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# Morphological, histopathological, and immunohistochemical changes in tissues of adult male Sprague-Dawley rats orally treated with isotretinoin

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ABSTRACT

**Background:** Isotretinoin (ISO) is the most effective drug prescribed by dermatologists for the treatment of acne vulgaris and other clinical skin cases. A significant obstacle to using ISO is concerns regarding its adverse effect profile. Despite the well-established reproductive toxicity in females, information on the effects on human male fertility is scarce, contradictory, and inconclusive. This study aimed to investigate the potential histological and histochemical effects of ISO.

**Materials and Methods:** Isotretinoin was administered orally for seven successive days to Sprague Dawley male rats in a 5-20 mg/kg/day dose range. Standard histological and immunohistochemical techniques were used to evaluate ISO side effects.

**Results:** High doses of ISO led to infiltration of inflammatory cells in hepatic tissues, atrophy of the kidney glomeruli, and collapse of testicular compartments. Decreased E2F4 production was positively correlated to a reduced rate of spermatogenesis.

**Conclusions:** The findings provide further evidence for ISO's cytotoxic and reprotoxic potencies. These effects are probably partly due to slowing down the expression of an E2F4 transcription factor. The dysregulated gene may play an essential role in spermatogenesis. The diagnostic value of the E2F4 gene needs to be further validated by different proteomics approaches, and its precise role in spermatogenesis needs to be investigated.



## 1. Introduction

Isotretinoin has revolutionized the treatment of problematic cystic acne.[1] Most harmful effects regarding the use of ISO are associated with the skin.[2] Since ISO is filtered from the bloodstream by the liver, taking it may exert an extra strain on this organ. Opposing data from human studies were encountered in the literature; some suggested hepatotoxicity,[3] while others claimed no changes in the parenchymal liver in patients who took it at 10–40 mg daily for at least six months.[4] In female mice, it was confirmed that ISO at a dose of 1 mg/kg considerably induced cytotoxicity in the liver tissues of pregnant mothers.[5]

Likewise, there are contradictory reports regarding the safety of oral ISO on the genitourinary systems.[6,7] Severe histological changes in the kidneys of ISO-treated animals have been established.[5,6,8] Contrary to this, there were no noteworthy differences in the baseline glomerular filtration rate between ISO and non-ISO users.[9]

In dogs given ISO for 30 weeks at 20 or 60 mg/kg/day, testicular atrophy and lower spermatogenesis were eminent.[10] Despite its several pronounced side effects

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and without the regulated use, ISO was and is still being used extensively.[11] Information about the direct effect of ISO on critical organs is scarce and debatable. For example, only a few immunohistological reports have been found in the literature.[12] The present study investigated the structure of the kidney, liver, and testis after ISO treatment. Since ISO affects the interphase of the cell cycle, especially $G_1$ and S, a second goal was added in the form of decreased DNA synthesis, stimulated p21 protein, and reduced Cyclin D1 protein leading to cell cycle arrest in G0/G1[13]—particularly, estimation of the effect of ISO on E2F4 expression in the testicular tissue.

The E2F4 gene is the main form of E2F, a group of genes that encodes a family of transcription factors.[14] E2F4 plays a role in the stability and function of different cell types in embryonic and adult tissues. These functions include angiogenesis, replication in normal and abnormal development of tissues, response to DNA damage, apoptosis, and differentiation.[14,15] The genes bound by E2F4 in the kidney, liver, and testis are clustered by transcription factor.[16]

## 2. Materials and Methods

### 2.1. Ethical considerations

All procedures were approved by the Institutional Animal Care and Use Committee at Yarmouk University (permit IACUC/2021/5) under the ethical committee guidelines and welfare considerations.

### 2.2. Animals

Twenty healthy 10-week-old male Sprague–Dawley rats (200-220 g) were used in this study. The animals were bred and maintained in the animal house facilities of the Department of Biological Sciences at Yarmouk University, Irbid, Jordan, under standard conditions. The food and tap water were supplied ad libitum.

### 2.3. Dosing

The ISO soft gelatin capsules (F. Hoffmann-La Roche Ltd, Basel, Switzerland), each containing 10 mg, were opened and diluted with soybean oil to obtain suspension at the desired concentrations. The rats were randomly allocated to five groups (4 animals each). Each animal in the first group was orally given soybean oil. The second, third, fourth, and fifth groups orally received a single oral gavage dose of freshly prepared ISO solutions at doses of 5, 10, 15, or 20 mg/kg b w), respectively, for seven consecutive days. The final application volume did not exceed 1 ml. The same controlled conditions were applied to each group to eliminate probable variation in the measured parameters. According to this, cumulative ISO doses achieved were 35-140 mg/kg. These dose levels were selected based on a previous study.[17] In this investigation, rats were orally gavaged with 13-cis-retinoic acid at 7.5 or 15 mg/kg for seven consecutive days (a cumulative dose of 52.5-105). The doses of 7.5 or 15 mg/kg/day were used to produce serum ISO concentrations equivalent to those of humans dosed with 1 mg/kg/day, as reported before.[17] The oral administration method was chosen for this study since these drugs are administered orally in a clinical setting. The rats were observed daily for clinical signs and physiological and behavioral changes.

### 2.4. Histological analysis

After termination of the experiments, animals were intraperitoneally euthanized with sodium pentobarbital (Sigma, St. Louis, MO, USA) at a dose of 100 mg/kg (20 mg/ml/animal). They were dissected, and the internal organs were grossly inspected. After that, biopsies (3–5 $mm^3$) of the kidneys, liver, and testes were cleaned with normal saline and stored in a 10% phosphate-buffered formalin solution (pH 7.4). The Formalin-fixed samples were processed in a programmed Spin Tissue Processor STP-2302 (Myr, EL Vendrell, Spain). The tissues were infiltrated in 1:1 Xylene: molten paraffin at 58–62 °C. Sections 4 to 6 $\mu$m thickness were cut from the paraffin blocks using a semi-automatic Rotary Microtome (Diapath Galileo, Italy). Slides were stained with hematoxylin and eosin (H&E) per the standard protocol. All the slides were coded, and five fields were blindly screened under the light microscope (Nikon, Japan).

### 2.5. Immunohistochemistry analysis

E2F4 expression was detected by using classic methods of immunohistochemistry.[12] Briefly, tissues were deparaffinized and rehydrated, and each section was encircled by a peroxidase-anti-peroxidase pen (Abcam) to make hydrophobic barriers on the glass slides and to hold the antibody solution within the target area on the slide. After drying for 10-15 sec at room temperature (RT), the slides were immersed in 10 mM sodium citrate buffer (pH 6.0) to expose the target protein and retrieve the antigen. The slides were heated to boiling in a microwave oven for 8-15 min. For quenching endogenous peroxidase activity, tissues were blocked in 3% $H_2O_2$-methanol for 15 min at RT and washed extensively with phosphate-buffered saline (PBS)-Tween 20 buffer. Then, tissues were probed with E2F4 Mouse Monoclonal Primary Antibody E2F4 (4E2F04 (WUF10), Invitrogen, USA) diluted in 3% bovine serum albumin (1:20) for one h at 37 °C in a humidified chamber. The same immunoglobulin isotype and light chain against E2F4 and a synthetic hapten, which is normally not present in animals, were used as the negative control. To detect the horseradish peroxidase activity, the 3,3'-diaminobenzidine (DAB Kit, USA) substrate was applied to the slides as

a chromogen. This was followed by hematoxylin as a counterstain according to the manufacturer's instructions. Tissues were covered with substrate and processed according to the manufacturer's instructions. All slides were examined under a light microscope (Nikon, ECLEPS, E200). The results were collected from repeated image analyses of tissue sections from at least three animals per treatment. Four eye fields were evaluated for each animal. For each treatment, 2000 cells in 10 seminiferous tubules were examined, and the number of immunopositive cells with brown-colored nuclei was recorded. Statistical analyses of the data were performed using a one-way analysis of variance and Tukey's post hoc test by SPSS for Windows 15.0. A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### *3.1. General health*

Signs of tiredness were noted in most of the ISO-treated animals. Rats dosed with low ISO doses displayed hostile tempers as evidenced by pitched voices and attempts at biting; treated animals showed a lower rate of taking food and water. No signs of hair loss or nose- or rectum bleeds were recorded.

### *3.2. Macroscopic analysis*

No gross massive swelling or discoloration was observed in any of the internal organs both in the control and the experimental rats at lower and intermediate ISO doses. However, the naked eye check discovered clear pale necrotic areas on a generally healthy kidney from an ISO-treated rat (Figure 1 A-2). Some ISO-treated rats presented a white "fat" body on the kidney (Figure 1 A-3). Further, compared to control livers (Figure 1 B-1), some experimental rats revealed hepatic necrotic lesions (Figure 1 B-2). Masses (0.5 to 1.0 inch) bulging from the liver and attached to the diaphragm were observed (Figure 1 B-3 and Figure 1 B-4, arrows). They appeared in one of the 15mg/kg-dosed rats and in the livers of all the 20 mg/kg administered rats.

The histopathology of the studied internal organs showed degeneration with increasing severity, especially at the doses of 15 and 20 mg $kg^{-1}$. The results obtained by H and E staining showed no pathological alterations in the kidney of the control group (Figure 2 a). The kidneys of rats treated with the highest ISO dose disclosed noticeable pathological lesions characterized by shrinkage of renal glomeruli and glomerular tuft atrophy (Figure 2 b).

Figure 3 a shows the typical microscopic liver architecture composed of hexagonal lobules and acini. Figure 3 b displays the histological changes in the liver, primarily congestion of the central vein and mononuclear cellular infiltration surrounding the bile duct following administration of ISO at a high dose (20 mg/kg) for seven

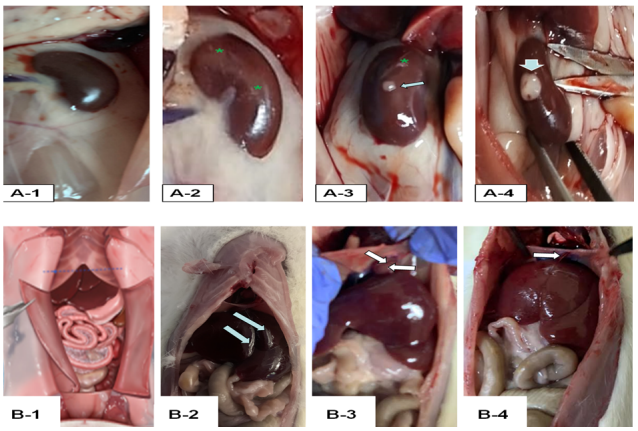


**Figure 1:** Gross anatomy of rat internal organs. Up: Kidney; **A-1**: Control rat showing a smooth and reddish kidney. **A-2**, **A-3**, and **A-4**: Isotretinoin-treated rats with abnormally-shaped enlarged kidneys, with mild necrosis (star), cyst (Thin arrow, or fatty body (Thick arrow). Bottom: Liver; **B-1**: Normal appearance of the liver in control rats; **B2-B4**: Isotretinoin-treated rats. Notice the development of hepatic lesions (blue arrows) and nodules or bulges (White arrows)

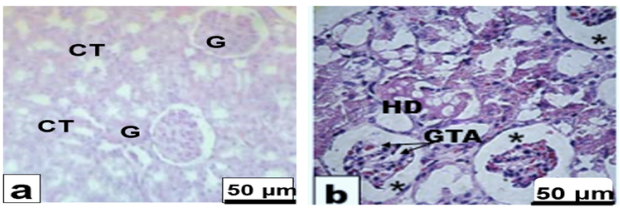


**Figure 2:** Photographs of a representative section of rat kidney tissue. (**a**): Control: Note the normal glomerulus (G) and convoluted tubules (CT). (**b**): Isotretinoin-treated rats: Note the glomerular atrophy (*), glomerular tuft atrophy (GTA), and hydropic degeneration (HD): Hematoxylin and Eosin stain. Magnification: 200 X

consecutive days. Kupffer cells became pronounced and increased in number due to ISO intoxication. Occasional necrotic lytic changes, correlated with loss of normal cytoplasmic eosinophilia, were seen in some rats exposed to ISO.

The testes of the control rats revealed a usual histological architecture with semicircular seminiferous tubules containing the different stages of spermatogonia cell differentiation (Figure 4 a). When sections of ISO treatment and the control group were compared, the former showed regressive alterations such as loosely arranged spermatogenic cells and atrophic interstitial compartments (Figure 4 b).

Table 1 and Figure 5 show that E2F4 expression was predominantly in the cells from the control. No statistically lower readings in the number of immunopositive nuclei were recorded at 5 mg/kg. In contrast, E2F4 was poorly

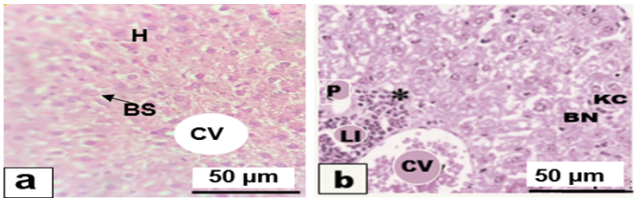


**Figure 3:** Photomicrographs of liver tissue of the differently treated rats (**a**): Control with normal hepatic architecture and usual central vein (CV), strands of hepatocytes (H) with well-defined nuclei and cytoplasm, radiate from CV toward the periphery of the hepatic lobule and separated by narrow blood sinusoids (BS, arrow). (**b**): Hepatic tissue from Isotretinoin-treated rat showing dilated CV) engorged with blood cells. Little leucocytic infiltration (LI, *) in the periportal area (P) was observed. Kupffer cell (KC), some cells are binucleated (BN)—Hematoxylin and Eosin (H&E) stain. Magnification: 200 X

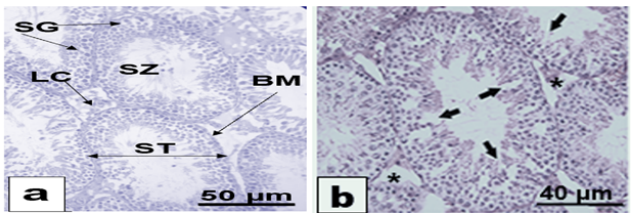


**Figure 4:** Representative images of Hematoxylin and eosin-stained rat testicular cross sections. (**a**): The Control group showed standard groups of seminiferous tubules (ST) with regular outlines and inter-tubular Leydig (interstitial) cells (LC) in between. The germinal epithelium was composed of spermatogonia, primary spermatocyte, rounded and elongated spermatids, and mature sperms in the lumen of the tubules. (**b**): Testis from isotretinoin-treated rats shows irregular tubular outlines with loosely arranged spermatogenic cells, inter-cellular vacuolation (arrows), and atrophic interstitial compartments (*). BM: Basement membrane; SG: Spermatogonia; SZ: Spermatozoa. Magnification: 400 X

expressed in the specimen from rats treated with ISO at 10, 15, or 20 mg/kg, and highly statistically significant reductions in E2F4 expression were calculated ($p<0.001$). In addition, the antibody against E2F4 stained nuclei was most strongly in the somatic cells (spermatogonia).

## 4. Discussion

In the present study, ISO caused apparent signs of a bad mood in the rats' behavior. There was a trend to reduce food and water intake. A similar reduction in food and water intake was observed[18] and linked to the consumption of ISO. The ISO-treated rats demonstrated increased neutrophils and macrophages in the blood. This increase agrees with the results reported in acne patients[19] and rats.[20]

We have demonstrated several changes in the standard architecture of the experimental rats' liver, kidney, and testes. The oregano-toxicity of ISO has been reported in several studies. Although underreported, the hepatic toxicity of ISO therapy is well-acknowledged.[21] The possible harmful effects of ISO on the renal system were reported in a handful of publications.[6] The overall analysis of histological sections of the liver and kidney of the ISO-treated rats showed general changes in the morphology, agreeing with that already found in rats,[22] and mice.[23]

The data from the present research confirmed that testicular tissues from animals treated with ISO exhibited degenerative changes. ISO stimulated toxicity at 20 mg/kg/day for seven days, including decreased Sertoli and Leydig cell numbers. Isotretinoin produced improper seminiferous tubules with a thick, abnormal basement membrane. Inspection of the histopathology of reproductive organs in repeated-dose toxicity investigations in rodents and nonrodents is one of the best assessment approaches executed before the first administration of a novel medication to humans.[2] The articles on the consequences of retinoids on spermatogenesis are opposing. The data from the present research confirmed that testicular tissues from animals treated with ISO at 20 mg/kg/day for seven

**Table 1:** Results of E2F4 expression in the testes of Isotretinoin-treated rats immunopositive nuclei. The count is based on screening of 2000 nuclei per dose

| Treatment | | E2F4 (Mean ± SEM) | % E2F4 Immunopositively Nuclei | P-value* |
|---|---|---|---|---|
| Soybean Oil | 0 | 1490 ±78.94 | 74.50 | - |
| Isotretinoin (mg/kg) | 5 | 1326±226.20 | 66.30 | 0.244 |
| | 10 | 554 ±81.91 | 27.70 | <0.0001 |
| | 15 | 482 ± 108.60 | 24.10 | <0.0001 |
| | 20 | 50± 6.50 | 2.50 | <0.0001 |

*ANOVA analysis and Tukey's post hoc test

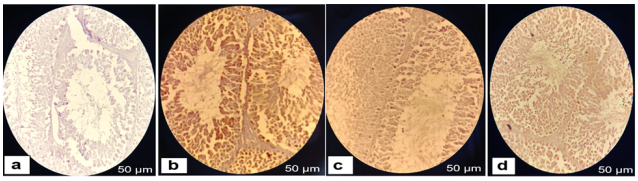


**Figure 5:** Photographs of representative immunohistochemistry stain of E2F-4 transcription factor showing staining patterns of the nuclei of formalin-fixed, paraffin-embedded testicular tissue using peroxidase-conjugate and diaminobenzidine. (**a**): Negative control without primary antibody; (**b**): vehicle control (Soybean oil) compared to testes; from 10 and 20 $\mu$g Isotretinoin-treated rats; (**c**): and (**d**): Respectively. More immunopositive nuclei (brown stain) are observed in the epithelial cells in the seminiferous tubules in the SBO control. The E2F4 protein expression was less pronounced in the 10 and 20 mg ISO-administered rat specimens. Magnification: 200 X

days exhibited degenerative changes. It stimulated toxicity, including a decrease in Sertoli and Leydig cell numbers. Isotretinoin produced improper seminiferous tubules with a thick, abnormal basement membrane. These findings are along with those encountered recently on rats that were given ISO at one oral daily dose of (1.0 mg/kg/day) for 21 consecutive days.[7] These researchers noticed seminiferous tubules lined by a few apoptotic spermatogenic cells with cytoplasmic vacuolation-associated shriveled spermatozoa. Earlier research showed that prolonged time and elevated doses of vitamin A were teratogenic, diminished testicular mass, produced lesions in the seminiferous epithelium, disturbed the normal spermatogenic cycle, and adversely influenced Leydig cells in dogs[10] and lizards.[24]

These findings may be explained by the fact that ISO activates neutrophils, inducing tissue injury by releasing reactive oxygen species (ROS) into the extracellular fluid.[21] Isotretinoin treatment caused a loss of biomembrane fluidity and lipid peroxidation with the generation of ROS.[19,20] Free radicals disturb the membrane integrity in mammalian testicles, possibly leading to testicular atrophy and seminiferous tubule collapses.[25] Isotretinoin impacts cell membrane permeability, resulting in the lysis of cells and allowing essential substances to escape.[21] It results in a loss of desmosomes and diminishes tonofilaments and glycocalyx cohesion of the cell.[2] Disruption of the Sertoli-germ cell junctions may cause failure of spermatogenesis. The blood-testis barrier is a system of tight junctions between adjacent Sertoli cells to form a vital microenvironment capable of supporting developing germ cells and helping preserve homeostasis. This microenvironment shields the developing cells in seminiferous tubules from obstructing molecules.[26]

In rats, ISO has been established to be reprotoxic;[27,28] administration of a high dose of ISO caused statistically significant declines in rat sperm motility, sperm viability, seminiferous tubule thickness, and germinal epithelium. Also, ISO was cytogenotoxic.[29] Cytotoxicity is one of the primary mechanisms that may cause acute or chronic damage.[5] Cytotoxicity may be followed by cell death, apoptosis, or necrosis,[29] leading to a drop in germinal epithelial cells. The results gained for the percentages of sperm aberrations[28] permit establishing a connection between the seminiferous epithelium changes and the decrease in the epididymal weight.[30] These workers demonstrated that ISO lowered the reproductive organ's weight and the amount of Sertoli and Leydig cells.

One of the outcomes of the present investigation was the decline in the immune marker E2F4 and spermatogenesis index after taking ISO for seven days. In contrast, significantly higher quantities of E2F4-positive cells were recorded in the control rats in spermatogonia and early-stage spermatocytes. The relatively prompted level of expression of E2F4 verified in the testicular epithelium during spermatogenesis was in general agreement with what was reported before.[12,28] It was consistent with its function identified concerning expression patterns.[31] As suggested previously, the relationship between E2F4 expression and tissues that are actively multiplying like testis proposes two conceivable alternative explanations.[23] The first is that E2F4 activity might be required for the proliferation of these cellular divisions. Hence, the absence of E2F4 activity in the testes of ISO-treated rats leads to declines in the cells that can produce mature spermatozoa. It was shown that the knockout of E2F4 initiates infertility in both sexes of mice, yet the gonads looked histologically normal.[32] Otherwise, E2F4 might not be necessary for vigorous proliferation but rather is required for a cell to leave the cell cycle upon reception of an appropriate signal. In the latter situation, the elevated level of E2F4 in these proliferative tissues would signify the differentiation capacity. It has been confirmed[14] that E2F4 has a broad array of effects on the production of the global transcriptome and the control of genes and routes of mitochondrial metabolism.

Elevated E2F4 concentrations and their translocation to the nucleus due to genotoxic stress may trigger the downregulation of numerous mitotic genes and subsequently stimulate a $G_0$-like state.[33] This proved that ISO given to rats may provoke damage of spermatogenesis and an escalated apoptosis in testicular germ cells by a drop in the amount of Cyclin $D_1$. The latter study registered a nonsignificant reduction in the number of E2F-positive cells in the testes.

What lets E2F4 control various gene programs in different cell types is not well-known. It is likely that ISO somehow repressed the expression of the E2F4. A cut in the expression of the gene indicates that high-dose ISO therapy may cause a slowing down of spermatogenesis. The loss of endogenous E2F4 activity can harm the testicular function. The reduction of E2F4¬positive cells may result from DNA injury, which seemed to be the primary source of decreased sperm since spermatozoa need undamaged DNA during fertilization. As an intranuclear polypeptide, E2F4 may be involved in DNA replication, excision, and repair. A cut in the expression of these genes indicates that high-dose ISO therapy may cause a slowing down of spermatogenesis.

A comparison of the immunohistochemical staining between normal liver and cancer cells revealed a vast difference in the E2F4-positive cells.[14] Cancer cells express E2F4 at greater levels than neighboring normal tissue. These results indicate that ISO may be involved in cellular impairment and death via the deactivation of E2F4. This observation strengthens the idea that E2F4 expression changes harmoniously with the cellular status. Some reports pointed out that E2F4 has a collection of functions in cells in addition to the cell cycle regulatory role, including embryonic and adult stem cells and in cancer.[13,14] In mature mouse tissues,[22] it was suggested that E2F4 is remarkably

abundant in hematopoietic tissues and the gut, contrary to the heart, kidney, liver, brain, and muscle. A non-canonical function for E2F4 has offered insights into the biology of actively dividing cells.[13] Based largely on the results obtained from in vitro studies, E2F4 has been classified as a key repressor of the cell cycle. In this respect, loss of E2F4 in the dividing stem and precursor populations of several tissue types has been found to reduce proliferation and DNA replication.[13]

This study has certain limitations. The first is the small number of animals used and the lack of long-term follow-up of the animals. The absence of a wash-out period after treatment is a second limitation. The other drawback is that ISO serum concentration was not measured. The duration of spermatogenesis is 54 days in the rat and 74 days in humans. Therefore, the normal spermatogenesis cycle should naturally be investigated for a more extended period to clarify the effects of ISO therapy.

## 5. Conclusions

The results of the present research, added to those published before,[28] suggest that subchronic exposure to high-dose therapy of ISO may have severe side consequences on the spermatogenic cycle as well as on liver and kidney functions. This outcome diverges from some reports on the protective effects of ISO against kidney damage in a rat model[6] and the amendment of sperm production in men with infertility.[34]It seems that E2F4 is not controlled by cell growth but alternatively can exist at almost equivalent levels in both quiescent and cycling cells. Collective data favor the idea that E2F4 has a new role in the control of spermatogenesis, and a reduction in the expression of the E2F4 gene signals that high-dose ISO treatment may lead to a decline in spermatogenesis. Currently, further research on reproductive toxicity in male rats is being carried out in our laboratory. The nature of the grossly "abnormal" masses close to the liver is under investigation. The role of E2F4 in undifferentiated embryonic stem cells and cancer cells remains a field to be further explored. Further, detailed research would be useful to clarify the effects of ISO therapy.

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## 7. Conflict of Interest

The authors have declared that no competing interest exists.

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