**Oxidative stress, apoptosis, inflammation, and proliferation modulator function of visnagin provide gonadoprotective activity in testicular ischemia-reperfusion injury**

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**Abstract.** – **OBJECTIVE:** Visnagin (Vis) is a compound found in the flowers and seeds of the Ammi visnaga plant with promising antioxidant and anti-inflammatory properties. We aimed to investigate the dose-dependent gonadoprotective effects of visnagin in rats while considering oxidative stress, apoptosis, and inflammation-related protein expression levels.

**MATERIALS AND METHODS:** Twenty-eight adult rats were divided into four groups of seven animals each; control, ischemia/reperfusion (I/R), I/R+30Vis, and I/R+60Vis. Animals in control received no surgical application and were sacrificed at the end of the experiment. The rats in I/R, I/R + Vis30, and I/R + Vis60 were exposed to testicular ischemia and the animals in I/R + Vis30, and I/R + Vis60 groups received either 30 or 60 mg/kg visnagin intraperitoneal. At the end of the experiment, testis tissues were used for the measurement of oxidative stress, apoptosis, and inflammation.

**RESULTS:** Our microscopic examinations indicated that I/R resulted in testicular degenerations and morphological alterations, which were improved in visnagin-treated animals. The biochemical analyses demonstrated that oxidative stress in the I/R group increased significantly (*p*<0.05) compared to the control group. The im-

**Graphical Abstract:** (1) Rats were divided into four groups: control, I/R, I/R+Vis30, and I/R+Vis60. (2) I/R, I/R+Vis30, and I/R+Vis60 groups were exposed to bilateral ischemia for 3 hours. (3) Rats in I/R, I/R+Vis30, and I/R+Vis60 received DMSO, 30 mg/kg, and 60 mg/kg visnagin, respectively, at the 1st hour of ischemia. (4) Reperfusion provided for 3 hours. (5) Animals were sacrificed with exsanguination. (6) Tissue samples were used for histological and biochemical analyses. (7) Oxidative stress, apoptosis, and inflammation increased, but PCNA in I/R group. (8) Visnagin improved pathological results, and a higher dose was more successful.

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Introduction

Testicular torsion ranks among the foremost urological emergencies. Testicular ischemia-reperfusion (I/R) injury is one of the most critical urological conditions that typically necessitate surgical intervention, primarily observed in newborns, young children, and adolescents. The degree of twisting and the duration of symptoms are prognostic factors that influence testicular salvage during episodes of testicular torsion. Testicular ischemia occurs as the spermatic cord twists upon itself, resulting in cessation of blood flow to the testis and accumulation of reactive oxygen species (ROS). Symptoms of testicular ischemia include scrotal and abdominal pain, swelling, skin redness, nausea, vomiting, and fever.

Surgical intervention can resolve testicular ischemia by untwisting the obstructed spermatic cord in the opposite direction; however, the underlying secondary mechanism of reperfusion may still necessitate treatment. One fundamental cause of testicular I/R damage is the overproduction of ROS. Hypoxia and increased ROS accumulation due to testicular torsion result in a reduction in sperm count and lead to infertility. Consequently, treatments aimed at reducing hypoxia and producing ROS have been developed. To date, researchers have investigated various treatment strategies, including antioxidants, calcium channel blockers, vasodilator drugs, plant-derived phenolic compounds, and amniotic fluid, among others.

These treatment strategies all target the revascularization of the disrupted vascular circulation and the reduction of oxidative stress. Although in the literature partial success was reported, the reduction of hypoxia and ROS caused by testicular torsion was not fully addressed. Hence, researchers are actively pursuing clinical research to understand the underlying mechanism of testicular I/R damage and to explore potential protective drug application treatment protocols.

Visnagin (Vis) is a compound obtained from the fruit and seeds of the plant *Ammi visnaga* and is known as 4,9-dimethoxy or 4-methoxy-7-methyl-furo[3,2-g] chromen-5-one. Khellin and visnagen extract are widely used as herbal medicine in the treatment of angina. Khellin is a spasmylytic agent and is utilized in kidney stone treatment. Khellin and visnagen extract significantly prolong the nucleation induction time of calcium oxalate. Khellin has been used in the photochemical treatment of vitiligo and psoriasis, and the photodynamic properties of khellin and visnagen in photo reactions with DNA have been studied. Furthermore, Vis and khellin mitigate oxidative stress, protect against oxalate-induced damage in renal epithelial cells, and exhibit protective effects against isoproterenol-induced acute myocardial injury.

Vis-containing N-isopropylacrylamide-methacrylic acid nanoparticles have been shown to prevent I/R-related heart damage and dysfunction in rats through apoptosis inhibition. Recent studies have demonstrated that a series of compounds derived from visnagin pyrimidine hybrids possess anti-inflammatory and analgesic properties. However, the detailed mechanisms of VIS’s effect on cerebral I/R damage have not yet been fully determined.

Our study aims to evaluate the protective effect of VIS on testicular tissue with testicular I/R injury and to investigate whether it plays a role in the effects on oxidative stress, inflammation, or apoptosis pathways.

Materials and Methods

Study Design

This study was conducted with 28 experimental animals, obtained after the ethical committee
approval by Harran University, (approval date: 10.05.2023 and number: 01-17). The animals were equally divided into four groups (Control, I/R, I/R + Vis30, I/R + Vis60). In the I/R, I/R + Vis30, and IR + Vis60 groups, a bilateral testicular ischemia model was created. Animals were administered general anesthesia, and a scrotal incision was made along the midline to expose the testes. They were then twisted 720° in opposite directions and secured to the tunica dartos to induce ischemia. After two hours of ischemia, the animals were re-anesthetized, the scrotums were reopened, and reperfusion was established. The scrotums were then sutured again, allowing reperfusion to occur for an additional two hours. Two hours after creating the experimental ischemia model, the I/R group was administered the solvent (25 mg/ml DMSO) that would be used for diluting visnagin. In the I/R + Vis30 and I/R + Vis60 groups, visnagin was dissolved in DMSO and administered intraperitoneonally at the indicated doses (30 mg/kg and 60 mg/kg, respectively) two hours after creating the ischemia model. After each animal was subjected to intraperitoneal injection, a waiting period of two hours was observed for the metabolism of visnagin. A literature review showed that the highest absorption of visnagin occurred at the 2nd hour when administered via oral gavage. Therefore, at the end of the total 2-hour application period, the scrotal sutures were reopened in the animals, and a reperfusion injury model was established. Animals in every group were sacrificed during the 3rd hour of reperfusion via intracardiac exsanguination. The scrotums were incised, and the left testis was fixed in 10% neutral buffered formalin (NBF) for microscopic examinations. The right testis of the animals was frozen at -80°C for biochemical analyses.

Biochemical Analyses

Tissue luminol, lucigenin chemiluminescence (CL) assays, malondialdehyde (MDA), and glutathione (GSH) levels were measured from frozen tissue samples for determination of oxidative stress and endogenous antioxidant activity among the groups. For that purpose, the luminol CL probe was used to specifically measure the hydrogen peroxide, hydroxyl radical, and hypochlorite radicals, and the Lucigenin CL probe was applied for the measurement of superoxide anion radical in testicular tissue samples. Tissue MDA levels were evaluated due to the end-up product of lipid peroxidation through thiobarbituric acid chromogenic reaction, and the GSH amount in tissue samples of the groups was measured. Luminol and lucigenin assay results are expressed as rlu/mg, and MDA and GSH levels were expressed as nmol/g and µmol/g, respectively.

Tissue Processing and H&E Staining

Formalin-fixed samples were dissected, washed under tap water, dehydrated through increasing alcohol series, and embedded into paraffin blocks. Five µm thick sections were obtained with a rotary microtome from paraffin-embedded tissue samples. Half of the obtained sections were stained with hematoxylin and eosin for routine pathological observations. 100 randomly selected seminiferous tubules were used for measurement of germinal epithelial thickness and Johnsen’s biopsy score in every group. The obtained results were analyzed statistically.

Immunohistochemistry and Quantification of Immunointensity

Serial sections from every testis sample were used for immunohistochemistry of Bax (Cat. No: sc-7480, Santa Cruz Biotechnology, Dallas, TX, USA), Caspase 3 (Cat. No: sc-56053, Santa Cruz Biotechnology, Dallas, TX, USA), tumor necrosis factor-alpha (TNF-α) (Cat. No: sc-52746, Santa Cruz Biotechnology, Dallas, TX, USA), and proliferating nuclear antigen (PCNA) (Cat. No: sc-25280, Santa Cruz Biotechnology, Dallas, TX, USA). Tissue sections were deparaffinized in xylene, rehydrated through decreasing alcohol series, and brought to distilled water. The samples were then washed in Phosphate Buffered Saline (PBS), the antigen retrieval was performed in citrate buffer (ph: 6.0), and endogenous peroxidase activity was inhibited by incubating the samples in 3% H₂O₂, which dissolved in methanol. A commercially produced immunohistochemistry (IHC) detection kit (Cat No: TP-125-RL; Thermo Scientific, Waltham, MA, USA) was used for the next steps. The blocking solution from the kit was used for inhibition of non-specific binding. The primary antibodies of Bax, Caspase 3, TNF-α, and PCNA were diluted in an antibody diluent with 1:100, 1:100, 1:250, and 1:100 ratios, respectively, and the diluted antibodies were dropped on the sections. Primary antibody incubation was performed at +4°C overnight, and the samples were washed at every intermediate step. Biotinylated secondary antibodies and enzymes from the IHC detection kit were used while considering the manufacturer’s instructions. The chromogenic reaction was developed.
with DAB chromogen, and sections were counterstained with hematoxylin. The slides were washed under tap water for discharge of excessive stain and dehydrated through increasing alcohol series, cleared in xylene, and mounted with Entellan. Prepared immunohistochemistry samples were evaluated under a light microscope, and micrographs were captured. Bax, Caspase 3, and TNF-α immunointensity analyses were performed in Image-J Software Version 1.46 (US National Institutes of Health, Bethesda, MD, USA) through threshold analysis. The threshold analyses of Bax, Caspase 3, and TNF-α were performed considering the ratio between DAB-positive seminiferous tubule area and total seminiferous tubule area. The PCNA analyses were performed by manually counting and considering DAB positive and total nuclei counts in every seminiferous tubule. A total of 70 randomly selected seminiferous tubules from every group were used for immunohistochemistry analyses. All measurements were converted to ratio and analyzed statistically to determine if there were any differences between the groups.

Statistical Analysis
All obtained raw data from biochemical, histochromatic, and immunohistochemical were evaluated statistically with SPSS Statistics for Windows version 24.0 (IBM Corp., Armonk, NY, USA). For that purpose, a parametric One-Way ANOVA test was used. Multiple comparisons between groups were performed with the Post-hoc Tukey test. Results are expressed as mean ± SD, and $p<0.05$ is considered significant.

Results

Biochemical Results
As a result of our biochemical examinations, we observed that the luminol level was lower in the control group. Measurements of luminol in the I/R group were found to be higher than the other groups and significantly different from all the groups ($p<0.001$). In the group where we applied a treatment of 30 mg/kg, the luminol level was numerically different from the control group, but no statistical difference was found ($p>0.05$). In the high-dose I/R + Vis60 group, the luminol level was seen to be similar to the control group and I/R + Vis30 groups ($p>0.05$) (Figure 1).

Looking at lucigenin levels, we found the highest level in the I/R group, and the control, I/R + Vis30, and I/R + Vis60 groups were found to be similar. The I/R group was found to be statistically significantly different from the other three groups ($p<0.001$). Similarly, in MDA levels, the highest level was in the I/R group, and the control, I/R + Vis30, and I/R + Vis60 groups were found to be similar. The I/R group was again statistically significantly different from the other three groups ($p<0.001$).

In terms of GSH levels, the lowest level was in the I/R group. There was a significant statistical difference between the control and I/R groups ($p<0.001$), as well as between the I/R and I/R + Vis60 groups ($p<0.05$). The statistical results of the biochemical analyses are shown in Table I.

Histopathological Results
The seminiferous tubule structure of the control group was normal, as expected. In this group, testicular parenchyma was filled with seminifer-

![Figure 1](image-url). Graphical demonstration of statistical analyses of biochemical measurements. Different superscript on every column indicates statistical significance. The symbols between the groups also indicate statistical significance. *$p<0.05$, **$p<0.001$. 

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ous tubules, and there was interstitial tissue between every tubular system. Germinal epithelium underwent spermatogenesis; leydig cell was visible (Figure 2 a1 and a2). In the I/R group, there were severe degenerations such as basement membrane perforation, testicular artery congestion, and hemorrhage, nuclear pyknosis was widespread within the germinal epithelial layers and interstitial tissue. Epithelial desquamation was very common in tissue samples of this group (Figure 2 b1 and b2). In the I/R + Vis30 group, the severity was improved, but vascular pathological changes, hemorrhage, and congestion were still existing. Epithelial desquamation in some of the seminiferous in this group was clear (Figure 2 c1 and c2). However, our microscopic examinations indicated the pathological changes in I/R + Vis60 groups were almost improved, and the tissue morphology of this group was very similar to that of the control group (Figure 2 d1 and d2). When we evaluated Johnsen’s Biopsy Score, the highest score level was observed in the control group. The biopsy result in the I/R group was significantly downregulated ($p<0.001$) compared to the control group. In the Vis60 group, the results were similar to the control group ($p>0.05$), but there was a significant difference ($p<0.001$) when compared to I/R and I/R + Vis30 groups. Johnsen’s Biopsy Score result in the Vis30 group was significantly different than ($p<0.001$) the rest of the groups. The statistical analysis results of Johnsen’s biopsy score are shown in Table II.

**Immunohistochemistry Results**

The micrographs of the immunohistochemistry samples are shown in Figure 3. Our analyses revealed that the highest level of Bax immunointensity was in the I/R group, and this group’s pro-apoptotic Bax level was significantly different from the Control, I/R + Vis30, and I/R + Vis60 groups ($p<0.001$). It was found that the Bax protein immunointensity levels in the Control, I/R + Vis30, and I/R + Vis60 groups were similar ($p>0.05$). When we examined the Caspase 3 immunointensity, sim-

<table>
<thead>
<tr>
<th>Table I. Statistical result analyses of biochemical measurements.</th>
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<tbody>
<tr>
<td><strong>Luminol</strong></td>
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<tr>
<td>(rlu/g)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>I/R</td>
</tr>
<tr>
<td>I/R + Vis30</td>
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<td>I/R + Vis60</td>
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Different superscripts between every column indicate statistical significance between the groups: $^{++}p<0.001$, $^++p>0.05$, $^{+}p<0.001$. Malondialdehyde (MDA), and glutathione (GSH).

**Figure 2.** Representative histopathological micrographs in control (a1-a2), I/R (b1-b2), I/R + Vis30 (c1-c2), I/R + Vis60 (d1-d2). The seminiferous tubule in the control group was normal. Germinal epithelium, leydig cells, and vascular structure in interstitial tissue were regular (a1-a2). In the I/R group, basement membrane perforation and undulation (arrow) were widespread. Most of the germinal epithelial cells were observed with pyknotic nuclei (arrowhead). Congestion in vascular space and hemorrhage (asterisk) in interstitial parenchyma were clearly visible (b1-b2). There was still widespread desquamated epithelium in the I/R + Vis30 group. Vascular pathological changes in this group (asterisk) were very similar to the I/R group (c1-c2). The seminiferous tubule and interstitium in the I/R + Vis60 group were very close to the control group. However, there was congestion in vessels of this group (d1-d2). Staining: Hematoxylin and eosin. Bar: 50 µm (a1, b1, c1, d1), 20 µm (a2, b2, c2, d2).
Similarly to the Bax results, the highest immunointensity was found in the I/R group, and the Caspase 3 immunointensity in the I/R group was significantly higher than in control and I/R + Vis60 groups (p<0.05), but similar to the I/R + Vis30 group (p>0.05). Looking at the TNF-α inflammatory cytokine levels, the lowest level was found in the control group, and the highest level was in the I/R group, with a great statistical difference between the control and I/R groups (p<0.001) and the I/R + Vis60 groups (p<0.001). The TNF-α level in the I/R group was found to be similar to the I/R + Vis30 group (p>0.05). When we examined PCNA immunointensity, the highest level was in the control group, and the lowest positive cell ratio was in the I/R group. Interestingly, a significant statistical difference was also found between the control group and the I/R + Vis60 group (p<0.05), the I/R + Vis30 treatment group (p<0.05), and between the I/R + Vis30 treatment groups (p<0.05). When the PCNA positive cell ratios were examined, the highest ratio was found in the Control group. This group’s PCNA-positive cell ratio significantly differed from the I/R, I/R + Vis30, and I/R + Vis60 groups (p<0.05). The graphical demonstration of the statistical results of the immunohistochemistry analyses is shown in Figure 4.

Table II. Statistical result analysis of histopathological and immunohistochemical observations.

<table>
<thead>
<tr>
<th></th>
<th>Johnsen's Biopsy Score (/10)</th>
<th>Bax immunointensity (%)</th>
<th>Caspase 3 immunointensity (%)</th>
<th>TNF-α immunointensity (%)</th>
<th>PCNA positive Cell ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.35±1.34a</td>
<td>22.23±3.93d</td>
<td>20.97±4.96d</td>
<td>24.60±5.85a</td>
<td>70.43±11.25d</td>
</tr>
<tr>
<td>I/R</td>
<td>6.35±1.62c</td>
<td>25.18±4.10f</td>
<td>23.43±5.25f</td>
<td>29.46±4.71p</td>
<td>54.13±8.15p</td>
</tr>
<tr>
<td>I/R + Vis30</td>
<td>7.67±1.81b</td>
<td>23.17±4.41d</td>
<td>22.79±5.43f</td>
<td>26.83±6.62a</td>
<td>60.61±15.89f</td>
</tr>
<tr>
<td>I/R + Vis60</td>
<td>8.89±1.38a</td>
<td>22.37±4.29d</td>
<td>20.96±4.28d</td>
<td>25.31±6.80a</td>
<td>66.88±15.33f</td>
</tr>
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Different superscript between every column indicate statistically significance between the groups. *p<0.001, **p<0.001, ***p<0.001, ****p<0.001, *****p<0.001, ******p<0.001, *******p<0.001, ********p<0.05, *********p<0.05, **********p<0.05, ***********p<0.05, ************p<0.05. Tumor necrosis factor-alpha (TNF-α).

Figure 3. Representative micrographs of Bax, Caspase 3, TNF-α, and PCNA in Control, I/R, I/R + Vis30, and I/R + Vis60 groups are as shown. Counterstaining: Hematoxylin. Bar: 50 µm.
Discussion

Although surgical intervention is accepted as the only treatment method, the accumulation of reactive oxygen species may continue the process of tissue degeneration, also referred to as I/R (Ischemia/Reperfusion) injury. This testicular complication can lead to infertility or sterility in patients over time. Therefore, researchers aim to discover not only the provision of reperfusion to the ischemic testis but also the most suitable additional treatment. Most previously published articles have examined antioxidant substances to suppress the adverse effects of I/R damage on the testes.

One of the most commonly observed changes during Ischemia/Reperfusion (I/R) injury is the accumulation of reactive oxygen species (ROS). Also, the increase in the release of inflammatory cytokines, the condensation (pyknosis) of germinal epithelial cells, cellular shedding, and the formation of multinucleated giant cells are observed. This is thought to be evidence of a disruption in cell proliferation and maturation. As reported in the literature, we observed severe pathological changes in testes exposed to I/R injury. Today, Johnsen’s biopsy is still one of the most accepted testicular tissues. Some studies showed that antioxidant supplementation before reperfusion can provide protection in I/R damage, and some treatment strategies have been discussed. In pre-clinical studies, it has been reported that testicular reperfusion after ischemia triggers lipid peroxidation, leading to an increase in serum and tissue MDA levels. In addition to its biochemical effects, interstitial edema, hemorrhage, and fibrosis have been reported among the morphological degenerative effects of I/R injury. In the testis, I/R damage results in the formation of multinucleated giant cells, known as coenocytes. These cells are believed to be associated with the failure of spermatogenesis. The formation of these cells has been associated with exposure to chemicals, and thermal or mechanical damage. If antioxidant applications are unsuccessful, these giant cells may remain in the testis. At the same time, excessive ROS production in the testis leads to an increase in caspase-3 activity, but a decrease in PCNA expression, and it has been reported that ROS scavengers reduce this apoptotic imbalance. However, in a previous study, it was reported that antioxidants only prevented apoptosis in the contralateral testis, and these results showed apoptosis at excessive ROS levels. The conflicting results of these studies indicate that the success chances of ROS scavengers depend on the degree of rotation of the spermatic cord, the duration of ischemia, the produced ROS level, and the inhibitory mechanism of the applied drugs.

In our study, when I/R (Ischemia/Reperfusion) injury was applied to the testis, we observed degenerations in the seminiferous tubules and germ cells, and a decrease in the Johnsen score. Additionally, an increase in serum MDA levels emerged due to lipid peroxidation. These findings are consistent with the literature. Interestingly, after I/R damage, multinucleated giant cells (MGCs) were often detected in the seminiferous tubules with limited shedding. Therefore, we believe that MGCs may be the first step in the infer-

Figure 4. Graphical demonstration of the statistical analyzes of Bax, Caspase 3, TNF-α immunointensity analyzes and PCNA positive cell ratio in seminiferous tubule of the groups. Different superscript on every column indicate significantly different compared to Control and I/R + Vis60 groups. Different symbols between the groups also indicate the statistically significance. *p<0.05, **p<0.001.
tility process in I/R damage. On the other hand, the ratio of PCNA expression, caspase-3 fragmentation, and serum MDA level were consistent with the literature. In the literature, we could not find any study examining the protective role of visnagin in testicular I/R damage. However, Rao et al. have reported that the application of visnagin after cerebral ischemia-reperfusion in rats improved brain cognitive levels, and also reduced caspase-3 activity, apoptosis, and morphological degenerations. In another study, raxofelast, a water-soluble analog of vitamin E, has alleviated morphological degenerations in 3-hour testicular ischemia.

In a study conducted by Duarte et al., it was concluded that low doses of visnagin inhibit contraction by blocking the entry of Ca+2 into the cell, while high doses of visnagin nonspecifically inhibit vascular smooth muscle contraction. This study also shows that high doses of visnagin inhibit vasoconstriction and provide conditions for reperfusion in cell damage. This situation explains the result we obtained in our study at 60 Vis.

Limitations

Although our observations provide promising results, there are some limitations in this study that should be considered in further studies. To improve the rationality of the statistical analysis, the sample size of this study should be increased. Second, although, our immunohistochemical measurement and analysis already provide semi-quantitative results, molecular biology methods such as western blotting and PCR should be used for the confirmation of the analysis of protein expression levels.

Conclusions

Our results show that 30 mg/kg visnagin, applied intraperitoneally, reduced reperfusion damage, morphological degenerations, and apoptosis. 60 mg of visnagin obtained almost similar results to the control group. Additionally, the decrease in MDA levels and the preservation of the integrity of the basal membrane at 60 mg visnagin is evidence of visnagin’s preventive effect on lipid peroxidation.

Acknowledgements

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Funding

This study did not receive any financial support.

Authors’ Contributions


Ethics Approval

This experimental study was performed after receiving approval of the Local Experimental Animal Ethics Committee of Harran University with approval date: 10.05.2023 and number: 01-17.

Informed Consent

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest to declare.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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