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Oxaliplatin-induced testicular toxicity is associated with transglutaminase-4 upregulation, hormonal dysregulation, and inflammatory responses

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Abstract

Oxaliplatin-induced toxicity presents a major challenge in cancer management because of its damaging effects on normal tissues, including the reproductive system. Transglutaminase 4 (TG4), a member of the transglutaminase enzyme family, is known for its role in protein cross-linking and cellular stress responses, but its role in chemotherapy-induced reproductive toxicity remains poorly understood. This study examines the impact of oxaliplatin, a platinum-based chemotherapeutic drug, on TG4 expression, enzymatic activity, and testicular toxicity in a rat model following intraperitoneal administration of oxaliplatin (10 mg/kg body weight weekly for six weeks). Biochemical analysis revealed significant hormonal and inflammatory alterations, including elevated serum interleukin-1β (IL-1β) levels, decreased testosterone concentrations, and increased follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels. These endocrine disturbances were accompanied by significant upregulation of TG4 expression in oxaliplatin-treated testicular tissue (OXP-TT), as demonstrated by quantitative real-time reverse transcription PCR (qRT-PCR) and immunohistochemical (IHC) analyses. Immunofluorescence (IF) further confirmed enhanced TG4 localization within both interstitial and seminiferous tubular regions. In addition, expression of the pro-inflammatory cytokines IL-6 and TNF was significantly increased. A marked elevation in total transglutaminase enzymatic activity was also detected in oxaliplatin-treated tissues. Collectively, these hormonal, inflammatory, and structural alterations occurred concurrently with increased TG4 expression and transglutaminase activity, suggesting that TG4 participates in the testicular response to oxaliplatin-induced stress. These findings indicate that TG4 is associated with chemotherapy-related reproductive toxicity and may represent a stress-responsive indicator of testicular injury. Further studies are required to determine whether TG4 plays a protective or pathogenic role in oxaliplatin-induced testicular damage and to evaluate its potential relevance in preserving fertility during platinum-based chemotherapy.

Keywords: Apoptosis; Gene Expression; Hormonal imbalance; Inflammation; Interleukin-1β; Male reproductive toxicity; Oxaliplatin; Spermatogenesis; Testicular damage; Testicular toxicity; Transglutaminase 4 (TG4)

Highlights:

- TG4 expression is significantly elevated in oxaliplatin-treated rat testicular tissue.
- Oxaliplatin disrupts reproductive hormone balance, reducing testosterone and increasing FSH and LH levels.
- Oxaliplatin increases total transglutaminase enzymatic activity in testicular tissue.
- Oxaliplatin treatment disrupts the seminiferous tubules' architecture and enhances inflammatory marker expression.
- IL6 and TNF upregulation suggest inflammatory involvement in testicular injury.
- TG4 is associated with oxaliplatin-induced testicular stress responses and may serve as a biomarker of reproductive toxicity.

Introduction

Transglutaminases (TGMs) are a family of enzymes that catalyze the cross-linking of proteins by creating covalent bonds between glutamine (Q) and lysine (K) residues in various polypeptides. The catalytic activity of transglutaminases is calcium-dependent, as binding of Ca^{2+} induces conformational changes that activate the enzyme and enable the formation of ε-(γ-glutamyl) lysine cross-links between substrate proteins (Eckert et al., 2014). These isopeptide linkages increase the mechanical stability of the modified proteins and make them highly resistant to proteolysis (Alvarez et al., 2020). TGMs are also involved in polyamine conjugation, lipid esterification, and glutamine residue deamidation, which contribute to various physiological and pathological processes (Chermnykh et al., 2020). To date, nine members of the transglutaminase family have been identified, including TGM1-7, Factor XIII, and Band 4.2 (Min and Chung, 2018). These enzymes play essential roles in cellular homeostasis, with some exhibiting cat-

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alytic activity while others, such as Band 4.2, are inactive due to specific mutations at their active sites (Eckert et al., 2014).

TG2 is the most studied member of the transglutaminase family due to its multifunctional nature, participating in transamidation, GTPase activity, and protein disulfide isomerization (Szondy et al., 2017; Tatsukawa and Hitomi, 2021). TG2 expression is commonly elevated in response to cellular stress and tissue injury, suggesting its role in cytoprotective mechanisms (Agnihotri et al., 2013). Other TG isoforms have specific tissue distributions and functions: TG1, TG3, and TG5 are predominantly found in epithelial tissues; TG4 is mainly expressed in the prostate gland; TG6 is present in the testes, lungs, and brain; and TG7 is primarily found in the testes and lungs (Odii and Coussons, 2014). Band 4.2, while catalytically inactive, is crucial for maintaining erythrocyte membrane integrity and overall cell stability (Almami et al., 2014; Odii and Coussons, 2014).

Chemotherapy remains a primary approach for treating cancer despite its adverse effects, including systemic toxicity and drug resistance (Norouzi et al., 2016). Platinum-based drugs such as cisplatin, carboplatin, and oxaliplatin are widely used but are limited by their high systemic toxicity (Zhang et al., 2022). Oxaliplatin, in particular, is known for its neurotoxic and testicular toxicity effects (Amer et al., 2020; Ashry et al., 2022).

The enzymatic activity of transglutaminases has been linked to toxicity responses, as seen in studies where TG2 overexpression exacerbates α-synuclein toxicity in neurodegenerative models (Tatsukawa and Hitomi, 2021). Conversely, inhibiting TG2 has shown protective effects in models of gliadin-induced toxicity (Rauhavirta et al., 2013). Additionally, TG2 involvement in organophosphate-induced neurotoxicity suggests its broader role in toxicity mechanisms (Aldubayan et al., 2022; Almami et al., 2020).

Furthermore, transglutaminase expression has been implicated in multiple cancers, with overexpression observed in glioblastoma, ovarian, pancreatic, lung, and breast cancers (Sima et al., 2022). High TG2 expression is correlated with poor prognosis in several malignancies, including ovarian, lung, and pancreatic cancer, due to its role in activating oncogenic pathways such as NF-κB, Akt, and FAK (Jeong et al., 2013). Importantly, TG2-mediated chemoresistance has been demonstrated in epithelial ovarian cancer cells, where it modulates NF-κB activity (Cao et al., 2008). Similarly, studies have shown that TG2 inhibition enhances the sensitivity of cancer cells to chemotherapy (Meshram et al., 2017).

Oxaliplatin is a third-generation platinum-based chemotherapeutic drug widely used to treat solid tumors, including colorectal, ovarian, lung, and gastric cancers (Klobučar et al., 2018; Yu et al., 2021). At the molecular level, oxaliplatin forms intra- and interstrand DNA crosslinks that interfere with DNA replication and transcription, activating DNA damage response pathways and leading to cell cycle arrest and apoptosis (Cao et al., 2008). Oxaliplatin induces DNA crosslinking and activates multiple downstream signaling pathways involved in stress responses, apoptosis, and chemoresistance (Virag et al., 2013). In addition to direct DNA damage, oxaliplatin has been reported to promote oxidative stress, mitochondrial dysfunction, and activation of inflammatory signaling cascades, which collectively contribute to tissue injury and apoptotic cell death (Zhang et al., 2022). Despite its therapeutic efficacy, oxaliplatin is associated with systemic toxicity, including peripheral sensory neuropathy, gastrointestinal disturbances, and hematological effects (Rogers et al., 2019). The nonspecific biodistribution of oxaliplatin therefore, limits its therapeutic efficiency and increases adverse effects on normal tissues (Tummala et al., 2016).

Transglutaminases have been implicated in both normal physiological processes and pathological conditions, including cancer progression, apoptosis, and chemoresistance (Elli et al., 2009). Elevated TG2 expression has been associated with enhanced cell survival in various cancer models, while its downregulation has been linked to increased sensitivity to apoptosis (Meshram et al., 2017). Moreover, TG2 overexpression is frequently observed in tumors and may contribute to chemoresistance (Kim et al., 2011). These findings suggest that transglutaminases may influence cell survival and stress responses, making them relevant candidates for investigating tissue responses to platinum-based toxicity.

Transglutaminases have been extensively studied in apoptosis and cell survival pathways (Elli et al., 2009). TG2, in particular, has been found to be upregulated in response to cellular stress, including chemotherapy exposure, where it may act as either a pro-survival or pro-apoptotic factor, depending on cellular context (Meshram et al., 2017).

Chemotherapy-induced testicular toxicity is often associated with endocrine disruption and inflammatory signaling. Alterations in reproductive hormones, including reduced testosterone and increased follicle-stimulating hormone (FSH) and luteinizing hormone (LH), reflect impaired spermatogenesis and dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis following exposure to cytotoxic agents (Kirkpatrick et al., 2025). Additionally, oxidative stress and inflammatory mediators such as interleukin-1β (IL-1β) contribute to reproductive injury by damaging germ cells and disrupting steroidogenesis (Walke et al., 2023). These stress-related responses may also influence transglutaminase activity, as transglutaminases participate in cellular stress adaptation and inflammatory signaling, suggesting a potential link between hormonal disruption, inflammation, and TG-mediated tissue responses during chemotherapy-induced testicular toxicity.

Oxaliplatin, while effective in treating cancer, induces systemic toxicity including testicular damage, which remains relatively underexplored in terms of its impact on transglutaminase activity and stress-associated signaling pathways (Zhang et al., 2022). This study aims to investigate the impact of oxaliplatin treatment on transglutaminase activity, gene expression, and protein localization in testicular tissue. Understanding these changes may provide insights into the mechanisms underlying chemotherapy-induced toxicity and inform strategies to mitigate its adverse effects.

Materials and methods

Biological samples and experimental design

Twenty adult male albino rats (150–200 g) were obtained from the Faculty of Pharmacy, Qassim University. All experimental procedures involving animals were conducted in strict accordance with institutional ethical guidelines and the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Qassim University [25-03-17].

Animals were housed in well-ventilated cages under controlled environmental conditions (temperature: 25 ± 1 °C; 12 h light / 12 h dark cycle) with free access to standard laboratory chow and water. Following a one-week acclimatization period, the rats were randomly assigned into two groups ($n = 10$ per group). Group I (Control): Received no treatment and were maintained on a standard diet. Group II (Oxaliplatin-treated; OXP-TT): Received intraperitoneal injections of oxaliplatin at a dose of 10 mg/kg body weight once weekly for six consecutive weeks. Oxaliplatin was administered using a ready-to-use injectable formulation (5 mg/ml; Accord Healthcare Ireland Limited, Ireland).

At the end of the treatment period, animals were fasted for 24 h and subsequently anesthetized using sodium phenobarbital (200 mg/kg body weight, intraperitoneally), diluted 1:3 in phosphate-buffered saline (PBS). Sodium phenobarbital was administered as a single terminal anesthetic dose immediately before sacrifice. Following adequate anesthesia, animals were euthanized by decapitation. Blood samples were collected, and sera were separated by centrifugation, then stored at −80 °C for subsequent biochemical analysis. Testicular tissues were harvested and snap-frozen in liquid nitrogen for molecular and enzymatic studies. Carcasses were disposed of through the Qassim University Veterinary Hospital in compliance with international biosafety and ethical disposal standards.

Tissue preparation and immunofluorescence

To identify the specific location of transglutaminases in the tissue of normal rat testes and oxaliplatin-treated samples (OXP-TT), the tissue was fixed in 10% formaldehyde followed by an embedding in paraffin. Tissue was sectioned at 3-μm thickened and then deparaffinized in xylene and finally hydrated in ethanol. The deparaffinized tissue sections were subjected to the Immunofluorescence technique according to the manufacturer's instructions (Elabscience, Texas, USA). Briefly, tissue section slides were fixed with 3.7% (w/v) paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.1% (v/v) Triton-X100 (Elabscience, Texas, USA) in PBS for 15 min at room temperature. Each step was followed by (3×) 5 min washes with PBS. Finally, the slides were blocked with 3% (w/v) BSA for 1 h at room temperature. The specificity of staining was confirmed using negative control sections from both control and oxaliplatin-treated groups, processed identically but with omission of the primary antibody. For detection, slides were incubated with TG4 primary antibodies pAb and anti-rabbit-Cy5 secondary antibody (red). Nuclei were stained with Counter Staining-DAPI (blue) and the slide viewed at 20× or 10× magnification using EVOS FL AMF4300 fluorescence microscope (Life Technologies, USA). Images were captured to assess protein localization and distribution, and positive staining was quantified with ImageJ software.

Tissue histology and immunohistochemistry

Tissue sections of normal rat testis and oxaliplatin-treated samples (OXP-TT) were prepared as described above. Slides were deparaffinized, and antigen retrieval was performed using a sodium citrate buffer (pH 6.0) at 100 °C for 10 minutes. Sections were blocked with 0.3% (v/v) hydrogen peroxide for 10 minutes at room temperature and then incubated in the protein-blocking buffer for 10 minutes. Primary antibodies (1:1000, v/v) targeting TG4 (Abbexa, Cambridge, UK) were applied and incubated with an HRP-conjugated polymer detection system. The specificity of staining was confirmed using negative control sections from both control and oxaliplatin-treated groups, processed identically but with omission of the primary antibody. Visualization was achieved using a DAB substrate, and tissue sections were counterstained with hematoxylin. Positive staining was quantified with ImageJ software.

RNA and protein isolation

Both total RNA and proteins were isolated using TRIzol® Reagent (Life Technologies: Cat. no. 15596-026). Frozen tissue was homogenized in 1 ml of TRIzol® Reagent, mixed with chloroform, incubated for 3 min, and centrifuged at 12,000× g for 15 min at 4 °C. The aqueous phase was transferred to a new tube, and 0.5 ml of 100% isopropanol was added. After incubation for 10 min, samples were centrifuged at 12,000× g for 10 min at 4 °C to precipitate the RNA. The supernatant was discarded, and the RNA pellet was washed and resuspended in RNase-free water. Total proteins were separated from the phenol-ethanol supernatant layer using protein precipitation methods according to the manufacturer's instructions. Both RNA and protein concentrations were determined using spectrophotometry.

Genomic DNA integrity assessment

Genomic DNA was extracted using the AFTSpin Blood/Tissue/Cell Fast DNA Extraction Kit (RK30110, ABclonal Technology) according to the manufacturer's instructions. Approximately 20–25 mg of testicular tissue was homogenized and digested with Proteinase K at 56 °C for 1–3 h. DNA was purified using spin columns and eluted in 60–100 μl Buffer EB2. DNA concentration and purity were measured using a NanoDrop™ spectrophotometer. For DNA integrity assessment, 5 μg of DNA from each sample was resolved on 1.5% agarose gel in 1× TBE buffer containing SYBR Safe. Electrophoresis was performed at 100 V for approximately 60 minutes. DNA integrity was evaluated qualitatively based on high-molecular-weight bands versus smear formation.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RNA concentration and purity were assessed using a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, USA). Rat-specific primers for TG2, TG4, TG6, TG7, TNF, IL6, CASP3 and GAPDH were designed using the PrimerQuest Tool (Integrated DNA Technologies) and are listed in Table 1. Quantitative RT-PCR was performed using the ABScript II One-Step SYBR Green qRT-PCR Kit (RK20404; ABclonal Technology) on an AriaMx Real-Time PCR System (Agilent Technologies, USA). Each reaction was performed in a total volume of 20 μl containing SYBR Green qRT-PCR buffer, 10 μl ABScript II enzyme mix, 0.8 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM), 0.4 μl ROX reference dye (50×), 0.4 μl RNA template, and RNase-free water to volume. Thermal cycling conditions consisted of reverse transcription at 42 °C for 5 min, initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30–34 s. All reactions were performed in duplicate. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH used as the internal reference gene.

Transglutaminase activity assay

Transglutaminase (TG) activity was determined using a colorimetric assay kit (Elabscience®, Cat. No. E-BC-K840-M) based on the enzyme-mediated acyl transfer between glutamine residues and primary amines. The resulting product forms a chromogenic compound, with absorbance measured at 525 nm using a microplate reader.

Samples (60 μl) were incubated with 200 μl of freshly prepared measuring solution at 37 °C for 1 h in the dark. After adding 200 μl of chromogenic working solution, tubes were incubated for 10 minutes, followed by centrifugation at

Table 1. Accession numbers and primer sequences for qRT-PCR of TG isoforms and related genes

Gene/Accession number		FWD Set 1	REV Set 1
TG2	NM_019386.3	TGTTGGTCAGAGGAGTGATTG	GGAGTGGACCTTGTGGTTATT
TG4	NM_022713.2	GATGCTGTGGAGCCTTAGTT	GCTCTTGAATCTGCCCTCATA
TG6	XM_063284796.1	CATCCTGAACATCTGCCTCTC	TCGGTCGTTGTTGCTGTT
TG7	XM_008762241.4	GGGTCTTCGCCTCTGTTATG	ATCTCGGCATTTCGGTCATAG
TNF	NM_012675.3	ACCTTATCTACTCCCAGGTTCT	GGCTGACTTTCTCCTGGTATG
IL6	NM_012589.2	GCCAGAGTCATTCAGAGCAATA	TAGGAGAGCATTGGAAGTTGG
CASP3	NM_012922.3	CCACGGAATTTGAGTCCTTCT	CACTCCCAGTCATTCCTTTAG
GAPDH	NM_017008.4	GACCACTTTGTCAAGCTCATTTC	CTCTCTTCCTCTTGTGCTCTTG

12,000× g for 10 minutes. Supernatants (200 µl) were transferred to a 96-well plate for absorbance measurement. TG activity (U/g protein) was calculated from a standard curve using the formula:

$$TG\ Activity = (\Delta A525 - b)/a \cdot Cpr \cdot \frac{T}{f}$$

where ΔA_{525} = OD of sample – OD of control, a and b are derived from the standard curve, C_{pr} is protein concentration, f is the dilution factor, and T is reaction time (1 h).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 11. Differences between control and oxaliplatin-treated groups were assessed using unpaired two-tailed Student's *t*-tests. Gene expression comparisons involving multiple targets were analyzed using two-way ANOVA followed by Tukey's *post-hoc* test. A *p*-value < 0.05 was considered statistically significant.

Results

Effect of oxaliplatin on serum hormone levels and inflammatory markers

To evaluate the impact of oxaliplatin on reproductive hormonal balance and systemic inflammatory status, serum levels of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and interleukin-1β (IL-1β) were measured in control and oxaliplatin-treated rats. As shown in Table 2 and Fig. 1, intraperitoneal administration of oxaliplatin (10 mg/kg body weight, once weekly for six consecutive weeks) resulted in a significant increase in serum IL-1β levels compared with control animals (12.888 ± 0.242 vs. 7.520 ± 0.563 ng/ml), representing an increase of approximately 71.4%. In contrast, a significant decline in serum testosterone levels was observed in the oxaliplatin-treated group (3.252 ± 0.046 ng/ml) compared with controls (6.15 ± 0.220 ng/ml), corresponding to a reduction of 47.12%.

Furthermore, oxaliplatin exposure resulted in a significant elevation in gonadotropin levels. Serum FSH levels increased from 102.06 ± 0.243 ng/ml in control animals to 121.35 ± 0.408 ng/ml in treated animals (18.9% increase). Similarly, serum LH levels increased from 1.248 ± 0.065 mIU/ml in controls to 1.90 ± 0.072 mIU/ml in oxaliplatin-treated animals, representing an increase of 52.24%. These results indicate that oxaliplatin disrupts endocrine regulation of the hypothalamic-pituitary-gonadal axis and promotes systemic inflammatory responses, both of which are consistent with chemotherapy-induced testicular dysfunction.

Gene expression of transglutaminases (TG2, TG4, TG6, and TG7) in normal and oxaliplatin-treated testicular tissue

To investigate the effects of oxaliplatin on the gene expression of transglutaminases, qRT-PCR analysis was performed to evaluate the transcriptional expression of multiple transglutaminase isoforms (TG2, TG4, TG6, and TG7) in testicular tissue following oxaliplatin treatment (Fig. 2). The results revealed differential regulation among these isoforms. Among the genes examined, TG4 was the only isoform showing a statistically significant (* $p < 0.05$) increase under the present conditions.TG2 also exhibited a moderate increase, whereas TG6 and TG7 displayed comparatively smaller or variable changes in expression. These results suggest that TG4 represents the most responsive transglutaminase isoform under the present experimental conditions, which guided our subsequent focus on TG4 protein localization and enzymatic activity analyses.

Table 2. Serum levels of IL-1β, testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in control and oxaliplatin-treated rats

Parameter	Control	Oxaliplatin (OXP-TT)	% Change
IL-1β (ng/ml)	7.520 ± 0.563	12.888 ± 0.242****	+71.4
Testosterone (ng/ml)	6.15 ± 0.220	3.252 ± 0.046****	–47.1
FSH (ng/ml)	102.06 ± 0.243	121.35 ± 0.408****	+18.9
LH (mIU/ml)	1.248 ± 0.065	1.90 ± 0.072****	+52.2

Note: Data are expressed as mean ± SEM ($n = 5$ per group). Statistical significance between control and oxaliplatin-treated groups was assessed using an unpaired two-tailed Student's *t*-test. **** $p < 0.0001$ vs control.

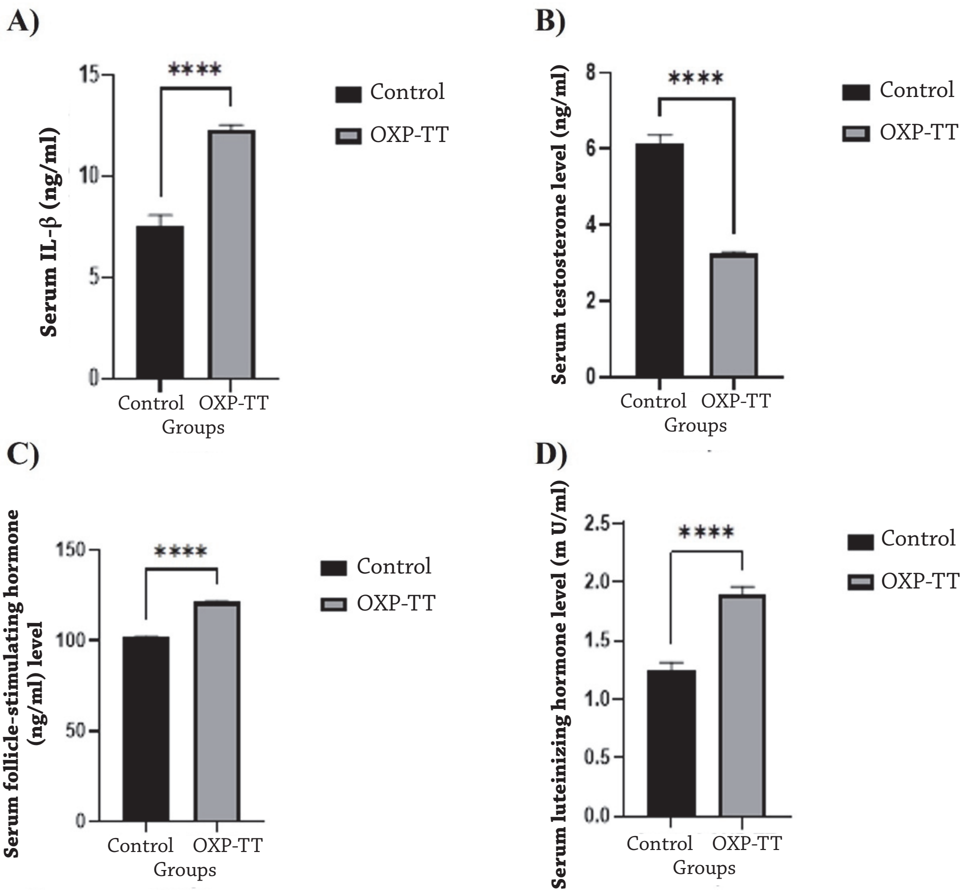


Fig. 1. Effect of oxaliplatin on serum hormone levels and inflammatory markers. Serum levels of (**A**) IL-1β, (**B**) testosterone, (**C**) FSH, and (**D**) LH in control and oxaliplatin-treated rats. Data are expressed as mean ± SEM ($n = 5$). Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. **** $p < 0.0001$ vs control.

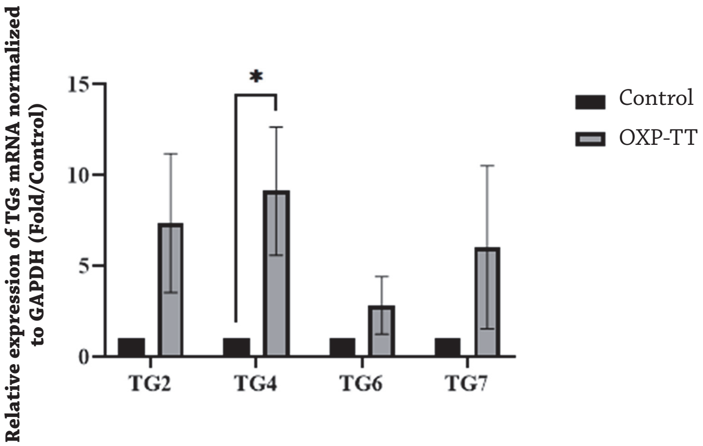


Fig. 2. Gene expression of TGs in normal and oxaliplatin-treated testicular tissue. Messenger RNA was extracted from normal and oxaliplatin-treated testicular tissue and subjected to qRT-PCR using TG2, TG4, TG6, and TG7-specific primers along with primers specific for a normalizing gene (GAPDH). Expression levels in Oxaliplatin-treated Testicular tissue samples (OXP-TT) were normalized to those of normal testicular tissue, which was arbitrarily set to 1. Data are indicated as the mean ± SEM from three independent samples and were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test to compare the mean difference of normal and Oxaliplatin-treated Testicular tissue samples ($n = 10$). Statistical significance was set at * $p < 0.05$. Among the analyzed isoforms, TG4 exhibited the most prominent transcriptional upregulation following oxaliplatin treatment.

Transglutaminase (TG4) protein expression and localization in normal and oxaliplatin-treated testicular tissue

To investigate the protein expression and localization of TG4 following oxaliplatin treatment, immunohistochemical (IHC) staining was performed on testicular tissue samples. The results revealed differential expression and localization of TG4 (Fig. 3). The presence of a positive IHC reaction in oxaliplatin-treated tissues (OXP-TT) compared to the control indicates an upregulation of TG4 protein expression in response to chemotherapy. The quantitative analysis of TG4-positive staining further supports this observation, showing a significant increase in the treated samples (* $p < 0.002$).

IHC staining revealed brown DAB-positive signals indicating TG4 protein localization within testicular tissue. Increased staining intensity was observed in oxaliplatin-treated samples compared with controls. Stronger brown staining intensity indicates higher TG4 protein accumulation within the affected regions. In normal testicular tissue, TG4 protein is predominantly localized between the seminiferous tubules, particularly within the interstitial spaces, where Leydig cells are present. In contrast, in oxaliplatin-treated tissue, TG4 expression appears intensified in both the interstitial regions and within the seminiferous tubules, suggesting its involvement in stress adaptation or tissue remodeling following chemotherapy exposure. The differential distribution may reflect specific cellular responses to chemotherapy-induced stress, requiring further investigation to elucidate its functional implications.

Additionally, tissue morphology and structural integrity were assessed through histological examination. In control samples, the seminiferous tubules appeared well-organized, with clearly defined germinal epithelium and intact Leydig cells. In contrast, oxaliplatin-treated samples exhibited disrupted tubular architecture, vacuolization, and increased apoptotic cells, indicating chemotherapy-induced testicular damage. These findings highlight the need for further investigations to determine whether TG4 expression correlates with protective or detrimental effects in response to oxaliplatin exposure.

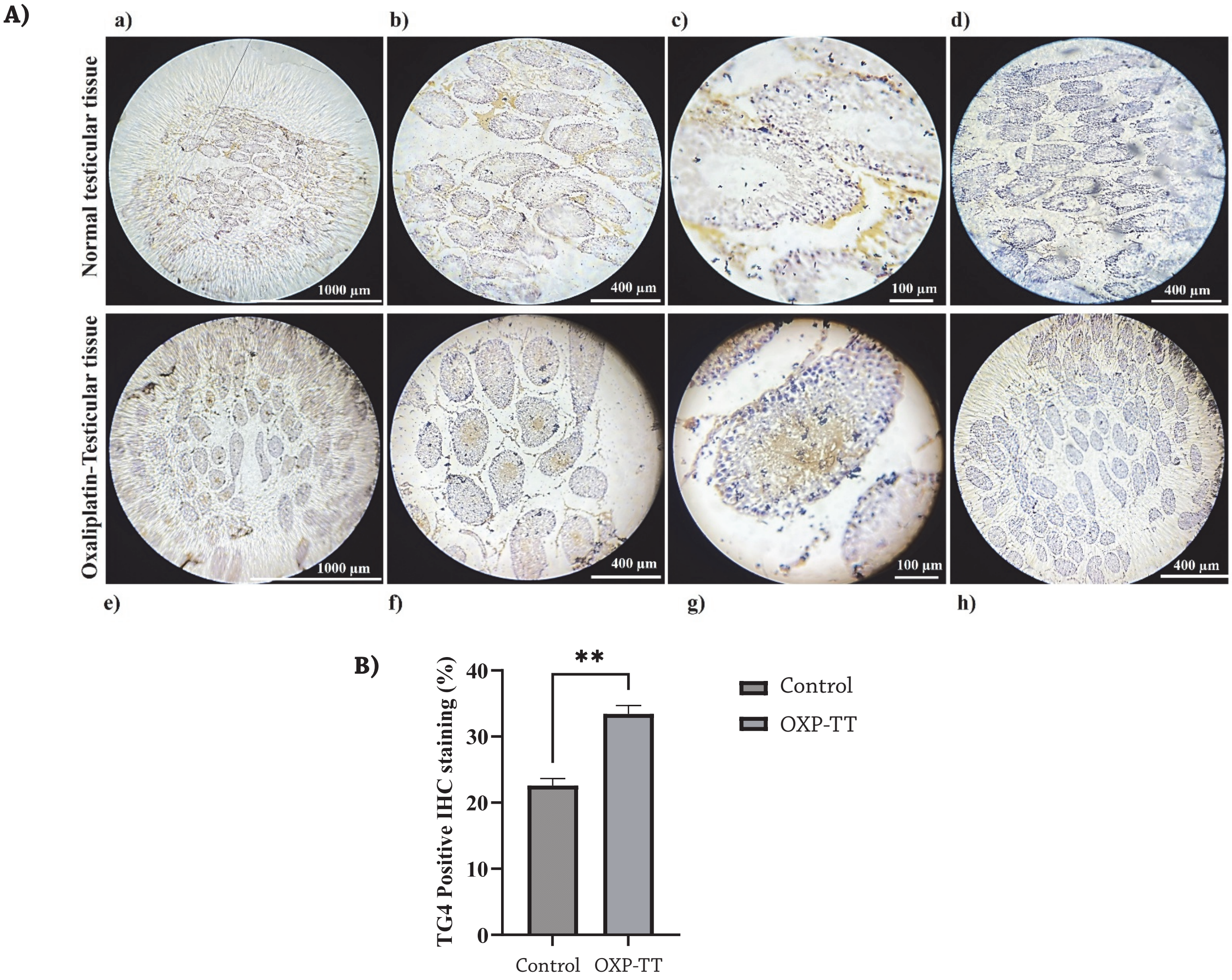
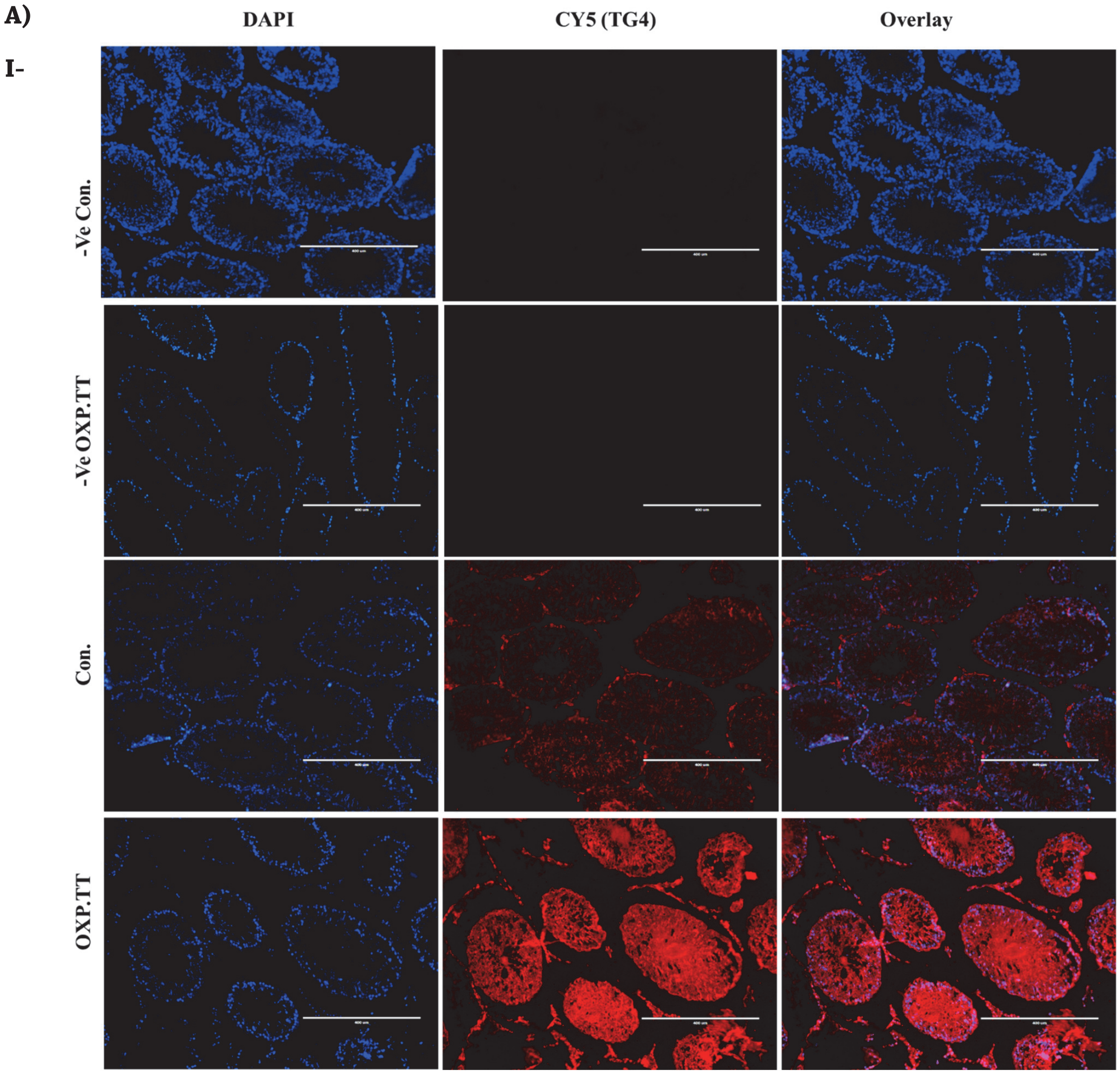


Fig. 3. Transglutaminase (TG4) protein expression, location, and distribution in rat normal and oxaliplatin-treated testicular tissue. (**A**) Immunohistochemical (IHC) staining of normal and oxaliplatin-treated testicular tissue for TG4 using a polyclonal antibody against TG4. Panels show representative images at 4× (**a, e**) (Scale bar = 1000 µm); 10× (**b, f**), (Scale bar = 400 µm); and 40× (**c, g**), (Scale bar = 100 µm) magnifications. Negative IHC reaction for TG4 in (**d**) normal and (**h**) Oxaliplatin-treated Testicular tissue (10×, Scale bar = 400 µm). Negative control sections processed without the primary antibody showed no detectable staining. (**B**) The percentage quantification of positive TG4 IHC staining. An unpaired *t*-test was used to compare the mean difference between normal and Oxaliplatin-treated testicular tissue samples ($n = 5$, ** $p < 0.002$).

Immunofluorescent analysis of TG4 in normal and oxaliplatin-treated testicular tissue

To further confirm the expression and localization of TG4 protein following chemotherapy, immunofluorescence (IF) staining was performed. The positive IF reaction in oxaliplatin-treated tissues (OXP-TT) compared to the control reinforces the notion that TG4 protein levels are upregulated following chemotherapy (Fig. 4). This suggests that TG4 may be actively involved in cellular responses to oxaliplatin-induced toxicity. The highly significant statistical difference (* $p < 0.0001$) in quantification analysis supports the notion that TG4 levels are markedly elevated in treated samples, warranting further investigation into its functional role in chemotherapy-induced stress responses. Additionally, IF analysis revealed the specific localization of TG4 in testicular tissue, providing insights into its spatial distribution and potential interactions within cellular compartments affected by chemotherapy. Consistent with the IHC findings, IF staining confirmed that TG4 expression is heightened in the interstitial regions and seminiferous tubules following oxaliplatin exposure. Structural changes in IF images included increased cellular disorganization, fragmented tubular structures, and intensified TG4 fluorescence signals, suggesting a role in oxidative stress response and tissue remodeling in chemotherapy-treated testes.



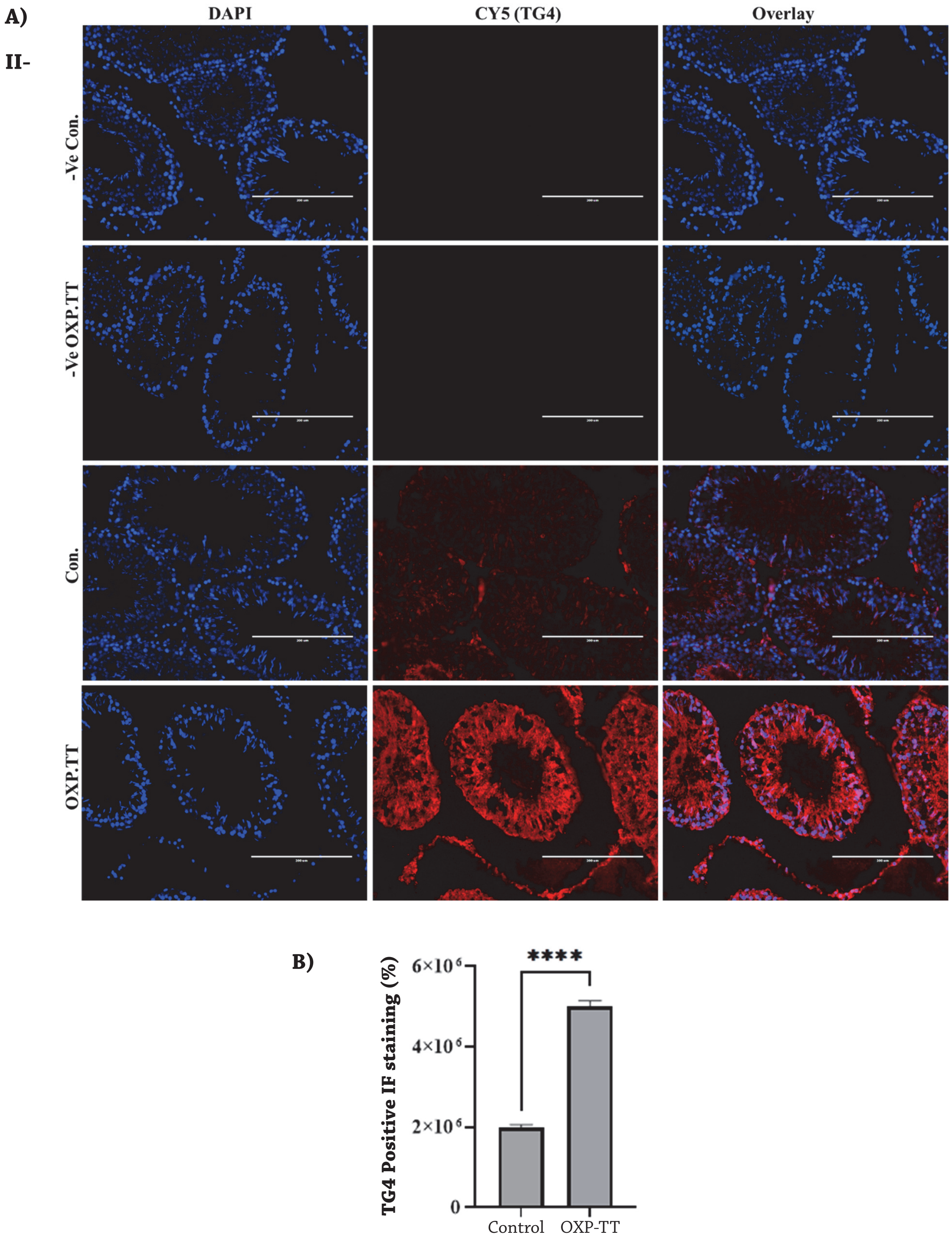


Fig. 4. Immunofluorescent of transglutaminase 4 (TG4) and its levels in normal and oxaliplatin-treated testicular tissue. Paraffin-embedded testicular tissue for TG4 using a polyclonal antibody against TG4. (**A**) Negative IF reaction (-Ve) no primary antibody for TG4 in (-Ve Con.) normal and oxaliplatin-treated testicular tissue (-Ve OXP-TT); Positive IF reaction for TG4 in (Con.) normal and oxaliplatin-treated testicular tissue (OXP-TT). Negative control sections processed without the primary antibody showed no detectable staining. The original magnification of the images was 10× (AI), and 20× (AII), respectively, and the scale bar was 200 μm or 400 μm. (**B**) The percentage quantification of positive TG4 IF staining. An unpaired *t*-test compared the mean difference between normal and oxaliplatin-treated testicular tissue samples ($n = 5$, $^{****}p < 0.0001$). DAPI (blue), TG4 [red (Cy5)].

Expression of inflammatory and apoptotic markers in oxaliplatin-treated testicular tissue

To evaluate inflammatory and apoptosis-related transcriptional responses, mRNA expression of IL6, TNF, and CASP3 was measured by qRT-PCR (Fig. 5A; $n = 10$ per group). Oxaliplatin treatment significantly increased IL6 and TNF expression compared to controls (*** $p < 0.0001$). CASP3 expression was also significantly elevated (* $p < 0.05$).

Genomic DNA integrity assessment (Fig. 5B) revealed predominantly intact high-molecular-weight DNA in control samples, whereas oxaliplatin-treated samples exhibited increased smear formation, consistent with reduced DNA integrity.

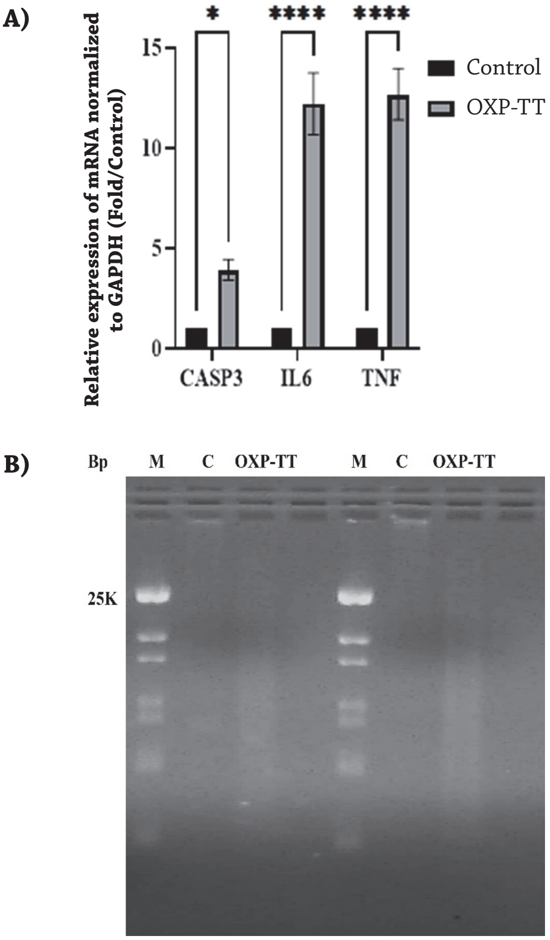


Fig. 5. Gene expression of inflammatory and apoptotic markers in oxaliplatin-treated testicular tissue. (**A**) Relative mRNA expression of CASP3, IL6, and TNF-α in normal and oxaliplatin-treated testicular tissue. Messenger RNA was extracted from normal (Control) and oxaliplatin-treated testicular tissue (OXP-TT) and subjected to qRT-PCR using CASP3, IL6, and TNF-α-specific primers along with primers specific for a normalizing gene (GAPDH). Relative mRNA expression of CASP3, IL6, and TNF-α normalized to GAPDH and expressed as fold change relative to untreated control tissue (set to 1). Data are presented as mean ± SEM ($n = 10$). Statistical analysis was performed using multiple unpaired two-tailed t-tests with Holm–Šídák correction for multiple comparisons. Statistical significance was set at * $p < 0.05$. (**B**) Oxaliplatin-associated genomic DNA fragmentation in rat testicular tissue. Genomic DNA was isolated from control C and oxaliplatin-treated (OXP-TT) rat testes and resolved on a 1.5% agarose gel (5 μg DNA loaded per lane). Treated samples exhibited increased DNA smearing/fragmentation compared with controls, consistent with enhanced DNA degradation. M, DNA molecular weight marker.

Transglutaminase (TG) activity in normal vs. oxaliplatin-treated testicular tissue

This figure demonstrates a statistically significant increase in TG activity in testicular tissues following Oxaliplatin treatment compared to the normal control group. The TG-specific activity was quantified using a colorimetric assay, and the result shows significant elevation in TG enzymatic activity in the treated group (** $p < 0.01$) (Fig. 6), suggesting that Oxaliplatin induces or enhances transglutaminase activation in testicular tissue. This may reflect a cellular stress response or apoptotic signaling pathway modulation, both of which are known to involve transglutaminase activity.

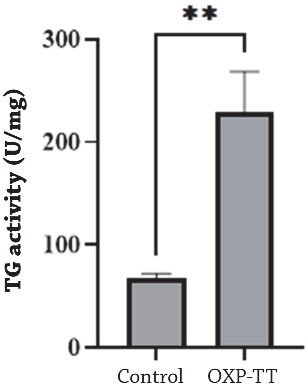


Fig. 6. Transglutaminase (TG) activity in normal vs. oxaliplatin-treated testicular tissue. Proteins extracted from normal and oxaliplatin-treated testicular tissue samples were subjected to a transglutaminase (TGs) colorimetric assay. Data points represent the mean ± SEM TG-specific activity. Data are indicated as the mean ± SEM and were analyzed using an unpaired t-test to compare the mean difference between normal and oxaliplatin-treated Testicular tissue samples ($n = 10$). Statistical significance was ** $p < 0.01$.

Discussion

Our experimental results indicate that oxaliplatin induces significant molecular, biochemical, and structural changes in testicular tissue. In addition to the histological and molecular alterations observed in the present study, oxaliplatin exposure was associated with clear endocrine and inflammatory disturbances, including reduced serum testosterone, increased FSH and LH levels, and elevated IL-1β. Together, these findings support the view that oxaliplatin disrupts testicular homeostasis at both the tissue and systemic levels. The marked increase in TG4 expression in treated samples (OXP-TT) compared with controls aligns with previous studies describing chemotherapy-associated modulation of transglutaminases in stress and cancer contexts (Sima et al., 2022; Tabolacci et al., 2019). The upregulation of TG4 suggests a potential role in cellular adaptation or resistance mechanisms in response to oxaliplatin-induced stress.

Transglutaminases catalyze calcium-dependent protein cross-linking reactions that enhance structural stability but may also promote irreversible protein aggregation under pathological conditions (Eckert et al., 2014; Tatsukawa and Hitomi, 2021). TG4 upregulation in oxaliplatin-treated testes may therefore reflect either an adaptive response aimed at stabilizing cellular structures under stress or a maladaptive process contributing to altered tissue remodeling. Similar dual roles have been reported for other TG isoforms in inflammatory and fibrotic conditions (Csobán-Szabó et al., 2021; Tatsukawa et al., 2015). Whether TG4 exerts protective or detrimental effects in chemotherapy-associated reproductive toxicity remains to be determined.

Histological and immunostaining analyses revealed disrupted seminiferous architecture, vacuolization, and altered TG4 localization in treated animals. These findings are consistent with reports describing oxaliplatin-induced testicular injury and impaired spermatogenesis (Akaras et al., 2024; Ghafouri-Fard et al., 2021; Lopes et al., 2021). Increased TG4 immunoreactivity within seminiferous tubules following oxaliplatin exposure suggests a shift in spatial distribution under stress conditions, which may reflect altered cellular compartmentalization or stress-associated signaling. However, the functional consequences of this altered TG4 distribution remain to be elucidated. Although other transglutaminase isoforms, such as TG7, have been implicated in testicular tumor biology (Altuwayjiri and Almami, 2025), the present findings specifically highlight TG4 as a stress-responsive isoform in non-neoplastic oxaliplatin-exposed testicular tissue.

A notable addition in the present study is the demonstration of oxaliplatin-associated hormonal dysregulation, characterized by a significant decline in testosterone together with compensatory increases in FSH and LH. This pattern is consistent with impaired Leydig cell function and disturbed hypothalamic-pituitary-gonadal (HPG) axis regulation, both of which are hallmarks of chemotherapy-induced testicular dysfunction (Ghafouri-Fard et al., 2021; Lopes et al., 2021). The concomitant increase in serum IL-1β further supports the presence of systemic inflammatory activation, which has been widely reported in models of chemotherapy-associated tissue injury (Zhang et al., 2022; Zhou et al., 2020). These endocrine and inflammatory changes are highly relevant to the TG4 findings, as transglutaminases are known to respond to cellular stress and inflammation-associated signaling pathways (Eckert et al., 2014; Ientile et al., 2007). Thus, the simultaneous elevation of IL-1β, gonadotropins, TG4 expression, and total transglutaminase activity suggests that TG4 upregulation may occur within a broader oxaliplatin-induced stress program linked to endocrine dysfunction and inflammatory injury.

In parallel, oxaliplatin exposure was associated with increased transcription of pro-inflammatory cytokines IL6 and TNF. Platinum-based chemotherapeutic agents are known to induce inflammatory responses through oxidative stress-dependent mechanisms (Zhang et al., 2022; Zhou et al., 2020). Persistent inflammatory activation within the testicular microenvironment may contribute to disruption of the blood-testis barrier, impaired spermatogenesis, and long-term reproductive dysfunction. Inflammatory signaling has also been reported to modulate transglutaminase activity in other tissues (Elli et al., 2009), raising the possibility that cytokine-driven pathways may influence TG4 regulation during chemotherapy-induced stress.

A novel and important observation of this study is the significant increase in transglutaminase enzymatic activity in the oxaliplatin-treated group (Fig. 6). This enzymatic activation was detected using a colorimetric assay and suggests that oxaliplatin triggers a biochemical response that promotes TG-mediated cross-linking processes. Increased TG activity has been associated with stress-related cellular events, including apoptosis, inflammation, and protein aggregation (Eckert et al., 2014; Ientile et al., 2007). The increase in total transglutaminase enzymatic activity observed in this study indicates enhanced TG-mediated cross-linking activity following oxaliplatin exposure. Because the assay measures overall transglutaminase activity, it cannot distinguish the contribution of individual isoforms. However, the observed transcriptional and protein upregulation of TG4 suggests that this isoform may contribute to the broader increase in transglutaminase activity under these conditions.

This elevation in TG activity may reflect a compensatory mechanism aiming to stabilize the cytoskeleton and mitigate cellular injury. Alternatively, excessive TG activity could exacerbate damage by inducing irreversible protein modifications and contributing to fibrosis or apoptotic cascades (Tatsukawa and Hitomi, 2021; Zhang et al., 2022). Therefore, the enzymatic findings complement the transcriptional and histopathological data, positioning TGs as central effectors in oxaliplatin-induced toxicity.

Furthermore, TGs have been shown to modulate immune signaling and cellular adhesion pathways. Their hyperactivation in response to stress might represent a maladaptive process that amplifies tissue injury rather than providing protection (Cao et al., 2008; Jeong et al., 2013). Targeting TG enzymatic activity using selective inhibitors, as previously demonstrated in cisplatin-resistant cancer cells, may provide a promising strategy to mitigate off-target toxicity during chemotherapy (Kim et al., 2011; Meshram et al., 2017; Virag et al., 2013).

The functional significance of TG4 upregulation in oxaliplatin-induced testicular toxicity remains unclear. Transglutaminase activation has been reported in multiple stress-related contexts, where it may participate either in adaptive cellular responses or in the progression of tissue injury. Previous studies investigating oxaliplatin-induced molecular alterations have demonstrated that prolonged drug exposure can trigger broad transcriptional and signaling changes associated with apoptosis, proliferation, and stress adaptation (Virag et al., 2013). Therefore, the observed increase in TG4 expression in the present study may reflect part of a wider stress-responsive signaling network activated during oxaliplatin exposure. Further mechanistic studies, including functional inhibition or tumor-based experimental models, will be required to determine whether TG4 contributes directly to tissue injury or represents a compensatory cellular response.

Consistent with prior findings in liver tissue (Alowss et al., 2026), oxaliplatin exposure in the present study was associated with increased transglutaminase activity. However, the predominant isoform in testicular tissue was TG4 rather than TG7, suggesting tissue-specific regulatory mechanisms. The concurrent elevation of TG4 expression and total TG activity suggests that TG4 may contribute to this enzymatic activation. However, because the assay measured total transglutaminase activity rather than isoform-specific activity, contributions from other TG family members cannot be excluded. Future studies employing isoform-selective inhibition or gene-silencing approaches will be necessary to define the specific enzymatic contribution of TG4.

Alterations in genomic DNA integrity further support the presence of cellular injury following oxaliplatin exposure. Oxidative stress-mediated genomic instability has been widely documented in various experimental models (Qin et al., 2026), supporting the concept that inflammatory and redox imbalance may contribute to oxaliplatin-associated tissue injury. Although classical apoptotic laddering was not observed, smear formation on agarose gels is consistent with DNA fragmentation and oxidative damage. Platinum compounds are known to induce DNA crosslinks and strand breaks, triggering DNA damage response pathways (Zhang et al., 2022; Zhou et al., 2024). Together with elevated CASP3 expression and oxidative stress markers, these findings reinforce the concept that oxaliplatin-induced testicular injury involves interconnected mechanisms of ROS generation, inflammatory activation, and apoptosis-related signaling.

From a translational perspective, dysregulation of transglutaminases has been implicated in male infertility and reproductive dysfunction (Yadav and Kim, 2024). If TG4 upregulation reflects sustained stress activation within testicular tissue, it may represent a potential indicator of chemotherapy-associated reproductive toxicity. However, direct effects on sperm parameters were not evaluated in the present study, and the functional consequences of TG4 modulation remain undefined.

Overall, these results contribute to the growing body of literature examining oxaliplatin-induced toxicity and provide a foundation for future studies investigating protective strategies against testicular damage. Further research is needed to explore the mechanisms underlying transglutaminase regulation and their role in oxaliplatin-induced stress responses. Additionally, understanding the functional consequences of TG4 overexpression and activity may aid in developing targeted interventions to minimize testicular toxicity and preserve fertility in cancer patients undergoing chemotherapy.

Conclusion

This study demonstrates that oxaliplatin induces significant testicular toxicity characterized by disrupted tissue architecture, hormonal dysregulation, and increased inflammatory responses. A notable finding is the upregulation of TG4 at both the gene and protein levels, accompanied by increased total transglutaminase enzymatic activity. The concurrent reduction in testosterone, elevation of FSH and LH, and increase in inflammatory mediators, including IL-1β, IL6, and TNF, indicate that oxaliplatin-associated testicular injury involves interconnected endocrine, inflammatory, and stress-related pathways. These findings suggest that TG4 is associated with oxaliplatin-induced testicular stress responses and may represent a stress-responsive marker of reproductive toxicity. Further mechanistic studies are required to determine whether TG4 contributes directly to tissue injury or reflects an adaptive response to oxaliplatin-induced damage.

Ethics approval

All experimental procedures were reviewed and approved by the Committee of Research Ethics at the Deanship of Scientific Research, Qassim University (Approval No. 25-03-17). All protocols were conducted following institutional guidelines and the ethical principles outlined in the Institutional Animal Care and Use Committee (IACUC).

Author contribution

Husah M. Alowss and *Ibtesam Almami*: conceptualization, methodology, data analysis, writing, reviewing, and editing. *Heba F. Gomaa*: experimental design, biochemical analysis, and manuscript review. All authors have read and agreed to this published version of the manuscript.

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Data availability

All data are available upon reasonable request.

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Conflict of interest

The authors have no conflict of interest to declare.

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