



## Expression of cyclin D1, p21, and estrogen receptor alpha in aflatoxin G1-induced disturbance in testicular tissue of albino mice

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### Abstract

**Background and purpose:** Aflatoxin (AF) is a mycotoxin produced by various strains of the *Aspergillus* family. AFG1 as one of the most important types is highly found in cereals and grains. AF affects sperm production or even its quality. This study was designed to test the effects of AFG1 on mice testicular tissue.

**Experimental approach:** Twenty-four Albino mice were divided into four groups of 6 each; a control group (0.2 mL corn oil and ethanol), three treatment groups with different periods (20 µg/kg AFG1 for 7, 15, and 35 consecutive days). All treatments were applied intraperitoneally. Biosynthesis of cyclin D1, p21, and estrogen receptor alpha (ERα) proteins was evaluated by immunohistochemistry (IHC) staining. Levels of cyclin D1, p21, and ERα mRNA were evaluated by the real-time polymerase chain reaction (RT-PCR) technique. Tubular differentiation index (TDI), reproductive index (RI), and spermiogenesis indices were also analyzed.

**Findings/Results:** AFG1 increased the percentage of seminiferous tubules with negative TDI, RI, and SPI compared to the control group ( $P < 0.05$ ). RT-PCR and IHC analyses illustrated time-dependent enhancement in p21 expression and cyclin D1 biosynthesis in AFG1-treated groups significantly ( $P < 0.05$ ). While the protein and mRNA levels of ERα were significantly ( $P < 0.05$ ) decreased in a time-dependent manner.

**Conclusion and implications:** The chronic exposure to AFG1 reduced the expression and synthesis of ERα, increased the expression and synthesis of p21 and cyclin D1, impaired apoptosis, which in turn could impair spermatogenesis.

**Keywords:** Aflatoxin G1; Apoptosis; Cyclin D1; Estrogen receptor alpha; p21.

### INTRODUCTION

Aflatoxins (AF) are secondary heterocyclic cancerous metabolites that are produced by *Aspergillus flavus* and *Aspergillus parasiticus* in agricultural foodstuffs such as peanuts, maize grains, cereals, and animal feeds (1). The most important types of AFs with molecular diversities are B1, B2, G1, G2, M1, and M2. The G-group (G1 and G2) has a lactone ring and presents yellow-green fluorescence under ultraviolet (UV) light radiation. This feature makes the use of fluorescence crucial for discrimination among G and other groups (2,3). AFG1 (molecular weight of 328, C17H12O2) is one of the most common types found in cereals and grains (4). According to the

published documents, due to the abundance of AFG1 contamination in cereals and food supplies, the north of China has the highest incidence of lung and esophageal cancers (5).

Estrogen receptor alpha (ERα) is a member of the nuclear steroid receptors family and binds to the estrogen 17-β as the related ligand. ERα has several domains including hormone binding, DNA binding, and activation site for transcription. This receptor is inactive in the absence of estrogenic compounds.

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DOI: 10.4103/1735-5362.310525

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Ligand could alter the conformation of ER $\alpha$  by the dimerization process. ER $\alpha$  is separated from their chaperones and binds to specific response elements known as estrogen response elements (EREs) in the promoters of target genes (6). Two forms of ERs including ER $\alpha$  and ER $\beta$  are found in testicular tissue of which the ER $\alpha$  is available in Leydig cells and spermatocytes (7). Since ER $\alpha$  receptors exist in testicular tissue, the estrogenic compounds can affect the function, synthesis, or structure of these receptors in a wide range (8).

Generally, cyclin proteins like cyclin D1 and cyclin E1 with profound roles in the interaction of estrogen and estrogen-like compounds with related receptors are involved in cell cycle regulation (4,9). Cyclin activity is mainly dependent on the cyclin-dependent protein kinases (CDKs). Increased expression of cyclin D1 protein leads to reduce the length of G1 phase duration, a process that occurs in tumor degeneration (10,11). The activity of the cyclin/ CDK complex is inhibited by p21, which in turn induces cell cycle arrest and/or apoptosis (12). In fact, this protein is coupled with the cyclin D1/CDK complex that leads to the onset of apoptosis following DNA damage (13).

Previous studies have shown that AFB1 affects the hypothalamic-pituitary axis disrupting the endocrine system of the testes (14,15). Since the AFG1 is one of the most toxic agents in the AFs category and its testicular toxicity has not been reported, in this experimental study, the ERs, testicular level of the cyclin D1 protein, and the relationship among cyclin D1 and kinase inhibitors (p21) were determined. The effects of AFG1 on different stages of spermatocytogenesis and spermiogenesis were investigated. Also, immunohistochemistry (IHC) staining for cyclin D1, ER $\alpha$ , p21 proteins, and real-time polymerase chain reaction (RT-PCR) for cyclin D1, p21, and ER $\alpha$  mRNA test was performed.

## MATERIALS AND METHODS

### *Chemicals*

AFG1 was purchased from Sigma Co. (USA; Cas No. 1162-39-5). The rabbit anti-mouse primary antibodies for cyclin D1, p21,

and ER $\alpha$  were obtained from Life-Teb Gen (Tehran, Iran). The RNA extraction kit was procured from Sinaclone Co. (Tehran, Iran). Secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen kit were purchased by Life Teb Gen Co. (Tehran, Iran). The mounting medium for IHC analysis (VECTASHIELD) was obtained from Vector Laboratories (Burlingame, CA, USA).

### *Animals*

Twenty-four mature male Swiss albino mice were used in this study from the laboratory animal center (Faculty of Medical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran). The animals were divided into four groups; one control and three treated groups. In the control group, corn oil and ethanol were administered (0.2 mL). The treated group received AFG1 with doses of 20  $\mu$ g/kg body weight daily for 7, 15, and 35 consecutive days. All procedures are applied peritoneally. Prior to injection, the AFG1 was diluted in corn oil and ethanol (14). All animal manipulations were approved by the Ethical Committee of Kermanshah University of Medical Sciences (Ethics code: IR.KUMS.REC.1395.652)

### *Histological analysis*

Each animal at the end of the treatments was euthanized by carbon dioxide gas (Adaco, Kermanshah, Iran). The testes were removed using a stereo zoom microscope (Olympus, Japan). Half of the tissue was fixed in Bouin for histological investigations and the other part was stored at -70 °C for further biochemical analysis. Testicular tissue underwent routine histological processes including ascending ethanol concentrations, paraffin infiltration, and tissue section preparation (5  $\mu$ m) by a rotary microtome (Microm, GMBH, Germany). They were stained with hematoxylin and eosin (H&E) and analyzed using a light microscope. Seminiferous tubules less than three germinal layers, percentage of tubules with abnormal spermiogenesis index (SPI), and percentage of tubules with negative repopulation index (RI) were considered as negative tubular differentiation index (TDI), negative SPI index, and negative RI, respectively.

**Table 1.** Nucleotide sequences for primers used in the real-time polymerase chain reaction.

Primers	Forward sequences (5'-3')	Reverse sequences (5'-3')
p 21 311bp	AGTGTGCCGTTGTCTCTTCG	ACACCAGAGTGCAAGACAGC
Cyclin D1 409bp	CTGACACCAATCTCCTCAACGAC	GCGGCCAGGTTCCACTTGAGC
ER $\alpha$ 128 bp	CCTCCCGCCTTCTACAGGT	CACACGGCACAGTAGCGAG
GAPDH 127 bp 236 bp	AGAACATCATCCCTGCATCCAC GAGAGTGTTTCCTCGTCCCG	GTCAGATCCACGACGGACACA TCCCGTTGATGACAAGCTTCC

### IHC staining

Tissue sections were heated at 60 °C for 25 min and deparaffinized in xylene and rehydrated in graded alcohol. The tissues were pretreated with 10 mM citrate buffer for 10 min for antigenic retrieval. Staining was performed following the manufacturer's instructions (Biocare and ScyTek, USA). The slides were washed with PBS and blocked with peroxidase blocking solution (0.03% hydrogen peroxide-containing sodium acid) for 5 min. Sections were incubated for 2 h at 4 °C in a humidified chamber with one of the following primary antibodies: ER $\alpha$  (1:600), p21 (1:500), cyclin D1 (1:700). The sections were incubated with a sufficient amount of streptavidin-HRP (streptavidin conjugated to horseradish peroxidase) in PBS containing an anti-microbial agent for 15 min. To visualize the reaction, slides were incubated for 10 min with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The slides were counterstained with Mayer's hematoxylin then dehydrated and mounted. The cyclin D1, p21, and ER $\alpha$  positive cells were counted in 1 mm<sup>2</sup> of the tissue. Moreover, the cellular distribution was evaluated by software.

### RT-PCR

Total RNA from testicles was extracted by TRIZOL method (13). The quantity and quality of the purified RNAs were verified by nanodrop spectrophotometer and electrophoresis using 1% agarose gel, respectively. Complementary DNA (cDNA) synthesis was carried out by taking 1  $\mu$ g RNA using a cDNA synthesis kit (Vivantis Technologies, Selangor DE, Malaysia) according to the manufacturer's protocol and RT-PCR was done as described earlier. All the primers (Table 1) were designed

using GeneRunner software, checked in NCBI Primer Blast, and purchased from CinnaGen (CinnaGen Co. Tehran, Iran). For PCR amplification, the following steps were undertaken; an initial step at 50 °C for 15 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s.

### Statistical analysis and image assessments

Data are presented as mean  $\pm$  SD of three independent experiments. The treatment groups were compared to the untreated control group using one-way ANOVA with Tukey's post hoc test. Differences were considered to be statistically significant when  $P < 0.05$ .

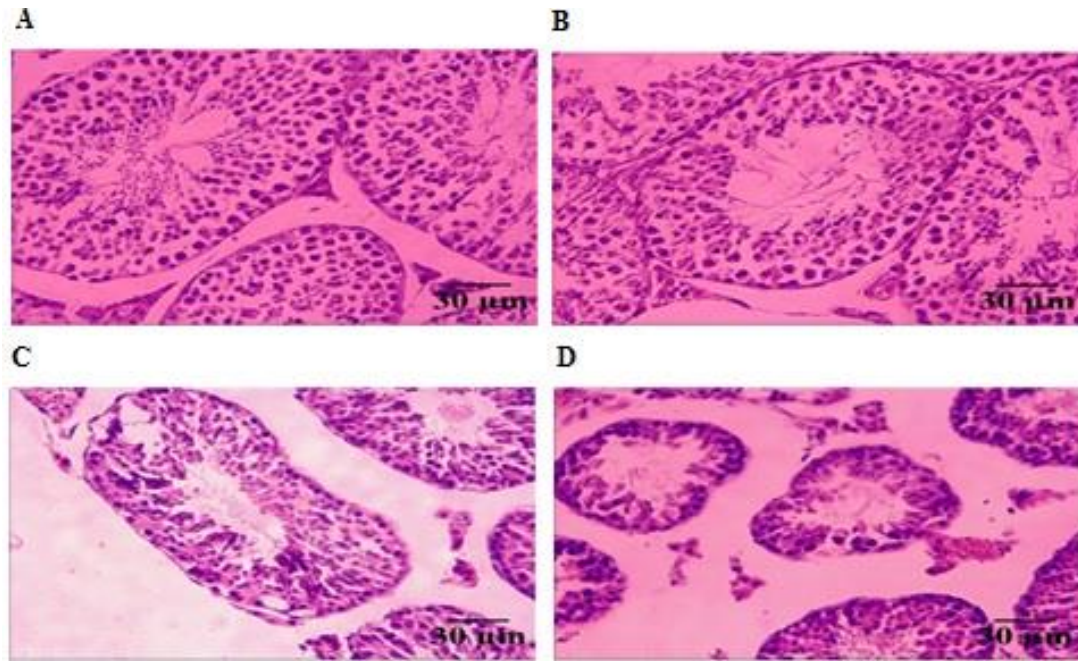
## RESULTS

### Histological examination of testicular tissue

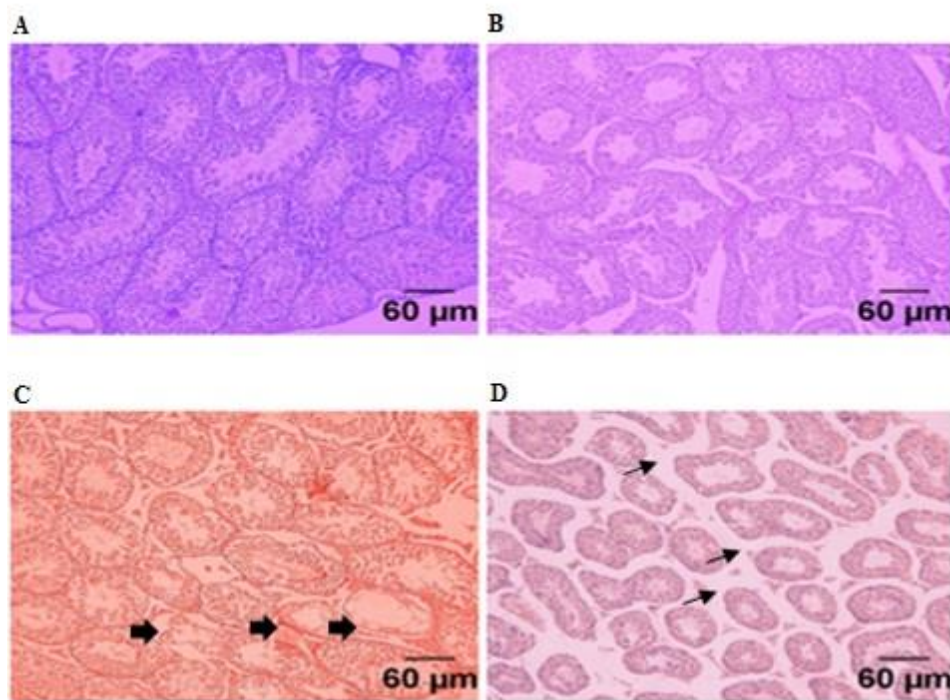
AFG1 caused spermatogenic tubules atrophy; significantly in the 35-day group compared to the other groups (Figs. 1 and 2). Spermatogenic tubules with negative TDI were increased significantly in groups of 7, 15 and 35 days compared to the control group ( $P < 0.001$ ). The percentage of spermatogenic tubules with SPI and negative RI showed a significant increase compared to the control group (Figs. 3-5).

### Gene expression of cyclin D1 and p21 following AFG1 exposure

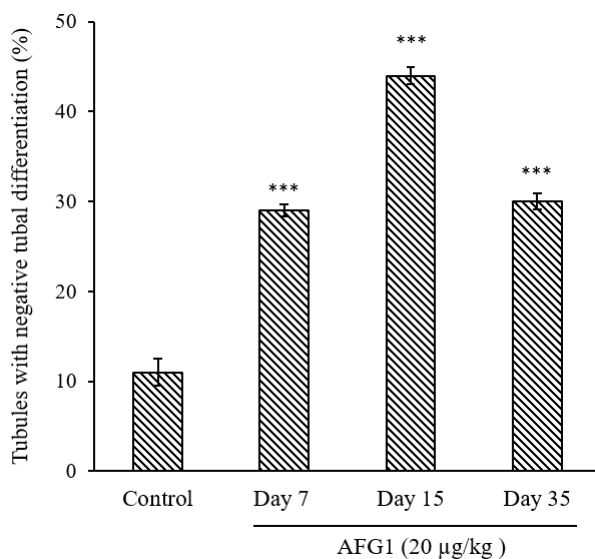
The expression and synthesis of the cyclin D1 protein, especially in the spermatocytogenic and spermatogenic cells in the AFG1 groups was increased significantly on the days of 7 and 15 compared to the control group ( $P < 0.05$ ). However, the highest level of cyclin D1 mRNA was observed in the mice receiving AFG1 for 15 days (Fig. 6).



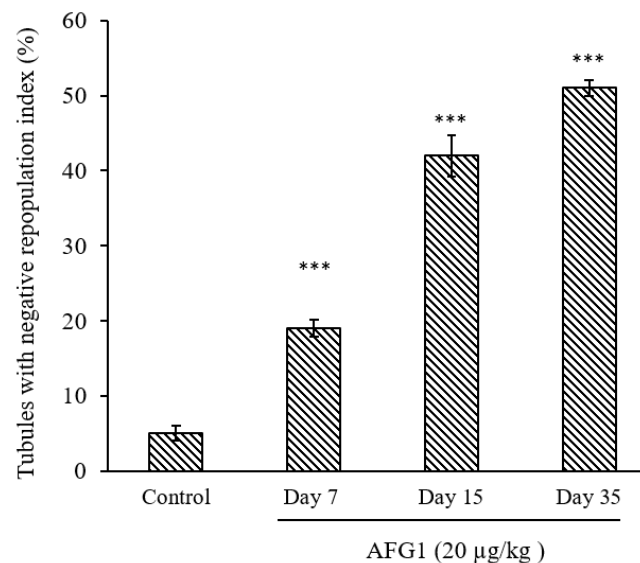
**Fig. 1.** Cross-sections of testicular tissue, (A) control group, (B-D) aflatoxin G1 receiving group after 7, 15, 35 days of treatment, respectively. aflatoxin G1 reduced the number of germ cell layers (C), cellular degradation along with arrest in spermatogenesis process (D). In the control group, the seminiferous tubules showed a normal spermatogenesis process. Also, the negative tubular differentiation coefficient along with interstitial edema was seen in 15-day and 35-day groups. Magnification: 40 $\times$ .



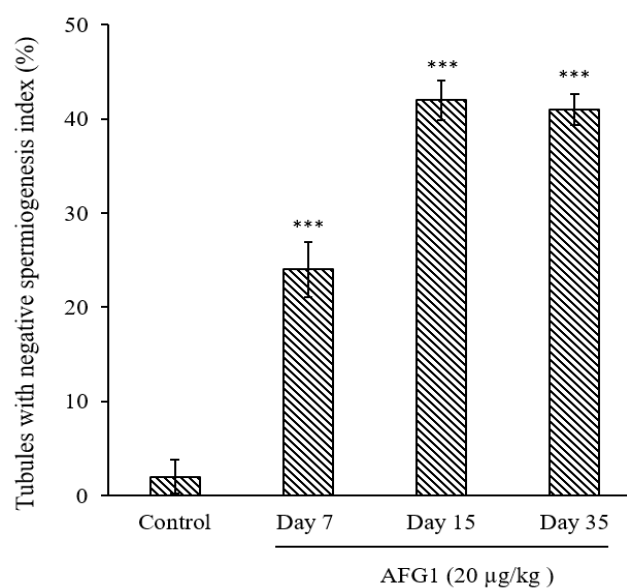
**Fig. 2.** Latitudinal sections of testicular tissue, (A) control group, (B-D) aflatoxin G1 receiving group after 7, 15, 35 days of treatment, respectively. A process of aflatoxin G1 induced atrophy of the spermatogenic tubules was significantly, especially in the 35-day group (D). Magnification, 10 $\times$ .



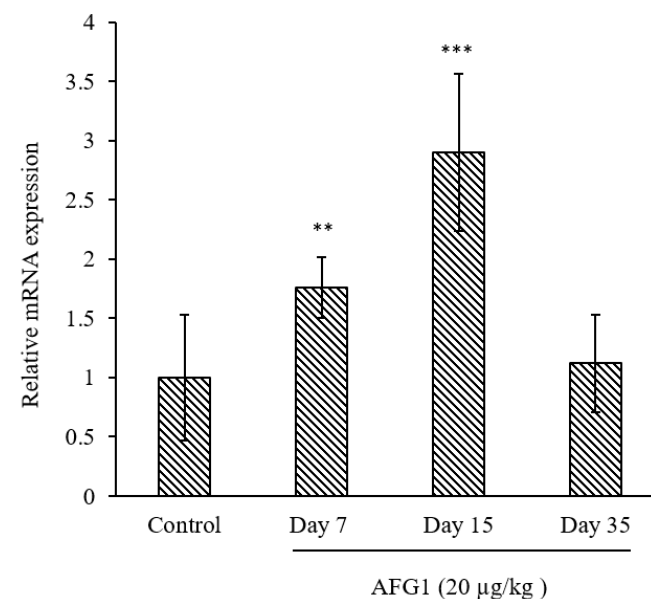
**Fig. 3.** Evaluation of the effect of AFG1 treatment (20 µg/kg body weight daily) after 7, 15, 35 days on the percentage of tubules with negative tubal differentiation coefficient. The data are expressed as the percentage of control cells as the means ± SD. \*\*\* $P < 0.001$  indicates significant differences compared with the control. AFG1, Aflatoxin G1.



**Fig. 4.** Evaluation of the effect of AFG1 treatment (20 µg/kg body weight daily) after 7, 15, 35 days on the percentage of tubules with negative repopulation index. The data are expressed as the percentage of control cells as the means ± SD. \*\*\* $P < 0.001$  compared with the control. AFG1, Aflatoxin G1.



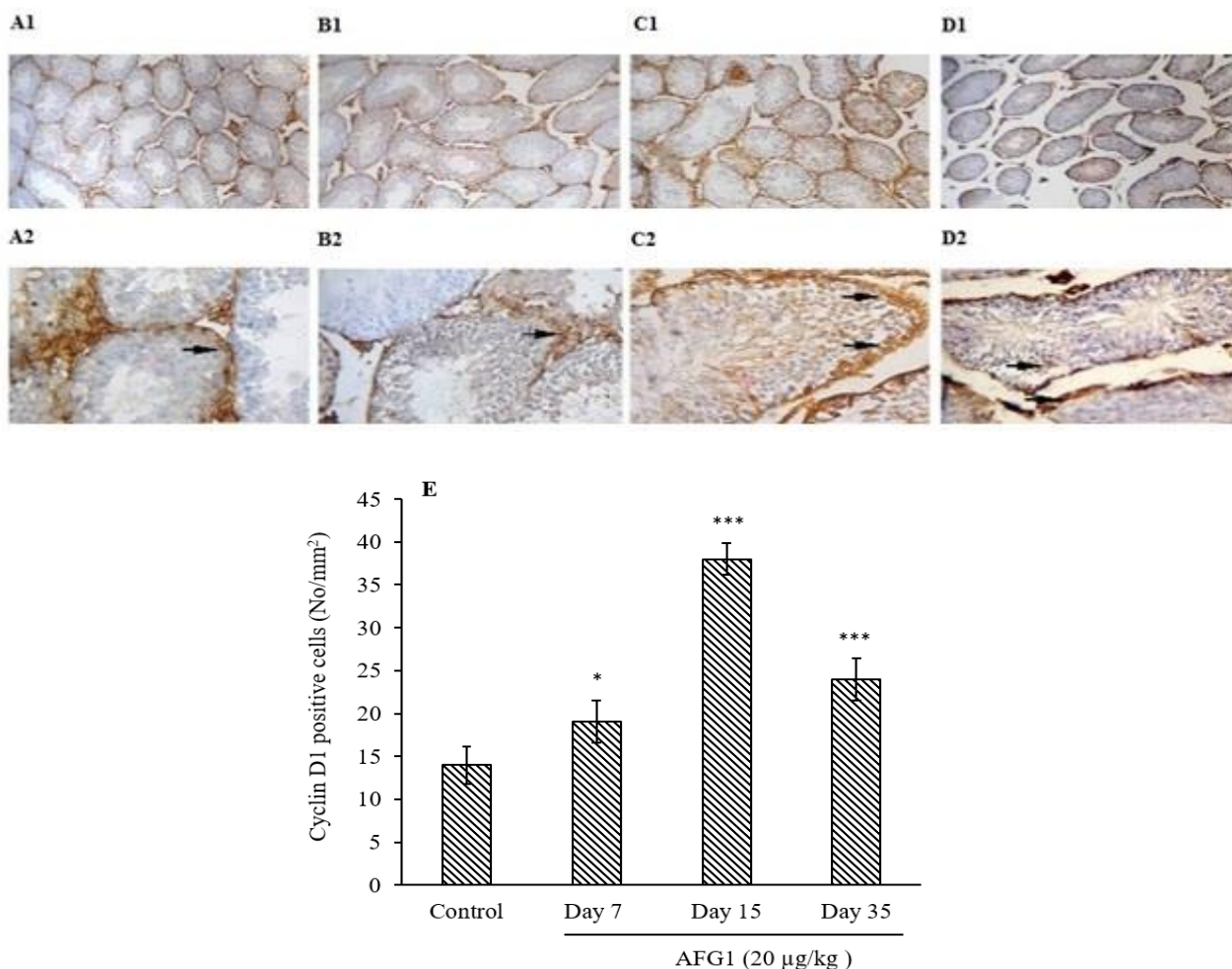
**Fig. 5.** Evaluation of the effect of AFG1 treatment (20 µg/kg body weight daily) after 7, 15, 35 days on the percentage of tubules with negative spermiogenesis index. The data are expressed as the percentage of control cells as the means ± SD. \*\*\* $P < 0.001$  indicates significant differences compared to the control. AFG1, Aflatoxin G1.



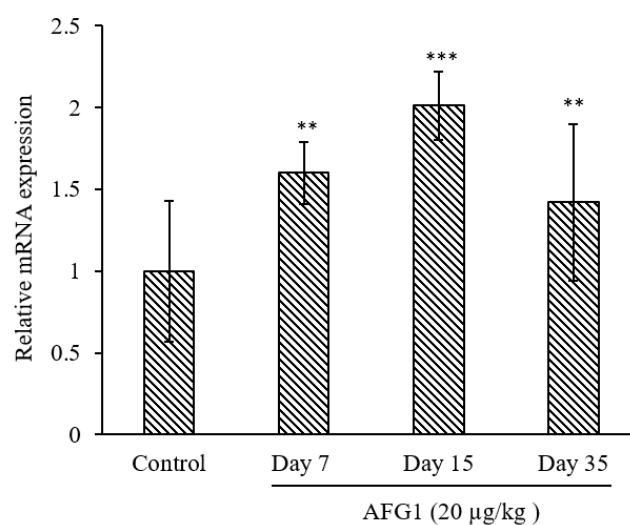
**Fig. 6.** Evaluation of the effect of AFG1 treatment (20 µg/kg body weight daily) after 7, 15, 35 days on mRNA expression of cyclin D1. The data are expressed as the percentage of control cells as the means ± SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  indicate significant differences compared with the control. AFG1, Aflatoxin G1.

The IHC studies showed that AFG1 increased the cyclin D1 synthesis in germ cells (Fig. 7). AFG1 increased the level of p21 mRNA in all groups,

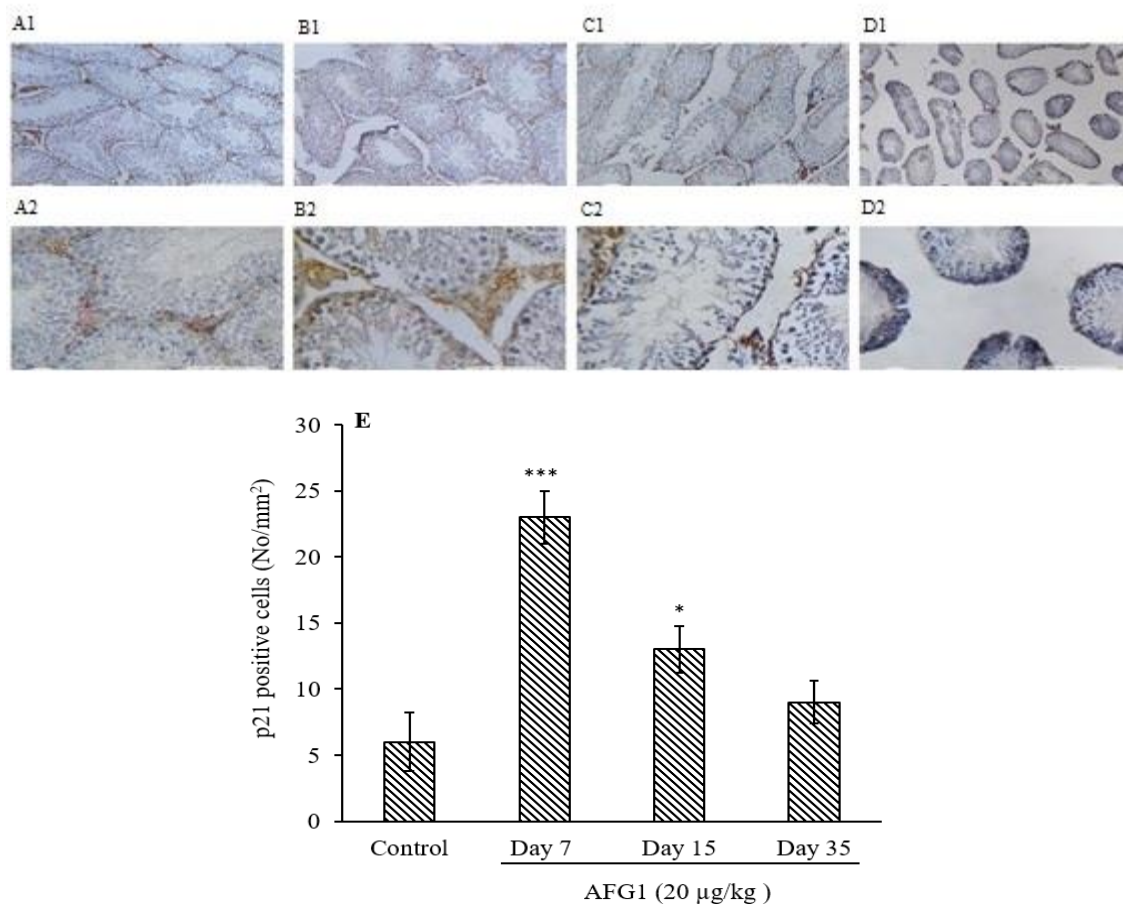
significantly compared to the control group (Fig. 8). Also, p21 was increased in all cell classes of germ cells after 7 and 15 days. (Fig. 9).



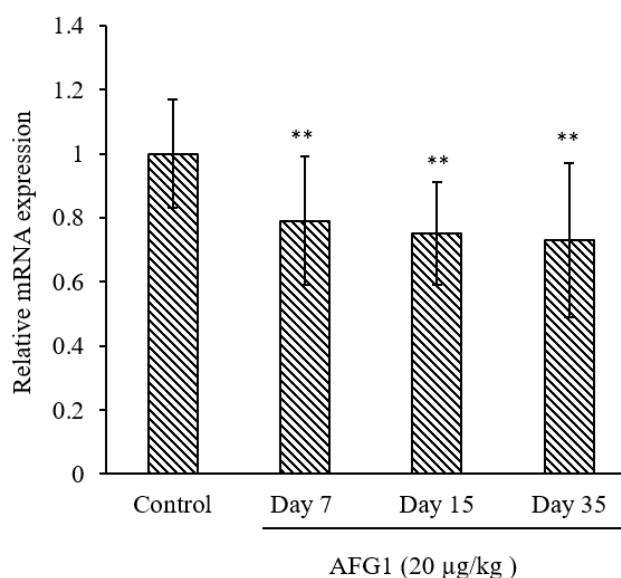
**Fig. 7.** Latitudinal sections of testicular tissue, (A) control group, (B) group receiving AFG1 after 7 days, (C) AFG1 receiving group after 15 days, (D) AFG1 receiving group after 35 days of treatment, and (E) the columns mean number of cyclin D1 positive cells from three independent experiments. The data are expressed as the percentage of control cells as the means  $\pm$  SD. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with control. AFG1, Aflatoxin G1. 1, Magnification: 10 $\times$ ; and 2, magnification: 40 $\times$ .



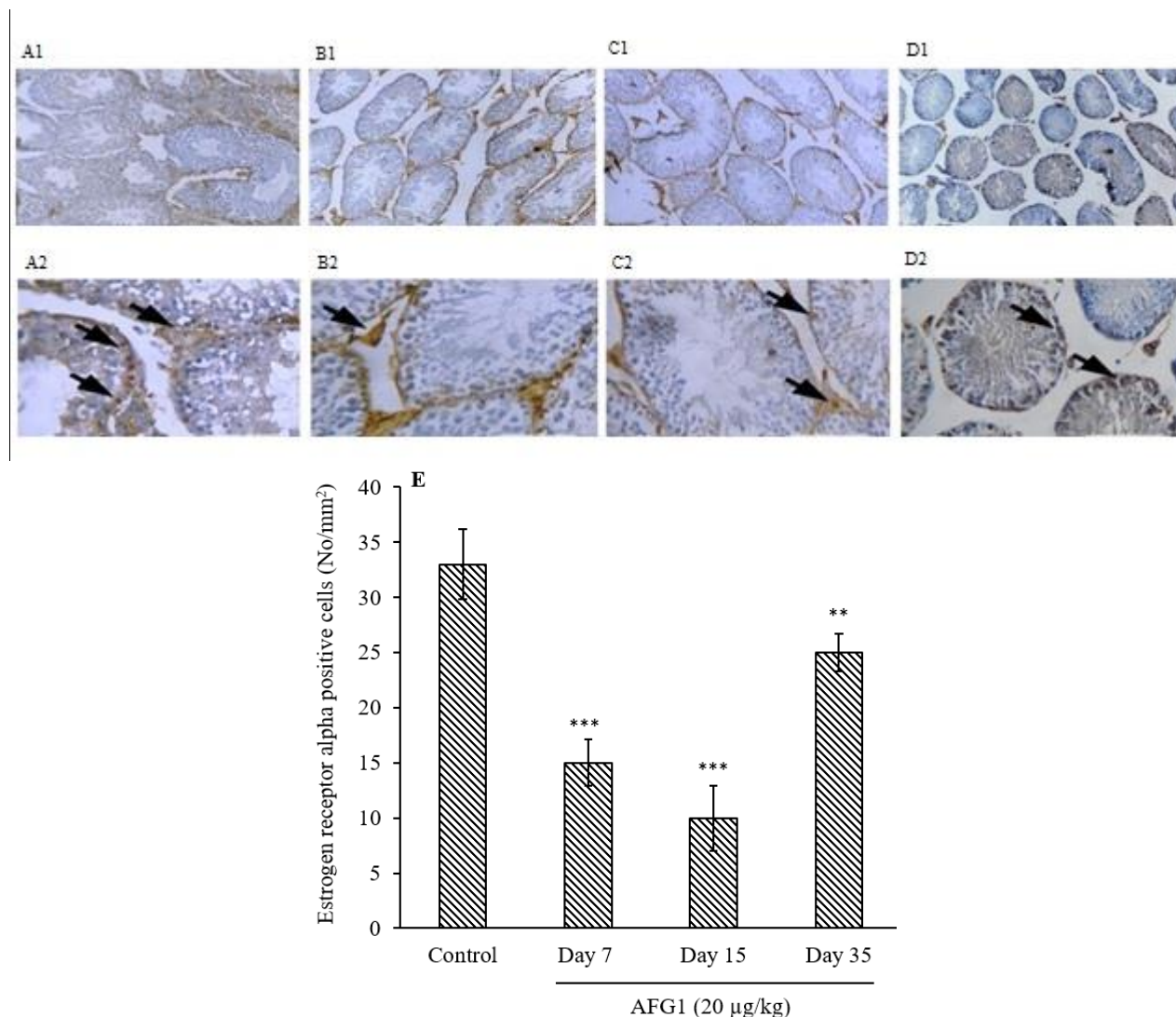
**Fig. 8.** Evaluation of the effect of AFG1 treatment (20  $\mu$ g/kg body weight daily) after 7, 15, 35 days on mRNA expression of p21. The data are expressed as the percentage of control cells as the means  $\pm$  SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with control. AFG1, Aflatoxin G1.



**Fig. 9.** Latitudinal sections of testicular tissue, (A) control group, (B) group receiving AFG1 after 7 days, (C) AFG1 receiving group after 15 days, (D) AFG1 receiving group after 35 days of treatment and (E) the columns mean number of p21 positive cells from three independent experiments. The data are expressed as the percentage of control cells as the means  $\pm$  SD. \* $P < 0.05$  and \*\*\* $P < 0.001$  indicate significant differences against the control. AFG1, Aflatoxin G1. 1, Magnification: 10 $\times$ ; and 2, magnification: 40 $\times$ .



**Fig. 10.** Evaluation of the effect of AFG1 treatment (20  $\mu\text{g/kg}$  body weight daily) after 7, 15, 35 days on mRNA expression of estrogen receptor alpha. The data are expressed as the percentage of the control cells as the means  $\pm$  SD. \*\* $P < 0.01$  compared with the control. AFG1, Aflatoxin G1.



**Fig. 11.** Latitudinal sections of testicular tissue, (A) control group, (B) group receiving AFG1 after 7 days, (C) AFG1 receiving group after 15 days, (D) AFG1 receiving group after 35 days and (E) the columns mean number of estrogen receptor alpha positive cells from three independent experiments. The data are expressed as the percentage of control cells as the means  $\pm$  SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  indicate significant differences *vs* the control. AFG1, Aflatoxin G1. 1, Magnification: 10 $\times$ ; and 2, magnification: 40 $\times$ .

### Rate of ER $\alpha$ synthesis following AFG1 exposure

ER $\alpha$  synthesis in the AFG1-treated groups decreased significantly in comparison with the control group. The lowest mRNA level was observed on day 35<sup>th</sup> following AFG1 administration (Figs. 10 and 11).

## DISCUSSION

According to the results of this study, AFG1 caused testicular tissue damage in a time-dependent manner. Also, it was shown that the AFG1 increased cyclin D1 expression and reduced the expression of ERs significantly. The expression of p21 was increased by AFG1 administration. Previous animal studies treated

with different doses of AFB1 revealed a significant reduction in the height of germ cells and an increased level of spermatogenic damage (sperm quality), significantly (16). In the previous study, it was shown that the percentage of spermatogenic tubules with differentiation coefficients, tubule replacement, and negative spermiogenesis in the recipient rats of AFB1 was increased time-dependently, and the diameter of the spermatogenic tubules and the thickness of epithelium germinal were decreased significantly (17). Another study showed that the administration of 15, 30, and 45  $\mu$ g of AFB1 in rats led to the reduction in the number of Leydig cells, apparent edema in the testicular interstitial tissue, decrease some spermatocytes and spermatids (18). In this



study, compared to the control group, the AFG1 caused a significant increase in the percentage of spermatogenic tubules with TDI, RI, and negative SPI. According to the published studies, estrogen in addition to anti-apoptotic effects can stimulate cell proliferation in the presence of ER $\alpha$  (19). Many phytoestrogens and mycotoxin compounds with external sources can disrupt this mechanism in the endocrine system of testicular tissue (20). In the present study, the ER $\alpha$  testicular biosynthesis of mice receiving AFG1 was reduced compared to the control group. Thus, the functional level of the ER $\alpha$  receptor was decreased by AFG1 administration. While testicular cells in experimental groups were still in the process of ER $\alpha$  transcription as a hemostatic mechanism. Also, testicular p21 biosynthesis in animals receiving AFG1 was significantly higher than the control group. Cyclin D1 in the complex form of cyclin D1-CDK-4 could facilitate the entrance to the phases of S and DNA synthesis in the cell cycle of the division process (21). In the present study, the evaluation of cyclin D1 expression in testicular tissue of rats exposed to AFG1 showed that the levels of cyclin D1 mRNA and protein in testicular tissue were increased as compared to the control group. In this manner, a significant increase in cyclin D1 mRNA of the 15-day rat group was detected which caused a disturbance in cell division and cell differentiation. These facts are proved by the increased percentage of spermatogenic tubules with differentiation coefficient, tubular replacement, and negative spermiogenesis coefficient clearly. The level of cyclin D1 expression is related to the presence of estrogen and receptors; thus, administration of any type of estrogen antagonists can significantly reduce the expression of cyclin D1 (22,23). On the other hand, with the increased expression level of cyclin D1 mRNA, the percentage of tubules with negative RI was also increased significantly, while in spite of decreasing the expression of the cyclin D1 mRNA, the percentage of spermatogenic tubules with negative RI was detected in high level. This contradictory expression of cyclin D1 reflects its dual role in proliferation and apoptosis in a way that a reduction in expression of cyclin D1 can decay the process of passing cells from

different stages of the cell cycle, and overexpression of cyclin D1 can activate apoptosis operators (24,25). In some studies, no significant relationship between the expression of p21 and cyclin D1 was found (26) while in others, the relationship has been emphasized (13,27).

In the present study, AFG1 increased the expression and the synthesis of p21, leading to increased levels of cyclin D1 expression and synthesis. Thus, it can be concluded that increased levels of cyclin expression can also lead to an increased rate of p21 expression, which finally can lead to disturbance of the cell division process.

## CONCLUSION

According to the obtained results from the present study, a reduction in the percentage of spermatogenic tubules with TDI and tube replacement (RI), negative spermatogenesis coefficient (SPI) in testicular tissue, as well as ER $\alpha$  receptor synthesis and increased expression and synthesis of cyclin D1 and consequently increased expression of p21 by AFG1 disrupted the process of cell division, which in turn could disrupt the operation of spermatogenesis.

## Acknowledgments

This work was financially supported by the Vice-Chancellor for Research of Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran (Grant No. 96068).

## Conflict of interest statement

The authors declared no conflict of interest in this study.

## Authors' contribution

C. Jalali was responsible for overall supervision. A. Abdolmaleki participated in statistical analysis and revised the manuscript. M. Pazhouhi participated in study design, data evaluation, and drafting. T. Zamir-Nasta and A. Ghanbari contributed to all the experimental work. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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