Royal jelly protection on flunixin meglumine-induced spermiotoxicity and testicular degeneration in mice

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Abstract

Current study was designed to investigate the protective effects of royal jelly on Flunixin meglumine (FM)-induced spermiotoxicity related to sperm concentration, abnormal spermatozoa count and histopathological changes in mice testis. The subjects were divided into five groups according to FM and/or royal jelly intake: Control group; group 1, FM alone (25 mg/kg, im); group 2, combination of FM (25 mg/kg, im) and royal jelly (200 mg/kg, oral); group 3, FM alone (50 mg/kg, im); and group 4, combination of FM (50 mg/kg, im) and royal jelly (200 mg/kg, oral). The animals were fed once daily for 15 days and they were sacrificed last day. Epididymal sperm concentration and abnormal spermatozoa count were noted. Testicular histological findings were evaluated. On purpose, organization of each animal was graded according to Johnsen’s scoring to assess the spermatogenesis relying on seminiferous tubule cross-section scores. Comparing to controls, FM administration caused a decrease in sperm concentration (p<0.05), an increase in total abnormal spermatozoa rates (p<0.05) and more degenerative changes in testes in mice.

Royal jelly supplementation ameliorated both sperm concentration and abnormal spermatozoa (p<0.05) comparing to the control group. In conclusion, we suggested that royal jelly might have protective effects in the FM-induced reductions in epididymal sperm concentration and increase in abnormal spermatozoa rate.

Key words: flunixin meglumine, royal jelly, male mice, sperm, testes toxicity
Introduction

Flunixin Meglumine (FM) is a non-steroidal anti-inflammatory drug used uniquely for animals with its secondary analgesic and antipyretic benefits (Wagner et al. 2017). FM shows its antiinflammatory and analgesic effects as a result of inhibition of cyclooxygenase (COX) enzyme which catalyzes the conversion of arachidonic acid into prostaglandins (Elmas et al. 2006, Hilton et al. 2017). FM shows its antiinflammatory and analgesic effects as a result of inhibition of cyclooxygenase (COX) enzyme which catalyzes the conversion of arachidonic acid into prostaglandins (Elmas et al. 2006, Hilton et al. 2011.) As one of the leading antiinflammatory agents in veterinary medicine, FM is used in the alleviation of inflammation and pain associated with musculoskeletal disorders and colic in horses (Naylor et al. 2014); the control of acute inflammation associated with infectious diseases in cattle and as an aid in the treatment of mastitis, metritis agalactia syndrome in sows (Kotowski et al. 2006). Although it is one of the most preffered drugs in veterinary medicine, surprisingly the toxic effects of FM on male reproductive system has not been investigated sufficiently.

Royal jelly (RJ) is a homogeneous substance secreted by the hypopharyngeal glands of worker bees (Teixera et al. 2017), containing considerable amounts of proteins, amino acids including eight essential amino acids (Ramadan and Al-Ghamdi 2012), lecithin, lipid, and sugars. In a recent tandem-mass spectrometry study the authors notified more than sixty constituents (Pina et al. 2018). Vitamin E, B complex vitamins, and other vitamins, mineral salts including zinc and copper (Kamakura et al. 2001, Nabas et al. 2014) are also found in its composition. Antioxidative (Nagai et al. 2006, Aksoy and Aslan 2017), antitumoral (Zhang et al. 2017), vasodilative (Nagai and Inoue 2004), and antihypercholesterolemic activities (Guo et al. 2008) may be attributable to its rich content. Several studies reported positive effects of RJ on animal reproduction (Kohguchi et al. 2004, Karacal and Aral 2008, Elnagar 2010). Its testosterone content, steroid hormone-type activities and stimulative effects on the production of luteinizing hormone, testosterone and progesterone, are thought to be the main mechanisms for beneficial effect of RJ on reproductive system (Husein and Kridli 2002), nevertheless the exact mode of action of RJ on reproduction is not clearly identified. Again, it is unclear even whether RJ could inhibit adverse effects of chemical toxicants, such as FM, to testis.

We planned current study to investigate toxic effects of high-dose FM on the male reproductive system and to examine the protective effects of RJ on the investigated parameters.

Materials and Methods

Chemicals and drugs

FM (2-²²-Methyl-3-(trifluoromethyl) phenyl²² amino²²-3-pyridinecarboxylic acid meglumine salt) which is a non-steroidal antiinflammatory drug, was purchased from DIF Co/Istanbul (Preperation name is Finadyne inj., containing 50 mg FM in 1 ml). RJ was obtained from Arijel Co., Ltd.

Animals and treatment

In this study, 40 healthy male swiss albino mice (12 weeks old weighing 23 ± 3.2 g) were used. Ethic committee of University of Harran, Faculty of Veterinary Medicine, approved the experiment protocol. The animals were kept under standard laboratory conditions (12-h light:12-h dark and 23 ± 2°C, 60–65% humidity) and fed with a diet consisting of 88% dry matter, 24% crude protein, 2600 kcal/kg, 7% crude cellulose, 8% crude ash, 1% calcium, 0.9% phosphorus, 0.5% sodium, 1.0% NaCl, 0.6% methionine. Food and water were provided adlibitum.

The mice were transferred to the experimental environment one week prior to the initiation of the trial so as to ensure their environmental adaptation. The mice were housed under controlled heating and ventilation conditions, and eight mice were placed into each cage. Feed and water were provided adlibitum to the animals. Five groups were established in the study. While planning study, the toxic dosage for mice was based on the information presented in EMEA Committee For Veterinary Medicinal Products, Flunixin, Summary Report (EMEA 1999). However, in our practice the mentioned dose and even half doses, were highly lethal. We titrated down the non-lethal dose—which was 50 and 25 mg/kg/day, intramuscularly. We designed our study according to this drug regimen. The final study groups were as follows:

Control Group: This group comprised eight mice and served as the control group.

Group 1: This group comprised eight mice. These mice were administered FM at a dose of 25 mg/kg/day for 15 days via intramuscular route.

Group 2: This group comprised eight mice. These mice were administered RJ at a dose of 200 mg/kg/day (El-Nekeety et al. 2007) for 15 days via gavage directly into the stomach and FM in a single dose of 25 mg/kg for 15 days via intramuscular route.

Group 3: This group comprised eight mice. These mice were administered FM at a dose of 50 mg/kg/day via intramuscular route.

Group 4: This group comprised eight mice. These mice were administered RJ at a dose of 200 mg/kg/day via intramuscular route.

Group 5: This group comprised eight mice. These mice were administered FM at a dose of 25 mg/kg/day via intramuscular route.
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Table 1. The sperm concentration of male mice (x10⁶/ml).

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<th>n</th>
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<th>Minimum</th>
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abc: There was statistically significant difference (p<0.05) between the groups.

Table 2. Abnormal spermatozoa rate (%).

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<td>1,98 a</td>
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<td>20,00</td>
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<td>17,10</td>
<td>0,97 a</td>
<td>5,00</td>
<td>35,00</td>
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</tbody>
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ab: There was statistically significant difference (p<0.05) between the groups.

for 15 days via gavage directly into the stomach and FM in a single dose of 50 mg/kg for 15 days via intramuscular route.

The mice included in the groups that were given FM and RJ (Groups 1-4) were weighed daily during the study protocol for 15 days and, accordingly, the dose to be administered per body weight was calculated and administered by intramuscular route and gavage was considered to be more accurate and safe. Intramuscular injections were administered to gluteal region of the animals by an experienced veterinarian and no local septic condition or inflammation was observed. All animals survived to the end of the study.

Epididymal sperm concentration and abnormal sperm

The mice were sacrificed by cervical dislocation. The epididymes were excised and placed in a Petri dish. Five microliters of the sperm suspension was transferred into an Eppendorf tube and diluted with 95 μL of PBS. After mixing, the sperm suspensions were counted. Sperm counts were made using a Thoma counting chamber and expressed as 10⁶/ml. A drop of sperm suspension was smeared onto a slide and stained with Papanicolaou stain. The percentages of sperm with normal and abnormal morphologies were estimated in five random fields in each slide selected at random (Karacal and Aral 2008).

Histopathological examinations

Testes were fixed in 10% buffered formaldehyde solution for histological evaluation. Formaldehyde-fixed testes were processed by routine automatic tissue processor, embedded in paraffin, sectioned into 4 μm slices, and stained with Hematoxylin-Eosin. The prepared slides were examined under light microscopy by using Johnsen’s Scoring System (Aral et al. 2008) for the status of spermatogenesis. Spermatogenetic activity and organization were graded, and Johnsen’s score was given for each animal. Johnsen’s score for assessing the spermatogenesis depends on scoring each seminiferous tubule cross-section. The criteria are as follows: 10, complete spermatogenesis; 9, many spermatozoa present but disorganized spermatogenesis; 8, only a few spermatozoa present; 7, no spermatozoa but many spermatids present; 6, only a few spermatids present; 5, no spermatozoa or spermatids present but many spermatocytes present; 4, only a few spermatocytes present; 3, only spermatogonia present; 2, no germ cell present; and 1, no germ cell or Sertoli cell present. Mean score count was given to each animal. From each testis in the group five seminiferous tubules were randomly selected.
All data were presented as the mean ± standard error of measurement (SEM). The values in the sperm concentration, abnormal sperm rate analyzed via ANOVA (SPSS 12.0) and followed by Duncan test. The significance level in comparisons was considered to be p<0.05. Histopathological scoring results were analyzed with a nonparametric Mann-Whitney U Test.

Results

Spermatozoa concentration values between control and experimental groups were statistically different (p<0.05, Table 1). Spermatozoa concentration was significantly lower in FM50 (third) group compared to control group (p<0.05). RJ supplementation to FM in both 25 and 50 mg/kg dose groups ameliorated the drop in spermatozoa concentration, however this effect was markedly and significantly higher in 25 mg/kg FM and 200 mg/kg RJ group (group 2), showing a better count even than in the control group (p<0.05) (Table 1).

From the point of abnormal spermatozoa count (Table 2), the most deteriorative effect was observed in FM50 (third) group. The highest abnormal spermatozoa count was in this group. There were no statistically significant differences between other groups. We observed that RJ supplementation to FM in both doses improved the abnormal spermatozoa counts, but these differences didn’t reach the statistical significance.

Histological observation of the testes showed normal spermatogenesis with spermatogenic cells at different stages of development in control mice (Fig. 1). Sertoli cells showed noticeable vacuolization of cytoplasm (Figs. 2, 3, 4, 5) compared to control testes. Furthermore, intertubular connective tissue stroma was less thick. Severe degenerative changes were found in histological specimens of testes exposed to FM. Moreover, a marked inhibition of spermatocyte maturation resulting in decreased number of mature sperms in the lumen of seminiferous tubules was found (Fig. 4).

According to histopathological evaluation, the mean value of Johnsen’s score in control group was 9.53±0.13. This score was significantly lower in experi-
mental groups compared to controls (p<0.01). Based on Johnsen’s scoring, values obtained from FM 25 and RJ group (8.13±0.40) were only similar to the the first group (7.53±0.46), but higher than both values obtained from third (4.93±0.54) and fourth (6.33±0.49) group (p<0.01). Considering histopathological changes, there were no statistically significant differences between the first and second group and third and fourth group (p>0.05). Histopathological changes with a Johnsen score lower than six were commented as severe ones (Aral et al. 2008).

**Discussion**

The present study revealed that higher doses of Flunixin Meglumine (FM) in mice reduced the sperm concentration, increased the abnormal spermatozoa rate and led to testicular degeneration. Furthermore, adding royal jelly (RJ) would partially reverse these toxic effects. Despite wide use of FM in veterinary medicine practice, it is the first report showing both the unfavorable effects of higher doses of FM in male reproductive system and partial capability of RJ to reverse these changes.

Cyclooxygenase (COX), which has COX-1 and COX-2 isoforms, is an enzyme responsible for the conversion of arachidonic acid to prostaglandins (PGs) (Danek 2006a,b, Matsuyama et al. 2018). COX-2 isoform was detected in Leydig cells of testis interstitium with immunostaining method and was suggested to contribute testosterone production directly (Balaji et al. 2007). On the other hand, in the same study, Balaji et al. (2007) claimed that rapid inhibition of COX-2 by nimesulide caused arachidonic acid accumulation and this accumulation, due to essential role of arachidonic acid in steroid hormone synthesis, would interfere with testosterone production, further negatively affecting sperm maturation steps. In another non-steroid antiinflammatory drug, vedaprofen, reduced levels of PGF and PGE in stallion seminal plasma compared to control group and claimed that this result was related to inhibi-
tion of testicular COX-2 activity (Janet et al. 2005). In a review article covering 1980-2017 period (Banihani 2017), paracetamol, which also inhibits COX pathway, besides its action over serotonergic system, was mentioned to change semen quality, particularly sperm morphology, and hence its fertilising ability when used at high doses. In the same article, this is explained by suppression of testosterone synthesis, inducing oxidative stress, provoking apoptosis of spermatocytes, reducing nitric oxide production and inhibiting prostaglandin synthesis that all seem to be related to COX inhibition. Also, FM shows inhibitory effects in COX pathway and despite being the most preferred medication in veterinary medicine, toxicity studies are scarce. In an early FM study, in an acute inflammatory model, Lees and Higgins (1984) showed that FM blocked prostaglandin E2-like activity, which has luteotrophic effects in ponies, for at least 24 hours. Another study showed PGE reduction as a result of 45 days of FM administration in rams (Archbald et al. 1990). In the present study, toxic doses of FM administered for fifteen days caused reduced spermatozoa concentration proportional to FM dosage and increased abnormal spermatozoa rate in mice. In the first of the therapeutic dose studies by Danek (2004) three healthy stallions were observed for acute effects of FM on male reproductive system and there was no significant change in testosterone and 17 beta-estradiol levels or seminologic parameters. The latter study (Danel 2006b) found recovery in sperm morphology and increased sperm concentration levels with single intravenous dose of FM in stallions infected with *E. coli* endotoxin. Corrective action of single therapeutic FM dose on sperm morphology might be explained by its antipyretic and antiinflammatory benefits rather than direct effects over reproductive system. Especially temperature increase due to *E. coli* endotoxin creates a spermotoxic environment, blockade of this condition might provide a rapid recovery. In our study we administered our animals highest FM dosage they could tolerate for 15 days and investigated long term.

Fig. 3. Representative microphotograph of a mouse testes from a group treated with 25 mg FM + 200 mg RJ (Group 2) showing disorganisation of germinal epithelium (star), x400, HE.
effect of higher FM doses. We could not find a literature about effects of long term high dose FM administration on reproduction system. We suggest that continuous PGE2 blockage provided by COX-2 inhibition by high dose of FM deprived the subjects of luteotropic benefits of PGE2. As Banihani (2017) remarked in his review suppression of testosterone synthesis, inducing oxidative stress, provoking apoptosis of spermatocytes, reducing nitric oxide production and inhibiting prostaglandin synthesis that all seem to be related to COX inhibition.

Despite devastating effects of high dose FM administration, RJ ameliorated sperm concentration and abnormal spermatozoa count. A decrease in the number of Leydig cells (which produce testosterone to control spermatogenesis), causes germ cell loss and consequently a decrease in number of spermatozoa (Karacal and Aral 2008, Ghanbari et al. 2015, Ghanbari et al. 2016). Besides, reactive oxygen species (ROS), can decrease spermatozoa number by stimulating lipid peroxidation, causing defects of spermatozoa cell membrane and DNA, testicular dysfunction (Doreswamy and Muralidhara 2005, Gandhi et al. 2017, Rizetti et al. 2017). It has been observed that addition of vitamin C and E to mice diet would lead a significant increase in spermatozoa number due to their antioxidative properties (El-Desoky et al. 2013, Min et al. 2016, Ourique et al. 2016).

A, E, C vitamin content of RJ provides protection against harmful endogeneous substances related to Leydig cells and germinative epithelium, thus this might increase spermatozoa concentration in our experimental subjects. Mitogenetic or cytotoxic substances show their effect by producing ROS (Baldissera et al. 2017) and these reactive oxygen radicals damage spermatozoal cell membrane (El-Desoky 2013, Ourique et al. 2016). Postmitotic spermatozoa types, spermatids and spermatozoa, are more sensitive to oxidative stress compared to other developmental stages (Kumar et al. 2002). ROS, mitotic and cytotoxic substance might cause abnormal spermatozoa formation by affecting different stages of spermatozoa formation, develop-

Fig. 4. Representative microphotograph of a mouse testes from a group treated with 50 mg/kg FM (Group 3) showing severe disorganisation of the germinal epithelium (star) of the seminiferous tubules, x400, HE.
Karacal and Aral (2008) notified that oral RJ administration to male mice would ameliorate changes in spermatozoa concentration, motility and abnormal spermatozoa formation. Elnagar (2010) confirmed these findings in his study. In a rat study Aksu et al. (2016) pretreated the animals one week with chrysin which is a component of honey products and propolis in two different doses, before creating acute Paracetamol toxication and suggested that principally antioxidative effect of chrysin mitigated side effects of acute Paracetamol toxicity in male reproductive system proportionally in a dose-dependent manner. In our study RJ might show its beneficial effect by correcting the balance of oxidant/antioxidant status.

Histological examination following administration of FM at different doses revealed degeneration of seminiferous tubules, incomplete spermatogenesis and severe decrease in the concentration of sperms in seminiferous tubules up to necrobiotic changes in spermatogonia cells (Figs. 2, 4). According to Johnsen scoring the group fed with FM 25 mg/kg and RJ (Group 2) showed better results than group 3 and 4, similar to group 1. In this study, the damages observed in the histological structure of testes may be explained by the direct effect of FM inducing impaired maturation or release of spermatozoa or by indirect effect of FM.

Again, according to Johnsen scoring, results of control group were significantly better to point out that this group was devoid of negative effects of high dose of FM. Besides, groups fed with RJ had better scores than FM only fed groups, however the difference did not reach statistical significance. Such a positive effect of RJ administration was demonstrated histopathologically by Karaca et al. (2015), revealing that antioxidant effect of RJ would exhibit a protective role in streptozotocin-induced testicular degeneration.

Conclusions

In conclusion, we suggest that higher doses of FM would cause testes degeneration and lead to reduced spermatozoa concentration, increased abnormal spermatozoa rate, however, addition of RJ to protocol...
would be preventive against negative effects of FM administration on male reproduction system.

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