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Protective Effect of Obestatin on Testicular Ischemia/Reperfusion Injury in Rats

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Abstract

Background: Testicular torsion (the most common cause of testicular ischemia) is one of the urologic emergencies occurring frequently in neonatal and adolescent period. Although reperfusion of the testis is the key treatment after ischemia, it enhanced the formation of reactive oxygen species (ROS) that result in testicular damage and cell apoptosis. The effect of obestatin on testicular ischemia-reperfusion injury has not been evaluated previously.

Aim of Study: This study was delineated to investigate the potential protective effect of obestatin on testicular ischemia/reperfusion (I/R) injury in rats.

Material and Methods: 30 healthy adult male albino rats weighting 194-217g were involved and divided into 3 equal groups, group (I): Sham operated control group, group (II): Testicular ischemia reperfusion (I/R) group and group (III): Obestatin treated ischemia reperfusion (I/R-obestatin) group; received obestatin (100 µg/kg) intravenously 15min before the testicular detorsion (reperfusion). Serum testosterone level was measured and orchiectomy was performed 6 hours after testicular detorsion and examined histopathologically and immunohistochemically for evaluation of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins expression. Testicular malondialdehyde (MDA), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) levels and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), caspase-8 and caspase-3 activities were also evaluated.

Results: This study demonstrated that administration of obestatin prior to detorsion significantly increased the reduced serum testosterone level and attenuated the testicular tissue damage observed in I/R group. It also reversed the increased MDA, TNF-α and IL-1β levels and significantly enhanced the antioxidant enzymes activities in the ipsilateral I/R testis. Moreover, it improved the significant high levels of caspase-8 and caspase-3 and alleviated the increased Bax and the reduced Bcl-2 proteins expressions noticed in I/R testis.

Conclusion: Obestatin has a protective effect against testicular I/R injury which can be attributed to its anti-oxidant, anti-inflammatory and anti-apoptotic properties.

Key Words: *Obestatin – Reperfusion injury – Testis – Oxidative stress.*

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Introduction

TESTICULAR torsion is one of the emergency conditions which requires immediate surgery to reperfuse the affected testis, however, attempt to reperfuse ischemic tissue may cause further damage to the testis. Many studies reported a loss of germ cells and disruption of the seminiferous epithelium after ischemia reperfusion (I/R) injury of the testis **[1,2]**.

Overgeneration ROS is thought to play a critical role in I/R injury **[3]**. Toxic free oxygen radicals, such as nitric oxide (NO^-), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) can lead to lipid peroxidation, protein denaturation, DNA damage and apoptosis **[4]**. Testes, also, are more sensitive to free radical damage due to their high content of polyunsaturated fatty acids **[5]**.

Moreover, Shih et al., **[6]** demonstrated the role of cytokines and infiltration of activated polymorphonuclear leukocytes in ischemia reperfusion injury in rats. The release of cytokines such as IL-1β and TNF-α after reperfusion results in recruitment of neutrophils and macrophages, causing germ cell apoptosis, disruption of spermatogenesis and testicular atrophy **[7]**.

In addition, as a consequence of testicular I/R-induced ROS generation, the caspase-dependent apoptosis pathways are activated leading to germ cell death. High levels of the initiator caspase-8 and the executioner caspase-3 were increased during testicular I/R **[8]**.

On the other hand, information from the literature indicates that antioxidant and anti-inflammatory treatment is inevitable before and after surgical intervention in I/R injury **[9]**. Obestatin is a circulating 23-amino-acid peptide hormone, encoded by the same gene as ghrelin and predom-

inantly produced in the stomach [10]. Apart from the stomach, obestatin expression was also found in other tissues, such as the endocrine pancreas, adipose tissue, liver, skeletal muscle and the male reproductive system [11].

Moreover, it has been demonstrated that obestatin can attenuate ischemia-reperfusion injury in other tissues subjected to I/R insults via reducing oxidative stress and inflammatory process and improving antioxidant activity [12,13]. However, to our knowledge, no studies have examined the effect of obestatin on testicular ischemia-reperfusion injury.

So, this study was designed to investigate whether obestatin can protect the testis from ischemia-reperfusion injury in rats. In an attempt to elaborate the mechanisms of the potential protective effects of obestatin, we investigated its effect on the redox status of the testes by assessing the levels of lipid peroxides and antioxidant enzyme activities in testes. We also assessed its probable anti-inflammatory and anti-apoptotic effects through estimation of some testicular inflammatory and apoptotic markers.

Material and Methods

The present study was carried out in Faculty of Medicine, Zagazig University, in the period from February to June 2019 and involved 30 healthy adult male albino rats weighing 194-217g (derived from the experimental animal services house of Faculty of Pharmacy). Rats were kept for 1 week for habituation in clean standard cages in groups of five. They received food and water ad libitum and housed at room temperature and in a 12h light/dark cycle. The experimental protocol was approved by the Institutional Research Board (IRB) and the ethics committee of Faculty of Medicine, Zagazig University.

Rats were equally divided into three groups: Group (I): Control group, rats in this group underwent sham operation without torsion and received 1Ml Normal Saline Intravenously, Group (II): The ischemia/reperfusion (I/R) injury group which underwent 2 hours of testicular torsion (ischemia) followed by 6 hours of detorsion (reperfusion) and received 1ml normal saline intravenously 15min before detorsion, and group (III): Obestatin treated I/R injury group (I/R-obestatin) which received obestatin (lyophilized powder form Sigma Aldrich Co.-USA) at a dose of 100 µg/kg intravenously 15 min before the testicular detorsion [12].

Surgical ischemia reperfusion (torsion / detorsion): Rats were anesthetized by intraperitoneal administration of 2% sodium phenobarbital (50mg/kg). The skin of scrotal area was shaved and then prepared with 10% povidone iodine solution. A right-sided mild scrotal vertical incision was performed to access the testis. Torsion was created by twisting the right testis 720° in a counter-clockwise direction and maintained by fixing it to skin of scrotum with a 4/0 silk suture. The testis was detorsioned to the natural position after 2 hours of torsion, and the scrotal skin was closed with 4/0 silk. During sham operation, the testis was localized through a right-sided scrotal incision, and then the incision was sutured with 4/0 silk without additional intervention [2].

Sample collection: After 6 hours of detorsion, blood samples were collected from the orbital sinus and serum was separated by blood centrifugation at 3000 rpm for 15 minutes and kept at 20ºc until measurement of serum free testosterone level. After blood sampling, laparotomy was done after sacrificing the rats by cervical dislocation under mild ether anesthesia. The right testis of each rat was removed and divided into two halves, with one half fixed in Bouin's fixative for 24 hours for histopathological and immunohistochemical examination, and the other half immediately frozen at –80°C then homogenated for biochemical analysis of malondialdehyde (MDA), tumor necrosis factor-α (TNF-α) and interleukin1 β (IL-1β) levels and superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), caspases-8 and -3 activities [2].

Preparation of testis homogenate: The testes halves were sliced into small pieces, and then homogenized in 1ml physiological saline. The homogenates were centrifuged at 20,000g-force for 30min at 4°C. The supernatants were collected and stored at 20°C until later analysis [14].

Serum free testostosterone level:

Serum free testostosterone level was measured using testosterone ELISA kit (BioCheck, Dr. Foster City, CA 94404) as described by Tietz, [15].

Evaluation of testicular redox status:

MDA level and SOD, CAT and GPX activities were measured using specific ELISA kits (Sigma Aldrich Co.-USA) according to Ohkawa et al. [16], Kakkar et al. [17], Luck [18] and Reddy et al. [19] respectively.

Measurement of testicular inflammatory biomarkers:

IL-1β and TNF-α concentrations were quantified according to the manufacturer's instructions

and guidelines using ELISA kit (Bio diagnostic company, Egypt).

Evaluation of apoptosis markers:

Activities of caspases-8 and -3 were measured using their specific colorimetric assays following their manufacturer's recommendations (Sigma Aldrich Co.-USA).

Histopathological examination:

Ipsilateral testes halves from all groups were fixed in Bouin's solution and followed by dehydration in a descending series of ethyl alcohol, then cleared in xylene and embedded in paraffin. Paraffin sections of testes were cut at 5 µm on a rotary microtome, mounted on slides and stained with hematoxylin eosin (H&E) and examined under a light microscope equipped with a digital camera [20].

Immunohistochemical study:

Paraffin sections were deparaffinized in xylene and rehydrated using ascending grades of alcohol and used to evaluate the expression of Bax and Bcl-2 proteins using monoclonal Bax and monoclonal Bcl-2 antibodies respectively (Sigma Aldrich Co.-USA) as described by Zhou et al., [21].

Statistical analysis:

Study results were represented as mean ± SD. The Statistical Package for the Social Sciences (SPSS), version 19.0 (SPSS Inc., Chicago, IL, United States) was used for performing the statistical analysis. Analysis of variance (ANOVA) followed by LSD post hoc test was performed to compare means of the different groups. p-values <0.05 were considered statistically significant.

Results

Testicular I/R resulted in histological changes in terms of disruptions of spermatogonia throughout the lumen of seminiferous tubules with spermatogenic arrest and expansion of interstitial tissue (Fig. 1B). However, treatment with obestatin resulted in a remarkable improvement in the structural and cellular morphology of the seminiferous tubules and interstitial spaces (Fig. 1C).

Serum free testosterone level was also decreased in I/R group compared to control, but this effect was improved by obestatin (p<0.001). In addition, it was observed that MDA, IL-1 β and TNF-α testicular levels, caspase 3 and 8 activities (p<0.001) and Bax protein expression were increased, while SOD, CAT and GPX activities (p<0.001) and Bcl-2 expression were decreased in I/R group. These effects were reversed by obestatin in I/R-obestatin group (p<0.001) (Table 1 and Figs. 2 & 3A-C).

Table (1): Serum free testosterone level and testicular TNF- α, IL-1 β, MDA, SOD, CAT, GPX, caspase-8 and caspase-3 in all studied groups.

Groups / Parameters	Group I	Group II	Group III
Serum free testosterone (ng/ml):			
X ± SD	4.82±0.40	1.02±0.17	3.13±0.49
p-value of LSD		p<0.001 **a**	p<0.001 **a,b**
TNFα (pg/mg protein):			
X ± SD	32.01±3.23	66.38±8.24	42.51±3.35
p-value of LSD		p<0.001 **a**	p<0.001 **a,b**
IL-1β (pg/ mg protein):			
X ± SD	34.33±3.47	57.3±4.74	39.80±3.77
p-value of LSD		p<0.001 **a**	p<0.01 **a**, p<0.001 **b**
MDA (nmol/mg protein):			
X ± SD	112.37± 6.26	198.26±16.93	133.91±7.19
p-value of LSD		p<0.001 **a**	p<0.001 **a,b**
SOD (U/mg protein):			
X ± SD	72.58±7.55	34.85±5.27	61.30±7.36
p-value of LSD		p<0.001 **a**	p<0.01 **a**, p<0.001 **b**
CAT (U/mg protein):			
X ± SD	16.98±2.36	7.28±1.62	12.76±1.41
p-value of LSD		p<0.001 **a**	p<0.001 **a,b**
GPX (U/mg protein):			
X ± SD	22.17±2.28	13.92±2.49	18.99±1.27
p-value of LSD		p<0.001 **a**	p<0.01 **a**, p<0.001 **b**
Caspase-8 (nmol/min/ml):			
X ± SD	0.41±0.06	2.95±0.61	1.23±0.31
p-value of LSD		p<0.001 **a**	p<0.001 **a,b**
Caspase-3 (µmol/min/ml):			
X ± SD	9.18±0.90	16.17±1.62	11.93±0.83
p-value of LSD		p<0.001 **a**	p<0.001 **a,b**

a = Significant versus group I. **b** = Significant versus group II.

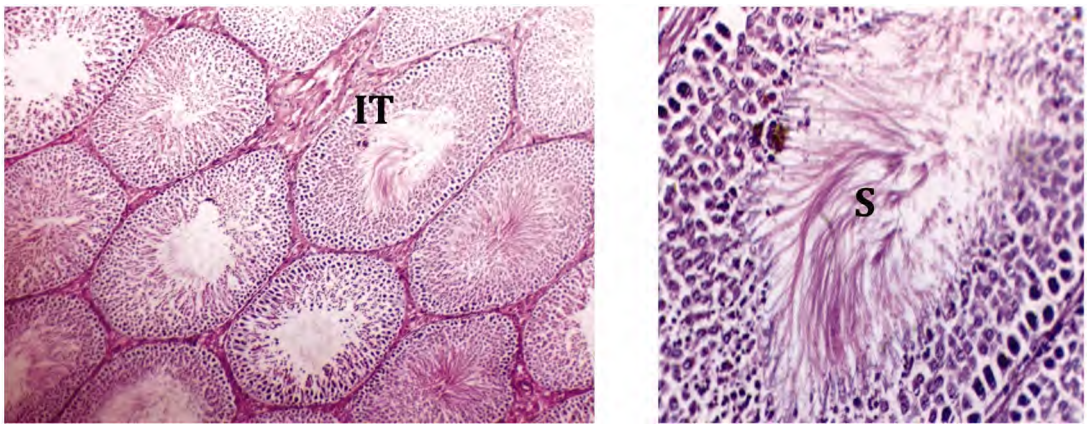

Fig. (1A): Testicular section from control group showing normal seminiferous tubules with normal spermatogenesis up to sperm formation (S) and normal interstitial tissue (IT) (H&E staining, Magnification X10 inset X40).

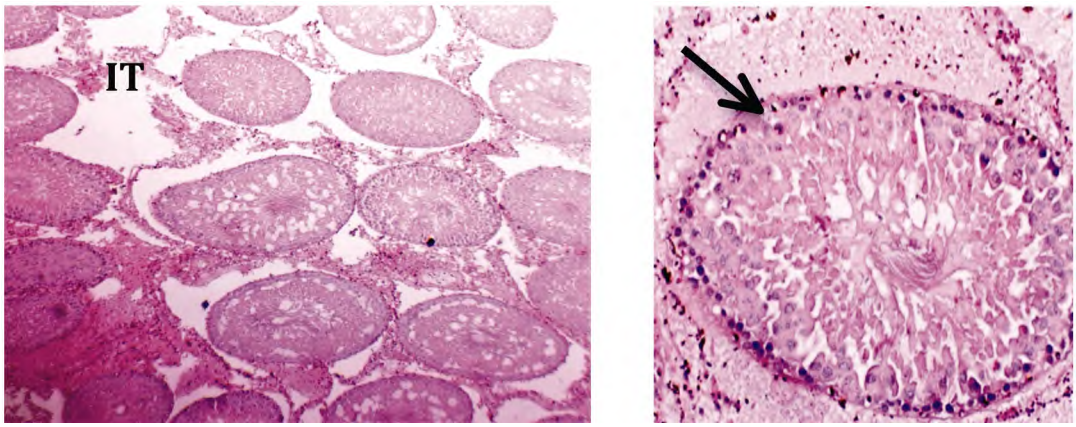

Fig. (1B): Testicular section from ischemia-reperfusion group showing disarrangement of spermatogonia throughout the lumen of seminiferous tubules with spermatogenic arrest (black arrow) and expansion of IT (H&E staining, Magnification X10 inset X40).

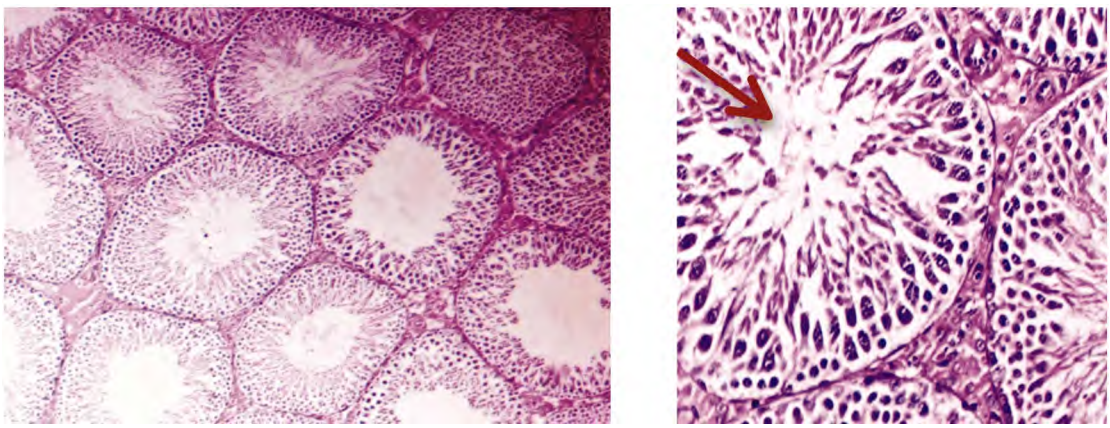

Fig. (1C): Testicular section from obestatin treated I/R group showing mild disruption of germ cell layers with recovery of spermatogenesis and spermatid formation (red arrow) (H&E staining, Magnification X10 inset X40).

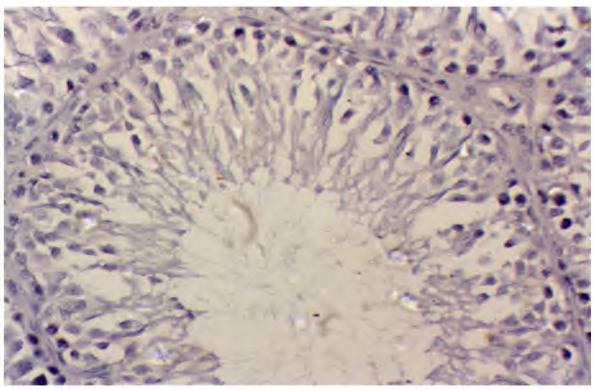

Fig. (2A): Testicular Bax immunohistochemistry from control group showing focal weak Bax expression in testicular control tissue (x400).

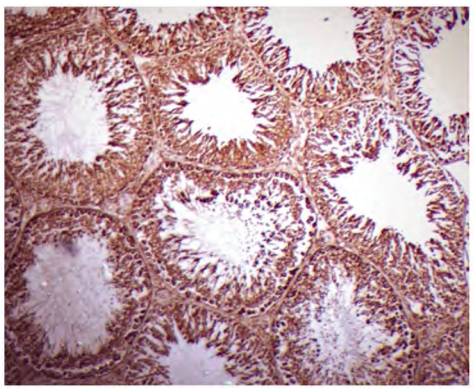

Fig. (3A): Testicular Bcl-2 immunohistochemistry from control group showing high Bcl-2 expression in testicular tissue (x200).

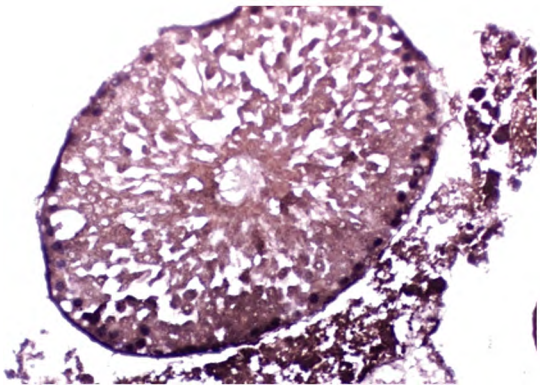

Fig. (2B): Testicular Bax immunohistochemistry from Ischemia reperfusion group showing marked Bax expression in testis exposed to I/R (x400).

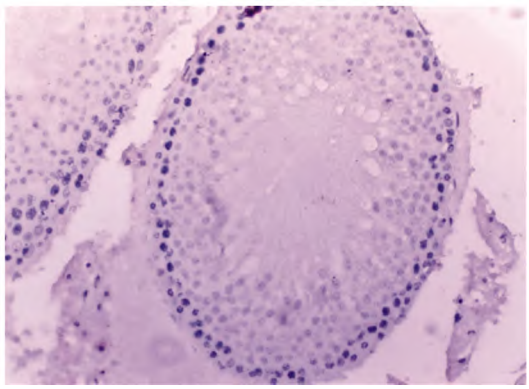

Fig. (3B): Testicular Bcl-2 immunohistochemistry from Ischemia reperfusion group showing absence of Bcl-2 in testis exposed to I/R (x400).

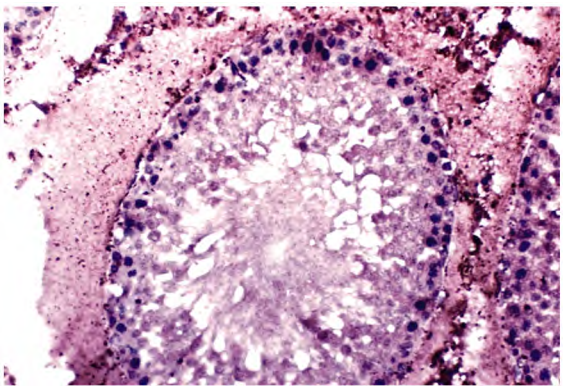

Fig. (2C): Testicular Bax immunohistochemistry from Obestatin treated I/R group showing decreased Bax expression in testicular tissue (x400).

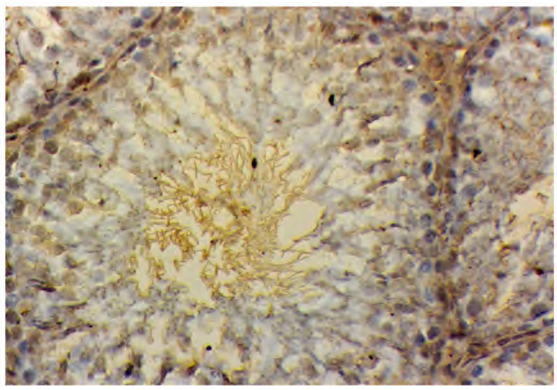

Fig. (3C): Testicular Bcl-2 immunohistochemistry from Obestatin treated I/R group showing increased Bcl-2 expression in testicular tissue (x400).

Discussion

Testicular torsion is one of the pediatric emergency conditions which require immediate surgical detorsion within 4-6 hours to avoid loss of testicular function [22]. Even if testicular torsion is corrected within this time period, testicular atrophy may develop subsequently [23]. The main cause of tissue injury after I/R is overproduction of ROS [24]. Several enzymes and drugs that were intended to inhibit oxidative stress were used to treat testicular reperfusion injury [25]. Obestatin has been reported to protect against I/R injury in other tissues [12,13]. Therefore, we attempted to use it for the treatment of testicular ischemia-reperfusion injury.

In the present study, untreated rats that were subjected to 2 hours of ischemia (torsion) followed by reperfusion (detorsion) for 6 hours showed a significant testicular damage which led to decrease in serum testosterone level. These results are similar to that demonstrated by other studies [2,26]. However, it was noticed that administration of obestatin prior to detorsion significantly increased serum testosterone level and attenuated testicular tissue damage after testicular I/R.

During the ischemic phase of I/R process, hypoxia lead to reduced tissue ATP production, increased calcium influx into the intracellular compartment, elevating superoxide generator enzyme in addition to chemotactic factors stimulation with polymorphonuclear leukocytes migration to the ischemic region, which could generate superoxide radicals after reperfusion that aggravate tissue damage [27].

The decline in free testosterone level could be due to reduced Leydig cell function. I/R injury plays a role in Leydig cell dysfunction, either acting directly, causing germ cell apoptosis or indirectly by inducing oxidative stress [28].

Furthermore, the present study confirms that testicular I/R increases oxidative stress as shown by the significantly increased MDA level and the significantly decreased antioxidant enzymes activities (SOD, CAT and GPX) in I/R group. Ischemia causes an increase in intracellular hypoxanthine as a result of ATP breakdown and then, during reperfusion, xanthine oxidase converts hypoxanthine to uric acid plus large quantities of superoxide radicals in the presence of oxygen [29].

It is widely accepted that ROS has a significant role in the testicular ischemia-reperfusion injury [30]. During I/R, the antioxidant enzyme levels rapidly decrease and ROS is produced and begins to damage various cellular molecules, contributing to further pathological complications [31].

On the other hand, obestatin treatment reduced MDA level in the ipsilateral testis and significantly enhanced the antioxidant enzymes activities. In accordance with our results the protective effect of obestatin in I/R injury in other tissues such as liver [12] and heart [13] by its antioxidant activity has been demonstrated.

Moreover, our results demonstrated increased testicular TNF-α and IL-1β in I/R untreated group, whereas obestatin treated I/R group showed a significant decrease in their levels in comparison to untreated group. Similarly, it has also been shown that obestatin decreases the pro-inflammatory factors level in colonic mucosa in rats with colitis [11]. This effect may be explained by previous reports which demonstrated that obestatin reduced the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in ischemia reperfusion [32]. NF-κB is a crucial nuclear transcription factor for the regulation of TNF-α, IL-1β, and IL-6 gene expression [33].

ROS stimulate the release and the formation of various inflammatory mediators with powerful chemotactic effect [34]. Neutrophil recruitment is also an important source of ROS production. The increase in proinflammatory cytokines after I/R facilitates, transmigration of neutrophils from endothelium into the testis interstitium, ROS overproduction, cellular dysfunction, and promotes apoptosis [35].

High apoptotic cell death may play significant roles in I/R cellular injury. Emerging data indicate that both distinct types of cell death, necrosis and apoptosis may take place simultaneously in oxidative conditions like I/R injury [30]. Apoptosis plays a significant role in maintaining homeostasis in spermatogenesis, however, testicular I/R injury usually leads to wide-spread spermatogenic cell apoptosis, which can cause impaired testicular function and male infertility [36].

High levels of the initiator caspase-8 and the executioner caspase-3 were increased in testicular I/R group in our study, but their levels were reduced by obestatin treatment in the treated I/R group. Caspase-3 is an inactive zymogen in the cytoplasm and the convergence point of multiple apoptotic pathways. Its activation is an irreversible sign to induce cell apoptosis, leading to cell shrinkage, chromatin condensation and DNA degradation [37].

In addition, the present study revealed a significant increase in Bcl-2 associated x (Bax) protein expression with a significant decrease in Bcl-2 expression (the second member of a range of proteins initially described in B-cell lymphoma; hence its nomenclature) in I/R testicular tissue, as measured by immunohistochemistry. The protective role of obestatin is also highlightened in the adjustment of Bax and Bcl-2 expressions in obestatin treated group. Bcl-2 is a prosurvival multidomain protein that regulates apoptosis by preventing the release of proapoptogenic factors from the mitochondria (e.g., cytochrome c) and subsequent caspase activation [14].

Other studies also demonstrated that obestatin administration decreased cellular apoptosis in other

degenerative diseases **[38,39]**. Zhang et al. **[13]** also showed that obestatin downregulated the expression of caspase-3 and Bax and upregulated the expression of Bcl-2 and can rescue cardiomyocytes from I/R-induced injury. When the apoptosis signaling pathway is activated, Bax, a pro-apoptotic protein, is translocated to the mitochondria with cytochrome C release that leads to the initiation of the apoptotic cascade. This can be prevented by the anti-apoptotic protein Bcl-2 via regulation of Bax **[40]**.

It has been demonstrated that the ratio of Bcl-2/Bax is crucial for normal spermatogenesis and determines whether apoptosis happens in cells exposed to damage **[41]**. Decrease in the Bcl-2/Bax ratio opens the mitochondrial permeability transition pore (MPTP), which further activates Caspase-3 and contributes to spermatogenic cell apoptosis following I/R injury in the rat **[2]**.

Conclusively, the present study demonstrated that obestatin has a benefit in reducing spermatogenic cell apoptosis and testicular damage that occurs after testicular ischemia reperfusion via attenuation of oxidative stress and inflammatory response and inhibition of the apoptosis pathway. Further clinical studies will be needed to evaluate its possible clinical applicability in patients with torsion.

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التأثير الوقائى للأوبستاتين على الإصابة بنقص وإعادة تروية الدم فى الخصية فى الجرذان

خلفية البحث: يعد التواء الخصية واحداً من حالات طوارئ المسالك التى تحدث بشكل متكرر فى فترة حديثى الولادة والمراهقين. وعلى الرغم من أن إعادة تروية الدم هو العلاج الأساسى بعد نقص التروية إلا أن زيادة تكوين الشوارد الحرة قد تؤدى إلى تلف الخصية وموت الخلايا. كما أنه لم يتم تقييم تأثير الأوبستاتين على الإصابة بننقص وإعادة تروية الدم فى الخصية من قبل.

الهدف من البحث: تم تصميم هذه الدراسة لدراسة التأثير الوقائى المحتمل للأوبستاتين على الإصابة بننقص وإعادة تروية الدم فى الخصية فى الجرذان.

مواد وطرق البحث: تم إجراء هذه الدراسة على عدد ثلاثين من ذكور الجرذان البيضاء البالغة التى يبلغ وزنها ١٩٤ ٢١٧ جم وقد تم تقسيمها بشكل عشوائى وعلى قدم المساواة إلى المجموعة الأولى التى إستخدمت كمجموعة ضابطة والمجموعة الثانية وهى مجموعة الإصابة بننقص وإعادة تروية الدم فى الخصية، والمجموعة الثالثة وهى مجموعة الإصابة بننقص وإعادة تروية الدم فى الخصية المعالجة بالأوبستاتين بجرعة ١٠٠ مايكروجرام / كجم أعطيت عن طريق الوريد قبل إعادة تروية الد م ب ١٥ دقيقة.

تم قياس مستوى هرمون التستوستيرون فى المصل وتم عزل الخصية بعد ٦ ساعات من إعادة تروية الدم لفحصها ميكرسكوبيا والتقييم المناعى للتعبير عن بروتينات Bax و Bcl-2 وقياس مستويات المالون داى الدهيد وإنترلوكين ١ بيتا وعامل نخر الورم ألفا وقياس نشاط سوبر أوكسيد ديسميوتيز والكاتاليز وجلوتاثايون بيروكسيداز وكاسباز ٨ وكاسباز ٣ فى الخصية.

النتائج: أوضحت نتائج الدراسة الحالية أن تناول عقار الأوبستاتين قبل إعادة تروية الدم أدى إلى تحسن ملحوظ وذو دلالة إحصائية فى الانخفاض الناتج لمستوى هرمون التستوستيرون فى الدم وتلف الأنسجة التى لوحظت فى مجموعة الإصابة بننقص وإعادة تروية الدم فى الخصية. كما أنه حسن مستويات المالون داى الدهيد وإنترلوكين ١ بيتا وعامل نخر الورم ألفا وعزز بشكل كبير من أنشطة الإنزيمات المضادة للأ كسدة فى الخصية. علاة على ذلك، فقد قام بتحسين المستويات المرتفعة من نشاط كاسباز ٨ وكاسباز ٣ وخفف من الزيادة فى بروتين Bax والانخفاض فى بروتين Bcl-2 اللذان لوحظا فى مجموعة الإصابة بنقص وإعادة تروية الدم فى الخصية.

الاستنتاج: الأوبستاتين له تأثير وقائى ضد الإصابة بنقص وإعادة تروية الدم فى الخصية والذى يمكن أن يعزى إلى خصائصه المضادة للأكسدة والإلتهاب والمضادة لموت الخلايا.