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Original Research

The Effects of N-Acetylcysteine on Asprosin Immunoreactivity in a Rat Ovarian Torsion-Detorsion Model

Gülay Bulu[1,*], Alpaslan Akyol[2], Tuncay Kuloğlu[3], Cengiz Şanlı[1]

[1]Department of Obstetrics and Gynecology, Fethi Sekin City Hospital, 23000 Elazig, Türkiye
[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Fırat University, 23119 Elazig, Türkiye
[3]Department of Histology and Embryology, Faculty of Medicine, Fırat University, 23119 Elazig, Türkiye
*Correspondence: gbulu@firat.edu.tr (Gülay Bulu)
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Abstract

Background: Ovarian torsion–detorsion injury induces ischemia–reperfusion–related oxidative stress, leading to ovarian tissue damage and impaired reproductive function. Asprosin has recently emerged as a metabolic hormone associated with oxidative stress and inflammation, but its role in ovarian ischemia–reperfusion injury remains unclear. This study aimed to evaluate the effects of N-acetylcysteine (NAC), a potent antioxidant, on reproductive capacity, as well as asprosin levels in a rat torsion–detorsion (T/D) model. **Methods:** 35 Wistar albino rats were randomly assigned to five groups (n = 7 per group): Group I (Control), Group II (Sham), Group III (NAC), Group IV (T/D), and Group V (T/D + NAC). Ovarian tissue and blood samples were collected to assess asprosin immunoreactivity, serum asprosin levels, anti-Müllerian hormone (AMH) concentrations, and total oxidant status (TOS). Histopathological changes in ovarian tissue were evaluated using immunohistochemical techniques. **Results:** Serum TOS levels were higher in the T/D group than in the Control group (12.16 vs. 2.28, $p = 0.016$) and lower in the T/D + NAC group than in the T/D group (5.38 vs. 12.16, $p = 0.032$); however, neither difference remained statistically significant after Bonferroni correction. Serum asprosin levels were significantly lower in the NAC (0.81 vs. 1.39, $p = 0.002$) and T/D (0.47 vs. 1.39, $p = 0.003$) groups compared with the Control group, whereas the difference for the T/D + NAC group (0.96 vs. 1.39, $p = 0.010$) did not remain significant after correction. Serum asprosin levels were higher in the T/D + NAC group than in the T/D group (0.96 vs. 0.47, $p = 0.008$), although this difference did not persist after Bonferroni correction. In ovarian tissue, asprosin immunoreactivity was lower in the T/D group than in the Control group ($p = 0.002$) and higher in the T/D + NAC group than in the T/D group ($p = 0.002$). AMH levels did not differ significantly among groups ($p > 0.05$). **Conclusion:** NAC administration was associated with reduced oxidative stress and attenuated degenerative changes in ovarian T/D injury. Asprosin levels reflected ischemia–reperfusion–related alterations, decreasing after torsion and showing partial recovery following NAC treatment. These findings suggest that asprosin may represent a potential biomarker of metabolic and oxidative stress in ovarian ischemia–reperfusion injury; however, the underlying mechanistic relationship warrants further investigation.

Keywords: asprosin; N-acetylcysteine; ovarian torsion-detorsion; ischemia-reperfusion injury; oxidative stress; anti-Müllerian hormone

1. Introduction

Ovarian torsion is defined as complete or partial rotation of the ovary, fallopian tube, or both around the vascular axis. It is a gynecological emergency with an estimated prevalence of 2.7% [1]. Delayed diagnosis may compromise follicular integrity and future fertility [2,3]. Ovarian masses and ovulation induction are common predisposing factors [4]. However, the mechanisms that ultimately lead to necrosis—including degree and duration of torsion—are not fully clarified [5]. Necrosis does not always occur even when the vascular pedicle is twisted, and in some cases infarction and necrosis can develop due to venous congestion and edema despite preserved arterial flow [5]. In many cases, when managed promptly, show preserved ovarian function [6,7]. For this reason, early detection and timely detorsion are central in management [8]. However, the present study is designed as a preclinical experimental model and does not directly evaluate clinical outcomes.

Nevertheless, detorsion introduces a second insult. Reperfusion triggers oxidative stress via sudden generation of reactive oxygen species (ROS) and recruitment of inflammatory cells [9]. Reperfusion itself is believed to be a major mechanism of tissue damage, independent of the ischemic period [10]. Although ovarian torsion has been widely studied, the downstream impact of reperfusion on reproductive capacity and energy metabolism is still not completely understood.

Anti-Müllerian hormone (AMH) and antral follicle count are widely used to assess ovarian reserve [11,12]. AMH is secreted by growing follicles and reflects functional ovarian capacity [12,13]. Therefore, monitoring AMH after torsion–detorsion (T/D) can provide insight into whether ovarian reserve is preserved. Serum AMH levels may thus indicate reproductive capacity after ovarian T/D.

Asprosin is a recently discovered protein hormone secreted by white adipose tissue and encoded by the Fibrillin 1 (*FBN1*) gene [14]. It plays a critical role in glu-

cose homeostasis and energy metabolism, particularly in the liver [15]. However, emerging evidence suggests that asprosin's biological functions extend beyond its metabolic effects. It has been implicated in oxidative stress regulation, mitochondrial activity, apoptosis, and inflammatory signaling via pathways such as Nuclear factor kappa B (NF-κB) and Toll-like receptor 4 (TLR4) [16,17,18]. Experimental data indicate that asprosin can modulate the production of ROS, endothelial dysfunction, and cytokine secretion, thereby linking energy metabolism to inflammatory and oxidative processes [17,18,19]. These properties suggest a potential pathophysiological role of asprosin in acute ischemia–reperfusion (I/R) injury, where sudden metabolic and oxidative stress occur during reperfusion. Previous experimental studies have also demonstrated the involvement of asprosin in ischemia- or oxidative stress–related injuries in other tissues. Asprosin has been shown to activate the TLR4–NF-κB–NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome pathway and promote vascular inflammation [16,17]. Inhibition of asprosin has been reported to attenuate oxidative stress and neointima formation following vascular injury [17]. It has been shown to enhance cytokine production and inflammatory signaling under oxidative conditions [18]. These findings collectively support the rationale for evaluating asprosin dynamics in ovarian I/R injury, where metabolic, inflammatory, and oxidative mechanisms coexist.

In an ovarian T/D model, ischemia and reperfusion may alter asprosin synthesis and release, reflecting both cellular energy demand and inflammatory activation. Evaluating asprosin expression in this setting is therefore mechanistically justified and may clarify how metabolic and inflammatory signals intersect during I/R. In this study, we primarily evaluated asprosin as a biomarker of metabolic and oxidative stress in ovarian I/R injury. Given its role in oxidative, mitochondrial, and inflammatory pathways, asprosin may also have mechanistic implications in tissue injury. However, this experiment was not designed to prove causality, and asprosin is interpreted here mainly as a stress-related biomarker rather than as an established causal mediator.

During ischemia and reperfusion, oxidative stress arises via mitochondrial dysfunction, Adenosine triphosphate (ATP) depletion, intracellular calcium overload, and cytoskeletal injury, all of which drive ROS production [20]. ROS can damage membranes, DNA, and proteins, and compromise tissue integrity. Antioxidants are therefore critical for limiting this injury [20,21]. N-acetylcysteine (NAC) is a thiol-containing antioxidant that replenishes intracellular glutathione, supports redox homeostasis, and reduces free radical–mediated damage [22,23]. In addition to its antioxidant effect, NAC also exerts antimutagenic and anti-inflammatory activity and has been used to mitigate I/R injury in various organs [22,23]. However, its effects in ovarian T/D models remain insufficiently characterized.

Unlike conventional oxidative or inflammatory markers such as Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), or malondialdehyde, asprosin lies at the interface of metabolic regulation and ischemia-related oxidative stress. As a glucogenic and proinflammatory adipokine, it can simultaneously reflect energetic stress and inflammatory activation. Measuring asprosin in ovarian T/D may therefore add information beyond classical cytokines and help clarify whether metabolic–endocrine pathways contribute to tissue recovery or damage.

Based on these considerations, we hypothesized that NAC may reduce oxidative injury and modulate asprosin levels in this experimental setting.

Although NAC has been previously shown to exert antioxidant and cytoprotective effects in various I/R models, its impact on metabolic–endocrine mediators such as asprosin has not been investigated in ovarian T/D injury. Therefore, this study was designed not merely to reconfirm the protective role of NAC, but to explore whether this protection may be linked to modulation of asprosin dynamics and oxidative stress balance.

This study aimed to investigate the potential protective effects of NAC against ovarian I/R injury induced by T/D, while also evaluating asprosin as a potential novel biomarker reflecting metabolic and oxidative alterations in this model. The findings of this study are expected to provide preliminary insights into the effects of NAC on oxidative stress and asprosin dynamics in an experimental ovarian I/R model, and to generate hypotheses for future mechanistic and translational research. Accordingly, the present study should be interpreted as an exploratory preclinical investigation aimed at understanding early biochemical and histopathological responses rather than establishing direct clinical applicability.

2. Materials and Methods

2.1 Animal Model

This randomized controlled experimental study was approved by the Experimental Animal Ethics Committee of Fırat University Faculty of Medicine (Number: 2019/02; meeting date: January 30, 2018) and conducted in accordance with ethical guidelines by the Department of Obstetrics and Gynecology at Fırat University. The experimental phase took place in April 2019 at the Experimental Animal Research Center of the Fırat University Faculty of Medicine. All experimental procedures were performed in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and institutional animal welfare regulations. Humane endpoints were predefined to minimize pain and distress, and all animals were monitored throughout the procedure for signs of suffering. Anesthesia and euthanasia were carried out under the supervision of a veterinary specialist, and every effort was made to reduce the number of animals used and their discomfort.

Animals were housed under standard laboratory conditions (22–25 °C, 12-hour light/dark cycle) with ad libitum access to food and water, in accordance with institutional and ARRIVE guidelines.

2.2 Experimental Protocol

Thirty-five female Wistar albino rats (n = 7 per group), aged 8–10 weeks, were randomly divided into five equal groups:

Group I (Control);

Group II (Sham);

Group III (NAC);

Group IV (torsion–detorsion; "T/D");

Group V (torsion–detorsion + NAC; "T/D + NAC").

We use the abbreviations "T/D" and "T/D + NAC" consistently in the Results, Tables, and Figures.

All animals were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (10 mg/kg). Adequate analgesia was provided by the combined use of ketamine and xylazine, which induces both anesthesia and muscle relaxation with a mild analgesic effect. No additional postoperative analgesic was administered, as all animals were euthanized immediately after tissue sampling in accordance with ARRIVE 2.0 ethical guidelines. Because the experimental protocol was acute in nature and all animals were euthanized shortly after the reperfusion period under anesthesia, additional postoperative analgesics were not administered, thereby minimizing potential pain and distress. This approach was selected to avoid potential confounding effects of analgesic agents on oxidative stress parameters and metabolic biomarkers, including asprosin levels. A midline laparotomy was performed to access the ovaries under sterile conditions.

Group I (Control group): We performed a midline lower abdominal incision of approximately 2.5–3 cm. After visualizing the uterus and adnexa, we closed the abdomen. Three hours later, we reopened the abdomen, excised both ovaries, collected blood samples, and euthanized the rats.

Group II (Sham group): We exteriorized the ovaries and placed sutures at the upper and lower poles without vascular occlusion. After 3 hours, we removed the sutures. At the 6th hour, we excised both ovaries, collected blood, and euthanized the rats.

Group III (NAC group): We administered a single intraperitoneal dose of NAC (100 mg/kg) 30 minutes before laparotomy. After visualizing the uterus and adnexa, we closed the abdomen. Three hours later, we reopened the abdomen, excised both ovaries, collected blood, and euthanized the rats. This group was included to control for the potential systemic or metabolic effects of NAC administration in the absence of I/R injury. It served as a negative control to differentiate NAC's intrinsic biochemical influence from its protective effects against T/D–induced oxidative stress.

Group IV (T/D group): After laparotomy, we rotated the bilateral adnexa and ovaries 720° clockwise and fixed them to the lateral abdominal wall with 5/0 polydioxanone suture to prevent spontaneous detorsion. After 3 hours, we reopened the abdomen, removed the fixation suture, and performed detorsion.

The T/D procedure and Sham design in this study were based on validated rat ovarian I/R models. In these models, the adnexa are rotated 720° (two complete turns) for 3 hours and subsequently detorsed for 3 hours, which provides a consistent and reversible ischemic injury without causing immediate necrosis [24,25].

In the present study, the Sham group was designed to reproduce surgical exposure and manipulation without inducing vascular occlusion. Therefore, the adnexa were exteriorized and temporarily fixed to the abdominal wall for the same duration (3 hours) without rotation, ensuring equivalent surgical and anesthetic stress across groups [25].

The 3-hour torsion and 3-hour detorsion periods were selected based on prior experimental rat models demonstrating that this duration is sufficient to induce ischemic changes, oxidative stress, and histopathological injury while avoiding irreversible necrosis or systemic deterioration [24,25]. Although significant hormonal alterations, such as AMH changes may not fully manifest within this short period, early oxidative and metabolic perturbations during reperfusion are known to precede longer-term functional alterations in ovarian reserve. After the reperfusion phase, bilateral ovaries were excised, blood samples were collected, and the rats were sacrificed under anesthesia for biochemical and histopathological analyses. This timing was intentionally chosen to model a clinically relevant, salvageable ovary rather than a frankly necrotic ovary. As such, the injury produced is expected to be predominantly biochemical/oxidative and histologically focal, rather than a global follicular loss severe enough to acutely suppress AMH secretion.

Group V (T/D + NAC group): We performed torsion as in Group IV (720° clockwise rotation and fixation). We administered a single intraperitoneal dose of NAC (100 mg/kg) 30 minutes before detorsion. Three hours after torsion, we reopened the abdomen, removed the fixation suture, and performed detorsion. Three hours after detorsion, we performed a final laparotomy, excised both ovaries, collected blood, and euthanized the rats.

2.3 Biochemical Study

All biochemical analyses were conducted in the Medical Biochemistry Laboratory at Fırat University Faculty of Medicine.

We collected blood in biochemistry tubes containing aprotinin, then centrifuged samples at 3500 rpm for 5 minutes to separate serum. We stored serum at −80 °C until analysis.

Table 1. Technical characteristics of ELISA assays used for biochemical analysis.

Parameter	Kit/Manufacturer	Catalog/REF No	Sensitivity	Measurement range	Intra-assay CV	Inter-assay CV
TOS	Rat TOS Enzyme-linked immunosorbent assay (ELISA) Kit (YL Biotechnology Co., Ltd., Shanghai, China)	Cat. No: YLA1392Ra	0.013 U/mL	0.02–60 U/mL	<10%	<12%
Asprosin	Rat Asprosin ELISA Kit (Mega Tıp Sanayi ve Tic. Ltd., Gaziantep, Türkiye)	REF: YX-011916R	0.054 ng/mL	0.10–30 ng/mL	<10%	<12%
AMH	Rat AMH ELISA Kit (Andygene Biotechnology Co., Ltd., Beijing, China)	—	0.500 ng/mL	0.50–50 ng/mL	<10%	<12%

Note: Abbreviations: TOS, total oxidative status; AMH, anti-Müllerian hormone; CV, coefficient of variation.

2.4 Serum TOS, Asprosin and AMH Levels Measurement

We measured serum total oxidant status (TOS) using an Enzyme-linked immunosorbent assay (ELISA) method (Rat TOS ELISA kit, Catalog No: YLA1392Ra, YL Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The kit range was 0.02–60 U/mL, intra-assay coefficient of variation (CV) <10%, inter-assay CV <12%, and sensitivity 0.013 U/mL. We washed plates using a Bio-Tek ELX50 automatic washer (BioTek Instruments, Winooski, VT, USA) and read absorbance with a ChroMate Microplate Reader P4300 (Awareness Technology Instruments, Palm City, FL, USA). Results are reported as U/mL.

We measured serum asprosin using an ELISA kit (LOT: 201806, Mega Medical Industry and Trade Ltd., Gaziantep, Türkiye) following the manufacturer's protocol. The intra-assay CV was <10%, inter-assay CV <12%, sensitivity 0.054 ng/mL. We washed plates with a Bio-Tek ELX50 washer and read absorbance at 450 nm with a Bio-Tek ELX800 ELISA reader (BioTek Instruments, USA). We plotted standard concentrations, generated a standard curve, and calculated sample values (ng/mL) after adjusting for dilution.

We quantified serum AMH using an ELISA kit (Andygene Biotechnology Co., Ltd., Beijing, China) according to the catalog protocol. Assay sensitivity was 0.5 ng/mL. We read absorbance at 450 nm using a Multiskan FC Microplate Photometer (ThermoFisher Scientific, Vantaa, Finland). Table 1 summarizes analytical performance (sensitivity, range, precision).

2.5 Preparation of Tissue Samples

Ovarian tissues were fixed in 10% formaldehyde, processed routinely (see Table 2), and embedded in paraffin. Sections (4–6 µm) were mounted on polylysine-coated slides, deparaffinized, and rehydrated through graded alcohols. Antigen retrieval was performed using citrate buffer (pH: 6).

Immunohistochemical staining was carried out using an anti-asprosin primary antibody (1:200; FineTest, Wuhan, Hubei, China), followed by appropriate secondary antibody and streptavidin–peroxidase application. Visualization was achieved using an 3-amino-9-ethylcarbazole (AEC) chromogen, and sections were counterstained with hematoxylin. Slides were examined and imaged using a Leica DM500 microscope (Leica Microsystems, Wetzlar, Germany).

Table 2. Histological processing series.

No.	Procedure	Duration
1	70% Alcohol	2 hours
2	80% Alcohol	1.5 hours
3	96% Alcohol I	30 minutes
4	96% Alcohol II	30 minutes
5	100% Alcohol I	30 minutes
6	100% Alcohol II	30 minutes
7	Alcohol + Xylene	15 minutes
8	Xylene I	15 minutes
9	Xylene II	15 minutes
10	Soft Paraffin + Xylene	45 minutes
11	Soft Paraffin	1 hour
12	Soft Paraffin + Hard Paraffin	1.5 hours
13	Hard Paraffin	3 hours
14	Inlaid	

Staining specificity was confirmed using positive and negative controls. All sections were evaluated by a blinded histologist. Asprosin immunoreactivity was assessed semi-quantitatively using a histoscore based on staining intensity and distribution, calculated from multiple high-power fields.

Semi-quantitative histopathological scoring was based on the evaluation of multiple non-overlapping high-power fields rather than a single representative image. All sections were examined at higher magnification, and the final histoscore represents the average of several fields assessed by a blinded histologist. Therefore, representative images may not fully reflect subtle but statistically significant differences detected through systematic scoring. Although digital image analysis software was not used, standardized semi-quantitative scoring criteria applied across multiple fields ensured consistency and reproducibility.

We calculated a histoscore by multiplying prevalence and severity. Prevalence categories were: 0.1 (<25%), 0.4 (26–50%), 0.6 (51–75%), and 0.9 (76–100%). Severity was scored as 0 (none), +0.5 (very little), +1 (mild), +2 (moderate), and +3 (severe).

Table 3. Serum TOS levels (U/mL).

Groups	TOS Median (IQR)	*p*	*p**
Group 1 (Control)	2.28 (1.29–3.12)	0.001	1–2: 0.931
			1–3: 0.063
Group 2 (Sham)	2.12 (1.35–2.95)		1–4: 0.016
			1–5: 0.008
Group 3 (NAC)	3.51 (2.49–4.66)		2–3: 0.038
			2–4: 0.010
Group 4 (torsion–detorsion – T/D)	12.16 (7.59–15.16)		2–5: 0.004*
			3–4: 0.029
Group 5 (torsion–detorsion + NAC – T/D + NAC)	5.38 (3.39–8.54)		3–5: 0.090
			4–5: 0.032

Values are presented as median (IQR). *p* values are derived from Kruskal–Wallis (*p*) and Mann–Whitney U (*p**) tests. Bonferroni correction was applied for multiple pairwise comparisons (adjusted significance threshold $p < 0.005$). For clarity, only statistically significant comparisons are marked with an asterisk (*). For exploratory analyses, false discovery rate (FDR)-adjusted q values were calculated ($q < 0.05$). Effect sizes (η^2 and r, with 95% confidence intervals) were calculated ($\eta^2 = 0.87$, 95% CI: 0.71–0.92). TOS, total oxidant status; NAC, N-acetylcysteine; IQR, interquartile range; T/D, torsion–detorsion.

2.6 Statistical Analysis

We calculated the minimum required sample size using G*Power 3.1 (Heinrich Heine University Düsseldorf, Düsseldorf, Germany) (F test, one-way fixed-effects Analysis of variance (ANOVA)). The primary outcome was serum TOS, based on prior studies of ovarian I/R showing large intergroup differences. Assuming a large effect size (Cohen's $f = 0.40$), power $(1–\beta) = 0.80$, and $\alpha = 0.05$ for one-way ANOVA across five groups, the required total sample size was 35 rats (7 per group). This provided adequate power to detect biologically relevant differences.

We performed statistical analysis in SPSS 22.0 (IBM Corp., Armonk, NY, USA). We used the Kruskal–Wallis test for overall comparison of more than two groups and the Mann–Whitney U test for pairwise comparisons. When Kruskal–Wallis indicated a significant overall difference ($p < 0.05$), we then conducted pairwise post hoc Mann–Whitney U tests with Bonferroni-adjusted significance thresholds. Continuous variables with non-normal distributions are expressed as median (IQR).

Following global group comparisons (ANOVA or Kruskal–Wallis tests), pairwise post hoc analyses were performed between all groups to identify specific intergroup differences and to evaluate the direction and magnitude of treatment effects. Although the primary comparison of interest was between the T/D and T/D + NAC groups to assess the effect of NAC treatment, additional comparisons (e.g., Control vs. T/D + NAC) were included to determine whether NAC administration restored oxidative stress parameters and histological features toward baseline control levels.

Because multiple pairwise intergroup comparisons were conducted across five experimental groups and several biochemical and histopathological parameters, corrections for multiple testing were applied to control the type I error rate. Bonferroni correction was used for primary pairwise comparisons by dividing the nominal α (0.05) by the number of simultaneous tests within each family of outcomes. For pairwise comparisons among five groups (10 comparisons), the Bonferroni-corrected significance threshold was set at $p < 0.005$ (0.05/10). Only *p*-values below this threshold were considered statistically significant after correction. For exploratory analyses involving multiple biochemical parameters (TOS, asprosin, and AMH), the Benjamini–Hochberg false discovery rate (FDR) procedure was also calculated, with $q < 0.05$ considered statistically significant. Both unadjusted and adjusted *p*-values are reported where relevant. Statistical significance was defined as $p < 0.05$ (two-tailed) after correction for multiplicity.

Effect sizes were calculated to evaluate the magnitude of group differences. For Kruskal–Wallis analyses, eta-squared (η^2) values were computed, while for pairwise Mann–Whitney U comparisons, effect sizes were expressed as $r = Z / \sqrt{N}$. Ninety-five percent confidence intervals (95% CI) for effect sizes were obtained using bias-corrected bootstrapping with 1000 resamples. Effect sizes were interpreted as small (0.1), medium (0.3), and large (≥0.5). All statistical procedures and effect size calculations were cross-checked using SPSS v22.0 (IBM Corp., Armonk, NY, USA) and verified with bias-corrected bootstrapping in R v4.2 (R Foundation for Statistical Computing, Vienna, Austria) for reproducibility.

Table 4. Serum asprosin levels (ng/mL).

Groups	Serum asprosin levels Median (IQR)	*p*	*p**
Group 1 (Control)	1.39 (1.04–1.78)		1–2: 0.181
			1–3: 0.002*
Group 2 (Sham)	1.66 (1.21–2.21)		1–4: 0.003*
			1–5: 0.010
Group 3 (NAC)	0.81 (0.16–1.26)[a]	0.001	2–3: 0.002*
			2–4: 0.004*
Group 4 (torsion–detorsion – T/D)	0.47 (0.36–0.61)[a]		2–5: 0.009
			3–4: 0.149
Group 5 (torsion–detorsion + NAC – T/D + NAC)	0.96 (0.74–1.22)		3–5: 0.755
			4–5: 0.008

Values are presented as median (IQR). *p* values are derived from Kruskal–Wallis (*p*) and Mann–Whitney U (*p**) tests. Bonferroni correction was applied for multiple pairwise comparisons (adjusted significance threshold $p < 0.005$). For clarity, only statistically significant comparisons are marked with an asterisk (*). For exploratory analyses, Benjamini–Hochberg FDR-adjusted q values were calculated ($q < 0.05$). [a] $p < 0.005$ vs. Control (Bonferroni-corrected). Effect sizes (η^2 and r, with 95% confidence intervals) were calculated ($\eta^2 = 0.75$, 95% CI: 0.58–0.87; $r = 0.88$). NAC, N-acetylcysteine; IQR, interquartile range.

3. Results

3.1 Biochemical Findings

In biochemical analyses assessing serum TOS levels across groups, Control, Sham, and NAC groups showed similar values. Compared with the Control group, the T/D group showed higher TOS levels ($p = 0.016$), and the T/D + NAC group showed lower serum TOS levels than the T/D group ($p = 0.032$); however, these pairwise comparisons did not remain statistically significant after Bonferroni correction (adjusted $\alpha = 0.005$) (Table 3). The intergroup difference in TOS levels demonstrated a large effect size ($\eta^2 = 0.87$, 95% CI: 0.71–0.92), indicating a large effect size, although the difference did not reach statistical significance after Bonferroni correction.

The Control and Sham groups showed comparable asprosin levels. Asprosin levels were lower in the NAC ($p = 0.002$), T/D ($p = 0.003$), and T/D + NAC ($p = 0.010$) groups compared with the Control group. After Bonferroni correction (adjusted $\alpha = 0.005$), the NAC and T/D groups remained significantly different from the Control group, whereas the T/D + NAC versus Control comparison did not. The T/D + NAC group tended to show higher asprosin levels than the T/D group ($p = 0.008$), but this comparison did not remain statistically significant after Bonferroni correction (Table 4). The serum asprosin difference among groups also indicated a large effect ($\eta^2 = 0.75$, 95% CI: 0.58–0.87), with a strong pairwise effect between the T/D and T/D + NAC groups ($r = 0.88$).

The Control and experimental groups showed similar AMH levels, with no statistically significant differences observed (Table 5). The AMH results showed negligible effect sizes ($\eta^2 \approx 0.00$, $r = 0.26$), consistent with non-significant *p*-values and the absence of intergroup differences. Importantly, the lack of change in AMH should not be interpreted as the absence of injury, but rather as the absence of measurable depletion of ovarian reserve within the short experimental window. AMH is predominantly secreted by small growing follicles and typically declines after more prolonged, extensive follicular loss. In the present T/D model, torsion was limited to 3 hours and followed by 3 hours of reperfusion, which is sufficient to induce acute oxidative and metabolic stress (as reflected by elevated TOS and altered asprosin) but may not be long enough to cause overt follicle depletion detectable by AMH. This pattern is consistent with subclinical/early injury rather than irreversible loss of reproductive potential.

Table 5. Serum AMH levels (ng/mL).

Groups	Serum AMH levels Median (IQR)	*p*
Group 1 (Control)	13.70 (11.94–15.07)	
Group 2 (Sham)	14.29 (12.71–16.53)	
Group 3 (NAC)	14.16 (11.43–17.04)	0.834
Group 4 (torsion–detorsion – T/D)	14.63 (11.44–16.97)	
Group 5 (torsion–detorsion + NAC – T/D + NAC)	13.84 (12.06–16.21)	

Values are presented as median (IQR). *p* values are from Kruskal–Wallis tests. For exploratory analyses, Benjamini–Hochberg FDR-adjusted q values were calculated ($q < 0.05$). Effect sizes (η^2 and r, with 95% confidence intervals) were calculated ($\eta^2 \approx 0.00$; $r = 0.26$). AMH, anti-Müllerian hormone; NAC, N-acetylcysteine; IQR, interquartile range.

After Bonferroni correction for multiple pairwise comparisons (adjusted $\alpha = 0.005$), only selected comparisons remained statistically significant. Specifically, the T/D versus T/D + NAC comparison remained significant

for ovarian asprosin histoscore, whereas the corresponding serum TOS and serum asprosin comparisons did not retain statistical significance after Bonferroni correction.

3.2 Immunohistochemical Findings

Asprosin Immunoreactivity

Immunohistochemical staining for asprosin immunoreactivity under light microscopy revealed asprosin expression in the ovarian stroma (indicated by black stars). Asprosin immunoreactivity in ovarian tissue was similar among the Control (Fig. 1), Sham (Fig. 2), and NAC (Fig. 3) groups (Table 6). Compared with the Control group, asprosin immunoreactivity was significantly lower in the T/D group (p = 0.002) (Table 6, Fig. 4). However, when compared with the T/D group, asprosin immunoreactivity was higher in the T/D + NAC group (p = 0.002), and this difference remained statistically significant after Bonferroni correction (adjusted α = 0.005) (Table 6, Fig. 5). Photomicrographs from each experimental group are presented in Figs. 1,2,3,4,5, with positive asprosin immunostaining indicated by black stars and negative areas by red stars. Appropriate positive and negative controls were included to confirm staining specificity.

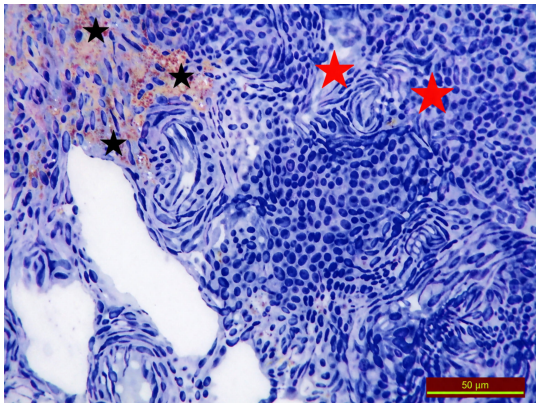

Fig. 1. Asprosin immunoreactivity in the ovarian stroma of the control group. Photomicrograph of ovarian tissue from the Control group showing weak cytoplasmic asprosin immunoreactivity (indicated by stars) in stromal cells. The black star denotes stromal cells with positive red 3-amino-9-ethylcarbazole (AEC) staining, whereas the red star indicates an area with absent or minimal staining (negative). Sections were counterstained with hematoxylin (Immunohistochemistry, AEC chromogen, hematoxylin counterstain). Scale bar = 50 µm.

The asprosin histoscore analysis demonstrated a large effect size (η^2 = 0.60) and a strong pairwise effect (r = 0.88) between the T/D and T/D + NAC groups.

4. Discussion

The findings of the current study provide preliminary insights into the effects of NAC on biochemical and

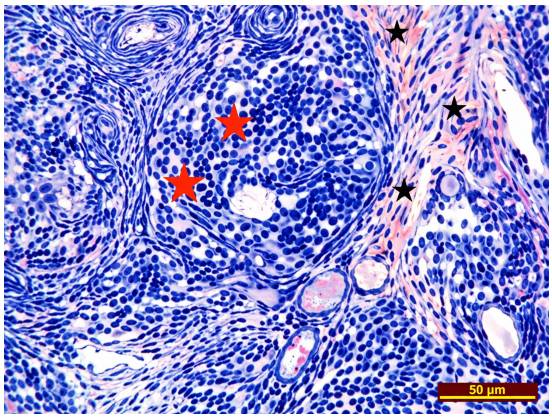

Fig. 2. Asprosin immunoreactivity in the ovarian stroma of the Sham group. Photomicrograph of ovarian tissue from the Sham group showing mild cytoplasmic asprosin immunoreactivity in stromal cells (stars). Sections were counterstained with hematoxylin. Scale bar = 50 µm.

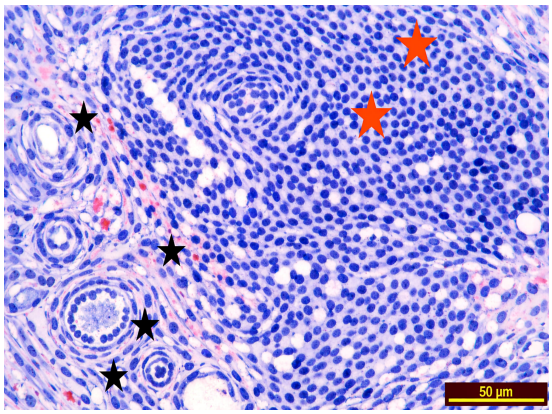

Fig. 3. Asprosin immunoreactivity in the ovarian stroma of the NAC group. Photomicrograph of ovarian tissue from the NAC-only group showing moderate cytoplasmic asprosin immunoreactivity in stromal cells (stars). Sections were counterstained with hematoxylin. Scale bar = 50 µm.

histopathological changes in an experimental ovarian T/D model. Ovarian torsion, a clinical condition leading to ischemia and reperfusion injury, may pose a risk to ovarian tissue integrity, and our study aimed to evaluate the potential protective role of NAC, an antioxidant known for its ability to combat oxidative stress.

The increase in oxidative stress associated with ovarian torsion and subsequent detorsion is a well-established phenomenon, primarily driven by the overproduction of ROS, which cause cellular damage. Previous studies have demonstrated that I/R injury in ovarian tissue is mediated through ROS generation, leading to damage to cell membranes, mitochondrial dysfunction, and follicular apoptosis [26,27]. NAC is a thiol-containing compound with well-established antioxidant and cytoprotective properties [28]. Acting as a precursor of glutathione, NAC mitigates ox-

Table 6. Asprosin histoscore.

Groups	Asprosin histoscore Median (IQR)	*p*	*p**
Group 1 (Control)	0.91 (0.80–1.20)	0.003	1–2: 0.699 1–3: 0.792 1–4: 0.002* 1–5: 0.180 2–3: 0.931 2–4: 0.002* 2–5: 0.310 3–4: 0.004* 3–5: 0.329 4–5: 0.002*
Group 2 (Sham)	0.83 (0.60–0.90)		
Group 3 (NAC)	0.88 (0.60–1.20)		
Group 4 (torsion–detorsion – T/D)	0.25 (0.20–0.45)[a]		
Group 5 (torsion–detorsion + NAC – T/D + NAC)	0.74 (0.45–0.90)[b]		

Values are presented as median (IQR). *p* values are from Kruskal–Wallis (*p*) and Mann–Whitney U (*p**) tests. Bonferroni correction was applied for multiple pairwise comparisons (adjusted significance threshold $p < 0.005$). For clarity, only statistically significant comparisons are marked with an asterisk (*). [a] $p < 0.005$ vs. Control (Bonferroni-corrected); [b] $p < 0.005$ vs. T/D (Bonferroni-corrected). Effect sizes (η^2 and r, with 95% confidence intervals) were calculated ($\eta^2 = 0.60$; r = 0.88). NAC, N-acetylcysteine; IQR, interquartile range.

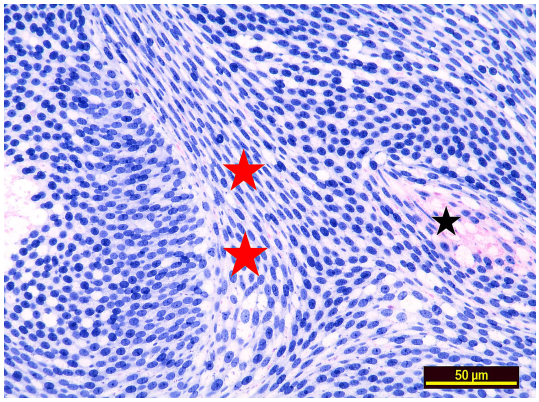

Fig. 4. Asprosin immunoreactivity in the ovarian stroma of the torsion–detorsion (T/D) group. Photomicrograph of ovarian tissue from the T/D group showing markedly reduced cytoplasmic asprosin immunoreactivity compared to the Control and NAC groups. Positive stromal cells (black stars) display weak red AEC staining, while most areas (red stars) remain negative for asprosin expression. Sections were counterstained with hematoxylin. Scale bar = 50 µm. Histopathological scoring was performed based on multiple high-power fields, and representative images may not fully reflect subtle quantitative differences detected by semi-quantitative analysis.

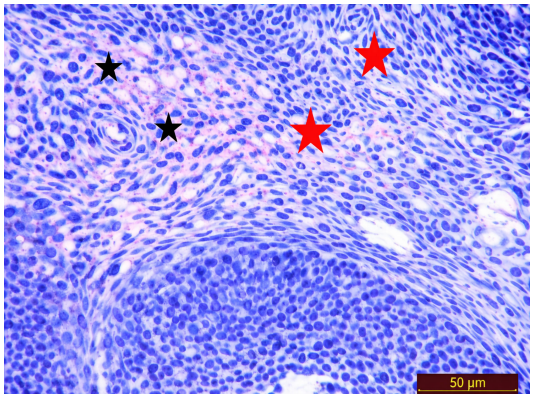

Fig. 5. Asprosin immunoreactivity in the ovarian stroma of the torsion–detorsion + NAC (T/D + NAC) group. Photomicrograph of ovarian tissue from the T/D + NAC group demonstrating stronger and more diffuse cytoplasmic asprosin immunoreactivity than in the T/D group, yet still lower than in the Control group. Positive stromal cells (black stars) show moderate to intense red AEC staining, while areas marked by red stars indicate regions with reduced or absent asprosin expression. The wider distribution of red staining suggests partial restoration of asprosin expression after NAC treatment. Sections were counterstained with hematoxylin. Scale bar = 50 µm. Histopathological scoring was performed based on multiple high-power fields, and representative images may not fully reflect subtle quantitative differences detected by semi-quantitative analysis.

idative stress by scavenging ROS and replenishing intracellular thiol pools [29]. In ovarian I/R models, NAC has been reported to attenuate lipid peroxidation, reduce interstitial edema, and preserve follicular morphology [30]. Experimental evidence also indicates that NAC enhances follicular viability by restoring mitochondrial redox balance and inhibiting pro-inflammatory cytokines, thereby protecting the ovarian stroma from reperfusion-associated injury [29,30]. Clinical and experimental data further support its potential to improve ovarian function in gynecologic contexts such as polycystic ovary syndrome [29]. Collectively, these findings suggest that the observed effects of NAC may be related to its antioxidant properties and its association with reduced oxidative stress. One of the notable findings in our study is the reduction in TOS in the

T/D + NAC group ($p = 0.032$) compared to the T/D group. This decrease may suggest an association between NAC administration and reduced oxidative stress, although it did not remain statistically significant after Bonferroni correction. These results are in line with prior research suggesting that NAC-mediated enhancement of intracellular glutathione and reduction of oxidative stress may be beneficial in various models of ischemia-reperfusion injury. Although NAC is known to enhance intracellular glutathione and stabilize redox homeostasis, we did not directly measure reduced or oxidized glutathione (GSH/GSSG). Therefore, the link between NAC and glutathione metabolism in this study is inferential and is based on the observed reduction in TOS rather than on direct biochemical confirmation.

Comparable protective effects have also been reported in other ovarian T/D models using different antioxidant and cytoprotective agents. Combined antioxidant treatments have been shown to significantly reduce total oxidant status and oxidative stress index, improve histological injury scores, and preserve primordial and primary follicle counts following ovarian I/R injury [31]. Similarly, mannitol and vitamin D, alone and particularly in combination, have been reported to attenuate oxidative stress, increase proliferative activity, and improve follicular integrity in a rat ovarian T/D model [32]. These findings support the biological plausibility of our results by indicating that pharmacological strategies targeting reperfusion-induced oxidative damage may be associated with preservation of ovarian architecture. Within this framework, the observed NAC-associated reduction in oxidative stress and attenuation of degenerative changes in our study are consistent with a broader pattern in which antioxidant interventions limit I/R–mediated loss of ovarian reserve.

Asprosin, the C-terminal cleavage product of profibrillin, is a recently identified adipokine with a potent regulatory role in metabolism [33]. In addition to stimulating hepatic gluconeogenesis, which elevates blood insulin and glucose levels, asprosin also directly promotes pancreatic β-cell apoptosis [19,33]. It influences glucose homeostasis and energy metabolism. The role of asprosin—a hormone linked to metabolic stress and energy regulation—emerged as another intriguing aspect of our study. In this context, considering that ovarian torsion and reperfusion affect energy metabolism, changes in asprosin levels in the T/D group were anticipated. Thus, we evaluated asprosin levels in reperfusion injury following ovarian torsion and detorsion, given its established role in energy metabolism and glucose homeostasis. Previous studies have demonstrated an association between altered asprosin levels and insulin resistance, diabetes, metabolic syndrome, obesity, and Polycystic ovary syndrome (PCOS) [34,35,36]. In our study, serum asprosin levels were significantly reduced in the T/D group ($p = 0.003$), reflecting metabolic disturbances and cellular stress resulting from ischemia. This reduction may indicate impaired glucose regulation and energy metabolism in ovarian tissue, as asprosin plays a critical role in maintaining blood glucose levels under stress conditions. Variations in asprosin levels observed between our study and previous studies involving different organs may be attributed to tissue-specific receptor expression and the influence of hormonal environments [37,38]. Furthermore, effects of asprosin on energy metabolism may vary depending on the tissue type [39,40]. Although there is a growing body of research on asprosin, further studies are needed to fully elucidate its functions and mechanisms of action.

The observed decrease in serum and tissue asprosin levels after torsion appears to contrast with reports describing increased asprosin secretion under systemic metabolic or hypoxic stress, such as in obesity or chronic ischemic disease [41]. However, these discrepancies likely reflect differences in the nature and duration of stress and the affected tissue type. While chronic metabolic stress enhances adipose and hepatic asprosin release as a compensatory glucogenic and proinflammatory response, acute localized I/R injury—as modeled in the ovary—may suppress local asprosin synthesis due to abrupt cellular energy failure, mitochondrial dysfunction, and loss of viable stromal cells. Therefore, the reduction observed here probably reflects acute local ischemic injury rather than systemic metabolic activation. Taken together, these findings support a model in which acute mitochondrial dysfunction and impaired local glucose handling during ovarian I/R suppress asprosin biosynthesis in stromal cells, in contrast to the compensatory elevation of circulating asprosin described under chronic systemic metabolic stress. The partial recovery with NAC treatment further supports this interpretation, suggesting that antioxidant protection may be associated with preservation of stromal cell integrity and corresponding changes in asprosin expression.

Some studies have suggested that NAC treatment may lead to elevated serum asprosin levels and has been linked to improvements in various disease states, particularly metabolic disorders [42,43]. In our study, serum asprosin levels ($p = 0.008$) and ovarian asprosin immunoreactivity ($p = 0.002$) were higher in the T/D + NAC group compared to the T/D group; however, only the difference in ovarian asprosin immunoreactivity remained statistically significant after Bonferroni correction. The increase in serum asprosin levels and asprosin immunoreactivity after NAC treatment suggests that NAC treatment was associated with changes toward control-like asprosin levels, potentially reflecting changes in metabolic and oxidative stress responses. However, since only asprosin was assessed as a metabolic biomarker in this study, the interpretation of ‘metabolic equilibrium’ should be considered preliminary and descriptive rather than confirmatory. This result is in line with prior studies that have suggested asprosin’s involvement in inflammatory processes and tissue repair, though its role in ovarian I/R injury remains under-

explored. These findings are descriptive and do not provide direct mechanistic or functional evidence regarding the role of asprosin in ovarian I/R injury.

Although these asprosin alterations were statistically significant and biologically plausible, the findings should be interpreted with caution because the present study did not include functional endpoints such as ovarian blood flow, follicular dynamics, or mechanistic pathway analyses. Therefore, the observed biochemical changes primarily reflect early metabolic and oxidative responses rather than confirmed functional recovery. Future experimental research integrating mechanistic and functional analyses (e.g., receptor-level signaling, apoptosis markers, and energy metabolism assays) is recommended to verify and expand upon these preliminary findings.

Interestingly, asprosin levels were not significantly different between the control and Sham groups, which further supports the specificity of the observed changes in the T/D and T/D + NAC groups. It is noteworthy that asprosin's role in the context of ovarian torsion and detorsion requires further investigation to fully understand its implications as a biomarker of metabolic stress.

Recent literature has highlighted a potential role of asprosin in both oxidative stress responses and reproductive physiology. In particular, Kacar et al. (2025) [44] demonstrated that circulating asprosin levels are modulated by female reproductive hormones, suggesting that asprosin may act at the interface between metabolic and reproductive pathways. Moreover, emerging evidence indicates that asprosin is responsive to oxidative stress and I/R–related metabolic disturbances [16,17,18], supporting its potential association with stress-related metabolic responses. Recent experimental and clinical studies have also demonstrated that asprosin is closely associated with oxidative stress and tissue injury–related metabolic responses [45]. This is consistent with our findings, in which asprosin levels changed in response to I/R injury and showed a partial increase following NAC administration.

In this context, the alterations in asprosin observed in our study may reflect not only oxidative injury but also broader endocrine and metabolic adaptations associated with ovarian I/R. AMH is widely used as a biomarker for assessing reproductive capacity, as it reflects the number of antral follicles and ovarian functionality [11,44]. Many studies have been conducted on serum AMH levels, indicating reproductive potential. Low AMH levels have been shown to be associated with reproductive dysfunction, including diminished reproductive capacity and impaired ovarian function [46,47]. Thus, we measured serum AMH values to evaluate ovarian reserve after the T/D and NAC treatment. We predicted that there would be changes in serum AMH levels in our study. In contrast to the changes observed in oxidative stress markers and asprosin levels, AMH levels did not show any significant differences among the groups. The lack of significant changes in AMH levels in this study could indicate that, despite the evident oxidative stress and changes in other metabolic markers, the degree of ovarian damage may not have been severe enough to influence AMH secretion. Thus, the current model appears to induce early metabolic and oxidative stress without immediate follicular depletion, which explains why AMH—a marker of follicular pool size rather than acute stress—remained unchanged at this short time point. Taken together, these findings suggest that the ovarian T/D insult produced predominantly an acute biochemical and histopathological stress response rather than an immediate, quantifiable depletion of ovarian reserve. In our model, torsion was maintained for 3 hours and detorsion/reperfusion for an additional 3 hours before tissue harvest. Prior experimental work indicates that such short-duration I/R is capable of triggering oxidative stress, edema, vascular congestion, and focal stromal damage without necessarily producing widespread follicular atresia or granulosa cell loss in the early phase. This is in line with our histological assessment, which demonstrated I/R–type injury in the T/D group and partial preservation/improvement in the T/D + NAC group, but did not show diffuse destructive loss of follicles. Therefore, the unchanged AMH levels in our study likely reflect that ovarian reserve was not yet measurably depleted, rather than indicating absence of injury. In other words, the injury at this time point appears to be subclinical with respect to long-term reserve. This interpretation is supported by previous experimental studies demonstrating that short-term I/R injury can induce early oxidative and histopathological changes without immediately affecting AMH levels, which typically decline only after more prolonged or severe follicular damage [32,48]. However, it should be emphasized that the present findings are limited to early biochemical and histopathological changes. Functional reproductive outcomes—such as ovarian blood flow, follicle count, estrous cycle dynamics, and fertility potential—were not evaluated in this study. Therefore, although NAC administration was associated with improvements in oxidative stress markers and histological features, these results cannot be directly interpreted as evidence of preserved reproductive function.

The findings of this study suggest that NAC may be associated with reduced markers of oxidative stress and tissue injury in ovarian T/D. Given the relatively large number of pairwise comparisons, even after statistical correction, some findings should be interpreted with caution as exploratory rather than definitive. However, these results should be interpreted with caution, as causal relationships cannot be established, and further mechanistic and clinical studies are required.

Limitations and Future Directions

While this study provides valuable information on the effects of NAC in a rat model of ovarian torsion-detorsion, there are certain limitations. First, the study was con-

ducted in an animal model, and while this provides valuable insights, further clinical studies are needed to translate these findings to human patients. Additionally, the mechanisms by which NAC influences asprosin expression and metabolic balance in ovarian tissues remain unclear and warrant further investigation. Finally, the duration of the torsion period and the use of NAC as a single dose may not fully replicate the clinical scenario, where delays in diagnosis and treatment are common. In clinical settings, ovarian torsion often persists for longer durations before surgical intervention, leading to more severe ischemic and follicular damage. Therefore, the 3-hour torsion model used in this study represents an acute and reversible stage of injury and may underestimate the degree of tissue necrosis or AMH decline typically observed in delayed clinical cases. In addition, the possibility of delayed follicular apoptosis and subsequent decline in ovarian reserve cannot be excluded, as the short duration of the experimental model may not fully capture long-term functional outcomes. Therefore, future studies should explore different treatment regimens, timing of administration, and longer follow-up periods to better understand the clinical potential of NAC in ovarian torsion.

Although Bonferroni and FDR corrections were applied to minimize the risk of type I error due to multiple comparisons, the possibility of residual false-positive results cannot be completely excluded. Therefore, some findings should be interpreted as exploratory rather than definitive. Despite the provision of effect sizes and confidence intervals to enhance clinical interpretability, the relatively small group sizes may limit the precision of these estimates. Another limitation is that ovarian reserve was assessed only by serum AMH at a single early post-reperfusion time point; therefore, delayed declines in reserve (e.g., due to later follicular atresia) could not be captured. In addition, functional reproductive outcomes—such as ovarian blood flow, follicle count, estrous cycle regularity, and fertility—were not assessed. Consequently, the present findings primarily reflect early biochemical and histopathological alterations rather than definitive evidence of functional ovarian preservation. Therefore, the clinical translatability of these results remains limited and requires confirmation in studies incorporating functional endpoints and longer follow-up.

Only TOS was measured as a global indicator of oxidative stress. The lack of specific antioxidant enzyme assays (such as superoxide dismutase or catalase) and lipid peroxidation markers (such as malondialdehyde) represents another limitation of this study. Moreover, specific antioxidant biomarkers such as reduced and oxidized glutathione (GSH/GSSG) were not assessed, preventing direct biochemical linkage between NAC administration and glutathione metabolism. Future studies should incorporate both oxidative and antioxidative parameters to provide a more comprehensive assessment of redox balance in ovarian ischemia–reperfusion injury.

Furthermore, all procedures were conducted in compliance with ARRIVE guidelines to ensure animal welfare, humane endpoints, and minimization of suffering; nevertheless, this experimental design remains subject to the inherent ethical limitations of animal research. The absence of additional postoperative analgesia may be considered a limitation; however, the experimental protocol was acute in nature, and all animals were euthanized shortly after the reperfusion period under anesthesia, thereby minimizing potential pain and distress. This approach was intentionally chosen to avoid potential pharmacological interference with oxidative stress parameters and metabolic biomarkers, including asprosin levels.

In addition, several methodological limitations should be acknowledged. First, the sample size was relatively small (n = 7 per group), which may reduce the statistical power to detect subtle differences and limit the generalizability of the findings. Second, the study was designed as an acute short-term experiment; therefore, no long-term follow-up was performed to evaluate the delayed or chronic effects of T/D injury and NAC treatment. Third, the evaluation of ovarian reserve relied on AMH and histoscore assessment without direct follicle counting or a specific histopathological scoring system for follicular density, which may limit the accuracy of reserve estimation. Finally, histopathological evaluation was performed by a single blinded histologist. However, the absence of inter-observer validation may be considered a limitation. These factors should be considered when interpreting the present results.

5. Conclusion

In this experimental rat model, NAC administration was associated with reduced oxidative stress (as reflected by lower TOS levels) and partial normalization of asprosin levels following ovarian T/D. These findings suggest an association between NAC treatment and changes in biochemical markers of I/R injury. However, in the absence of mechanistic or functional endpoints, causality cannot be established, and the observed associations should be interpreted with caution. Future studies incorporating longer-term follow-up and mechanistic analyses are needed to determine whether NAC confers meaningful functional protection of ovarian reserve.

Abbreviations

T/D, torsion-detorsion; TOS, total oxidant status; ROS, reactive oxygen species; NAC, N-acetylcysteine; AMH, anti-Müllerian hormone; I/R, ischemia-reperfusion.

Availability of Data and Materials

The data supporting the findings of this study are included within the article.

Author Contributions

GB, AA, TK and CŞ contributed to the study concept and design, data analysis and interpretation, and critical revision of the manuscript. GB and CŞ contributed to the acquisition of data and drafting and critical revision of the manuscript. GB, AA and TK were involved in the technical or material support and study supervision. All of the authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agree to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Experimental Animal Ethics Committee of Fırat University Faculty of Medicine (Number: 2019/02) and conducted in accordance with ethical guidelines by the Department of Obstetrics and Gynecology at Fırat University. All experimental procedures were performed in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and institutional animal welfare regulations.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this manuscript, AI-assisted tools (e.g., ChatGPT, OpenAI) were used only in the final stage for language editing purposes, including grammar correction, improving clarity, and enhancing overall readability. These tools were used solely as supportive instruments for linguistic refinement. They were not used in the study design, data collection, statistical analysis, interpretation of results, or in the generation of scientific content, tables, or figures. All scientific content, analyses, and conclusions presented in this manuscript are entirely the responsibility of the authors.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/CEOG52123.

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