 3 hr. of detorsion, testis tissues were collected to evaluate glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA) concentrations for oxidative stress, and nuclear factor kappa B (NF-κB) expression. There was statistically significant difference in all parameters [SOD ($p < 0.001$), GPx ($p< 0.001$), MDA ($p < 0.001$)], except NF-κB expression when the experimental groups compared to sham group. Tissue GPx and SOD levels were significantly higher, while MDA level was significantly lower in the ischemia / reperfusion + Vit B12 group than in the ischemia / reperfusion group. This study showed that Vit B12 can maintain testis structure against ischemia reperfusion injury. Moreover, Vit B12 attenuated inflammation and related oxidative stress and also helped to preserve testis function following IR damage.

اثرات محافظتی ویتامین B12 بر آسیب پیچش/دتورشن بیضه در موش صحرایی

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چکیده

پیچ خوردگی بیضه یک اورژانس اورولوژیک در مردان است که می تواند منجر به ایسکمی در بافت بیضه شود. هدف از این کار ارزیابی اثرات ویتامین B12 بر آسیب پیچش بیضه پس از پیچش/دتورشن بود. ۳۵ سر موش صحرایی نر به طور تصادفی به پنج گروه شامل شم، ایسکمی، ایسکمی + ویتامین B12 (۴ میلی‌گرم/کیلوگرم داخل صفاقی)، ایسکمی-دتورشن و ایسکمی-ریپرفیوژن + ویتامین B12 (۴ میلی‌گرم/کیلوگرم داخل صفاقی) تقسیم شدند. سه ساعت ایسکمی (پیچش) و ۳ ساعت ریپرفیوژن در هر دو بیضه ایجاد شد. بعد از ۳ ساعت از ریپرفیوژن ، بافت‌های بیضه برای ارزیابی غلظت گلوتاتیون پراکسیداز (GPx)، سوپراکسید دیسموتاز (SOD)، مالون دی‌آلدئید (MDA)، و بیان فاکتور هسته‌ای کاپا B (NF-kB) جمع‌آوری شدند. در همه پارامترهای SOD، GPx و MDA به جز بیان NF-κB در گروه‌های تجربی در مقایسه با گروه شم تفاوت معنی‌دار وجود داشت ($p<0.001$). سطوح GPx و SOD بافتی به طور قابل توجهی بالاتر بود، در حالی که سطح MDA در گروه ایسکمی/ ریپرفیوژن + ویتامین B12 به طور قابل توجهی کمتر از گروه ایسکمی/ ریپرفیوژن بود. این مطالعه نشان داد که ویتامین B12 می تواند ساختار بیضه را در برابر آسیب خونرسانی مجدد ایسکمی حفظ کند. علاوه بر این ویتامین B12 التهاب و استرس اکسیداتیو مرتبط را کاهش داد.

واژه های کلیدی: ویتامین B12، پیچ خوردگی بیضه، ایسکمی/ریپرفیوژن، آسیب اکسیداتیو

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INTRODUCTION

Acute scrotum syndrome is a urological emergency, the symptoms of which include sudden pain, redness, and swelling of the scrotum. Twisting of the spermatic cord on itself accounts for approx. 35-40% of the pathological cases of the scrotum. In this uropathology, the testicular cord twists around its axis and disrupts testicular perfusion. Tissue damage is caused by blocked and impaired perfusion to the testicular parenchyma [1]. The primary and essential germ cell structures, such as intracellular genomic content, proteins, and membrane lipids, are disrupted by reperfusion after torsion, which has a negative impact on sperm quality and natural production [2]. In this mechanism, oxygen free radicals produced during ischemia-reperfusion are crucial. Lipid peroxidation in cell membranes and mitochondria is brought on by an excess of oxygen free radicals; nevertheless, lipid peroxidation alters membrane permeability and compromises the integrity of cell membranes [3]. One of the methods to reduce oxidative stress on torsion of the scrotum s the use of antioxidants [1]. The compound alpha-(6,5-dimethylbenzimidazolyl) cobamide cyanide, which is known as vitamin B_{12} or cyanocobalamin, as a coenzyme plays a role in several important enzyme systems, namely methyl malonyl-COA isomerase, leucine mutase, each of which requires adenosylcobalamin, and the third one is methionine synthetase, which requires methylcobalamin. Vitamin B_{12} is vital in the metabolism of nucleic acids and proteins and participates in the metabolism of lipids and carbohydrates [4]. Vitamin B_{12} and its derivatives (cobalamin) have shown antioxidant activity in medicinal concentrations and reduced the level of reactive oxygen species (ROS) [5]. The balance between methionine as an essential amino acid and homocysteine (Hcy), synthesized by methionine synthetase or betaine homocysteine methyltransferase (BHMT), is always maintained in mammals. These enzymes stop homocysteine from building up, which causes ischemia-reperfusion (I-R) harm when it accumulates in the heart, brain, and kidney after reperfusion [6]. Folate and vitamins B_6/B_{12} are important cofactors in homocysteine metabolism [1]. Subclinical B_{12} deficiency reduces Hcy conversion into methionine and consequently contributes to the increase of intracellular Hcy [1]. It is believed that Hcy manages ROS accumulation through multiple mechanisms, such as the autoxidation of Hcy, leading to the production of H_2O_2 [7, 8]. Due to the effects of vitamin B_{12} on inflammatory indicators, this study aimed to investigate the protective effects of vitamin B_{12} on the I-R-induced damage of rat testicular tissue using biochemical indicators.

MATERIALS AND METHODS

Animals

35 adult healthy male rats 3-4 months old (weight 200 ± 20 g) were provided from a certified laboratory animal center (Tabriz, Iran). The animals were housed in polypropylene cages under a temperature-controlled, 12-hour light/dark cycle and free access to food and water.

Rats were randomly divided into five groups:

- Sham group (n = 7): After anesthetizing all the experimental animals, their scrotums was surgically opened and then closed.

- Ischemia group, IS (n = 7): The scrotum of the anesthetized rats was surgically opened and both testes underwent

ischemia for 3 hr. by a 720° rotation, then samples were collected.

- Ischemia-Vitamin B_{12}, IVit (n = 7): by testicular rotation, followed by the same procedure as the second group.

- Ischemia-reperfusion, IR (n = 7): was subjected to reperfusion for 3hr by modifying the testis rotation after ischemia induction similar to Group 2.

- Ischemia-reperfusion-vitamin B_{12}, IRVit (n = 7): was administered with a dose of 4 mg/kg IP injection 1 hr. after administration of vitamin B12 similar to Group 3.

Experimental protocol

All animal procedures and experiments were performed in accordance with the Islamic Azad University Code of Practice for the Care and Use of Animals and approved by the research ethics committees of Islamic Azad University-Tabriz Branch. In all of the groups, in order to create ischemia in the IR group, each testis was twisted 720 and fixed to the inner wall of the scrotum with nylon suture for 3 hr. For reperfusion, the suture was removed after 3hr and detorsion was performed. The animals received an intraperitoneal injection of 4 mg/kg vitamin B_{12} (PBJ Pharma, 1000 μg/4 mL) daily for 1 week before ischemia induction, in the IVit and IRVit groups. After reperfusion, animals in all groups were sacrificed by decapitation and their testes were removed. All procedures were performed following injection i.p. of Ketamine 50 mg/kg and Xylazine 5 mg/kg anesthesia; additional injections of the same anesthetic were administered as needed during the procedures. Obtained tissue samples were subjected to biochemical analyses.

The left testes were sent to the laboratory to test the concentrations of superoxide dismutase (SOD), glutathione peroxidase (GPX), malondialdehyde (MDA), and nuclear factor kappa B (NF-κB) expression.

Tissue preparation for oxidative stress biomarkers

A method for tissue preparation is to perfuse the tissue with PBS solution with pH 7.4 containing 0.16 mg/ml of heparin before dissection to dissolve red blood cells and clots. The tissue was then homogenized in 5-10 mL of cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) per gram of tissue. It was centrifuged at 10,000 xg at 4 °C for 15 min and the supernatant was extracted for biochemical analysis.

Measurement of GPX activity

The GPx activity was measured by a colorimetric assay kit (ZellBio GmbH, Germany). The enzymatic reaction was initiated by the addition of cumene hydroperoxide (CuOOH) to the reaction mixture containing GSH, NADPH, EDTA, $NaNO_3$, and glutathione reductase. The change in the absorbance at 340 nm was monitored.

Measurement of SOD

Measurements were performed according to the method described by Sun et al [9]; when xanthine is converted into uric acid by xanthine oxidase, SOD forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT and a purple-colored formazan dye appears. The sample was weighed and homogenized in 2 ml of 20 mmol/l phosphate buffer containing 10 mmol/l EDTA at pH 7.8. The sample was centrifuged at 6000 rpm for 10

min, finally the resulting brilliant supernatant was used as an assay sample. The measurement mixture containing 2450 μL measurement mixture (0.3 mmol/l xanthine, 0.6 mmol/l EDTA, 150 μmol/l NBT, 0.4 mol/l Na2CO3, 1 g/l bovine serum albumin), 500 μL supernatant, and 50 μL xanthine oxidase (167 U/l) was vortexed. It was then incubated for 10 min. At the end of the reaction, formazan appeared. The absorbance of the purple-colored formazan was measured at 560 nm. The more enzymes present, the less O2− radical that reacts with NBT appears [10].

Measurement of MDA

To measure MDA, 500 μl of the tissue mixture was dissolved in 3 ml of 1% phosphoric acid. After the vortex, 1 ml of a 0.67% thiobarbituric acid solution was added to the test tube, vortexed completely, and heated inside a boiling bain-marie for 45 min. After the required period, the test tubes were cooled under cold water, 3 ml of normal butanol was added to the tubes, vortexed for 1-2 min, and then centrifuged at 3000 rpm for 10 min. The organic phase (supernatant) was separated and the optical density was measured at a wavelength of 532 nm against normal butanol as a blank. The MDA concentration of the tissue mixture of the samples was determined after transferring the results to a standard curve.

Measurement of the NF-κB

NF-κB protein expression was verified in testis tissue samples by Western blot, and β-actin was used as a loading control. Protein aliquots (50 μg) from ovaries treated with VCD, VCD, and ginseng or with no treatment were loaded onto a 12% sodium dodecyl sulfate gel, and electro transferred to a PVDF membrane. Membranes were blocked with 10% nonfat dried milk. The membrane was incubated with an Anti-NF-kB p65 antibody (ab1652; abcam, Boston, MA, USA, 1: 1000) at 4∘C for 12h. Blots were incubated with HRP-conjugated IgG (sc-2357, Santa Cruz Biotechnology, Boston, CA, USA, 1: 1000) at room temperature for 1 h. The NF-kB protein was detected by chemiluminescence.

Statistical analysis

Statistical analysis was performed using the SSPS v. 23.0 statistical software (SPSS Inc., Armonk, NY, United States). Means ± SD were subjected to the one-way ANOVA test. Values for $p \leq 0.05$ were considered statistically significant.

RESULTS

GPx

The tissue levels of GPx decreased significantly in all groups undergoing torsion surgery compared to group C. GPx levels were similar in the IR, IRVit, and IS groups. Compared to the IR and IRVit groups, GPx levels in the testicular tissue increased in the ISVit group ($p < 0.01$) (Table 1).

MDA

A comparison of the results showed that the levels of MDA increased significantly in all experimental groups compared to group C ($p < 0.001$). The levels of MDA were similar in the IR and IS groups. However, of administration of vitamin B_{12} before IR treatment significantly alleviated ($p< 0.001$). The levels of MDA compared to the groups IS and IR. The lowest MDA level belonged to the ISVit group ($p < 0.001$) (Table 1).

SOD

Table 1 shows that SOD levels decreased significantly in all experimental groups compared to the group C ($p < 0.001$). The SOD activity significantly was decreased in the groups IS, IR, and IRVit compared to the ISVit group ($p < 0.001$). The lowest SOD level was measured in the IR group. SOD levels in IS, IR, and IRVit groups were similar (Table 1).

Table 1: Effects of torsion/detorsion and administration of Vit B12 on MDA level and SOD and GPx activities of testis tissue.

group	GPx (U/mg protein)	SOD (U/mg protein)	MDA (µmol/ mg protein)
C	17.27 ± 1.01 [s]	9.50 ± 0.96 [a]	1.00 ± 0.17 [d]
ISVit	12.17 ± 1.70 [b]	5.70 ± 0.60 [c]	2.07 ± 0.15 [c]
IRVit	8.30 ±1.65 [c]	5.00 ± 0.75 [c]	2.56 ± 0.15 [b]
IS	10.13 ± 0.95 [bc]	7.53 ± 1.19 [b]	3.10 ± 0.20 [a]
IR	8.30 ± 2.06 [c]	4.28 ± 0.81 [c]	3.47 ± 0.50 [a]
Sig.	0.001	0.001	0.001

[a-c] $p < 0.001$ compared with sham

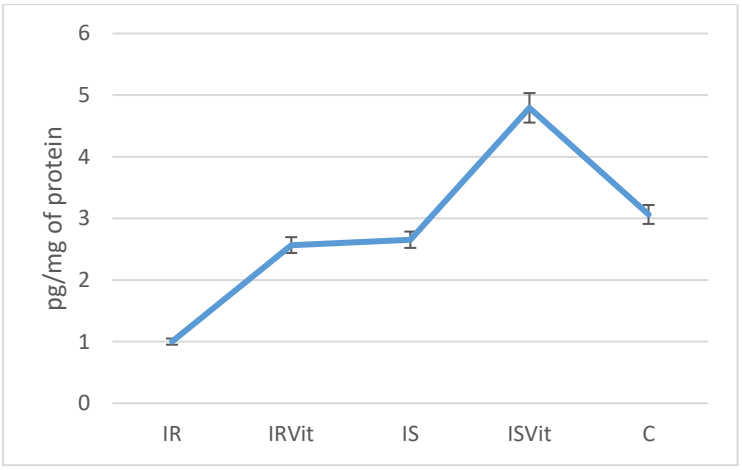

Figure 1: NF-κB protein expression average values by groups.

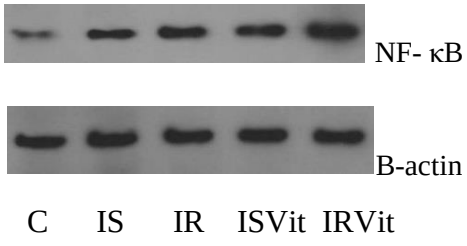

Figure 2: NF-κB and B-actin protein expression, western blotting band from tissue.

NF-κB levels in testicular tissue

The results of NF-κB protein expression in all treatments are shown in Figure 1. The NF-κB protein expression increased non-significantly in all experimental groups compared to group C. The use of Vit B_{12} decreased NF-κB protein expression in both IS and IR groups (Figure 2).

DISCUSSION

IR damage in several organs has been linked to an excess of reactive nitrogen species and ROS. Particularly vulnerable to IR damage are the lungs, liver, heart, gut, and kidney. O_2 is unavailable during ischemia for use as a reactant in non-enzymatic processes or as a terminal electron acceptor in the respiratory chain of the mitochondria. Mitochondrial function is restored upon reperfusion, but it produces plenty of ROS. Free radicals caused by the oxidation of unsaturated lipids and cholesterol in membranes and lipoproteins are currently known as the most IR-induced destructive process [11]. Testicular torsion causes tissue damage, including decreased germinal epithelium thickness, the diameter of seminiferous tubules, and the Ranson score, as well as a decrease in sperm count and an increase in abnormal sperm. In terms of oxidative stress factors, testicular ischemia increases MDA levels and decreases SOD, GPx, and CAT activity [12]. Shokohi et al. (2018) showed that the excessive ROS levels released in the tissues during reperfusion of hypoxic tissues led to oxidative stress in the testicular parenchyma, damage to the cellular genome content, and apoptosis induction through the activation of caspase cascades; all these changes directly resulted in an increase in necrosis in testicular tissue [13]. The damage following IR leads to decreased TAC, GPx activity, and increased MDA levels, as well as greatly reduced spermatogenesis indices and elevated damage to the cell genome content [14]. Spermatozoa are adversely affected by IR damage in the testis, which is directly linked to elevated lipid and protein peroxidation and reduced SOD antioxidant enzyme activity [1]. The SODs convert superoxide anion into hydrogen peroxide (H2O2), which is a key component of the antioxidant defense system [15]. A wide spectrum of agents have been used in the literature for IR damage including; Vit E, Curcumin, N-acetyl cysteine, and Vit B12 [16, 17]. In mammals, vitamin B_{12} is an essential component of two enzyme systems involved in several metabolic reactions, such as the metabolism of carbohydrates, lipids, some amino acids, DNA synthesis, adenosylcobalamin, and methylcobalamin, as well as coenzymes of methylmalonyl coenzyme, Amutase, and methionine synthetase enzymes [18]. Vitamin B_{12} stimulates the conversion of homocysteine to methionine, and vitamin B_{12} deficiency causes an increase in homocysteine levels, which is easily oxidized to hydrogen peroxide, thereby increasing ROS in the body. Due to its role in homocysteine metabolism, therefore, it indirectly protects against oxidative stress. Vitamin B_{12} plays a key role in the oxidative stress cycle. Subclinical vitamin B_{12} deficiency causes ROS production, which in turn disrupts vitamin B_{12} absorption. Oxidative stress can contribute to the formation of glycosylated end products, which can reduce vitamin B_{12} absorption [19]. Testicular IR dramatically lowered the levels of SOD and GPx in testicular tissue in the current investigation. Conversely, testicular tissue samples from the IR groups showed considerably higher levels of MDA and NF-κB than those from the sham group. This was likely due to the heightened ROS levels generated by ischemia.

CONCLUSION

Our results obtained through MDA, GPx, and SOD evaluation confirm that ischemia reduces oxidative stress by increasing lipid peroxidation and reducing total antioxidant power. Through oxidative stress induction and lipid peroxidation, ischemia causes a decrease in oxidative phosphorylation enzymes and ATP depletion in the cell, ultimately leading to a decrease in cell viability..

ETHICS

The study was approved by research ethics committees of Islamic Azad University-Tabriz Branch (Ethical number IR.IUA.TABRIZ.REC.1402.194).

CONFLICT OF INTEREST

None.

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