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BOLDENONE-INDUCED APOPTOTIC, STRUCTURAL, AND FUNCTIONAL ALTERATIONS IN THE LIVER OF RABBITS

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Abstract: Boldenone undecylenate (BOL) is an anabolic androgenic steroid used in livestock to improve growth and food conversion. This study investigated the actions of BOL on structure and functions of rabbit liver as well as the effects of its withdrawal. Eighteen mature male New Zealand rabbits were divided into 2 groups: Control group (n=6) were injected with 0.25 mL corn oil/kg body weight (BW), while BOL group (n=12) received 3 intramuscular injections, 2 wk apart, of BOL (4.5 mg/kg BW). Animals were scarified 1 d after last injection except for 6 rabbits from BOL group that served as the BOL-withdrawal group (4 wk after the 3rd injection). Intramuscular injection of BOL increased ($P<0.05$) malondialdehyde (MDA) level, but markedly lowered activities of superoxide dismutase (SOD) and catalase (CAT) and reduced glutathione (GSH) concentration compared to both control and BOL-withdrawal groups. Treatment with BOL significantly ($P<0.05$) increased serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) compared to the control group. BOL injection caused different histopathological alterations and apoptosis in liver, but these changes were less evident in the BOL-withdrawal group. Expression of p53 and tumour necrosis factor-α (TNF-α) genes was up regulated in BOL compared to control group, while the expressions of p53 and TNF-α were down regulated in BOL-withdrawal group in comparison with BOL group. In conclusion, BOL injection induced structural and functional changes in the liver of rabbits, increasing oxidative stress and mediators of apoptosis such as ROS, p53 and TNF-α. All these parameters returned to near the control values after withdrawal.

Key Words: boldenone, liver, oxidative stress, apoptosis, rabbits.

INTRODUCTION

Anabolic androgenic steroids (AAS) are synthetic derivatives of male sex hormone, testosterone, that have been modified to improve its anabolic rather than androgenic activity. The AAS promote protein synthesis, muscle growth, and erythropoiesis and thus are used to enhance strength and endurance in canine, equine and human athletes via increasing muscle protein production (Guan *et al.*, 2010).

Boldenone (1,4-androstadiene-17beta-ol-3-one; BOL) and its precursor boldione (1,4-androstadiene-3-17dione) are testosterone derivatives used as synthetic anabolic androgenic steroids in livestock under the trade names Equipoise, Ganabol, Equigan and Ultragan (Cannizzo *et al.*, 2007). BOL, which exhibits a strong anabolic and moderately androgenic property, is used mainly as undecylenate ester by bodybuilders but is also administered illegally to racehorses (Soma *et al.*, 2007). It improves growth and food conversion in food producing animals such as veal calves and beef cattle (Kicman, 2008) by promoting positive nitrogen balance and protein synthesis and reducing protein catabolism. In addition, BOL induces retention of body water, nitrogen, sodium, calcium, and potassium ions. In most countries worldwide, this anabolic steroid is forbidden for meat production and human uses (Cannizzo *et al.*, 2007). Misuse of AAS causes several adverse health effects such as liver dysfunction (Amsterdam *et al.*, 2010),

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tendon damage (Battista *et al.*, 2003), disturbance of endocrine and immune functions, sebaceous system and skin alterations and changes of the haemostatic system and urogenital tract (Hartgens and Kuipers, 2004).

To our knowledge, there are relatively few studies investigating the detrimental effects of BOL administration on the hepatic antioxidant defence mechanism as well as on the enhancement of pro-inflammatory cascades and the induction of apoptosis in the liver. Therefore, this study was carried out to evaluate the hepatotoxic effects of BOL injection in rabbits as well as the effects of its withdrawal through estimation of serum concentrations of hepatic transaminases, antioxidant enzyme activities, histopathological examination of liver and determination of the expression levels of tumor protein p53 and tumor necrosis factor (TNF-α) genes using semi-quantitative RT-PCR.

MATERIAL AND METHODS

Animals

Eighteen apparently mature New Zealand male rabbits weighing 2.8-3.5 kg and 5- to 6-mo old were obtained from the Laboratory Animal farm at the Faculty of Veterinary Medicine, Zagazig University, Egypt. Animals were housed separately in metal cages and left to acclimatise for 2 wk before the experiment. Pelleted commercial feed (Abou Amer Co., Cairo, Egypt) and water was supplied *ad libitum*. The experimental protocol was approved by the ethical committee of Cairo University and the experimental procedures were performed according to the guidelines of the National Institutes of Health for the care and use of laboratory animals.

Chemicals

Equi-gan® vial was purchased from Laboratorios Tornel, Co., S.A. Mexico.

Experimental design

After 2 wk of acclimatisation, rabbits were divided into 2 groups, control (n=6) and BOL-treated group (n=12). The control group was injected with i.m. corn oil (vehicle) in a dose of 0.25 mL/kg BW. The treated group received 3 intramuscular injections of BOL (4.5 mg/kg BW) 2 wk apart, for 4 wk. The dose of BOL was selected according to Paget and Barnes (1964). Six rabbits from the BOL group were withdrawn from the experimental treatment and served as the BOL withdrawal group (4 wk after the 3rd injection). Rabbits of control and BOL groups were sacrificed 1 d after the last injection of vehicle and BOL respectively, while rabbits of the withdrawal group were sacrificed 4 wk later.

Biochemical investigations

After decapitation, blood samples were collected from jugular vein and centrifuged at 3000 rpm for 15 min to separate serum which was stored at –20°C until biochemical analyses. Activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined spectrophotometrically using commercial diagnostic kits provided from Biodiagnostic Co. (Giza, Egypt). After dissection, the livers were removed and weighed with the attached fat trimmed off. Some samples were homogenised (10% w/v) in potassium phosphate buffer solution (pH 7.4) and centrifuged at 3000 rpm for 15 min. The resulting supernatant was used to determine the parameters of oxidative status using assay kits of catalase (CAT; CAT100-1KT) and superoxide dismutase (SOD; 19160-1KT-F), glutathione (GSH; CS0260-1KT) and lipid peroxidation by malondialdehyde (MDA; MAK085-1KT) provided by Sigma (St. Louis, MO, USA). Other liver samples were immediately frozen in liquid nitrogen for the determination of p53 and TNF-α gene expressions using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Histopathological examination

Liver specimens were fixed in 10% neutral buffered formalin, processed and stained with hematoxylin and eosin dyes for standard histopathological examination using light microscope

Expression levels of liver p53 and TNF-α mRNAs

Total RNA was extracted from liver tissue using protocol provided by RNeasy Mini Kit, Cat. No. 74104 (Qiagen, Heidelberg, Germany). The amount of RNA extracted was quantified and qualified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA). Only high purity samples (OD 260/280 >1.8) were further used.

One µg of total RNA was reverse transcribed into cDNA using Qiagen 2Step RT-PCR Kit, Cat. No. 205920 following the manufacturer instructions in a 20 µL total volume.

The mixture contained 2 µL cDNA, 0.2 mM of each dNTP, and the Taq polymerase buffer which contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 7.5 pM of each primer (Table 1) and 1.5 U of Taq polymerase was placed in a 2720 thermocycler (Applied Biosystems, USA). Expression was normalised by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression which was used as an internal housekeeping control. PCR amplification conditions were as follows: denaturation at 95°C for 4 min followed by 28 cycles of 95°C, 1 min; 55°C, 1 min; 72°C, 1 min for TNF-α and annealing temp. 58°C for p53 and GAPDH. The 10 µL of PCR products were analysed on 2% agarose gel stained with ethidium bromide in 1× Tris acetate ethylenediaminetetraacetic acid (EDTA) buffer (TAE, Tris 0.04 M, acetate 0.04M, EDTA, 0.001M), pH 8.3-8.5 (Stock solution was 50× from Bioshop® Canada Inc. Burlington). The electrophoretic picture was visualised by gel documentation system (Bio Doc Analyze, Biometra, Germany). The expression levels of the gene bands intensity on gel were analysed by use of Image J software (version 1.24).

Statistical analysis

The results were expressed as mean±standard error (SE). The data were analysed for a statistical significance between the control and treated groups by one-way analysis of variance (ANOVA) using IBM SPSS Statistics computer program (version 21). *P*-Values lower to 0.05 were considered statistically significant.

RESULTS

Biochemical analyses

Serum ALT and AST activities were significantly (*P*<0.05) increased in rabbits injected with BOL in comparison to control group. In the BOL-withdrawal group, their levels were lower than those of the BOL group, but still higher than those of controls (Figure 1). The hepatic SOD and CAT activities and GSH concentration were significantly decreased in BOL group, while MDA concentration was significantly elevated in comparison to control group (Table 2). The BOL-withdrawal group showed an increase (*P*<0.05) in hepatic SOD and CAT activities and GSH content and a decrease (*P*<0.05) in MDA concentration when compared to the BOL group (Table 2).

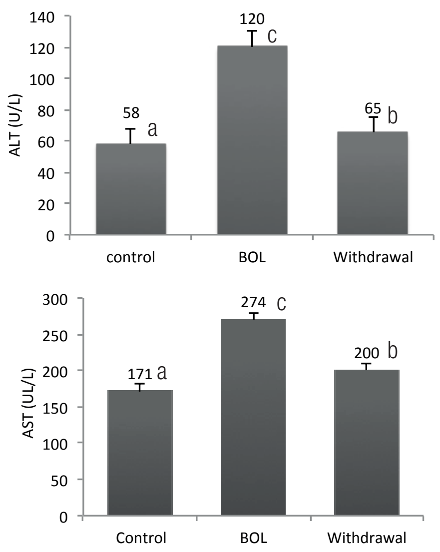

Figure 1: Effect of BOL i.m. injection on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations of male New Zealand rabbits (mean±standard error, n=6). Bars carrying different letters are significantly different at *P*<0.05.

Histopathological examination

The liver of control rabbits revealed normal and intact hepatocyte and sinusoidal architectures (Figure 2A), while that of BOL group showed focal areas of coagulative necrosis. The latter were partially replaced by lymphocytes (Figure 2B) or lymphocytes and extravasated erythrocytes (Figure 2C). In addition to focal haemorrhages, congestion of hepatic blood vessels and sinusoids was also noticed (Figure 2D). Hydropic degeneration and vacuolations in the

Table 1: Oligonucleotide primers used for semi-quantitative RT-PCR analysis of GAPDH, p53, and TNF-α genes.

Gene	Sequence	Product size/pb	References
GAPDH	Forward: 5' ACCACAGTCCATGCCATCAC -3' Reverse: 5'CTGGAAGATGGTGATGGGTT-3'	455	Jones *et al.*, 1995
p53	Forward: 5'TCATCTTGGGCCTGTGTTATCT-3' Reverse: 5'GTGCAGGGTGGCAAGTGG-3'	332	Bromidge and Howe, 2000
TNF-α	Forward: 5'CTCAGCCTCTTCTCAICTTCC3' Reverse: 5'GCAGAGAGGAGGTTGACT/CT3'	421	Farges *et al.*, 1995

GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

hepatocytes were widely spread in the hepatic lobules, in addition to activation of apoptosis with numerous apoptotic bodies throughout the hepatic parenchyma (Figure 2E). The portal areas showed mild to moderate hyperplasia in the lining epithelium of bile ducts and few lymphocyte infiltrations (Figure 2F). In comparison with the above-mentioned BOL group findings, the liver of BOL-withdrawal group revealed milder lesions with intact hepatic cells and cords. Mild vacuolations in the cytoplasm of some hepatocytes were observed (Figure 2G). Sometimes, the portal areas showed few lymphocyte infiltrations with no evidence of the hyperplasia in biliary epithelia (Figure 2H).

Expression levels liver p53 and TNF-α mRNAs

The expression of p53 and TNF-α mRNAs in liver of BOL group was up regulated in comparison to control group, while that of the BOL-withdrawal group was down regulated compared to BOL-treated one. The GAPDH gene almost show stable patters in all group (Figure 3).

DISCUSSION

The results revealed that BOL injection in rabbits induced liver dysfunction, indicated by significant elevation in serum transaminases activities (AST and ALT) compared to the control group. Our results are in agreement with previous studies (El-Moghazy *et al.*, 2012; Tousson *et al.*, 2013). Moreover, similar results were recorded on AAS abuse (Urhausen *et al.*, 2003). These results are also consistent with Welder *et al.* (1995) who cited that AAS have toxic effects in primary rat hepatic cultures. Controversially, Molano *et al.* (1999) reported that prolonged administration of anabolic steroid stanozolol had no significant effect on classical serum markers of liver function. The increment in serum AST and ALT activities may be attributed to the release of these enzymes from the cystol of liver cells into the blood circulation as a result of hepatocytes damage (Navarro *et al.*, 1993). The activity of hepatic enzymes showed a significant reduction during the withdrawal period compared to BOL group and returned to near the control values. These findings are consistent with the suggestions of Peters *et al.* (1997) that elevation in liver enzymes usually tend to return to normal once the drug is stopped and with the findings of Urshausen *et al.* (2003) who found that the negative effects of massive consumption of AAS on liver function were restored to normal after discontinuation of abuse.

Our results showed a significant increase in MDA level and markedly lowered activities of SOD, CAT and GSH concentrations in BOL rabbits compared to those of control rabbits. These results support the findings of El-Moghazy

Table 2: Effects of BOL injection on MDA and GSH concentrations and the antioxidant enzyme activities (SOD and CAT) in the liver of male New Zealand rabbits (mean±standard error, n=6).

Parameters Groups	MDA (μmol/g tissue)	SOD (U/mg protein)	CAT (μmol H_2O_2 decomposed/g tissue)	GSH (mg/g tissue)
Control	10.99±0.22[c]	2.80±0.10[a]	147.50±0.01[a]	16.13±1.40[a]
BOL	16.81±0.49[a]	1.70±0.30[c]	145.00±0.01[c]	7.03±0.66[c]
BOL-withdrawal	13.10±0.18[b]	2.20±0.01[b]	146.00±0.01[b]	14.13±1.20[b]

MDA: malondialdehyde. SOD: superoxide dismutase. CAT: catalase. GSH: reduced glutathione.
Means within the same column having different superscripts are significantly different at $P<0.05$.

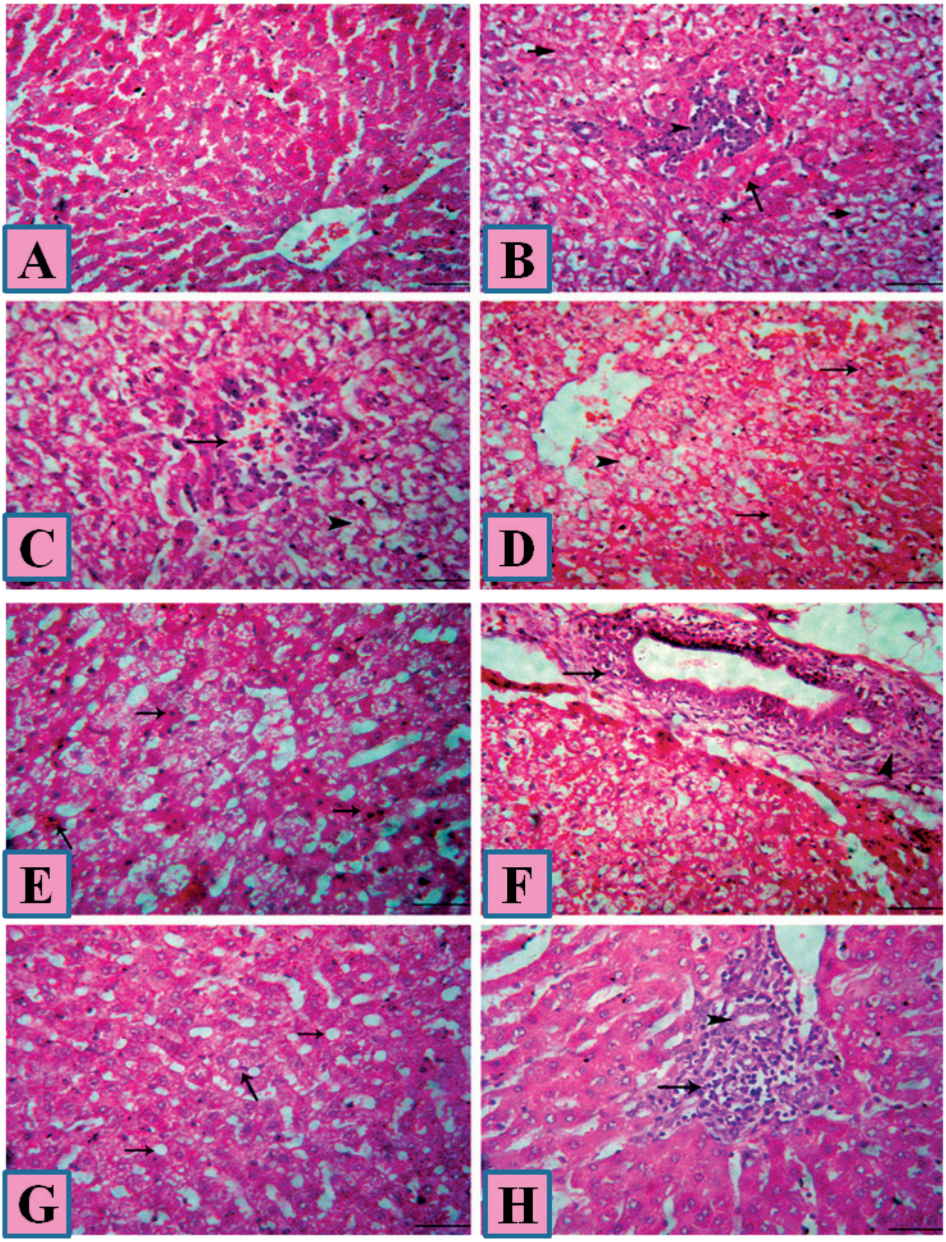

Figure 2: Photomicrographs of rabbit liver from: A: control shows normal hepatocyte and sinusoidal architectures. (B-F): BOL- treated group shows: B: coagulative necrosis (arrow) with lymphocyte infiltrations (arrowhead) and hydropic degeneration (short arrows). C: lymphocytes and erythrocytes replaced the necrosis (arrows). D: congestion of hepatic sinusoids and haemorrhage (arrows). E: numerous apoptotic bodies throughout the hepatic parenchyma (arrows). F: portal area with hyperplasia in the lining epithelium of bile ducts (arrow) and few lymphocyte infiltrations (arrowhead). (G and H): BOL- withdrawal group shows: G: vacuolations in the hepatocytes (arrows). H: portal area with lymphocyte infiltrations (arrow) besides normal bile duct (arrowhead). HE (Bar=100 µm).

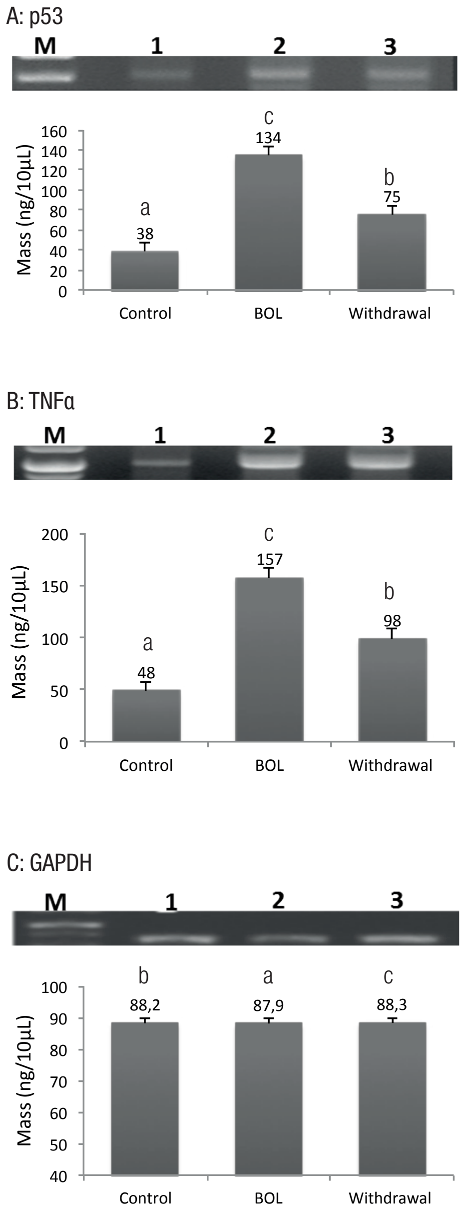

Figure 3: The expression level of mRNAs of A: p53, B: TNF-α and C: GAPDH genes in the liver tissue of male New Zealand rabbits. M; DNA marker (100 pb), lane 1; control group, lane 2; BOL- treated group, lane 3; The BOL- withdrawal group. GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

et al. (2012) who reported that BOL injection in rabbits caused hepatic oxidative stress. These findings are also concordant with previous studies by Sadowska-Krepa *et al.* (2011), where they cited that AAS treatment induced oxidative stress in rats. The ability of BOL to induce lipid peroxidation supports the hypothesis of Langfort *et al.* (2010) that testosterone, by elevating hormone-sensitive lipase activity and stimulating the lipolysis of rat cardiomyocytes, enhances the availability of long chain fatty acids for ATP synthesis which, in turn, elevates oxygen utilisation and thus enhances reactive oxygen species (ROS) generation. Interestingly, the withdrawal of BOL for 4 wk after the last injection restored the hepatic oxidative stress markers to near the control values.

The biochemical parameters confirmed the liver histopathological findings. The histopathological examination of liver of BOL group revealed coagulative necrosis with lymphocytic infiltration, congestion of hepatic blood vessels, hydropic degeneration and vacuolations of hepatocytes, as well as activation of apoptosis and numerous apoptotic bodies throughout the hepatic parenchyma. These findings are in agreement with those of El-Moghazy *et al.* (2012) who reported that intramuscular injection of BOL to rabbits has a marked adverse effect on hepatic structure. Similar changes in hepatic tissue of cattle have been reported by Groot and Biolatti (2004). These pathological changes became milder in BOL-withdrawal group.

The histopathological lesions were correlated with expression of p53 and TNF-α genes which was up regulated in BOL rabbits compared to the control group. Our results are in line with that described by Tousson *et al.* (2011), who reported an increase in the expression of p53 after BOL injection in rabbits. These findings are also in agreement with Du Toit *et al.* (2005) who found that AAS increased levels of TNF-α in myocardial tissue in basal and post ischemic periods. Moreover, exogenous androgen supplementation has been indicated to increase apoptosis in rat ventricular myocytes (Zauyg *et al.*, 2001). Moreover, our findings also revealed that the expression level of p53 and TNF-α genes was down regulated in the BOL-withdrawal group compared to BOL.

The p53 proved to have regulatory responses to a variety of cellular stressors including DNA damage, nucleotide depletion, chemotherapeutic drugs, oxidative stress, genotoxic damage, oncogene activation and hypoxia (Grawish, 2008). These stressors can trigger p53 to induce cell growth arrest or apoptosis, as p53 regulates the transcription rate of some different genes responsible for cell cycle regulation, DNA repair and apoptosis (Wang *et al.*, 2005). p53 was associated with the production of oxygen radicals (Buzek *et al.*, 2002). p53 is implicated in the activation of aspartate-specific cysteine proteases (caspases) that mediated apoptosis. One of the signalling pathways by which p53 induce apoptosis is the engagement of particular (death) receptors that belong to the tumour necrosis factor receptor (TNF-R) family (Ashkenazi and Dixit, 1998). Moreover, TNF-α is a cytokine capable of initiating a broad range of biological effects, including apoptosis of some tumour cells (Wong *et al.*, 1997). These effects are mediated through TNF receptor oligomerisation, which activates a caspase cascade through cytoplasmic proteins and TNF-receptor complexes. Therefore, all these results suggest that BOL could induce inflammatory cascades in liver. Furthermore, TNF-α has been shown to induce apoptosis and accumulation of p53 in various cell types, suggesting the potential involvement of p53 in TNF-α-induced cell death, as previously reported in prostate carcinoma cell line LNCaP (Rokhlin *et al.*, 2000).

CONCLUSION

Our data suggest that the anabolic androgenic steroid BOL induced hepatotoxicity in rabbits through induction of some mediators of apoptosis such as ROS, p53, and TNF-α which interplay together and resulted in alterations in the function and structure of hepatic tissue. However, these alterations were restored to near the control levels after its withdrawal. In addition, misuse of this drug may contribute to continuous damage of hepatic function and structure that may lead to a progressive liver injury.

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