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Original Research

Ghrelin and NUCB2/Nesfatin-1 expression in unilateral testicular torsion-induced rats with and without N-acetylcysteine

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Abstract: Testicular torsion (TT) is a common urological problem in the field of pediatric surgery. The degree and duration of torsion determines the degree of testicular damage; however, its effects on the expression of octanoylated ghrelin and nucleobindin 2 (NUCB2) /nesfatin-1 synthetized from testicular tissue remain unclear. We explored the effects of experimentally induced unilateral TT on serum and contralateral testicular tissue ghrelin and NUCB2/nesfatin-1 levels, and determined whether N-acetyl cysteine (NAS) treatment had any effects on their expression. A total of 42 Wistar Albino strain rats were divided into 7 groups: Group (G) I control, GII sham, GIII 12-hour torsion, GIV 12-hour torsion + detorsion + 100 mg/kg NAS, GV 24-hour torsion, GVI 24-hour torsion + detorsion + 100 mg/kg NAS, and GVII 100 mg/kg NAS. Octanoylated ghrelin and NUCB2/nesfatin-1 concentrations were evaluated in serum using the ELISA method and in testicular tissue with immunohistochemical methods. Immunoreactivity of octanoylated ghrelin significantly increased in GI compared to GIII, GV, and GVI ($p<0.05$). NUCB2/nesfatin-1 immunoreactivity increased in GV and GVIII relative to GI ($p<0.05$). In the 12-hour torsion group, a significant decrease in octanoylated ghrelin levels with NAS treatment was observed; however, in the 24-hour torsion group, a significant decrease was not observed. In the 12-hour torsion + NAS treatment group, a significant change was not observed in NUCB2/nesfatin-1 expression. Following 24-hour torsion, an increase in NUCB2/nesfatin-1 levels was observed, and NAS treatment did not reverse this increase. It was determined that increases in the expression of octanoylated ghrelin and NUCB2/nesfatin-1, the latter of which was a result of TT, reflect damage in this tissue. Importantly, NAS treatment could prevent this damage. Thus, there may be a clinical application for the combined use of NAS and octanoylated ghrelin in preventing TT-related infertility.

Key words: Testicular torsion; Octanoylated ghrelin; NUCB2/nesfatin-1; N-acetylcysteine; Rat.

Introduction

Testicular torsion (TT) is a urological emergency that is most commonly found in newborns and adolescents. Although testicular damage changes with the degree and duration of TT, the main cause of infertility is destructive changes in contralateral testis. However, the mechanism of damage in contralateral testis remains unclear (1,2). Twisted testis should be detorsioned, reperfusion should be confirmed, and viability should be controlled as soon as possible to prevent testicular damage. Excision of the testis with suspect viability or leaving it *in situ* to determine whether its hormonal function recovers is a debatable issue. In addition, after ischemia and reperfusion (IR), neutrophil infiltration and the severity of damage increases, which are mediated by free oxygen radicals. Various agents can be experimentally applied to prevent this damage. These structural changes in testes also affect the biochemistry and physiology of circulation (3).

The important roles of tissue hormones such as leptin, resistin, adiponectin, ghrelin, and nucleobindin 2(NUCB2)/nesfatin-1 in the regulation of testicular functions have been reported (4–8). Although intensive production of ghrelin and NUCB2/nesfatin-1 in testicular tissue has been recognized, changes in testicular tissue secondary to IR injury remain unclear. Ghrelin has been found in four different forms in biological fluids and tissues: octanoylated ghrelin, decanoic ghrelin, decanoyl ghrelin, and desacyl ghrelin. Among these four forms of ghrelin, octanoylated ghrelin has the most important biological functions as it plays a role in the regulation of appetite, release of growth hormone, restriction of inflammation, and antimicrobial activities. Therefore, in our study we evaluated this peptide hormone (9,10).

N-acetyl cysteine (NAS), which is used as a mucolytic agent, has antioxidant, anti-inflammatory, and cell-protective effects. It also increases microvascular blood flow, provides endothelial protection through its thiol group, and can prevent or decrease the severity of IR injury (11). It is possible that octanoylated ghrelin, which is synthetized in testicular tissue and has antioxidative effects, and NUCB2/nesfatin-1 with its opposing effects, may be involved in preventing IR injury in TT. Accordingly, exploring the effects of NAS supplementation on octanoylated ghrelin and NUCB2/nesfatin-1 hormones will increase our understanding of the underlying mechanisms in TT.

In this study, we explored how serum and contralat-

eral testicular tissue ghrelin, total oxidative stress (TOS) and total antioxidant status (TAS) as well as NUCB2/nesfatin-1 levels, are affected in rats subjected to unilateral TT, and determined the effects of NAS treatment on the expression of these molecules.

Materials and Methods

Approval by the ethics committee for investigations on experimental animals was obtained for this study (Decree No: 05.11.3014-2014/22-210-2014/112). A total of 42 male Wistar Albino rats weighing 200–250 g were used, which were separated into 7 groups (6 rats/group).

Experimental groups

Group I (GI): Control group that only underwent bilateral orchiectomy; Group II (GII): (Sham group) Lower and upper parts of the testes were sutured. After 12 h, the sutures were removed and after 72 h, the right testes were removed. Group III (GIII): 12 h of torsion (ischemia) group: Left testes of rats were rotated clockwise 720 degrees with their chord elements, and an extravaginal testis model was formed. Torsioned testis was fixed to the inner surface of the scrotum from two points with 6-0 propylene sutures. After 12 h, the incision was opened and the testis was detorsioned; after 72 h, right orchiectomy was performed. Group IV (GIV): Detorsion group, where the IR model was constituted after 12 h of torsion. In this group, rats received NAS (100 mg·bwt/d) through the intraperitoneal route. Group V (GV): 24-hour torsion (ischemia) group. In this group, detorsion was applied after 24 h of experimental torsion. Group VI (GVI): The IR model was formed in testes detorsioned after 24 h of torsion. This group received NAS at 100 mg/kg/d through the intraperitoneal route. Group VII (GVII): This group received NAS at 100 mg/kg/d through the intraperitoneal route.

Type of anesthesia and surgical procedures

Prior to the surgical procedure, all of the rats received Xylazin through the intramuscular route (Rompun Vet; Bayer AG, Istanbul, Turkey) at doses of 5–10 mg/kg, and received ketamine hydrochloride (Ketalar; Eczacibasi, Istanbul, Turkey) at doses of 50–70 mg/kg for general anesthesia. After cleansing and disinfecting the skin of the anterior abdominal wall with 10% povidone-iodine solution, a 2 cm midline vertical skin and subcutaneous incision was performed. The left testis was bluntly dissected away from the tunica vaginalis, spermatic cord, and gubernaculum, and removed from the abdominal cavity. After every procedure, the testis was placed into the scrotum and an incision wound was re-closed with 2-0 silk sutures.

Biochemical tests

Blood samples obtained through cardiac puncture were placed in tubes containing aprotonin and delivered to the laboratories of clinical chemistry. Blood samples were centrifuged at 4000 rpm for 5 min, and serum samples were transferred into Eppendorf tubes and stored at -80°C until analysis. Levels of ghrelin (Cat No: YHB0466Ra) and NUCB2/nesfatin-1 (Cat No: YHB0765Ra) were analyzed using the ELISA method with kits provided by GENTAUR Co [Kampenhout, BELGIUM]. The manufacturing firm did not indicate intra-assay and inter-assay CV values, but measurements performed in our laboratory determined the intra-assay and inter-assay CV values of the kits as follows: intra-assay CVs for ghrelin and NUCB2/nesfatin-1 were <10% vs. <10%, and inter-assay CVs for ghrelin and NUCB2/nesfatin-1 were < 15% vs. < 12%. Blood glucose, total cholesterol, HDL-C, LDL-C, and triglyceride levels were measured using an autoanalyzer. Serum total antioxidant (TAS) and total oxidant (TOS) level were also measured on an OLYM-POS AU2700 autoanalyzer using a Rel assay Test Kit (Mega Medical Inc, Gaziantep, Turkey)

Immunohistochemical tests

Immunohistochemical analysis of tissues was performed in compliance with the ABC method previously reported by Hsu *et al*. (12) for the evaluation of immunohistochemical staining. A histoscore was formed based on the percentage of the area stained (0.1: <25%; 0.4: 26–50%; 0.6: 51–75%; 0.9: 76–100%) and intensity of the immunoreactivity (0: none; +0.5: scarce; +1: mild; +2: moderate; +3: severe) as indicated (histoscore = extent × intensity of staining).

Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences 21" (SPSS 21, Chicago, IL, USA) program was used. Numerical data are expressed as the mean ± standard deviation. For comparison of multiple independent groups that did not fit into the normal distribution, the Kruskal-Wallis test was used. Among groups with significant outcomes determined based on the Kruskal-Wallis test results and for the detection of paired groups that caused intergroup differences, the Mann-Whitney-U test was used. Based on the results of the analysis, $p<0.05$ was considered statistically significant.

Results

Biochemical findings

General lipid profiles, glycemic levels, and intergroup comparisons of these parameters are shown in Table 1. According to the Kruskal-Wallis test, cholesterol levels were significantly higher in GIII, GIV, and GV compared to GI ($p<0.05$). HDL levels significantly increased in Groups III and V ($p<0.05$) and LDL levels significantly increased in Group V ($p<0.05$). Glycemic levels significantly decreased in GV ($p=0.03$), and any intergroup difference was not detected for triglyceride levels ($p=0.076$). Serum levels of octanoylated ghrelin were comparable in GI and GIII. Compared to GI, ghrelin levels significantly increased in GIII ($p<0.05$). Ghrelin levels significantly decreased in GIV and GVII compared to GIII ($p<0.05$). However, ghrelin levels markedly increased in GV and GVI relative to GIV ($p<0.05$). Ghrelin levels in GVII significantly decreased compared to GV ($p<0.05$). Similarly, a significant decrease in ghrelin levels was detected in GVII compared to GVI ($p<0.05$). Serum NUCB2/nesfatin-1 levels did not significantly differ among GI, GII, GIII, and GIV. However, compared to GI, NUCB2/nesfatin-1 levels

Table 1. Comparison of lipid profiles and glycemic levels in the experimental groups.

	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	*p**
Cholesterol (mg/dL)	30.5 ± 4.5	43 ± 10.6	55.5 ± 10.7	52 ± 8.7	66.25 ± 3.7	46 ± 4.9	39.25 ± 3.4	< 0.001
HDL (mg/dL)	15.25 ± 1.5	19.5 ± 3.1	26.25 ± 2.8	20.5 ± 1.0	23.75 ± 5.3	20.5 ± 2.6	20.75 ± 3.2	0.003
LDL (mg/dL)	14.75 ± 2.7	20 ± 5.5	24.5 ± 7.1	25.25 ± 9.3	38.25 ± 3.3	22.25 ± 3.3	17.25 ± 2.2	< 0.001
Glucose (mg/dL)	100.5 ± 7.1	95 ± 4.3	97.25 ± 3.3	90.5 ± 6.4	84 ± 2.1	102.25 ± 6.8	109 ± 4.0	< 0.001
Triglyceride (mg/dL)	42.75 ± 7.5	54.25 ± 17.5	73.75 ± 10.5	67.25 ± 11.0	73 ± 16.0	50.75 ± 16.5	47 ± 22.0	0.076

Group I = Control; Group II = Sham; Group III = 12 hour TT; Group IV = 12 hour TT + DT + NAS; Group V = 24 hour TT; Group VI = 24 hour TT + DT + NAS; Group VII = NAS; p* = Kruskal-Wallis Test results. TT: Testicular torsion; DT: Detorsion; NAS: N-Acetyl Cysteine.

were increased in GV and GVII ($p<0.05$). Compared to GVI, a significant decrease in NUCB2/nesfatin-1 levels was observed in GVI ($p<0.05$). Compared to GVII, a significant increase in NUCB2/nesfatin-1 levels was observed in GVI ($p<0.05$). Changes in serum octanoylated ghrelin (Fig. 1A) and NUCB2/nesfatin-1 levels (Fig. 1B) are shown in Figure 1.

Total antioxidant status (TAS); GI and GII were observed similarly in TAS values. Compared with GI; GIII, GV and GVI were found statistically decreased significant. ($p <0.05$). Compared with GIII, GIV, GVI and GVII were observed to increase statistically significant ($p <0.05$). Compared with GV, GVII was significantly increased ($p <0.05$). TOS values were observed similarly in GI and GII. Compared with GI; GIII and GV were found statistically increased significant ($p <0.05$). Compared with GIII; GIV, GVI and GVII were found statistically decreased significant ($p <0.05$). Compared with GV, GVI and GVII were observed significantly decreased ($p <0.05$). Changes in serum TAS (Fig. 2A) and TOS levels (Fig. 2 B) are shown in Figure 2.

Immunohistochemical findings

Immunohistochemical staining was examined under the light microscope, and immunoreactivity of octanoylated ghrelin (Fig. 3) and NUCB2/nesfatin-1 (Fig. 4) was observed in testicular Leydig cells and spermatogenic series.

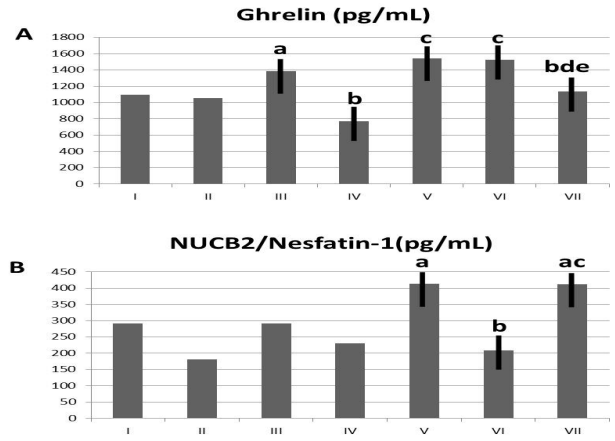

Figure 1. Variation in serum octanoylated ghrelin (**A)** and NUCB2/nefsatin-1 (**B**) secondary to testicular torsion in experimental groups. **A. Ghrelin:** [a] Increase in GIII relative to GI ($p<0.05$). [b] Decrease in GIV and GVII relative to GIII ($p<0.05$). [c] Increase in GV and GVI relative to GIV ($p<0.05$). [d] Decrease in GVII relative to GV ($p<0.05$). [e] Decrease in GVII relative to GVI ($p<0.05$). **B. NUCB2/nesfatin-1:** [a] Increase in GV and GVII relative to GI ($p<0.05$). [b] Decrease in GVII relative to GV ($p<0.05$). [c] Increase in GVII relative to GVI ($p<0.05$).

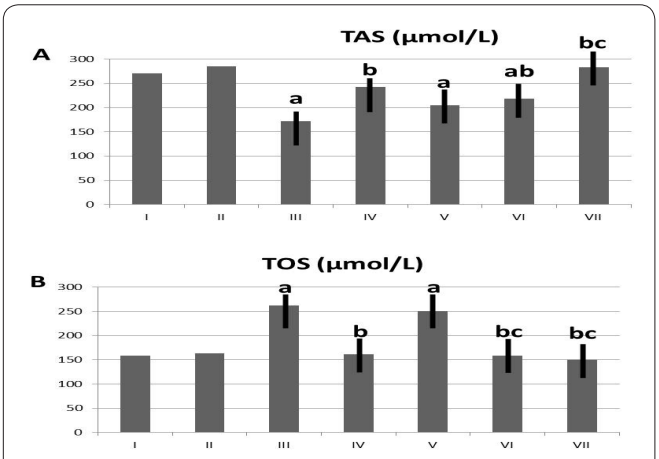

Figure 2. Variation in serum TAS (A) and TOS (B) secondary to testicular torsion in experimental groups. **A. TAS:** [a] Decrease in GIII, GV and GVI relative to GI ($p<0.05$). [b] Increase in GIV, GVI and GVII relative to GIII ($p<0.05$). [c] Increase in GVII relative to GV ($p<0.05$). **B. TOS:** [a] Increase in GIII and GV relative to GI ($p<0.05$). [b] Decrease in GIV, GVI and GVII relative to GIII ($p<0.05$). [c] Decrease in GVI and GVII relative to GV ($p<0.05$).

Comparison of experimental groups for immunoreactivity of octanoylated ghrelin

In the negative control group (Fig. 3a), immunoreactivity of octanoylated ghrelin was not observed. Immunoreactivity of octanoylated ghrelin was comparable in both G1 (Fig. 3b) and GII (Fig. 3c). Compared to GI, a significant increase in the immunoreactivity of octanoylated ghrelin was observed in GIII (Fig. 3d) ($p<0.05$). Compared to GIII, immunoreactivity of octanoylated ghrelin significantly decreased in GIV (Fig. 3e) and GVII (Fig. 3h) ($p<0.05$). Relative to GIV, an increase in the immunoreactivity of octanoylated ghrelin was observed in GV (Fig. 3f) and GVI (Fig. 3g) ($p<0.05$). Compared to GV, a significant decrease was observed in the immunoreactivity of octanoylated ghrelin in GVII ($p<0.05$). Compared to GVI, a significant decrease was observed in the immunoreactivity of octanoylated ghrelin in GVII ($p<0.05$).

Comparison of experimental groups regarding immunoreactivity of NUCB2/nesfatin-1

In the negative control group (Fig. 4a), NUCB2/nesfatin-1 immunoreactivity was not observed. NUCB2/nesfatin-1 immunoreactivity was comparable in GI (Fig. 4b), GII (Fig. 4c), GIII (Fig. 4d), and GIV (Fig. 4e). Compared to GI, a significant increase was observed in NUCB2/nesfatin-1 immunoreactivity in GV (Fig. 4f) and GVII (Fig. 4h) ($p<0.05$). Compared to GV, a significant decrease was observed in NUCB2/nesfatin-1 immunoreactivity in GVII (Fig. 4g) ($p<0.05$). Compared

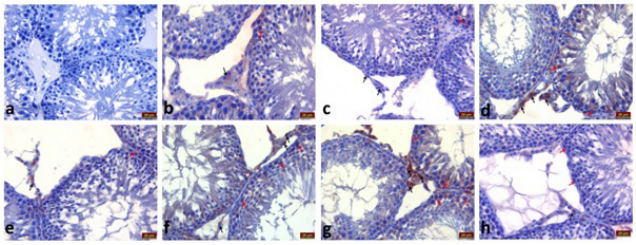

Figure 3. Immunoreactivity of octanoylated ghrelin in leydig cells (black arrow) and cells of spermatogenic series (red arrow) in the experimental groups.

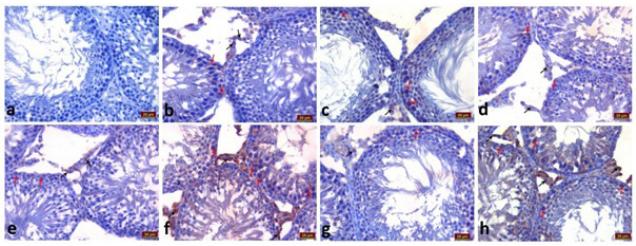

Figure 4. Immunoreactivity of NUCB2/nesfatin-1 in leydig cells (black arrow) and cells of spermatogenic series (red arrow) in the experimental groups.

to GVI, a significant increase was observed in NUCB2/nesfatin-1 immunoreactivity in GVII ($p<0.05$). The histoscores of octanoylated ghrelin and NUCB2/nefsatin-1 immunoreactivities are summarized in Table 2.

Discussion

TT is a common urological problem in pediatric surgery and may lead to testicular atrophy, germ cell injury, and infertility. Therefore, timely emergency surgery is critical to prevent testicular atrophy (3). In experimental animal studies, many agents have been used to decrease the adverse effects of IR injury including allopurinol, polyethylene glucol, oxypurinol, ibuprufen, vitamin E, caffeic acid phenethyl ester, gingko biloba, vardenafil, and tadalafil; however, a completely effective agent has not yet been identified (13,14). Currently, efforts are underway to identify a novel agent that will decrease or completely abolish IR injury in TT (15). In this report, we investigated the effects of NAS (11) on cell protective activity and on the expression of octanoylated ghrelin, which has antimicrobial activity (16), and on NUCB2/nesfatin-1 (4), which has effects that partially oppose those of octanoylated ghrelin.

In previous experimental studies, different degrees of torsion (360° and 720°) have been applied for IR (17,18). However, for an indication of complete damage, testis should be twisted 720°. Therefore, in this study, reperfusion was administered to rats during 12 h and 24 h of ischemia induced by 720° TT, and again after 72 h. Although in the short term (12 h) NAS treatment decreased octanoylated ghrelin levels, in the long term (24 h), this did not occur. The production of free oxygen radicals as hydroxyl radicals and hydrogen peroxide increase during ischemia, and these released free oxygen radicals damage testicular tissue (19). In addition, the production of greater amounts of free oxygen radicals associated with the increase in blood flow following reperfusion has deleterious effects on testicular cells (13). In our study, the decrease in endogenous antioxidant octanoylated ghrelin levels with the administration of NAS following testicular detorsion after 12 h of torsion (GIV) (20) is related to suppression of ghrelin synthesis through the antioxidant activity of NAS. Indeed, in the presence of high levels of exogenous antioxidant, the need for endogenous antioxidant is decreased. Based on the principle of maximum economy (Kleiber's law), the body does not synthesize proteins without a purpose. Thus, in the presence of NAS, it does not spend energy on the synthesis of octanoylated ghrelin (21,22).

In this study, the synthesis of octanoylated ghrelin increased following 12 h of torsion (GIII). This supports the use of octanoylated ghrelin for the diagnosis of TT. Similarly, in the 24-hour-torsion group (GV), levels of octanoylated ghrelin increased; however, this differed from the 12-hour TT group (GIV) after administration of NAS (GVI) and a decrease in octanoylated ghrelin levels was not observed. We believe that this may be due to more severe cellular damage due to longer periods of torsion and the destructive changes in receptors responsive to NAS. Moreover, a decrease in octanoylated ghrelin levels was not observed because of the release of cellular contents into circulation secondary to tissue necrosis. In a previous study by Rifaioglu *et al.* (17), sloughing, decreased cellularity, hemorrhage, and necrosis were observed in testicular tissue as a result of torsion. In addition, Somuncu *et al.* (23) reported interstitial edema and hemorrhagic foci in testicular tissue. In addition, the authors reported a similarity between serum and testicular tissue octanoylated ghrelin levels. Accordingly, the synthesis of octanoylated ghrelin in testicular tissue and the regulation of octanoylated ghrelin synthesis by NAS have been observed.

In the 24-hour torsion group (GV), compensatory synthesis of octanoylated ghrelin induced a decrease in glycemia. Thus, our results were suggestive of an

Table 2. Immunoreactivity histoscores of octanoylated ghrelin, and NUCB2/Nesfatin-1in experimental.

Experimental Groups	Octanoylated Ghrelin	NUCB2/Nesfatin-1
Group I	0.31 ± 0.09	0.30 ± 0.08
Group II	0.30 ± 0.08	0.32 ± 0.06
Group III	2.20 ± 0.77 [a]	0.27 ± 0.09
Group IV	2.10 ± 0.46 [a,c]	0.37 ± 0.08
Group V	2.10 ± 0.46 [a,c]	2.34 ± 0.49 [a,b,c]
Group VI	2.25 ± 0.49 [a,c]	0.33 ± 0.06 [d]
Group VII	0.34 ± 0.16 [b,d,e]	1.95 ± 0.36 [a,b,c,e]

a: Groups when compared with GI ($p<0.05$). b: Groups when compared with GIII ($p<0.05$). c: Groups when compared with GIV ($p<0.05$). d: Groups when compared with GV ($p<0.05$). e: Groups when compared with GVI ($p<0.05$).

inverse correlation between octanoylated ghrelin and blood glucose levels. In the literature, a negative correlation between octanoylated ghrelin and glucose has been reported (24). In addition, NUCB2/nesfatin-1 mainly decreases food intake. As indicated in previous reports, NUCB2/nesfatin-1 has these effects through leptin-independent mechanisms (25). Accordingly, increases in NUCB2/nesfatin-1 levels may be responsible for this decrease in glycemic levels after prolonged TT. In our study, the administration of NAS without inducing TT (GVII) also increased NUCB2/nesfatin-1 levels. Levels of an appetite-suppressing hormone (i.e., NUCB2/nesfatin-1) increase with the administration of NAS, which support the use of this drug for the treatment of obesity.

Our results showed that 12-hour torsion (GIII) and the administration of NAS therapy (GIV) did not affect NUCB2/nesfatin-1 expression. This may be due to specific physiologic events after the expression of NUCB2/nesfatin-1 and through the regulation of thirst (26). In addition, to conserve energy related to the functions of NUCB2/nesfatin-1 in energy metabolism, the organism may have some restrictions, which may explain this condition (27). However, over time (GV), levels of NUCB2/nesfatin-1 levels increased because of the release of cellular contents into circulation to decrease inflammation due to IR (28). In addition, NAS treatment in these cases did not decrease NUCB2/nesfatin-1 levels because the doses of NAS were likely inadequate to resolve oxidative damage or there is an inadequate number of receptors in necrotic tissues, which will limit the activity of NAS. In this study, although testicular synthesis of NUCB2/nesfatin-1 has been confirmed, as demonstrated in the 12-hour torsion groups, NAS treatment did not markedly affect NUCB2/nesfatin-1 synthesis. Although NAS treatment has an impact on the levels of octanoylated ghrelin, further investigations should be performed to explore why NAS treatment had no effects on NUCB2/nesfatin-1 levels in the *de novo* 12-hour torsion groups.

In conclusion, this is the first study to explore the effects of NAS on levels of octanoylated ghrelin and NUCB2/nesfatin-1 in TT. We observed that NAS did not affect levels of NUCB2/nesfatin, but did affect levels of octanoylated ghrelin. It is possible that through their antioxidant effects (1), NAS and ghrelin decrease the severity of IR injury in testicular tissue following detorsion. Because in this study it was observed that because in this study it was observed that TOS values of NAS statistically decreased the increasing values in GIII and GV (ischemia groups).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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