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Protective effect of esterified glucomannan on aflatoxin-induced changes in testicular function, sperm quality, and seminal plasma biochemistry in rams

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ABSTRACT

The aim of this study was to determine the effect of aflatoxin (AF) on spermatologic, biochemical, and testis parameters in rams, and the protective efficiency of esterified glucomannan (EG) co-administered with AF. Thirty-two Merino rams (12–14 months old) were used. The experimental design consisted of four dietary treatments. The control group was fed commercial feed. The AF group was fed with commercial feed plus 250 μg/d of total AF. The EG group received commercial feed plus 2 g/d of EG. The AF + EG group was given commercial feed plus 250 μg/d of total AF and 2 g/d of EG. There were treatment, time, and treatment-by-time interaction effects on sperm motility, abnormal spermatozoa, damaged acrosome, and dead spermatozoa ($P < 0.01$). The percentage of motile sperm was lower and the percentages of abnormal sperm, sperm with damaged acrosomes, and dead sperm were greater in the AF group than in the control, AF+EG, and EG groups, as from week 3 until the end of week 12 ($P < 0.05$). As from week 3, hyaluronidase activity in the seminal plasma increased significantly in the AF group, compared with the control. The co-administration of AF+EG was found to be effective in preventing the increase in hyaluronidase activity. As week 4, malondialdehyde (MDA) levels were significantly higher in the AF group compared with the control. The combined administration of AF+EG was found to be effective in lowering the MDA levels, increased by AF, to the levels measured in the control ($P < 0.05$). Although glutathione (GSH) levels were determined to have significantly decreased in the AF group in comparison to the control, it was observed that, in the group co-administered with AF and EG, particularly after week 7, the GSH levels, which had decreased owing to AF, were largely ameliorated ($P < 0.05$). In conclusion, AF adversely affected spermatologic, biochemical, and testis parameters, and the combined administration of EG with AF reversibly eliminated these adverse effects in rams.

1. Introduction

Silage production is an increasing practice in many countries because it enables fodder to be used as a feed source over an extended period. Poor management of the silage procedure can result in excessive moisture or dryness, condensation, heating, and leakage of rainwater,

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leading to the growth of undesirable microaerobic acid fungi. Fungal growth in silage can give rise to the loss of nutritive compounds and contamination with aflatoxin (AF), which contains several molecules that are dangerous for livestock and humans [1,2]. In ruminants, AFs have a wide variety of effects, including weight loss, poor performance, decreased fertility, abortion, hepatotoxicosis, and immunosuppression [3–5]. Young sheep are more susceptible to aflatoxicosis than adult sheep [6]. Feed contaminated with these toxins causes severe economic problems in ruminant breeding. Aflatoxins also create potential public health risks by their transmission from livestock to humans through milk and meat consumption [7].

Aflatoxins are a group of metabolites produced by fungi belonging to the genus *Aspergillus*, and in particular by *A flavus* and *A parasiticus* [8]. The growth of *Aspergillus* species and the production of AFs require the presence of certain favorable environmental conditions. These conditions include, among others, the relative humidity of the feed material stored and the storage area, storage temperature, rate of oxygen in the environment, duration of storage, and quality of the feed stored [8–11]. Because AFs are fat soluble, they are readily absorbed from the gastrointestinal tract and transferred in blood to their site of biotransformation, which is also their main site of accumulation, namely the internal organs, primarily the liver and kidneys [8]. Similar to all mycotoxins, the general mode of action of AFs is based on the inhibition of DNA, RNA, and protein synthesis [12]. The toxicity of the most toxic type of AFs, AFB_1, is reported to arise not from the toxin itself, but from its metabolites generated as a result of the activity of certain enzymes, including cytochrome P-450 and aryl hydrocarbon hydroxylase [13,14]. Research has shown that AFB_1 metabolites react with the cell DNA, resulting in mutation [14,15].

The maximum residue limit of AFs allowed in food, as adopted in August 1966 by the Codex Alimentarius Commission, jointly formed by the Food and Agriculture Organization and the World Health Organization, is 30 ppb [16]. However, this limit has been reduced to 20 ppb by the US Food and Drug Administration. It has been reported that, when administered to rats at sublethal doses, AFB_1 causes the degeneration of the testes and the mortality of germinal cells, which in return results in decreased sperm production, concentration, and motility of spermatozoa [17,18]. Some researchers [19] indicated that AFs induced adverse effects on sperm count and morphology in male buffaloes. Research in poultry [20,21] has shown that exposure to AF decreases testes weight and plasma testosterone levels, and delays sexual maturity. To reduce the adverse effects of AFs, it is required that indigestible binders, which are inert in nature and have no nutritional value, be added into the ration. These substances are not absorbed from the gastrointestinal tract and bind AFs, thereby reducing the absorption of these mycotoxins from the gastrointestinal tract [22–27]. A different approach to biological detoxification is based on the use of *Saccharomyces cerevisiae* and its cell wall component (glucomannan) to ameliorate the adverse effects of AF [28,29]. Esterified glucomannan (EG) has a capacity of binding AF at very high levels (80%–97%) [26,28]. Glucomannan is reported to have immunoregulatory [30], anti-inflammatory, antitumoral, antidiabetic, cholesterol-reducing, antifibrotic, and hypoglycemic effects [31], which bear significance for animal health. It has been reported that, in previous studies in which it was administered as an adsorbent at varying doses, glucomannan eliminated the adverse effects of AF on biochemical [26] and hematologic parameters [32], performance [29], and immune response [5] either partly or completely [25,29,33]. The aim of this study was to determine the effect of AF on spermatologic, biochemical, and testis parameters, and the protective effect of EG co-administered with AF against the adverse effects of this mycotoxin.

2. Material and methods

2.1. Animals and diet

This study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of Selçuk University (2008/061) and included thirty-two 12- to 14-months-old Merino rams. Animals were examined for general health. Ivermectin (Avromec-F, 1 mL/50 kg) and oxfendazole (Okzan-F, 1 tablet/50 kg) were administered for antiparasitic treatment. In addition, enterotoxemia (Pluritoxiven-8, 1 mL) and smallpox vaccinations were given. For the adaptation of the animals to the environment and the new feeding regimen, a 15-day acclimatization program was applied before the start of the study. Individually weighted rams were divided into four equal groups. The experimental feeding regimen was continued for ninety-two days. The duration of the treatment (92 days) was based on a possible cumulative toxicity as well as on the duration of spermatogenesis and spermiogenesis in rams. Water and alfalfa were given *ad libitum*. Each morning, before given feed the animals were administered with AF and EG, incorporated into 250 g of commercial feed.

2.2. Experimental design

The experimental design consisted of four dietary treatments. The control group was fed with commercial feed. The AF group received commercial feed plus 250 μg/d of total AF. The EG group was given commercial feed plus 2 g/d of EG (Mycosorb, Alltech, Victoria, Australia). The AF + EG group was fed with commercial feed plus 250 μg/d of total AF and 2 g/d of EG.

2.3. Aflatoxin

The AF used in this study was produced (on the premises of the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Selçuk University, Konya, Turkey) from the *A parasiticus* NRLL 2999 culture (USDA, Agricultural Research Service, Peoria, IL) via the fermentation of rice using the method of Shotwell, et al. [34] with minor modifications by Demet, et al. [35]. Fermented rice was sterilized in an autoclave, dried at 70 °C, and ground to a fine powder. In compliance with the method described by Vicam [36], the extraction and purification of AF in fermented rice was performed by applying the rice to an immunoaffinity column (Down test; Vicam). The amount of

AF was measured by high-performance liquid chromatography according to the method described by Stroka, et al. [37]. The amount of total AF in the fermented rice was 73.96 ppm. The AF in the rice consisted of 84.15% AFB_1, 6.29% AFB_2, 9.13% AFG_1, and 4.25% AFG_2 (rate of return method, 97.4%; sensitivity, 0.4 ppb).

2.4. Semen collection and spermatologic examination

In the study, ejaculates were obtained from rams twice a week for 12 week, using an electro-ejaculator (P-T Electronics, OR, USA). An ejaculate volume of 2 to 3 mL was obtained with each application.

Progressive motility was assessed using a phase-contrast microscope (original magnification, ×100), with a warm stage maintained at 37 °C. A wet mount was made using a 5-μL drop of extended semen (1:10) placed directly onto a microscope slide and covered with a coverslip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score.

For assessment of sperm morphology, at least three drops of semen were placed into Eppendorf tubes containing 1 mL of Hancock's solution (62.5 mL formalin, 150 mL sodium saline solution, 150 mL buffer solution, and 500 mL double-distilled water) [38]. One drop of this mixture was placed onto a microscope slide and covered with a coverslip. The percentage of abnormal sperm (detached heads, acrosomal aberrations, abnormal mid-pieces, and tail defects) was recorded by counting a total of 200 spermatozoa under a phase-contrast microscope (original magnification, ×1000; oil immersion).

Sperm viability in the samples was assessed by staining with nigrosin–eosin [39]. The stain was prepared by dissolving 1.67 g of eosin-Y, 10 g of nigrosin, and 2.9 g of sodium citrate in 100 mL of distilled water. The sperm suspension smears were prepared by mixing a drop of the extended semen sample with two drops of the stain on a warm slide and immediately spreading the stain with a second slide. Viability was assessed by counting 200 cells under a phase-contrast microscope (original magnification, ×1000). Sperm showing partial or complete purple colorization were considered nonviable and only sperm showing strict exclusion of the stain were considered to be viable.

2.5. Histologic examination of samples

At the end of Day 92, the testes were weighed, and tissue samples were taken by scarification and fixed in 10% neutral buffered formaldehyde, embedded in paraffin wax, and stained with Crossman's modification of trichrome stain to determine the histologic structure [40]. Tissue samples were also applied AgNOR (Ag nucleolar organizer regions) staining techniques. Five serial sections were cut from the testis samples of each animal, and applied trichrome staining. Ten photographs were taken of each section with a video camera (Leica DFC 320, Heerbrugg, Switzerland). The diameter of ten seminiferous tubules in each photograph was calculated with the IM 50 package program (Leica). The AgNOR staining of the sections was performed as described by Platon, et al. [41] and Korek, et al. [42]. Briefly, tissues were processed and paraffin sections (6 μm) were stained with a solution containing one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate (Merck, Whitehouse Station, NJ). Staining was performed at 37 °C in the dark for 20 to 30 minutes [43]. Histologic preparations were examined under a light microscope (Leica DM-2500 attached to a DFC-320 digital camera). Twenty-five nucleated Sertoli cells were evaluated in each animal. The nuclear area and the *AgNOR* area were analyzed with an image analysis program (IM-50). Also, the ratio of the *AgNOR* area to the nuclear area was calculated.

2.6. Determination of hyaluronidase activity, malondialdehyde, and glutathione levels in the seminal plasma

Hyaluronidase activity was measured using the methods described by Wilkinson, et al. [44] and Tanyıldızı and Türk [45]. *N*-Acetylglucosamine solutions (50, 100, and 200 mg/L in water) were used to construct a calibration curve. Semen samples were centrifuged at 500*g* for 5 minutes to separate the sperm cells, and the supernatant was discarded. The semen samples were diluted 1:5 with 0.15 mol/L sodium chloride. One milliliter of the diluted samples was added to 0.1 mL of acetate buffer (0.3 mol/L containing 0.45 mol/L sodium chloride). Later, 0.1 mL hyaluronic acid substrate was added to this mixture, followed by incubation for 24 hours at 37 °C in an incubator. Subsequently, 60 μL of potassium tetraborate (0.8 mol/L in water, pH 10) was added. The reaction was terminated using a heating block for 5 minutes. Later, the mixtures were cooled in an ice-water bath before adding 2 mL dimethylaminobenzaldehyde (Stock DMAB reagent-10% w/v in 12.5% v/v concentrated hydrochloric acid in glacial acetic acid; stock reagent diluted 1 in 10 with glacial acetic acid before use) and then incubated for 20 minutes at 37 °C in a water bath. The reaction mixtures were immediately centrifuged at 1500*g* for 10 minutes and the absorbance of the supernatant was read at 582 nm within 30 minutes using a spectrophotometer.

Glutathione (GSH) levels in seminal plasma were measured as described by Beutler, et al. [46]. Accordingly, 0.2 mL of seminal plasma and 1.8 mL of distilled water were mixed with 3 mL of precipitating solution (metaphosphoric acid 1.67 g, disodium EDTA 0.2 g, NaCl 30 g in 100 mL distilled water). After allowed to stand for 5 minutes, the solution was filtered. We then added 1 mL clear filtrate, 4 mL freshly prepared disodium hydrogen phosphate (4.6 g/L) solution, and 0.5 mL DTNB reagent (5,5'dithiobis-2-nitro benzoic acid, 20 mg in 100 mL citrate buffer). The absorbance of the yellow color was read in a spectrophotometer at 412 nm.

Malondialdehyde (MDA) levels were measured using the thiobarbituric acid method described by Rao, et al. [47]. Absorbance was measured at 534 nm in a spectrophotometer (Chebios s.r.l. Optimum-1 UV-1).

2.7. Statistical analysis

The data were analyzed using the mixed model of SAS, and the effects of treatment duration and treatment-by-time interaction were determined. Biochemical parameters for

four experimental groups were analyzed by analysis of variance, followed by Duncan's post hoc test to determine significant differences between the groups. Differences with values of $P < 0.05$ were considered significant. Statistical analyses were performed by using the SPSS 10.0 package (SPSS, Inc., Chicago, IL).

3. Results

3.1. Spermatologic parameters

The rates of progressive sperm motility, abnormal spermatozoa, damaged acrosome, and dead spermatozoa by weeks are given in Figure 1A–D. There were treatment, time, and treatment-by-time interaction effects on sperm motility, abnormal spermatozoa, damaged acrosome, and dead spermatozoa ($P < 0.01$). Although no difference was observed between the experimental groups for spermatologic parameters during the first 2 week of the study, the percentage of motile sperm was lower and the percentages of abnormal sperm, sperm with damaged acrosomes, and dead sperm were greater ($P < 0.05$) in the AF group than in the control, AF+EG, and EG groups, as from week 3 to the end week 12. The combined administration of AF+EG resulted in the amelioration of AF-induced damage at a significant level, improving the rates of sperm motility, sperm with intact acrosome and viable sperm ($P < 0.05$). Compared with the control group, the incorporation of EG into feed alone did not induce any major effect.

3.2. Histologic findings

Histologic examination of the testes demonstrated that the spermatogenic lines of the seminiferous tubules were of a regular structure in the control group. The spermia were located in the part of the seminiferous tubules near the lumen and were in a position facing the Sertoli cells. In the testes of the rams, which were administered with AF alone, it was observed that the number of spermatogenic cells in the seminiferous tubules had decreased, losses had occurred in the spermatogenic lines, and in some tubules the spermatogenic cells had been sloughed into the lumen.

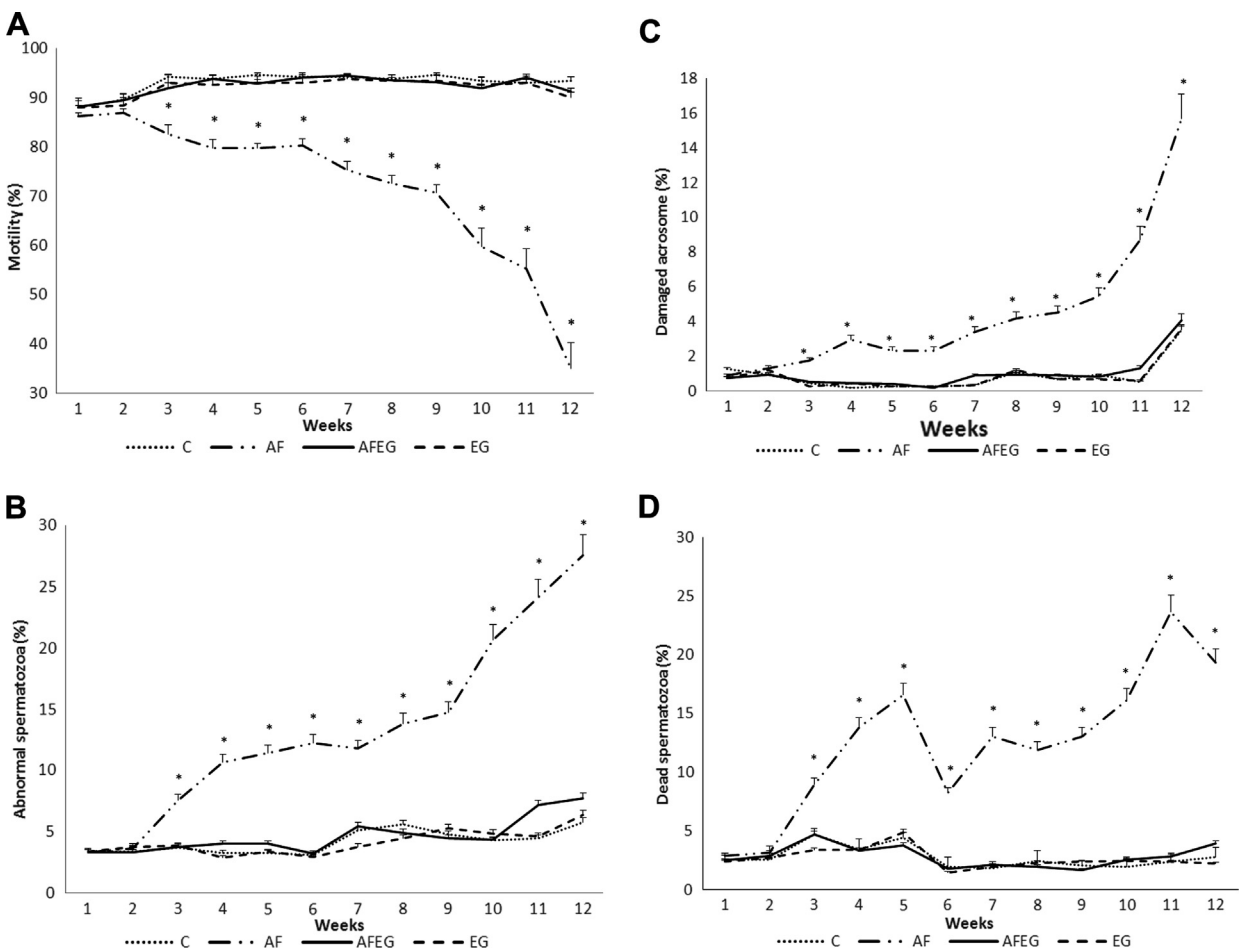

Fig. 1. (A) Rates of progressive sperm motility by weeks. * Means are different within week ($P < 0.05$; $n = 8$ per group). (B) Rates of abnormal spermatozoa by weeks. * Means are different within week ($P < 0.05$; $n = 8$ per group). (C) Rates of damaged acrosome by weeks. * Means are different within week ($P < 0.05$; $n = 8$ per group). (D) Rates of dead spermatozoa by weeks. * Means are different within week ($P < 0.05$; $n = 8$ per group).

In the group administered with both AF and EG, in the lumen of some of the seminiferous tubules, spermatogenic cells other than spermia were also observed, yet in general, the spermatogenic lines were regular in structure. In the group given EG alone, it was observed that the histologic structure of the testes was normal and displayed similarity to that of the control group.

The paired weight of the testes, gonadosomatic index, the mean diameter of the seminiferous tubules, and the AgNOR parameters measured in the Sertoli cells are presented in Table 1. It was ascertained that the paired weight of the testes, gonadosomatic index, and diameter of the seminiferous tubules had reduced significantly in the group that received AF alone, compared with the control group ($P < 0.05$); these parameters in groups given AF+EG and EG alone did not differ from those of the control group.

3.3. Biochemical findings

As shown in Figure 2A, as from week 3 of the trial, hyaluronidase activity in the seminal plasma had significantly increased in the AF group, compared with the control group. The combined administration of AF and EG was observed to have prevented the increase in enzyme activity caused by AF ($P < 0.05$). As can be seen in Figure 2B, as week 4 of the study, MDA levels in the seminal plasma were significantly higher in the AF group, compared with the control group. The combined administration of AF and EG was found to be effective in reducing the MDA levels, increased as a result of AF exposure, to the levels of the control group ($P < 0.05$). Although the GSH levels had decreased significantly in the AF group compared with the control group, it was observed that in the group, which was administered with AF+EG, the GSH levels that had decreased owing to AF were significantly ameliorated as from week 7 of the study (Fig. 2C, $P < 0.05$).

4. Discussion

Several environmental, physiologic, and genetic factors are influential on the impairment of sperm functions and the development of infertility. Of these factors, oxidative damage induced by free radicals that have effect on spermatozoa has gained increased importance in recent years. Oxidative stress leads to the disorder of reproductive functions by increasing the level of free radicals and weakening the antioxidant protective mechanism [48,49]. Oxidative damage causes deleterious effects on the functions of cellular components (i.e., enzymes, nucleic acids, membrane, and proteins) [50]. Glucomannan, an adsorbent that enables biological detoxification, has found common use in recent years [25,27]. In a previous study, in which feed containing AF was added glucomannan, it was observed that the adverse effects of AF were ameliorated in broiler chickens [26].

In the present study, the effects of AF on the reproductive organs of rams, and the potential effects of EG co-administered with AF, in favor of either the reduction or the prevention of AF-induced adverse effects in the testes, were investigated. Some authors [51] indicated that, in rabbits, AFs adversely affected spermatologic parameters (decrease in motility, acrosomal disorders, increase in the rates of nonviable and abnormal spermatozoa, and inhibition of spermatogenesis). In the present study, the rather high rate of abnormal spermatozoa in the AF group was attributed to the impairment of spermatogenesis as a result of chronic aflatoxicosis. In a previous study [19] in male buffaloes, it was reported that sperm count and morphology were adversely affected by AF. Some researchers [52] reported that, in animals exposed to aflatoxicosis, the body/testis weight and serum testosterone levels, and motility of spermatozoa decreased, although the rate of abnormal and nonviable spermatozoa increased. Similarly, in the present study, it was observed that the rate of nonviable and abnormal spermatozoa had increased and the rate of motility had decreased. Furthermore, it was determined that the relative testis weight had significantly decreased in the group administered AF alone, compared with the other experimental groups.

Deterioration of the spermatologic parameters having been observed as from week 3 of the experimental feeding regimen could be an indicator of the effects of AF becoming apparent within this time period. Furthermore, the absence of adverse effects in the groups administered with EG alone and a combination of AF+EG is considered as an indicator of the protective effect of EG. The present study demonstrated that chronic aflatoxicosis accelerated the decrease in motility and increased the rate of abnormal and nonviable spermatozoa.

Both body weight and paired testis weight of the rams included in the AF+EG group having been determined to be close to the values of the control group is in support of previous studies, which suggest that EG could be used as an adsorbent to reduce or eliminate the toxic effects of AF [26,27,53].

Table 1
Live weight of animals, paired testes weight, gonadosomatic index, diameter of seminiferous tubules and ratio of AgNOR area to nuclear area (%) in Sertoli cells after 12 week.

Groups (n = 8)	Live weight of animals (kg)	Paired testes weight (g; ±SD)	Gonadosomatic index	Diameter of seminiferous tubules (μm)	Ratio of AgNOR area to nuclear area (%) in Sertoli cells
C	80.94 ± 1.51	654.26 ± 24.00[a]	0.404 ± 0.02[a]	153.00 ± 1.90[a]	7.61 ± 0.52[a]
AF	75.69 ± 3.46	491.04 ± 76.47[b]	0.329 ± 0.06[b]	137.31 ± 2.52[b]	5.86 ± 0.23[b]
AF+EG	81.96 ± 1.86	694.96 ± 94.30[a]	0.415 ± 0.06[a]	154.29 ± 2.46[a]	7.04 ± 0.33[ab]
EG	80.65 ± 1.18	668.25 ± 99.83[a]	0.411 ± 0.05[a]	148.33 ± 1.13[a]	6.68 ± 0.47[ab]

Abbreviations: AF, aflatoxin; AF+EG, aflatoxin+glucomannan; C, control; EG, glucomannan.
[ab] $P < 0.05$.

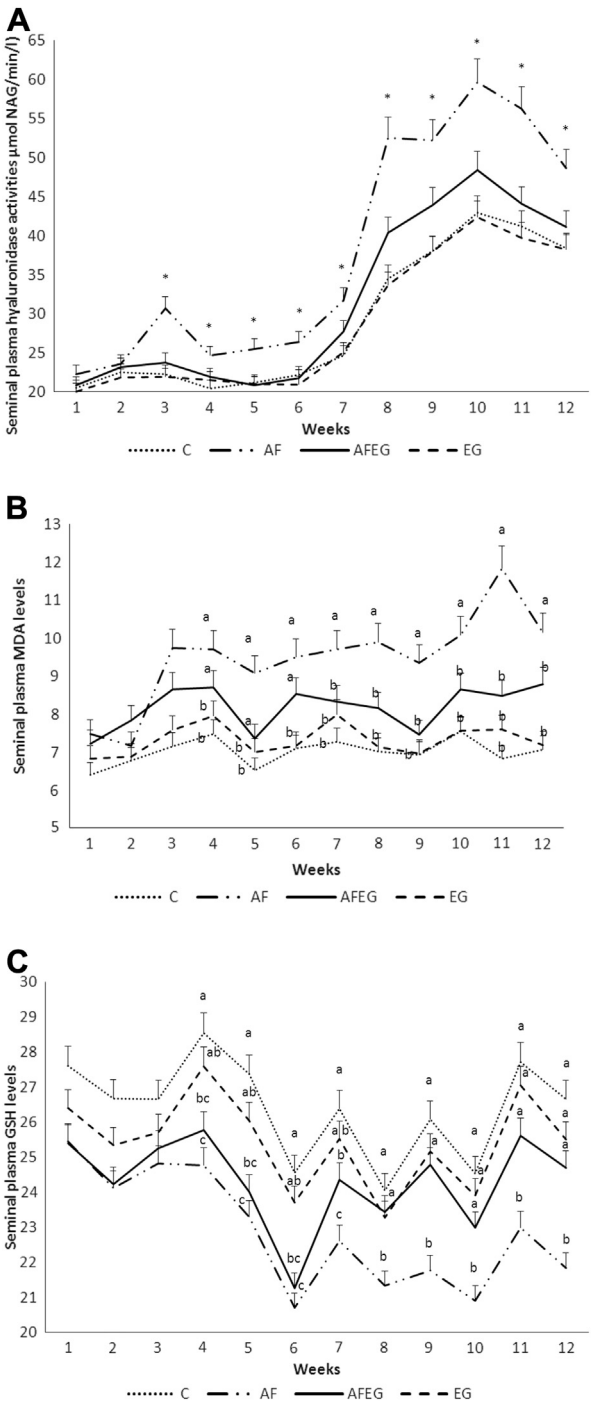

Fig. 2. (A) Seminal plasma hyaluronidase activities in ram semen by week. * Means are different within week ($P < 0.05$; $n = 8$ per group). (B) Seminal plasma MDA levels in ram semen by week. [a,b] $P < 0.005$ ($n = 8$ per group). (C) Seminal plasma GSH levels in ram semen by week. [a,b]; [a,c] and [b,c] $P < 0.005$ ($n = 8$ per group).

It was reported [54] that the administration of AF to mice resulted in the decrease of the diameter of seminiferous tubules. These researchers also pointed out an increase in the severity of toxic effects in parallel with longer exposure periods. In this study, the administration of AF for a period of 12 week resulted in the sloughing of the cells lining the wall of seminiferous tubules, loss of integrity, and decrease in the diameter of seminiferous tubules. The reason underlying the decrease in the diameter of seminiferous tubules could be the degeneration and desquamation in the wall of these tubules [22,54].

The silvering technique enables the staining of NORs (rDNA regions) actively involved in transcription. Significant increase is observed in the expression of the genes found in the NORs of actively proliferating cells with high synthetic activity [55]. To the authors' knowledge, no previous study exists on the investigation of the effects of AF on the ratio of AgNOR area to nuclear area in Sertoli cells. In the present study, the ratio of AgNOR area to nuclear area in the nucleus of Sertoli cells was significantly lower in the AF group compared with the control group. This suggests that the administration of AF in feed has an inhibitory effect on the proliferation and synthetic activity of Sertoli cells. It was noted that EG, used as an AF binder, eliminated this effect to a certain extent.

In the present study, MDA and GSH levels in the seminal plasma of the group administered with EG alone did not display any significant differences from that of the control group. On the other hand, during the first weeks of the study, in the group that received AF+EG, GSH levels in the seminal plasma, although statistically insignificant, were higher than that of the AF group. However, as from week 7, these increased levels were observed to draw closer to the values of the controls and the EG group. The increased levels of MDA in the seminal plasma, observed in the AF group from the start of the study, became more pronounced as from week 4 ($P < 0.05$). An increase was also observed in the AF+EG group, compared with the controls and the EG group, yet this difference was statistically insignificant. This demonstrates that glucomannan ameliorates AF-induced toxicosis.

The acrosome contains the enzymes required for the penetration of the spermatozoon through the zona pellucida into the ovum during fertilization [56]. Hyaluronidase is an enzyme found in the acrosome of spermatozoa, and has significant functions in the fertilization process [57]. In the present study, it was ascertained that exposure to AF significantly increased semen hyaluronidase activity in group AF rams as from week 3 of the trial ($P < 0.05$). Increase in semen hyaluronidase activity could either arise from an increase in the rate of abnormal or nonviable spermatozoa or could be explained by the transfer of hyaluronidase from the serum into the seminal plasma as a result of chronic intoxication. In the present study, in rams suffering from chronic toxicosis, glucomannan incorporated into the feed ration together with AF ameliorated the adverse effects of AF on hyaluronidase activity.

This study revealed that chronic aflatoxicosis has adverse effects on spermatologic parameters. It was determined that chronic aflatoxicosis led to a decrease in the diameter of seminiferous tubules, and resulted in the loss of the histologic integrity of the testes, as well as in an increase in MDA levels and a decrease in GSH levels in the seminal plasma, and an increase in the plasma hyaluronidase

activity. In several previous studies, different binders have been used to determine the effectiveness of these substances. However, of these binders, glucomannan has been studied only to a very limited extent in rams. It is clear that the prevention of peracute, acute, and chronic intoxications in both livestock and laboratory animals is of great significance. Various agents can be used for this purpose. The present study demonstrated that, of these agents, glucomannan, which has adsorbent activity, can be used with clinical success. In the present study, it was concluded that EG is an agent that can be used to prevent chronic intoxication with AF.

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