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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(2): 1202-1212. **OPEN ACCESS**

The histological and physiological potential of obestatin on testicular ischemia-reperfusion induced injury in adult albino rats

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Testicular ischemia reperfusion (IR) is a critical trouble in males in different ages. This trouble takes place due to the torsion and detorsion of testis. It has been manifested that obestatin have anti-inflammatory and protective effects on ischemia reperfusion injury in the kidney, brain and heart. However, its actions on testicular ischemia reperfusion injury have not been evaluated. We seek to examine the actions of obestatin on rat testicular ischemia/ reperfusion induced by torsion/ detorsion and the potential following mechanisms. In this study, we divided 40 adult male albino rats weighing (150-180) gm equally into 4 groups as follow: (I) control "sham" group"; (II) testicular ischemia reperfusion (IR) group, 2 hours of ischemia followed by 2 hours of reperfusion, (III) ischemia reperfusion group treated by saline (0.5 m) administered after ischemia reperfusion and (IV) ischemia reperfusion group treated by obestatin (8 nmol/Kg/body weight). After doing surgery to all rats, some testicular oxidative stress markers and serum free testosterone were measured and histopathologic study. Significant decrease in serum testosterone levels, glutathione peroxidase (Gpx) activity and testicular superoxide dismutase (SOD) and increase in tissue malondialdehyde (MDA) and increase in testicular weight were observed in ischemia reperfusion group comparing with the control group. These antioxidant levels were increased in obestatin treated groups with significant decrease in MDA levels. Also, obestatin increase serum free testosterone. These results manifested that obestatin guards against the damage caused by ischemia reperfusion on testicular structure and function when used for 7 days by dose of 8 nmol/kg/body weight through its anti-oxidant mechanism.

Keywords: obestatin, ischaemia, reperfusion, testis.

INTRODUCTION

Testicular torsion is mainly happen in mature men and leads to abnormally blood flow in testis (Asadi et al., 2017). It is one of the disaster cases which necessitate immediate surgery. However, testicular reperfuse after ischemia may cause extra damage. Testicular ischemia reperfusion (IR) injury leads to damage of germ cells and disruption of the seminiferous epithelium (Taati et al., 2016). The damage which caused due to IR is a result of Reactive oxygen species (ROS) produced throughout *IR* **(***Asadi* et al., 2017). ROS affect on cell replication and metabolism so, the tissue must use its antioxidant capacity (Gillham et al., 1997).

Avoidance of reperfusion injury using a combination of enzymes and drugs has been studied along with the assessment of histopathological changes after testicular torsion/detorsion (Akondi et al., 2011). For increasing the ability of body to remove all ROS, antioxidant supplements must be used (Asadi et

al., 2017). Curcumin and dexamethasone reduce IR injury and increase the activity of antioxidant enzyme systems (Wei et al., 2009).

Obestatin is a 23-amino acid peptide encoded by the same gene as ghrelin (Zhang et al., 2005). Obestatin has a special structure which is needed for binding to its receptor (Cowan et al., 2016). It is produced principally in stomach and induces the expression of genes mainly regulating pancreatic beta-cell differentiation and insulin biosynthesis (Zhao et al., 2008). Obestatin is localized in almost of all parts of the male reproductive system. In leydig cells of the testis, it is co-localized with preproghrelin (Zhao et al., 2008). It is expressed in epididymis, rete testis, vas deferens and seminal vesicles (Moretti et al., 2014).

The possible involvement of obestatin in the protection of renal, cardiac and intestinal tissues against IR injury via its anti- apoptotic and anti-oxidant has been proved (Akondi et al., 2011; Koç et al., 2014). In spite of obestatin anti-oxidative properties in different tissues, insufficient studies have been reported on testicular IR injury. Therefore, our present study was performed to elucidate the possible effects of obestatin on the testes of adult albino rats following an IR injury and its possible mechanism/s of action.

MATERIALS AND METHODS

Animals

Forty adult male albino rats weighing 150 to 180 gm were gained from the animal house of college of Veterinary Medicine, Zagazig University. Rats were protected in cages of steel in the Lab of Physiology, college of Medicine, Zagazig University. They were housed in a controlled temperature and lighting and they received water and food normally. They were fed the uniform commercial rat laboratory chow and were kept for 2 weeks to accommodate with the lab environment (Akondi et al., 2011). Rats were treated in a humane way according to the rules of the use and care of research animals. The experimental protocol was agreed by the Institutional Research Board (413/2017).

Experimental groups: Rats were separated into equal four groups:

Group I: Control (sham-operated) group: in this group, animals was subjected to sham operation without the application of the torsion and scarified 4 hours after operation.

Group II: ischemia-reperfusion (IR) group: in this group, the testicular torsion was prepared and lasted for 2 hours then detorsion for a new 2 hours (Dogan et al., 2016) and then scarified.

Group III: IR treated with saline group: in this group, rats were injected IV in their tails with a single daily dose of saline (0.5ml) for 7 days.

Group IV: IR treated with obestatin: testicular torsion/detorsion (Dogan et al., 2016). Rats were injected IV in rat tail with a single daily dose of obestatin (8 nMol/kg/body weight) dissolved in 0.5ml saline for 7 days. This dose of obestatin was selected after Bukowczan et al., 2015 who proved the great protective action of this dose in the pancreas IR.

In all groups, evaluation of testicular weight, testicular antioxidant system and testicular histopathological examination were done in addition to serum free testosterone.

Surgical procedure

Rats were injected with thiopental sodium (25 mg/ kg) subcutaneously for anesthesia (Dogan et al., 2016). We remove all hairs from the scrotal skin area and then a scrotal vertical cut was performed. At the stage of sham operation, the testis were brought through the cut and then replaced without twisting. Torsion was designed by twisting the testes 720° in a counter-clockwise direction and fixing it to scrotum by suture. After 2 hours of ischemia, we removed the suture and the testes were detorted and replaced into scrotum. After each surgery, the cut was closed using silk sutures (Dogan et al., 2016).

After an overnight fasting after the last injection, rats breezed ether for blood sampling from retro-orbital venous plexus and then they were allowed to clot for 2 hours at room temperature before centrifuging at approximately 3000 rpm for 10 minutes (Peters et al., 1996). The serum after separation was used for free testosterone level estimation. Animals were scarified and testes were collected. After weighing of the testes, the left ones were obtained for histopathological studies and the right ones were homogenated for biochemical estimations (Akondi et al., 2011).

Histopathological examination of testes

After carefully removing of tunica vaginalis of left testes, the testes were dissected out and cleaned with cold saline. Then they were put in Bouin's solution and followed by dehydration, then cleared in xylene and fixed in paraffin. 5 µm Paraffin sections of testes were cut and stained with hematoxylin – eosin (H&E) and examined under a light microscope (Murthy et al., 1988).

Biochemical investigations:

Serum free testostosterone levels were measured using testosterone ELISA kit (BioCheck,A 450)**.**

Testicular antioxidant system evaluation:

The right testes were sliced and homogenized in cold 50 mM phosphate buffer (pH 7.0) composed of 0.1 mM EDTA to make 10% homogenate (w/v). The homogenates were centrifuged for 10 min at 1000 r.p.m. The supernatants were divided and used for enzymes activity assays: Glutathione peroxidase (GP_X), Catalase (CAT), Superoxide dismutase (SOD) and lipid peroxidation levels determination (Schlorff et al., 1999).

STATISTICAL ANALYSIS:

The IBM SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) software was used. Data are offered by standard deviation and means. Comparisons between groups were done with one-way ANOVA and LSD post hoc test**,** Significance was accepted at P< 0.05.

RESULTS

Table 1: explain the results of serum free testosterone levels, testicular mass, MDA, GPx and catalase. Regarding serum free testosterone levels (pg/ml), results proved that the mean values of serum free testosterone levels in group II and group III were significantly low compared with group I (P< 0.001), whereas the mean value of group IV showed a non-significant difference comparing with control group (P> 0.05).

Regarding testicular mass, results proved that the mean values of testicular mass (gm) in group II were significantly higher than that of group I (P< 0.001), whereas the mean value of group III and group IV showed a non-significant difference compared with that of group I (P> 0.05). In addition, group III and group IV had a significant decrease in the testicular mass when compared to group II (P< 0.05), whereas group IV had a non-significant difference comparing with group III (P> 0.001).

Regarding MDA, results proved that the mean values of testicular MDA (nmol/1mg protein) in group II and group III were significantly higher than that of group I (P< 0.001), whereas the mean value of group IV showed a non-significant difference when compared with the control group (P> 0.05). In addition, group III and group IV had a significant decrease in testicular MDA comparing to IR group (group II) (P< 0.05), whereas group IV had a significant low level of testicular MDA comparing with group III (P< 0.001).

Regarding SOD, GPx and catalase, results proved that the mean values of SOD (U/mg protein), GPx activity (U/mg protein) and catalase (U/mg protein) activities in group II and group III were significantly lower comparing with group I (P< 0.001), whereas The mean values of group IV showed a non-significant difference comparing with control group (P> 0.05). In addition, group III had a significant decrease in the mean value of SOD, GPx & catalase comparing to IR group (group II) (P< 0.05), whereas group IV had a significant high level of testicular SOD, GPx and catalase comparing with group II (IR group) and group III (P< 0.001).

Table 1: serum free testosterone, testicular weight, MDA, SOD, CAT and GPx in all studied groups

Parameters		Group I	Group II	Group III	Group IV
Serum free testosterone (Pg/ml)	$\overline{X}$ ±SD	4.98±0.317	3.22±0.54	3.59±0.46	4.98±0.27
	P value of LSD		P < 0.001[a]	P < 0.001[a,b]	P < 0.001[b,c]
Testicular weight (gm)	$\overline{X}$ ±SD	1.092±0146	1.3±0.128	1.152±0.072	1.178±0.0954
	P value of LSD		p<0.001[a]	P<0.05[b]	P<0.05[b]
MDA (nmol/mg protein)	$\overline{X}$ ± SD	108.58±3.86	144.19±7.34	123.25±6.228	110.96±5.05
	P value of LSD		p<0.001[a]	P<0.001[a,b]	P<0.001[b,c]
SOD (U/mg protein)	$\overline{X}$ ±SD	71.79±2.923	53.75±8.49	44.79±6.42	72.33±3.815
	P value of LSD		p<0.001[a]	P<0.001[a] P<0.05[b]	P<0.001[b,c]
CAT (U/mg protein)	$\overline{X}$ ±SD	18.715±1.75	8.3±1.17	12.048±1.729	18.339±1.77
	P value of LSD		p<0.001[a]	P<0.001[a,b]	P<0.001[b,c]
GP_X (U/mg protein)	$\overline{X}$ ±SD	25.187±2.28	19.48±4.26	16.06±1.9	25.51±2.895
	P value of LSD		P<0.001[a]	P<0.001[a] p<0.05[b]	P<0.001[b,c]

a=significant VS group I, b= significant VS group II, c= significant VS group III

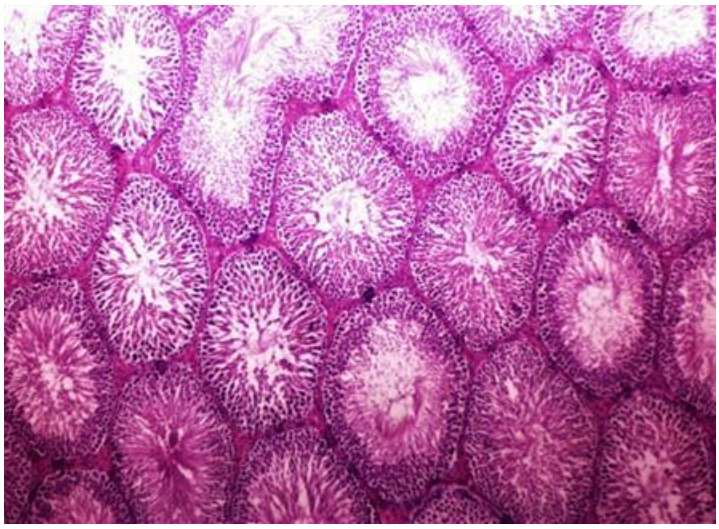

Figure1a: Photo microscopic picture of normal testis of control rate showing normal spermatogenesis and interstitial tissue (H&E X100).

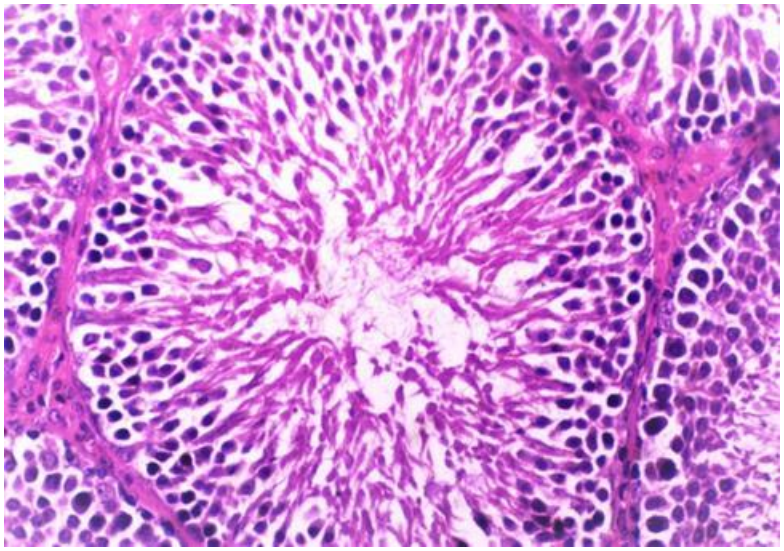

Figure1b: Photo microscopic picture (High power view) of the previous case showing normal spermatogenic maturation and normal interstitial tissue (H&E X400)

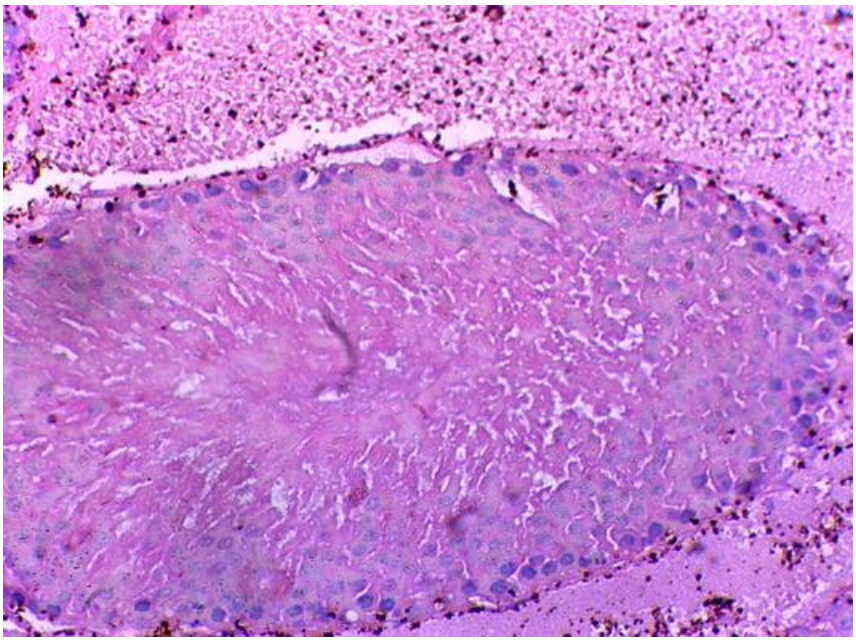

Figure 2: Photo microscopic picture (High power view) of the previous case showing disruption of tubule with necrosis of spermatogonia and interstitial hemorrhage (H&E X400).

Figure3a: Photomicroscopic of asection in testis with reperfusion showing mild disturbance of germ cell arrangement and regeneration of spermatogonia. (H&E X100).

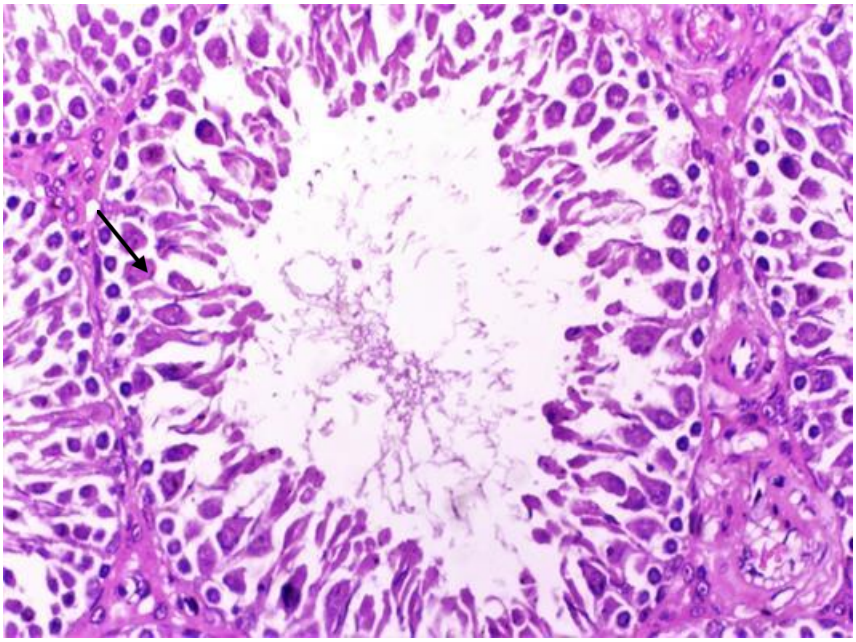

Fig.3b: Photo microscopic (High power view) of the previous case showing tubule with prominent Sertoli cells (arrow) and regeneration of some spermatogonia with arrest at 2ry spermatogonia. (H&E X400).

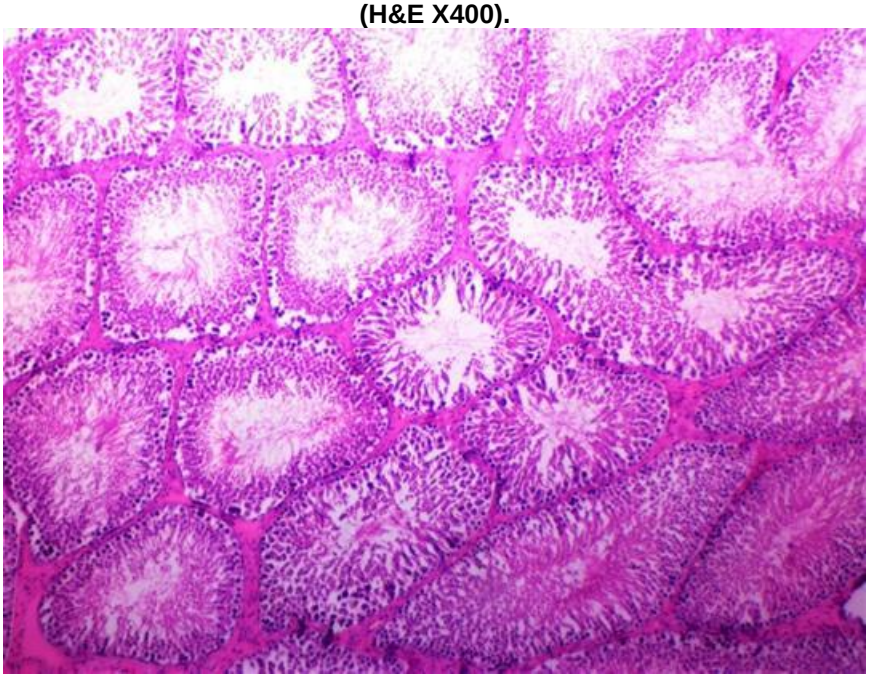

Figure 4a: Photomicroscopic of asection in testis with reperfusion showing regeneration of spermatogonia in most of tubules. (H&E X100).

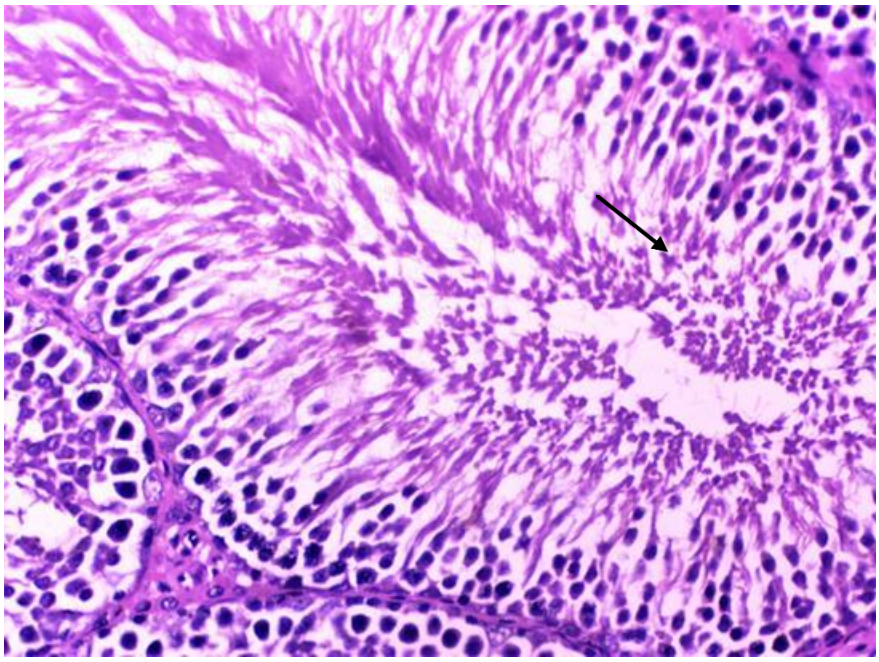

Figure 4b:Photomicroscopic (High power view) of the previous case showing regeneration spermatogonia more than non- treated group up to spermatid (arrow). (H&E X400)

Histological investigation:

Group I showed normal spermatogenic maturation and normal interstitial tissue, showed in figure (1a, 1b). Group II, "IR" showed disruption of tubule with necrosis of spermatogonia and interstitial hemorrhage and edema, showed in figure (2a, 2b). Group III" IR saline treated group" showed mild disturbance of germ cell arrangement and regeneration of spermatogonia with prominent Sertoli cells (arrow) and regeneration of some spermatogonia with arrest at 2ry spermatogonia, showed in figure (3a, 3b). Group IV "IR obestatin treated showed a regeneration of spermatogonia in most of tubules, showed in figure (4a, 4b).

DISCUSSION

Testicular torsion is a widespread urologic disaster in infants, children, and adolescents. It has been evaluated at 1 out of 160 to 4000 males under 25 years old (Lee et al., 2019). Reduction of oxygen supply due to ischemia results in germ cell death and oxidative stress. Reperfusion causes excessive production of reactive oxygen and nitrogen species causing cell and tissue injury (Abdel-Gaber et al., 2018). Testicular damage depends on the degree and duration of torsion and detorsion (Soltani et al., 2018). Rapid diagnosis and surgical detorsion are mandatory. Return of flow of blood after detorsion, may cause more damage (Wei et al., 2017). More studies reported that 30 min to 1 hour of torsion followed by 1 to 4 hours of detorsion is enough duration to successfully form an IR model (Ning et al., 2018). Therefore, we established a model in which 2 hours testicular torsion model followed by detorsion for 2 hours.

Obestatin is a recently discovered hormone and the majority of it, produced by stomach, has decreased the hazard of diabetes on testicular function (Khalefa and Raafat, 2017). (Bukowczan et al., 2015) reported that obestatin reduced severity of IR-induced acute pancreatitis and accelerated the recovery by its anti-inflammatory properties. Also, (Koc et al., 2014) reported that anti-apoptotic, anti-oxidative and anti-inflammatory properties of obestatin reduce the renal IR-injury. Moreover, (Zhang et al., 2017) indicated that obestatin has a protective effect against cardiomyocyte injury after IR.

However, so far, the effect of obestatin on testicular IR is not widely reported. Therefore, we aimed to explore the effect of obestatin on testicular IR. The present study was conducted over 40 male adult albino rats and were equally divided into 4 groups (n=10). It aimed to investigate the effect of 8 nMol/kg/body weight obestatin intravenous injection on 2 hr testicular

ischemia followed by 2 hr reperfusion.

According to the histopathological findings, in the IR group a necrosis of spermatogonial interstial and interstitial hemorrhage was seen. Moreover, the edema in the interstitial area was widespread. These results are matching with (Dogan et al., 2016) who reported that there was an observed edema and hemorrhages in the interstitial area in the IR group. In addition to (Refaie, 2018) who recorded disarrangement of testicular structure due to decrease in number of germinal epithelium in the IR group. In line with the present results (Abozaid and Ahmed, 2019) reported that while the testis of control group showed normal spermatids, spermatogonia and interstitial tissue, the IR group showed interstitial edema, degeneration and vacuolization of spermatogonia.

In the third IR group which treated with saline for 7 days, a mild disturbance of germ cell arrangement and regeneration of spermatogonia were seen which indicated the beginning of recovery. The fourth group treated with obestatin showed regeneration of spermatogonia in most of tubules with restoration of the normal component of interstitial without any signs of edema. This result is similar to control group. These findings go along with that of (Raafat et al., 2019).

The results of the current study showed a significant decrease in serum free testosterone level in IR group. This result goes along with these of (Wei et al., 2017; Refaie, 2018; Abdel-Gaber et al., 2018) who reported that IR led to edema and resulted in tissue damage such as Leydig cells, which is the source of testosterone secretion. The current study which investigated the effect of obestatin administration on testicular IR and found that obestatin increased the free testosterone level. This finding goes along with that of (Khalefa and Raafat, 2017).

Furthermore, we found a significant increase in testicular masses after reperfusion. This increase can be demonstrated by the edema, inflammation and hyperemia (the vasodilatation induced increase in blood supply) occurred due to ischemia and reperfusion. However, (Refaie et al., 2017) who reported a significant decrease in testicular weight does not agree with the current result. Also, the current study reported a non-significant difference in testicular weights in obestatin-treated group comparing with the control group.

The enzymatic antioxidant defense system includes catalase, superoxide dismutase (SOD) and glutathione (GSH) (Refaie et al., 2017). Malondialdehyde (MDA), an end product of lipid peroxidation generated by ROS (Zhang et al., 2016). The end product of lipid peroxidation, MDA is used as marker to evaluate the increased ROS formation in testicular IR tissue injury (Takhtfooladi et al., 2013). SOD and catalase are anti-oxidant enzymes in the defense system of testis (Celik et al., 2016) and glutathione is another anti-oxidant enzyme for detoxification of oxygen metabolites (Elshaari et al., 2011).

The present study had found an increase in MDA and decrease in SOD, GPx and catalase in IR group. These results agree with the previously published data of (Kara et al., 2016; Ning et al., 2018; Refaie et al., 2018) who reported that testicular IR is due to overproduction of proinflammatory cytokines (interleukin-1β and TNF-α), which recruit macrophages and neutrophils (Lysiak, 2004). Neutrophils are the source of ROS, which causes the testicular IR injury. ROS reacts with membrane lipids and results in lipid peroxidation and loss of cellular components of the tissue (Kara et al., 2016). However, our results do not agree with that of (Lee et al., 2019) who reported that levels of MDA, SOD and catalase were significantly higher in IR group than the control group.

The beneficial effect of obestatin which was the proved by histopathological and testicular tissue oxidative stress evaluation can be explained by many mechanisms.

First: obestatin anti-oxidant effect. The current study proved that SOD, GPx and catalase had increased, while MDA had decreased in obestatin treated group. This anti-oxidant effect of obestatin was proved in many studies such as (Mirarab et al., 2019) who reported that obestatin prevented lipid peroxidation (decrease MDA) and also increased antioxidant enzymes (SOD and GSH) and thus caused DNA damage in cerebral IR.

Moreover, (Zhang et al., 2017) reported that obestatin antagonized oxidative stress induced by IR injury. He added that obestatin decreases the levels of MDA and increases the levels of SOD. This result goes along with that of (Koc et al., 2014) who reported that obestatin guards against renal IR injury by anti-oxidative properties. (El-Gohary, 2017) reported that obestatin treatment improved the IR injury of the liver by decreasing total oxidative status (TOS) and oxidative stress index (OSI) and increasing total antioxidant status (TAS).

Second: The possible anti-inflammatory effect of obestatin on IR. Previous studies reported that inflammation reaction was the common response

to injury of tissue (Zhang et al., 2017). The study of (Mirarab et al., 2019) proved that ischemia induced neuroinflammation in the hippocampal CA1 neurons whereas obestatin reduced TNF-α production induced by IR. This result goes along with that of (Pamukcu et al., 2013) who the study which showed that obestatin had an anti-inflammatory effect in an experimental model of colitis. In addition, (Zhang et al., 2017) suggested that obestatin had anti-inflammatory effect in cardiomyocytes injury after IR. Also, they found that obestatin pretreatment inhibited the expression of inflammation factors (TNF-α, IL-6, ICAM-1, and iNOS).

Furthermore, (El-Gohary, 2017) reported that hepatic IR resulted in release of inflammatory cytokines such as TNF-α and interleukins after reperfusion. These mediators activated the neutrophils, which produce more ROS and increased the production of TNF-α from Kuppfer cells and consequently caused more hepatocyte damage. Obestatin treatment significantly improved the IR injury of liver by reducing the pro-inflammatory cytokines (TNF-α and IL-6).

Third: the possible anti-apoptotic effect of obestatin on different tissues. The anti-apoptotic effect of obestatin was detected by (Zhang et al., 2017) who reported that obestatin decreased cardiomyocyte apoptosis after IR injury by its anti-apoptotic effect. In addition, (Raafat et al., 2019) proved that obestatin alleviated the increased Bax and the reduced Bcl-2 proteins expressions, while it decreased the significant high levels of caspase-8 and caspase-3in I/R testis.

CONCLUSION

Obestatin can reduce the hazard effect of IR on testicular structure and function when used for 7 days by dose of 8 nmol/kg/body weight. This beneficial effect of obestatin is due to its anti-oxidant effect as it decreased MDA and increased GSH, SOD and catalase significantly comparing with the saline treated group. These results suggest that obestatin can have promising contributions in future treatments in clinical testis torsion/ detorsion damage

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

To Dr/ Mona Mostafa Ahmed, Pathology Department, colleague of Medicine, Zagazig University for performing the histopathological study.

AUTHOR CONTRIBUTIONS

The supervision of prof. Dr. Nabil A. Soliman and prof. Dr. Abeer Albiomy Khalefa are designed experiments and reviewed the manuscript. Raghda Mohamed Abou elfotoh performed the experiments, data analysis and also wrote the manuscript. All authors read and approved the final version.

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