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Original Communication

Relationship Between Cell Death Parameters and Changes in MicroRNA-125b Expression During Chlorambucil-Induced Testicular Toxicity and the Protective Potential of Cobalamin

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Abstract

Background: Chemotherapy-related male infertility is a major concern among cancer survivors. Chlorambucil, an alkylating agent, induces oxidative damage that may disrupt the balance between autophagy and apoptosis in testicular tissue. Thus, this study aimed to investigate the role of microRNA-125b (*miR-125b*) in chlorambucil-induced testicular injury and evaluated the protective effects of cobalamin (vitamin B12). **Methods:** A total of 32 adult male Wistar rats were randomly assigned to four groups (n = 8): control, chlorambucil (2 mg/kg/day), chlorambucil plus cobalamin (3 µg/kg/day), and cobalamin alone. Histological, spermatological, biochemical, hormonal, and molecular assessments were performed at 30 days of treatment. **Results:** Chlorambucil administration significantly impaired sperm quality, reduced spermatogonial cell numbers, decreased seminiferous tubule diameter and epithelial height, and lowered Johnsen's score ($p < 0.05$). These changes were accompanied by elevated malondialdehyde (MDA) levels, reduced superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and decreased serum testosterone concentrations. In addition, chlorambucil increased *miR-125b, Bax, and caspase-3* expression while suppressing *Bcl-2* and autophagy-related genes (*Atg5, Atg6, Atg7, Atg8,* and *Atg12*) ($p < 0.05$). Cobalamin co-treatment significantly mitigated chlorambucil-induced reproductive toxicity, increasing sperm count by approximately 48.3% compared with the chlorambucil-treated group, restoring testosterone levels toward normal, suppressing apoptosis, enhancing the expression of autophagy-related genes, and partially reversing *miR-125b* upregulation ($p < 0.05$). **Conclusions:** Chlorambucil-induced testicular toxicity appears to be mediated, at least in part, through oxidative stress–associated *miR-125b* dysregulation, promoting apoptosis and suppressing autophagy. Cobalamin exerts significant gonadoprotective effects and may represent a promising therapeutic approach for preserving male reproductive function during chemotherapy.

Keywords: chlorambucil; cobalamin; cell death parameters; reproductive toxicity; *microRNA-125b*

1. Introduction

Although advances in cancer therapy have substantially improved patient survival, chemotherapy-induced male infertility remains a major long-term complication that compromises reproductive health and quality of life among cancer survivors [1]. Germ cells are particularly vulnerable to chemotherapeutic agents because of their rapid proliferation and tightly regulated differentiation. Consequently, treatment-induced activation of cell death pathways may impair spermatogenesis, disrupt steroidogenesis, and ultimately reduce male fertility [2,3].

Chlorambucil, a widely used alkylating agent for the treatment of chronic lymphocytic leukemia and certain lymphomas, exerts its antineoplastic activity primarily through DNA crosslinking and subsequent inhibition of cell proliferation [4]. However, these therapeutic effects are accompanied by undesirable toxicity in normal tissues, including the testis. Experimental evidence indicates that chlorambucil enhances reactive oxygen species (ROS) generation, weakens antioxidant defenses, and induces extensive DNA damage, thereby promoting testicular dysfunction and germ cell loss [5,6].

Among the molecular mechanisms involved in chemotherapy-induced reproductive toxicity, apoptosis and autophagy have attracted considerable attention. These tightly interconnected pathways are essential for maintaining germ cell homeostasis under physiological conditions, whereas their dysregulation under oxidative stress contributes to impaired spermatogenesis and testicular injury [7]. The balance between apoptosis and autophagy is further regulated by microRNAs (miRNAs), which act as critical post-transcriptional modulators of gene expression

and influence cellular responses to oxidative and genotoxic stress [8].

MicroRNA-125b (*miR-125b*) has emerged as an important regulator of germ cell development, spermatogenesis, and stress-responsive signaling pathways [9,10,11]. Previous studies have shown that *miR-125b* expression is altered in response to oxidative stress and DNA damage, suggesting its involvement in regulating apoptosis and autophagy [12,13]. Nevertheless, its contribution to chlorambucil-induced testicular injury and its relationship with apoptosis- and autophagy-related signaling remain largely unexplored.

Cobalamin (vitamin B12) is an essential micronutrient involved in DNA synthesis, methylation reactions, and cellular redox homeostasis. Beyond its established metabolic functions, accumulating evidence suggests that cobalamin exerts antioxidant and cytoprotective effects by limiting oxidative stress, supporting cellular proliferation, and preserving testicular function [14,15]. Whether these protective actions involve modulation of *miR-125b* and its downstream regulation of apoptosis and autophagy has not yet been determined.

To the best of our knowledge, no previous study has simultaneously evaluated *miR-125b* expression together with apoptosis- and autophagy-related molecular markers in a chlorambucil-induced model of testicular injury. We hypothesized that cobalamin attenuates chlorambucil-induced testicular damage by modulating *miR-125b* expression and restoring the balance between apoptosis and autophagy. Accordingly, the present study aimed to investigate the association between *miR-125b* dysregulation and alterations in apoptosis- and autophagy-related genes in chlorambucil-induced testicular injury and to evaluate the gonadoprotective potential of cobalamin.

2. Materials and Methods

2.1 Experimental Design

This investigation was conducted using thirty-two healthy adult male Wistar rats (8 weeks old, weighing 200–250 g) obtained from the Animal Research Center of Gonabad University of Medical Sciences, Gonabad, Iran. Before the initiation of the experiment, the animals were acclimatized to the laboratory environment for one week. Rats were housed in polypropylene cages (four animals per cage) under standard laboratory conditions at a controlled temperature of 22 ± 2 °C, with adequate ventilation and a 12 h light/12 h dark cycle. Standard laboratory pellet diet and tap water were provided ad libitum. Following acclimatization, animals were randomly allocated into the experimental groups using a simple randomization method.

Healthy adult male Wistar rats, approximately 8 weeks old and weighing 200–250 g, were included in the study. Animals were considered eligible if they appeared healthy and showed no apparent signs of illness or physical abnormality during the acclimatization period. Animals showing signs of illness, physical injury, or other conditions that could interfere with the experimental procedures or outcome assessment were excluded. Eligible animals were randomly assigned to the experimental groups.

All experimental procedures were approved by the Ethics Committee of the Ministry of Health and Medical Education of the Islamic Republic of Iran (Approval No.: IR.GMU.AEC.1404.001; Date: 2025-05-07). All procedures were designed to minimize distress and discomfort to the animals. All efforts were made to minimize animal suffering and reduce the number of animals used. The study was conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines and the principles of Replacement, Reduction, and Refinement (3Rs), **Supplementary Table 1**.

Sample size determination was carried out before the initiation of the study using a statistical formula for comparing two independent means. Sperm concentration was selected as the primary endpoint. Based on a significance threshold of 0.05 and a study power of 90%, the calculated minimum number of animals was 7.73 per group; therefore, eight rats were included in each experimental group [16].

Using a random allocation schedule, the animals were distributed into four experimental groups (n = 8):

1-Control: received intraperitoneal administration of physiological saline.

2-Chlorambucil: treated with chlorambucil at 2 mg/kg/day via the intraperitoneal route.

3-Chlorambucil + Cobalamin: received chlorambucil (2 mg/kg/day, IP) together with cobalamin (3 µg/kg/day, IP).

4-Cobalamin: administered cobalamin alone at a dose of 3 µg/kg/day intraperitoneally.

Chlorambucil and cobalamin were freshly dissolved in sterile normal saline (0.9% NaCl) immediately before administration. Animals in the control group received an equivalent volume of normal saline via the same route of administration.

The treatment regimen continued for 30 consecutive days. Twenty-four hours after the final injection, anesthesia was induced using ketamine (80 mg/kg, IP) combined with xylazine (10 mg/kg, IP) [17]. Following anesthesia, both testes were surgically removed. The right testis was designated for histopathological and histomorphometric analyses, whereas the left testis was snap-frozen at −70 °C for molecular assays. The cauda epididymis was collected for sperm evaluation, and approximately 3 mL of blood was obtained by cardiac puncture. Serum was separated after centrifugation (3000 rpm, 12 min) for biochemical analyses. All sample collection and outcome assessments were performed under blinded conditions. Following sample collection, animals were euthanized under deep anesthesia in accordance with the approved institutional animal ethics guidelines.

2.2 Histopathological and Histomorphometric Analysis

Tissue processing: The right testes were fixed in 10% neutral-buffered formalin for 48 h and processed routinely through graded ethanol dehydration, xylene clearing, and paraffin embedding. Paraffin blocks were sectioned at a thickness of 5 µm using a rotary microtome and stained with hematoxylin and eosin (H&E). All subsequent histological and histomorphometric analyses were performed using these sections under a light microscope at 400× magnification [18].

Histological examination: Histological examination was performed to evaluate the overall architecture of the seminiferous tubules and the integrity of the germinal epithelium.

Spermatogonial quantification: For each animal, approximately 25 ± 3 circular seminiferous tubule cross-sections were selected from different tissue regions using a systematic random-sampling method. To minimize biological variability, only tubules at stages VII–VIII of the seminiferous epithelial cycle were analyzed. Spermatogonial cells were identified according to established morphological criteria [19]. Type A spermatogonia exhibited oval nuclei with finely dispersed chromatin and prominent nucleoli, whereas type B spermatogonia displayed round nuclei with peripheral heterochromatin condensation. Cell counting was independently performed by two investigators blinded to the experimental groups. The total number of recognizable spermatogonia within each tubular cross-section was recorded and expressed both as the mean number of cells per tubule and as the number of cells per 100 µm of basement membrane length.

Seminiferous tubule diameter and germinal epithelial thickness: Morphometric measurements were performed using calibrated ImageJ software (version 1.53; National Institutes of Health (NIH), Bethesda, MD, USA). Seminiferous tubule diameter was measured as the distance between two opposite points of the basement membrane, whereas germinal epithelial thickness was measured from the basement membrane to the luminal border.

Johnsen score: The quality of spermatogenesis was assessed using the Johnsen scoring system. Thirty seminiferous tubules per animal were evaluated and assigned scores ranging from 1 to 10 according to the degree of germ-cell maturation. A score of 10 represented complete spermatogenesis with abundant mature spermatozoa, whereas a score of 1 indicated complete absence of germ cells and Sertoli cells. Intermediate scores reflected progressive degrees of spermatogenic impairment as originally described by Johnsen [20].

2.3 Evaluation of Sperm Characteristics

To obtain sperm suspensions, the cauda epididymis was dissected and finely minced in 1 mL of physiological saline maintained at 35–37 °C. The resulting suspension was gently mixed to facilitate sperm release.

For assessment of sperm morphology, aliquots (10 µL) were spread onto poly-L-lysine-coated glass slides. Following air drying and fixation in 70% ethanol, slides were stained using the Papanicolaou technique comprising hematoxylin, Orange G, and EA-50 solutions. Two hundred spermatozoa per specimen were examined microscopically at 400× magnification and categorized as morphologically normal or abnormal according to head and tail defects.

Sperm viability was determined using eosin-Y staining. Following a 30-s exposure period, unstained spermatozoa were considered viable, whereas eosin-positive cells were classified as nonviable. Viability was reported as the percentage of living sperm cells.

For quantification of sperm concentration and motility, 10 µL of diluted sperm suspension was loaded into a Neubauer hemocytometer. Sperm concentration was calculated from counts obtained in five major squares using the standard hemocytometric formula. Motility was assessed by determining the percentage of immotile spermatozoa, which was recorded for statistical analysis [21].

2.4 Quantitative Real-Time PCR Analysis

Immediately after dissection, the left frozen testicle was homogenized and processed for molecular investigations. Total RNA was isolated using a commercially available extraction kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's recommendations. The purified RNA served as the template for complementary DNA (cDNA) synthesis using a reverse-transcription reaction.

Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR) employing YTA SYBR Green Master Mix (2×) and a StepOne™ Real-Time PCR instrument (Applied Biosystems, USA). Each amplification reaction was prepared in a final volume of 10 µL consisting of 5 µL SYBR Green master mix, 1 µL cDNA template, 0.2 µM of each primer, and nuclease-free water to volume. GAPDH was selected as the endogenous reference gene.

The thermal cycling program consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles including denaturation at 95 °C for 15 s, primer annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. A final dissociation stage was performed at 95 °C for 15 s and 55 °C for 60 s to verify amplification specificity. Relative transcript abundance was determined using the comparative $2^{-\Delta\Delta Ct}$ method.

The expression profiles of autophagy-associated genes (*Atg5, Atg6, Atg7, Atg8,* and *Atg12*) and apoptosis-related genes (*Bax, Bcl-2,* and *Caspase-3*) were evaluated [22]. Target genes included autophagy-related genes (*Atg5, Atg6, Atg7, Atg8, Atg12*), apoptosis-related genes (*Caspase-3, BAX, Bcl-2*), and *miR-125b*.

Primer sequences are listed below:

Atg6 (F: CGAAAGGTGGTGGCAGAAAAC; R: ACTATATTCTCGCTGGTACTGAGC).

Atg8 (F: TCAGTGAGAGCTGCCTCTGTC; R: AGCAGTGGGGATTTACACAGTG).

Atg5 (F: AGATCACAGTTCTGGGATGC; R: TCAGGCGGTAGAGATCGTAG).

Atg7 (F: TGTCTTGCAGCATCCTGAG; R: TCAAGAACTTTGGATGAACAGG).

Atg12 (F: GCACTCATCGACTTCATCAG; R: ACTGCCAAAACACTCATATAGAG).

GAPDH (F: GGTCTACATGTTCCAGTATGACTC; R: CATTTGATGTTAGCGGGATCTCG).

Bax (F: TTGCTACAGGGTTTCATCCA; R: GAGTACCTGAACCGGCATCT).

Bcl-2 (F: GCTACCGTCGTGACTTCGC; R: CCCCACCGAACTCAAAGAAGG).

Caspase-3 (F: TGTGCTCCAGGCTTCCTTAATC; R: AGGCTTATGGGAAATGCTGGAC).

miR-125b (F: GCGGCTCCCTGAGACCCTAAC; R: GTGCAGGGTCCGAGGT).

U6 (F: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAAT; R: GCTTCGGCAGCACATATACTAAAAT).

2.5 Determination of miRNA Expression

The expression level of *miR-125b* was assessed using stem-loop reverse transcription followed by quantitative PCR amplification. Reverse transcription reactions were carried out with miRNA-specific stem-loop primers incorporating a universal sequence to facilitate amplification using a common reverse primer.

For cDNA synthesis, a 10-μL reaction mixture was prepared containing reverse transcriptase (0.5 μL), RT buffer (2 μL), stem-loop primer (1 μL), dNTP mixture (1.5 μL), total RNA (~500 ng; 1 μL), and nuclease-free water (4 μL). The reverse-transcription program consisted of sequential incubations at 16 °C for 30 min, 42 °C for 30 min, and 82 °C for 5 min.

All oligonucleotide primers were synthesized by Oligogen (South Korea). Expression data were normalized against U6 small nuclear RNA (snRNA), which served as the internal control [23].

2.6 Biochemical Assays

2.6.1 Assessment of Oxidative Stress Biomarkers

Serum malondialdehyde (MDA) concentrations were determined as an indicator of lipid peroxidation using the thiobarbituric acid reactive substances (TBARS) assay. Briefly, serum samples (0.2 mL) were combined with glacial acetic acid and thiobarbituric acid solution prepared in sodium hydroxide. The reaction mixtures were heated in a boiling-water bath for 15 min and subsequently cooled to room temperature. Absorbance values were recorded spectrophotometrically at 532 nm.

The antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) were quantified using commercially available assay kits (Randox and Ransod, UK) according to the manufacturers' instructions. Measurements were carried out using an ELISA microplate reader (ABER-2, China). GPx activity was determined based on glutathione oxidation in the presence of cumene hydroperoxide, whereas SOD activity was estimated through its inhibitory effect on formazan dye generation in the xanthine/xanthine oxidase reaction system [24].

2.6.2 Measurement of Circulating Testosterone

Serum testosterone concentrations were quantified using a rat/mouse testosterone ELISA kit (DEMEDITEC, Germany). The assay operates on the principle of competitive enzyme-linked immunosorbent detection.

Standards and serum samples were loaded in duplicate into microplate wells and incubated with the corresponding reagents according to the manufacturer's protocol. Following sequential incubation, washing, and substrate development steps, the enzymatic reaction was terminated by adding stop solution. Optical density was measured at 450 nm using a microplate reader. Hormone concentrations were subsequently calculated by interpolation from a standard calibration curve generated from known testosterone standards [25].

2.7 Statistical Analysis

All quantitative data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism software (Version 8.0; GraphPad Software Inc., San Diego, CA, USA). Differences among experimental groups were assessed by one-way analysis of variance (ANOVA). Whenever significant overall effects were detected, Tukey's post hoc multiple-comparison test was applied to identify pairwise group differences. Adjusted p-values obtained from Tukey's procedure were used to control family-wise error rates for each outcome measure. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 Histological Findings and Sperm Characteristics

Histopathological examination revealed marked structural alterations in the testes of rats treated with chlorambucil (Fig. 1). Morphometric analysis demonstrated significant reductions in Johnsen's score, seminiferous tubule diameter, germinal epithelial thickness, and spermatogonial cell count compared with the control group ($p < 0.05$; Table 1). These histological abnormalities were markedly attenuated by cobalamin co-administration, resulting in partial restoration of normal testicular architecture and significant improvement in all measured histomorphometric parameters relative to the chlorambucil-treated group ($p < 0.05$).

Consistent with the histological findings, chlorambucil markedly impaired sperm quality (Fig. 1; Table 2). Rats receiving chlorambucil exhibited significantly lower sperm count and viability together with higher percentages of abnormal and non-motile sperm than the control group ($p <$

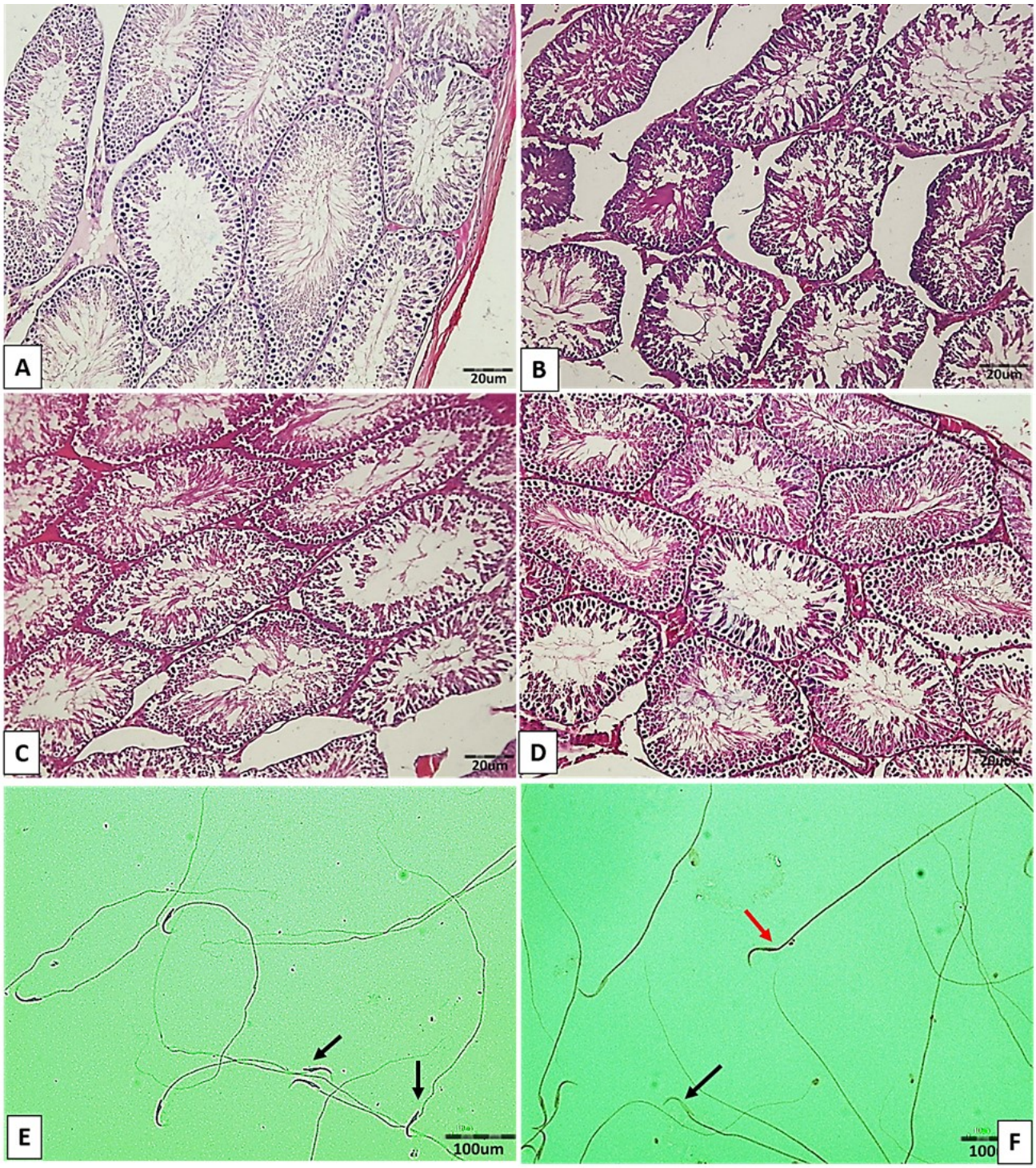

Fig. 1. Histological and sperm morphological assessment of the experimental groups. Representative hematoxylin and eosin (H&E)-stained testicular sections from the (A) control, (B) chlorambucil (Chl), (C) chlorambucil + cobalamin (Chl + Cob), and (D) cobalamin (Cob) groups. (E) Representative Papanicolaou-stained sperm smears showing sperm morphology; arrows indicate abnormal spermatozoa. (F) Representative eosin Y-stained sperm samples for viability assessment; red arrows indicate dead sperm and black arrows indicate viable sperm. The scale bar is 20 µm for the histological images and 100 µm for the sperm images.

0.05). Co-treatment with cobalamin significantly improved all evaluated sperm parameters compared with chlorambucil treatment alone ($p < 0.05$), although complete recovery to control values was not observed.

3.2 Effects on Oxidative Stress Parameters

Chlorambucil administration significantly disrupted the oxidative balance by decreasing glutathione peroxidase

Table 1. Histomorphometric parameters of testicular tissue in rats treated with chlorambucil and cobalamin.

Group	Johnsen score	Seminiferous tubule diameter (µm)	Germinal epithelial thickness (µm)	Spermatogonial count (cells/tubule)
Control	9.01 ± 1.11	257.51 ± 5.62	131.20 ± 5.33	36.52 ± 3.11
Chl	6.12 ± 1.35*	176.21 ± 7.12*	91.23 ± 4.12*	16.33 ± 2.18*
Chl + COB	8.42 ± 1.12$^{\#}$	231.10 ± 4.33$^{\#}$	118.02 ± 6.52$^{\#}$	28.61 ± 2.81$^{\#}$
COB	8.79 ± 2.11	263.34 ± 5.89	131.81 ± 5.71	37.12 ± 2.37

*Data are presented as mean ± SD (n = 8 rats per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ versus the control group; $^{\#}p < 0.05$ versus the chlorambucil (Chl) group. Chl, chlorambucil; COB, cobalamin. SD, standard deviation; ANOVA, analysis of variance.

Table 2. Effects of chlorambucil and cobalamin on sperm parameters in experimental groups.

Group	Sperm count (×10^6/mL)	Viability (%)	Abnormal morphology (%)	Immotile sperm (%)
Control	88.18 ± 4.72	85.13 ± 5.32	14.66 ± 2.20	5.93 ± 1.02
Chl	19.33 ± 2.50*	45.34 ± 3.31*	53.61 ± 4.61*	40.80 ± 4.11*
Chl + COB	28.66 ± 1.10$^{\#}$	68.41 ± 5.11$^{\#}$	41.00 ± 2.01$^{\#}$	15.99 ± 3.62$^{\#}$
COB	87.13 ± 5.22	89.33 ± 6.12	16.33 ± 1.71	6.45 ± 1.40

Values are expressed as mean ± SD (n = 8 animals per group). Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant. * Indicates a significant difference compared with the control group ($p < 0.05$), whereas $^{\#}$ indicates a significant difference compared with the Chlorambucil (Chl) group ($p < 0.05$). Chl, chlorambucil; COB, cobalamin.

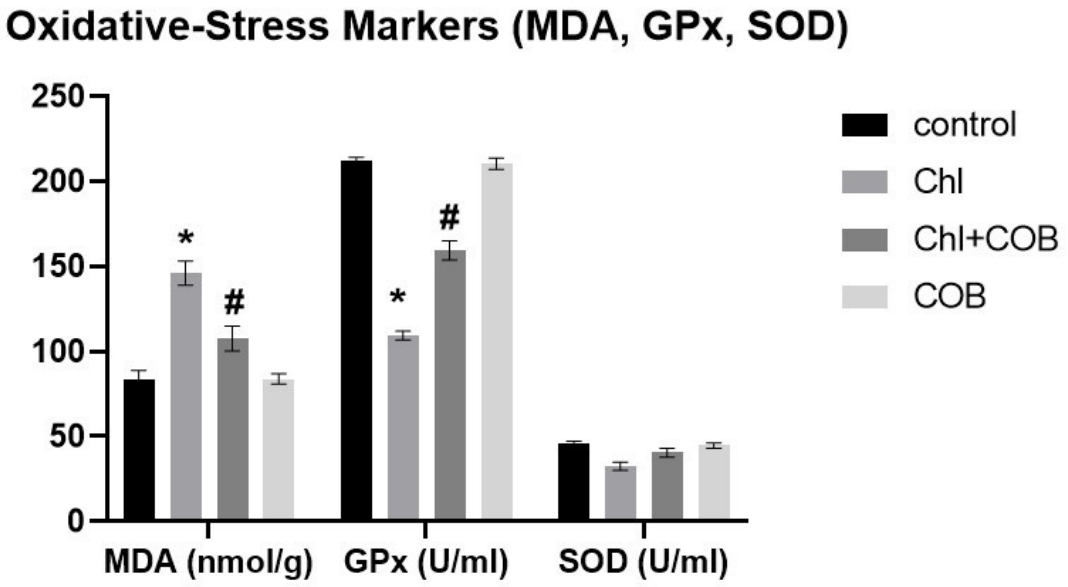

Fig. 2. Effects of chlorambucil and cobalamin on oxidative stress biomarkers. Levels of malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD), in the experimental groups. Data are presented as mean ± SD (n = 8). *$p < 0.05$ versus the control group; $^{\#}p < 0.05$ versus the chlorambucil group.

(GPx) activity and increasing malondialdehyde (MDA) levels compared with the control group ($p < 0.05$; Fig. 2). Although superoxide dismutase (SOD) activity showed a decreasing trend following chlorambucil treatment, this change did not reach statistical significance. Cobalamin co-treatment significantly restored GPx activity and reduced MDA concentrations relative to chlorambucil-treated animals ($p < 0.05$). While SOD activity tended to increase in the cobalamin-treated group, this effect was not statistically significant.

3.3 Effects on Serum Testosterone

A significant reduction in serum testosterone concentration was observed following chlorambucil treatment compared with the control group ($p < 0.05$; Fig. 3). This decline was significantly alleviated by concurrent admin-

istration of cobalamin, which partially restored circulating testosterone levels relative to rats receiving chlorambucil alone ($p < 0.05$).

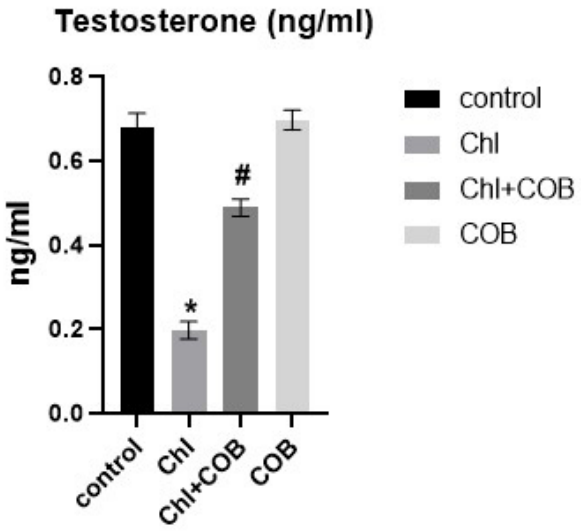

Fig. 3. Effects of chlorambucil and cobalamin on serum testosterone levels. Data are presented as mean ± SD (n = 8). *$p < 0.05$ versus the control group; #$p < 0.05$ versus the chlorambucil group.

3.4 Expression of Apoptosis-Related Genes

As shown in Fig. 4, chlorambucil significantly altered the expression of apoptosis-related genes in testicular tissue. The mRNA expression levels of the pro-apoptotic genes *Caspase-3* and *Bax* were significantly upregulated, whereas expression of the anti-apoptotic gene *Bcl-2* was markedly downregulated compared with the control group ($p < 0.05$). Cobalamin co-administration significantly reversed these changes by suppressing *Caspase-3* and *Bax* expression while increasing *Bcl-2* expression relative to the chlorambucil-treated group ($p < 0.05$), suggesting attenuation of apoptosis.

3.5 Expression of Autophagy-Associated Genes

The expression of autophagy-associated genes was markedly affected by chlorambucil treatment (Fig. 5). Relative mRNA levels of *Atg5, Atg6, Atg7, Atg8,* and *Atg12* were significantly reduced compared with those in the control group ($p < 0.05$). In contrast, cobalamin co-treatment significantly increased the expression of these genes relative to chlorambucil alone ($p < 0.05$), indicating partial restoration of autophagy-related molecular signaling.

3.6 miR-125b Expression Profile

Testicular *miR-125b* expression was significantly elevated in chlorambucil-treated rats compared with the control group ($p < 0.05$; Fig. 6). Co-administration of cobalamin significantly reduced *miR-125b* expression relative to chlorambucil treatment alone ($p < 0.05$), bringing its expression closer to that observed in the control group.

Overall, no significant differences were observed between the control and COB (cobalamin-treated control) groups across any of the evaluated biochemical, histological, immunohistochemical, or molecular parameters, indicating that cobalamin administration alone did not induce measurable alterations in healthy animals. Furthermore, the control and COB groups exhibited similar patterns when compared with the experimental groups, supporting the conclusion that the observed changes were attributable to chlorambucil exposure and the experimental interventions rather than to cobalamin administration under normal physiological conditions.

4. Discussion

This study investigated the interplay between cell death pathways and *miR-125b* expression in chlorambucil-induced testicular toxicity and evaluated the protective role of cobalamin (vitamin B12). The findings indicate that chlorambucil disrupts testicular homeostasis through oxidative stress–mediated injury, leading to impaired spermatogenesis, enhanced apoptosis, suppression of autophagy, hormonal disruption, and dysregulation of *miR-125b*. Cobalamin co-administration effectively counteracted these alterations, suggesting a coordinated cytoprotective mechanism involving redox regulation and modulation of cell death signaling networks.

Chlorambucil, a nitrogen mustard alkylating agent, exerts its cytotoxic effects primarily through DNA crosslinking and excessive production of reactive oxygen species (ROS) [26]. A notable finding was the significant reduction in spermatogonial cell populations following chlorambucil exposure, suggesting selective vulnerability of proliferating germ cells to alkylating damage. Loss of spermatogonial stem and progenitor cells compromises subsequent stages of spermatogenesis, leading to reduced sperm output and defective sperm quality [27]. Histological evidence in the present study further supports a global suppression of spermatogenic activity.

Chlorambucil also induced a significant decline in serum testosterone levels, indicating Leydig cell dysfunction. Oxidative stress–mediated mitochondrial damage and disruption of steroidogenic enzymes likely contribute to impaired testosterone biosynthesis. Because adequate intratesticular testosterone concentrations are essential for normal spermatogenesis, hormonal insufficiency may have further exacerbated germ cell loss and structural degeneration [28].

A central molecular finding of this study is the upregulation of *miR-125b* in chlorambucil-treated testes. *miR-125b* is known to regulate cell fate decisions, including apoptosis, proliferation, and stress responses [29,30]. Its increased expression may represent a stress-responsive adaptation to DNA damage and oxidative injury. However, it

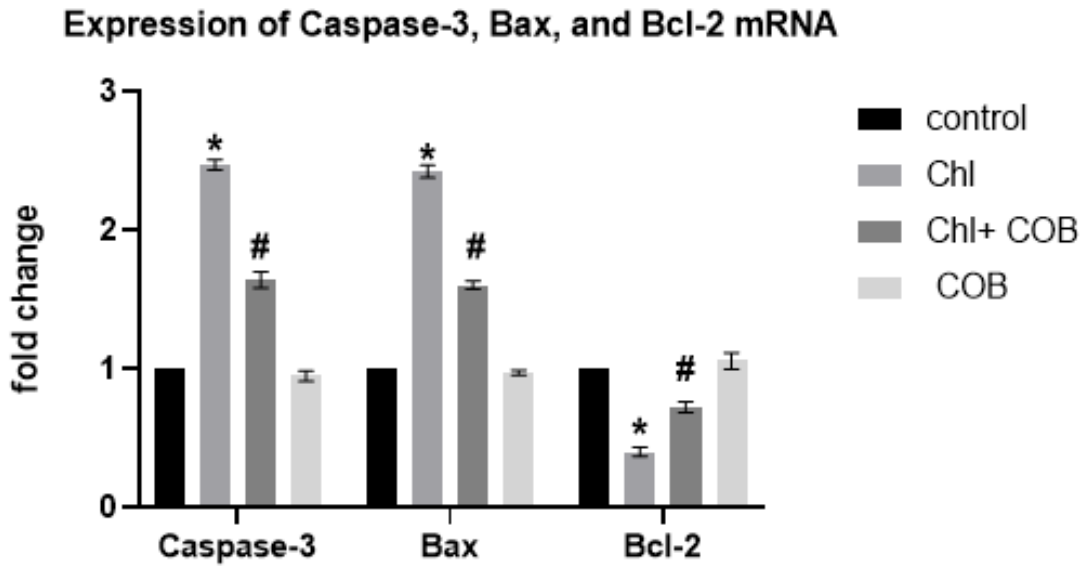

Fig. 4. Relative expression of apoptosis-related genes in testicular tissue. Relative mRNA expression levels of *Caspase-3*, *Bax*, and *Bcl-2* in the experimental groups. Data are presented as mean ± SD (n = 8). *$p < 0.05$ versus the control group; $^{\#}p < 0.05$ versus the chlorambucil group.

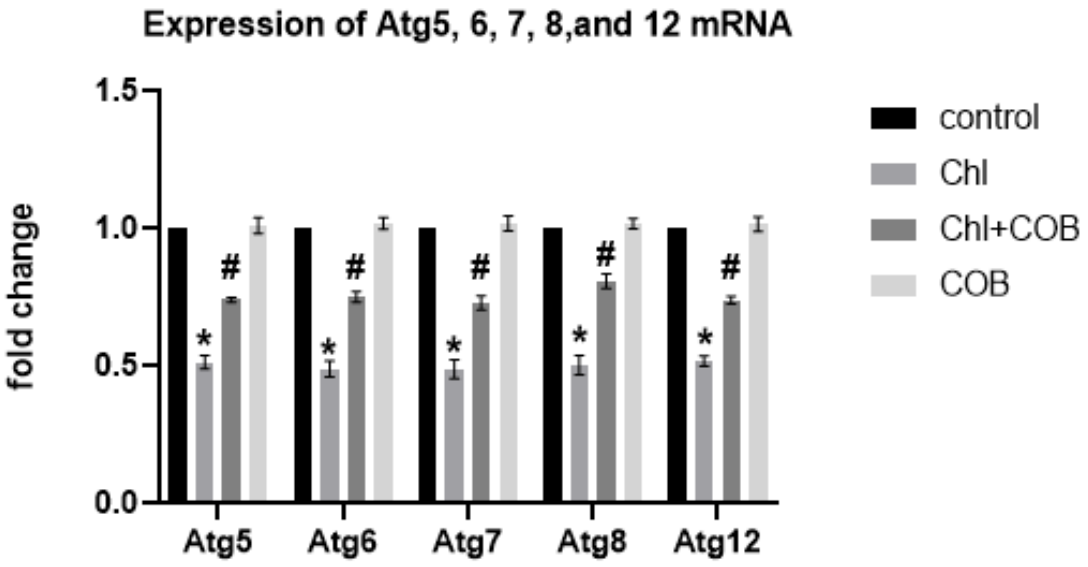

Fig. 5. Relative expression of autophagy-related genes in testicular tissue. Relative mRNA expression levels of *Atg5, Atg6, Atg7, Atg8*, and *Atg12* in the experimental groups. Data are presented as mean ± SD (n = 8). *$p < 0.05$ versus the control group; $^{\#}p < 0.05$ versus the chlorambucil group.

may also contribute to pathological signaling by modulating key survival pathways.

Mechanistically, *miR-125b* has been shown to suppress anti-apoptotic proteins such as *Bcl-2*, thereby facilitating apoptotic signaling [31]. This is consistent with the increased apoptotic activity observed in the present study.

In addition to its pro-apoptotic influence, *miR-125b* may negatively regulate autophagy-related genes, including ATG7 [32]. The downregulation of autophagy-related transcripts observed in this model suggests impaired autophagic flux under chlorambucil exposure. Since autophagy serves as a cytoprotective mechanism for removing damaged organelles and maintaining cellular homeostasis [33], its suppression likely shifts the balance toward apoptosis, accelerating germ cell loss and worsening testicular injury [33].

Interestingly, our findings also suggest a dose-dependent dual role of chlorambucil in autophagy regulation. While lower doses have been reported to activate autophagy-related gene expression, the present study demonstrates that a higher dose (2 mg/kg) suppresses

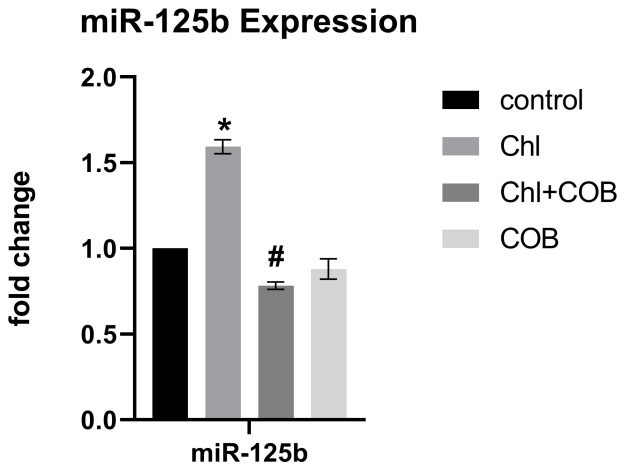

Fig. 6. Relative expression of *miR-125b* in testicular tissue. Relative *miR-125b* expression in the experimental groups. Data are presented as mean ± SD (n = 8). $*p < 0.05$ versus the control group; $^{\#}p < 0.05$ versus the chlorambucil group. MicroRNA-125b, *miR-125b*.

autophagy-related transcripts. This biphasic response may reflect a threshold-dependent transition from adaptive cellular protection to irreversible cellular damage.

Beyond apoptosis and autophagy, *miR-125b* may further impair spermatogenesis by targeting genes involved in cell-cycle regulation and stem cell self-renewal [11]. Such effects could explain the marked depletion of spermatogonial populations observed in this study and contribute to long-term impairment of germ cell regeneration.

Cobalamin co-administration significantly ameliorated chlorambucil-induced testicular toxicity across all evaluated parameters. The observed protective effects are likely mediated through its antioxidant properties and its role in maintaining cellular redox balance [14,34].

Restoration of autophagic activity suggests reactivation of cellular quality-control mechanisms, facilitating the removal of damaged organelles and limiting pro-apoptotic signaling. Correspondingly, reduced apoptotic signaling further supports the cytoprotective role of autophagy in limiting cell death under stress conditions [35,36].

Importantly, cobalamin also restored serum testosterone levels, suggesting preservation of Leydig cell function. Maintenance of mitochondrial integrity and support of steroidogenic pathways may underlie this effect. Restoration of hormonal homeostasis likely contributed to the recovery of spermatogenesis and improved histological organization of the testes [15,37].

The partial normalization of *miR-125b* expression following cobalamin treatment represents a novel and important finding. This effect may be attributed to reduced oxidative stress, improved homocysteine metabolism, and enhanced glutathione-mediated antioxidant defense [14,34]. Additionally, decreased DNA damage may suppress stress-responsive signaling pathways that regulate miRNA expression, including *miR-125b* [38].

Previous studies support the protective role of vitamins in chemotherapy-induced gonadotoxicity. For example, vitamin E has been shown to attenuate cyclophosphamide- and busulfan-induced reproductive damage through antioxidant and anti-apoptotic mechanisms, preserving seminiferous tubule integrity and improving sperm parameters [39,40].

These findings align with the present results and further support the therapeutic potential of antioxidant supplementation in chemotherapy-associated infertility.

Strengths and Limitations

This study has several strengths, including a multilevel evaluation of testicular toxicity incorporating histological, biochemical, hormonal, and molecular endpoints. Additionally, this is among the first studies to simultaneously investigate *miR-125b* expression alongside apoptosis and autophagy pathways in chlorambucil-induced testicular injury, providing a more integrated mechanistic perspective.

However, several limitations should be acknowledged. The experimental duration (30 days) may not fully represent the complete spermatogenic cycle in rats. Furthermore, molecular analyses were limited to mRNA expression without protein-level validation. Finally, as an animal study, extrapolation of these findings to human reproductive physiology should be made with caution.

5. Conclusion

Chlorambucil-induced testicular toxicity is closely associated with oxidative stress–mediated upregulation of *miR-125b*, leading to dysregulation of cell death signaling and impaired spermatogenesis. Cobalamin effectively mitigates these effects by restoring redox balance, modulating *miR-125b* expression, and rebalancing apoptosis and autophagy pathways, thereby preserving endocrine and germ cell function. Collectively, these findings highlight *miR-125b* as a potential biomarker of chemotherapy-induced reproductive toxicity and support cobalamin as a promising gonadoprotective adjunct.

Availability of Data and Materials

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

Author Contributions

AE: Conceptualization, Methodology. MM: Data curation, Writing- Original draft preparation. SAE: Visualization, Investigation. MS: Conceptualization, Methodology, Supervision, Writing – Review & Editing. ZR: Software, Validation. All authors contributed to editorial changes in

the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Animal experiments were conducted following approval from the Institutional Ethics Committee of the Ministry of Health and Medical Education of the Islamic Republic of Iran (Approval No.: IR.GMU.AEC.1404.001; Date: 2025-05-07). All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals, Eighth Edition (National Research Council, 2011) for laboratory animal care and use. Measures were implemented throughout the study to minimize discomfort and suffering. The study design complied with the principles of Replacement, Reduction, and Refinement (3Rs) and followed ARRIVE recommendations for reporting animal research.

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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 manuscript preparation, ChatGPT was used exclusively for language refinement, text editing, reduction of textual similarity, and assistance with manuscript condensation. No artificial intelligence tool contributed to study design, data processing, statistical analysis, interpretation of findings, or formulation of conclusions. All AI-assisted outputs were carefully reviewed, verified, and revised by the authors. Full responsibility for the accuracy, originality, and integrity of the manuscript remains with the authors.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/IJVNR53516.

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