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RESEARCH ARTICLE

Assessment of tebuconazole exposure on bovine testicular cells and epididymal spermatozoa

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ABSTRACT

This study is the first to investigate the effects of tebuconazole (TEB) on the physiological functions of bovine testicular cells and epididymal spermatozoa. Motility and plasma membrane integrity of spermatozoa exposed to TEB (0.001–100 μM) were evaluated at different incubation times (0–6 h), while TEB-induced spermiotoxicity was assessed after 24 h in cell cultures. Testicular cells, obtained from the parenchyma of bovine testes, were seeded at 1.0×10^4 and 1.5×10^6 cells/well in 96- and 12-well culture plates and incubated for 48 h in culture media containing TEB (0.001–100 μM) to evaluate cytotoxicity and hormone release, respectively. TEB did not affect the motility and plasma membrane integrity. However, significant spermiotoxicity occurred at higher TEB (1–100 μM) concentrations ($P < 0.05$) compared to control and lower doses. Although no dose caused cytotoxicity in testicular cells ($P > 0.05$), 1 and 100 μM TEB caused a significant increase in testosterone secretion ($P < 0.05$). As a result, high doses of TEB (1–100 μM) had slightly suppressive effects on spermatozoa; however, these doses had stimulatory effects on testosterone secretion by testicular cells. It appears that the disruption of hormonal homeostasis of testicular cells after TEB exposure may result in metabolic and especially reproductive adverse effects in bulls.

KEYWORDS

bovine reproduction, fungicide, sperm motility, testis, testosterone synthesis, viability

INTRODUCTION

Fungicides, a type of pesticides, are widely used for killing or inhibiting fungi or fungal spores in crops. They disrupt fungal membranes by inhibiting ergosterol biosynthesis, thus resulting in the death of fungi (FAO, 2000; Ugurlu, 2009). Triazole fungicides are among the most widely used pesticides worldwide. In the last few decades, many kinds of triazole fungicides have been developed to cure fungal diseases in agricultural products and living organisms. Tebuconazole (TEB) is a common triazole fungicide that is used for controlling local and systemic fungal infections, rust and mildew of seeds, crops, fruits, vegetables, and leaves, or other plant diseases (FAO, 2000). This particular fungicide is also preferentially used for the treatment of human fungal infections, such as vaginal mycoses in women and cankers in babies (Kjærstad et al., 2010).

Increasing agricultural and industrial activities have greatly contributed to environmental pollution by contaminating natural resources, such as soil, air, and water (Fu et al., 2003). Tebuconazole is one of the fungicides most frequently found in agricultural soil samples of some European countries (Silva et al., 2019). It was persistent in the soil for 600 days according to a previous report (Cui et al., 2018). It has also been reported that the

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presence of TEB in rivers has increased in recent years (Montuelle et al., 2010), and it has been detected in surface waters in concentrations up to 175–200 g/L (Elsaesser and Schulz, 2008). Therefore, the main routes of exposure of living organisms to these environmental pollutants are via ingestion, inhalation, or direct contact with contaminated resources (Gore et al., 2014). Every year, livestock are also exposed to fungicides due to the excessive use of the latter in veterinary medicine and agriculture or their presence in the waste products of industrial plants. Oruc (2010) has stated that farm animals are accidentally poisoned by fungicides applied to agricultural materials. Also, it has been reported that farm animals are usually exposed to fungicides including TEB through the consumption of contaminated roughage, fresh fodder, other feed items or water (EFSA, 2008; Drážovská et al., 2016). Due to their lipophilic features, azole compounds can also enter the body by penetrating through the skin depending on their resorption kinetics which is 60% within 24 h for TEB (Zarn et al., 2003). The use of fungicides can reduce the costs of feed production; however, they can cause some adverse effects harmful to human and animal health (Frank et al., 2015).

TEB potentially interferes with the function of cytochrome p450 enzyme (CYP) systems that play critical roles in steroid biosynthesis in mammals. Thus, it is also termed an endocrine disruptor (Sanderson et al., 2002; Kjærstad et al., 2010). According to some previous studies (Sanderson et al., 2002; Zarn et al., 2003; Cao et al., 2017), TEB might adversely affect female reproduction in humans by inhibiting aromatase (CYP19A1) activity, which converts oestradiol to testosterone. In addition to these effects, Chen et al. (2019) reported that TEB administered to rats by oral gavage increased serum testosterone and decreased serum oestradiol levels. Also, it was stated that TEB led to a decrease in progesterone production in bovine luteal cell cultures (Atmaca et al., 2018). Furthermore, Joshi et al. (2016) demonstrated that TEB caused a reduction in reproductive organ weights in male rats and a decrease in sperm density and motility. Results from a recent study demonstrated that TEB caused both a reduction in epididymal sperm count and oxidative stress in the rat testis (Yang et al., 2018).

Because of its common use and persistence in the environment, TEB causes growing concern in the public (Yang et al., 2018). However, according to current scientific data, the toxic effects of TEB on the reproductive system of male livestock are still unclear. Primary testicular cultures and spermiotoxicity assays offer valid and valuable data in *in vitro* studies in which the species-specific effects of environmental pollutants on the reproductive system were screened (Yurdakok-Dikmen et al., 2019). Thus, the objective of the present study was to evaluate the effects of TEB on the male reproductive system in terms of physiological functions and cytotoxicity by the use of bovine testicular cell cultures and epididymal spermatozoa for evaluation.

MATERIALS AND METHODS

Animals and chemicals

All study procedures were conducted with the approval of the Local Ethics Committee of Kirikkale University (2020-E.4807). Chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. Tebuconazole (TEB) was prepared as a 1 M stock solution by dissolving it in 10% dimethyl sulphoxide (DMSO) that was diluted in culture medium DMEM/F12. The final concentrations of TEB used for the exposure of cells were 0.001, 0.01, 0.1, 1, 10, and 100 µM. These concentrations were guided by the data from the previous *in vitro* experimental/toxicological studies (Kjærstad et al., 2010; Bulbul and Ozhan, 2012; Roelofs et al., 2014; Cao et al., 2017). As experimental material, we used testes of healthy 2- to 5-year-old Simmental bulls without reproductive problems ($n = 4$), collected from the local abattoir. The testes were immediately transferred to the laboratory in pre-warmed sterile phosphate-buffered saline (PBS) containing an antibiotic-antimycotic solution.

Epididymal sperm collection

The transported testes of each animal were washed three times with 70% alcohol and sterile PBS containing 1% antibiotic-antimycotic solution, respectively. The segment containing the cauda epididymidis and vas deferens was isolated from the whole epididymis dissected from the testes. Spermatozoa in this segment were collected via retrograde flushing, a method previously described by Martinez-Pastor et al. (2006). Briefly, in this technique, the epididymal segment was washed with Salamon's Tris-citrate (Tris) extender and air was injected from the vas deferens to the cauda epididymidis by using a blunted 21-gauge needle to flush out all sperm into a Falcon tube. The sperm concentration of the sample was determined using a Thoma chamber, and it was diluted with Tris at 200 × 10^6 spermatozoa/mL (Salamon and Maxwell, 2000). Five tubes served as experimental groups, each containing 1 mL of spermatozoa diluted in PBS (native control), 0.1% DMSO (vehicle control), 0.01 µM TEB, 1 µM TEB, and 100 µM TEB, respectively. The tubes were then put into a shaking water bath at 37 °C to assess the motility and plasma membrane integrity of sperm at different incubation times (2, 4, and 6 h). All processes from the collection of testicles to placing of the diluted sperm into the water bath were completed within 2 h.

Sperm motility

The percentage of sperm motility was assessed subjectively using a phase-contrast microscope (DM1000, Lecia, Germany) with a heated stage at 37 °C (Varisli et al., 2013). For this purpose, 5 µL of sperm sample from each experimental group was put on a microscope slide and covered with a coverslip. Motile spermatozoa were then assessed on four non-overlapping fields at ×200 magnification. The first

measurement of motility after completing the sperm collection represented 0 h, which was the time control. The measurements were also repeated at 2, 4, and 6 h of incubation.

Plasma membrane integrity

After 2, 4, and 6 h of incubation, the plasma membrane integrity of spermatozoa was determined with a commercial live/dead sperm viability kit (catalogue no. L-7011, Molecular Probes, USA), which contains SYBR-14/propidium iodide (PI) fluorescent dyes, as previously described by Varisli et al. (2015). According to this method, 50 µL of sperm sample was transferred to another microtube, and 10 µL of 1 µM SYBR-14 was added to sperm samples in each group and incubated for 10 min at 37 °C. Five µL of PI (5 µM) was added. After a 5-min incubation period, the motility of spermatozoa was stopped with 3 µL Hancock's solution. To determine the viability of spermatozoa, 3 µL of this suspension was put on between two coverslips. A total of 200 spermatozoa were counted under a fluorescence microscope (DM IL LED FLUO, Leica, Germany). Spermatozoa stained with PI or SYBR-14 were seen as red or green fluorescence, and accepted as dead or live, respectively. The percentage of sperm viability was calculated as live cells/total counted cells. All of these steps were repeated three times for each group during each incubation period.

In vitro spermiotoxicity assays

Epididymal sperm samples were also diluted with Tris at a concentration of 3.0×10^5 spermatozoa/100 µL Tris containing 0.01% DMSO or TEB ranging from 0.001 to 100 µM. Experiments were conducted in a 96-well plate in triplicate for each group. Sperm cells were incubated in CO_2 incubator (BINDER GmbH, Germany) for 24 h at 37 °C, and spermiotoxicity was measured using the WST-1 assay kit according to the manufacturer's instructions (Abcam, USA, catalogue no. ab155902). At the end of TEB exposure, 10 µL WST-1 solution was added to each well, including the blanks that contained only medium, and incubated for 4 h under the same conditions. Following this additional incubation period, the absorbance of yellow formazan crystals, converted from tetrazolium salt in microplates, was measured with a microplate reader (Multiskan Go, Thermo Scientific, Finland) at 450 nm, while the culture medium without sperm cells was used as the blank. The percentage of cytotoxicity was then calculated using corrected absorbance as follows: 100 × (control or sample)/control.

Isolation of testicular cells

First the capsule of the testes was removed and then the testes were washed with 70% alcohol and sterile PBS, respectively. A small piece of tissue was extracted from the parenchyma of the testes after splitting them. These small testicular tissue pieces were placed in a Petri dish and thoroughly minced with a single-sided razor blade. Testicular cells were dissociated by trypsinisation overnight at 37 °C in a CO_2 incubator, as described previously (Yurdakok-Dikmen et al., 2019). Afterward, the cell suspension was filtered via 100-µm cell strainers (Falcon, USA) in order to remove undigested pieces and then centrifuged twice with the medium. The cells were resuspended in 1 mL of culture medium and counted by trypan blue staining to determine their viability.

Incubation of testicular cells

Before TEB treatment, 1.5×10^6 and 1.0×10^4 viable cells/well were seeded into 12- and 96-well culture plates with 1 mL and 100 µL DMEM/F12 media containing 20% fetal calf serum and 1% antibiotic-antimycotic solution, respectively. Plates were incubated for 24 h to allow cells to attach to the plates. Following that period, the medium was replaced with fresh serum-free medium containing 1% of 1 mg/mL insulin, 0.55 mg/mL transferrin, 0.5 µg/mL sodium selenite (ITS) and 0.1% DMSO or different TEB concentrations (0.001–100 µM). Testicular cells were exposed to the fungicide for 48 h, after which time the culture media were collected and stored at −20 °C to be used for steroid hormone analysis.

Viability assay of testicular cells

At 48 h, 10 µL WST-1 reagent was added to each well. Also, 100 µL culture media plus 10 µL WST-1 reagent were used as blank. The plate was then incubated for an additional 4 h in a 5% CO_2 aerated incubator at 37 ºC. During this time, the WST-1 tetrazolium salts were converted into formazan crystal, a coloured dye, via mitochondrial dehydrogenase enzymes. Afterward, the absorbance of this coloured dye in each well, which varies according to the metabolically active cell density, was measured using a microplate reader at 450 nm. The blank absorbance was then subtracted from the sample's absorbance for determining the corrected absorbance. Finally, the cell viability of the treatment groups was calculated by comparing the viability to the control, whose viability was accepted as 100%.

Testosterone analysis

An electrochemiluminescence immunoassay kit (Elecsys Testosterone II, Roche Diagnostic, USA) was used for measuring testosterone levels released by cells into the culture media during incubation according to the manufacturer's recommendations in an auto-analyzer (Roche Cobas E800). The sensitivity of the measurement range was between 0.025 and 15.0 ng/mL.

Statistical analysis

All the data defined as percentage were expressed as means and standard errors of the mean. Testosterone levels and cytotoxicity assay results were analysed with one-way analysis of variance (ANOVA) using SPSS 18.0 package program for Windows, while the results obtained from sperm motility and plasma membrane integrity experiments were analysed with a general linear model and repeated measures ANOVA.

Tukey's test was used for the *post-hoc* tests. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

Effects of tebuconazole on epididymal spermatozoa

Epididymal spermatozoa, which were exposed to different TEB concentrations (0.01–100 µM) in culture for 2, 4, and 6 h, were used to evaluate the motility and plasma membrane integrity. The percentage of sperm motility was not altered by low (0.01 µM), medium (1 µM), or high (100 µM) TEB doses compared to the control groups ($P > 0.05$) at any of the incubation times (2, 4, and 6 h). However, the motility was significantly reduced in a time-dependent manner in the control and in all treatment groups ($P < 0.001$) as shown in Table 1.

As shown in Table 2, the plasma membrane integrity of epididymal spermatozoa of the TEB-treated groups was not different from the control groups at any incubation period (0, 2, 4, and 6 h; $P > 0.05$). However, a significant decrease was observed in sperm membrane integrity in each group, including the control at 6 h of incubation compared to the first incubation time ($P < 0.05$). Figure 1 also illustrates the time-dependent effects of TEB on plasma membrane integrity.

The spermiotoxic effects of different doses of TEB were investigated under culture conditions after a 24-h incubation period. A dose-dependent reduction in the viability of epididymal spermatozoa was observed. This effect was not statistically significant from 0.001 to 0.1 µM concentrations. However, the decrement was significant at 1 µM ($P < 0.05$), 10 µM ($P < 0.01$), or 100 µM ($P < 0.01$) of TEB (Fig. 2).

Effects of tebuconazole on testicular cells

As shown in Fig. 3, none of the TEB concentrations used had cytotoxic effects on testicular cells. Even if the viability of the cells decreased at the lowest dose, the decrease was not statistically significant compared to the other doses ($P > 0.05$).

The effects of TEB on the function of testicular cells was investigated at low (0.01 µM), medium (1 µM), and high (100 µM) doses by comparing these cells with the vehicle control. Although testosterone levels, secreted by the testicular cells into the culture media, increased at the 1 and 100 µM doses compared with the control group and the 0.01 µM TEB group ($P < 0.05$), the hormonal activity of cells was not altered by exposure to the lowest dose of TEB compared with the control group ($P > 0.05$) as shown in Fig. 4.

DISCUSSION

Many pesticides are known as endocrine-disrupting chemicals (EDC), and 31% of these are fungicides (Mnif et al., 2011). TEB is one of the triazole fungicides, and it has been detected in different environmental components (Elsaesser and Schulz, 2008). Its adverse effects on reproduction have been previously reported in some *in vivo* and *in vitro* studies (Joshi et al., 2016; Cao et al., 2017; Atmaca et al., 2018; Yang et al., 2018; Chen et al., 2019). However, no study exists about the effects of TEB on the reproduction of male livestock animals which are also exposed to fungicides including TEB during grazing, drinking or dermal contact with contaminated environmental sources (EFSA, 2008; Oruc, 2010; Drážovská et al., 2016). As reproductive activity is critical for livestock animals for sustaining their offspring, disruption of this activity causes economic losses for the breeder. Therefore, this study was

Table 1. Effects of different concentrations of tebuconazole on bovine epididymal sperm motility

	0 h Mean ± SEM	2 h Mean ± SEM	4 h Mean ± SEM	6 h Mean ± SEM	*P* value
Native control	79.17 ± 1.93^{a}	71.67 ± 1.67^{a}	42.50 ± 1.79^{b}	29.17 ± 0.83^{c}	<0.001
0.1% DMSO	83.33 ± 1.42^{a}	76.67 ± 2.84^{a}	42.50 ± 1.79^{b}	25.00 ± 1.95^{c}	<0.001
TEB 0.01 µM	80.83 ± 2.60^{a}	70.83 ± 1.49^{a}	40.83 ± 1.93^{b}	30.00 ± 2.13^{c}	<0.001
TEB 1 µM	81.67 ± 2.07^{a}	76.67 ± 1.88^{a}	40.83 ± 1.93^{b}	29.17 ± 1.49^{c}	<0.001
TEB 100 µM	80.00 ± 2.46^{a}	72.50 ± 1.79^{a}	40.83 ± 0.83^{b}	27.50 ± 1.31^{c}	<0.001
P value	NS	NS	NS	NS	

Different letters (a, b, c) within the same row indicate significant differences at $P < 0.001$. DMSO: dimethyl sulphoxide, TEB: tebuconazole.

Table 2. Effects of different concentrations of tebuconazole on plasma membrane integrity of bovine epididymal spermatozoa

	0 h Mean ± SEM	2 h Mean ± SEM	4 h Mean ± SEM	6 h Mean ± SEM	*P* value
Native control	79.33 ± 3.84^{a}	75.67 ± 4.98^{a}	69.33 ± 5.04^{a}	65.00 ± 2.52^{b}	<0.05
0.1% DMSO	83.67 ± 1.33^{a}	82.33 ± 2.91^{a}	71.33 ± 4.33^{a}	57.33 ± 3.53^{b}	<0.05
TEB 0.01 µM	79.67 ± 1.33^{a}	74.33 ± 4.10^{a}	67.33 ± 4.48^{a}	54.00 ± 2.52^{b}	<0.05
TEB 1 µM	79.33 ± 1.33^{a}	71.33 ± 1.86^{a}	64.66 ± 3.33^{a}	58.33 ± 7.67^{b}	<0.05
TEB 100 µM	83.33 ± 0.67^{a}	73.00 ± 0.58^{a}	68.33 ± 1.20^{a}	63.67 ± 1.76^{b}	<0.05
P value	NS	NS	NS	NS	

Different letters (a, b) within the same row indicate significant differences at P < 0.05. DMSO: Dimethyl sulphoxide, TEB: Tebuconazole.

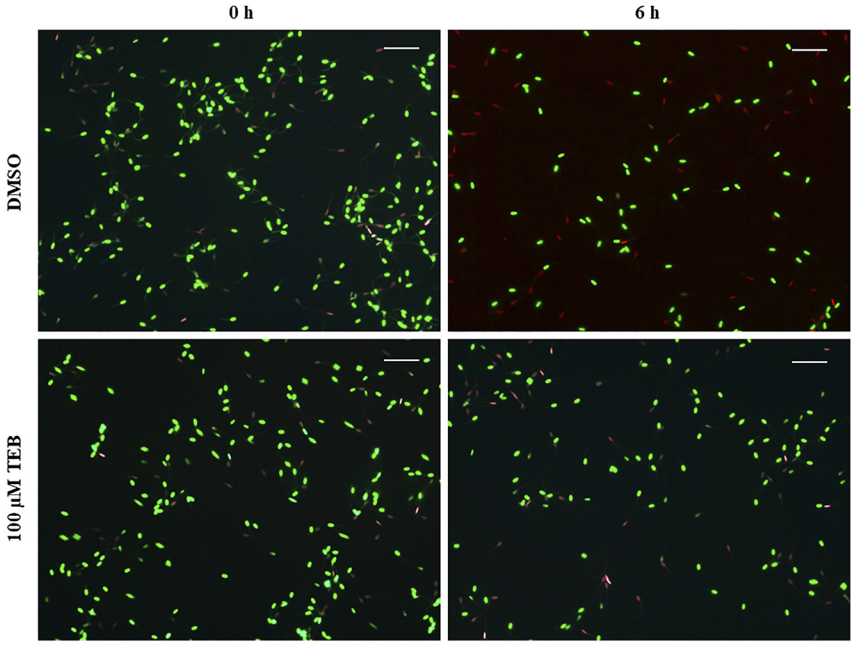

Fig. 1. The plasma membrane integrity of bovine epydidymal spermatoza after *in vitro* exposure to vehicle (0.1% DMSO) and different TEB concentrations. Double staining method via PI and SYBR14 was used to evaluate the viability, in which live cells were seen in green while dead cells in red by fluorescence microscopy at ×200 magnification. DMSO: dimethyl sulphoxide, TEB: tebuconazole. Scale bar: 50 µm

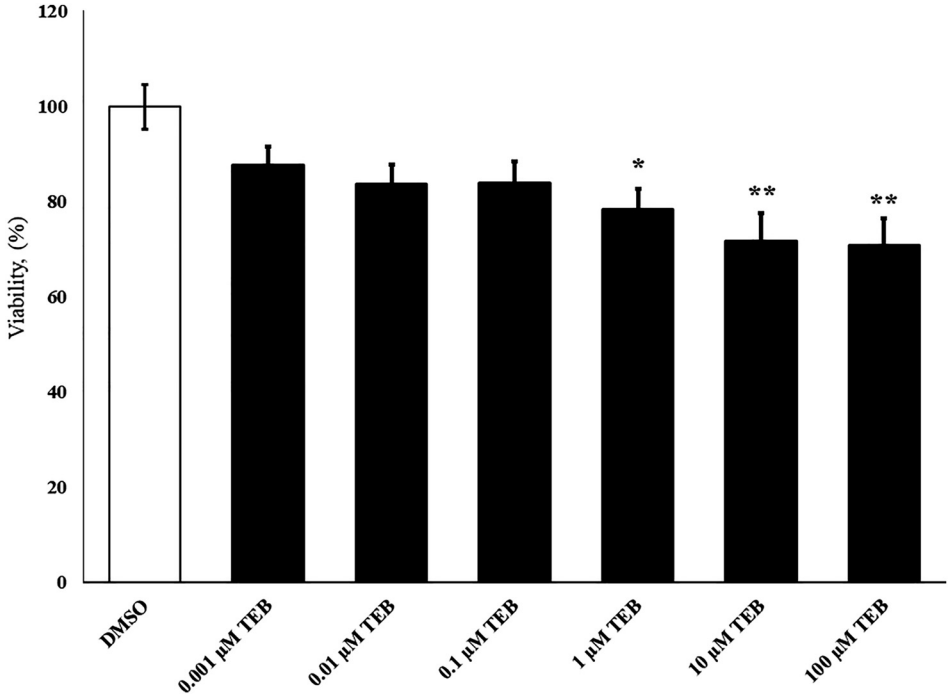

Fig. 2. Effects of different concentrations of TEB on the viability of bovine epididymal spermatozoa following a 24-h exposure period. The data obtained from % of control values were expressed as the mean ± standard error of the mean. The viability of spermatozoa at higher TEB doses was significantly different from that found with 0.1% DMSO. *$P < 0.05$ and **$P < 0.01$. DMSO: dimethyl sulphoxide, TEB: tebuconazole

designed to investigate the effects of TEB on the physiological functions of bovine testicular cells and epididymal spermatozoa. Also, we preferred to use bovine epididymal spermatozoa as an experimental material because of their easy availability from slaughtered bulls at the abattoir.

According to our results, none of the tested concentrations of TEB had a dose-dependent effect on epididymal sperm motility. Similarly, Goetz et al. (2007) did not find any differences in sperm morphology or motility of rats fed 100–2,500 ppm triazole fungicides as compared to the

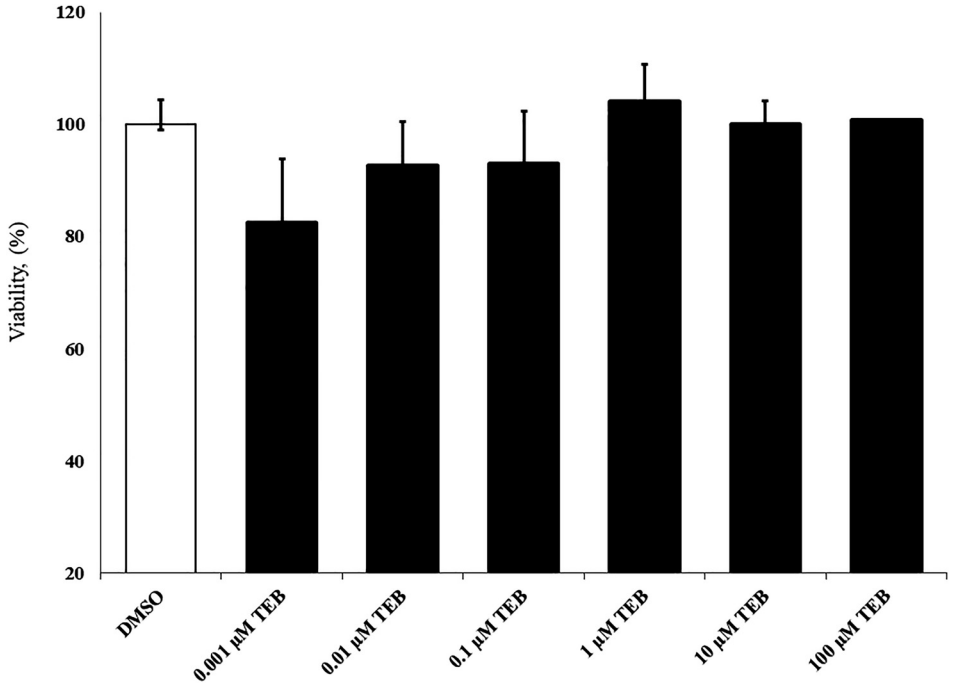

Fig. 3. Effects of different concentrations of tebuconazole on the viability of bovine testicular cells following a 48-h exposure period. The data obtained from % of control values were expressed as the mean ± standard error of the mean. The viability of testicular cells treated with different TEB doses was not significantly different from that obtained with 0.1% DMSO. $P > 0.05$. DMSO: dimethyl sulphoxide, TEB: tebuconazole

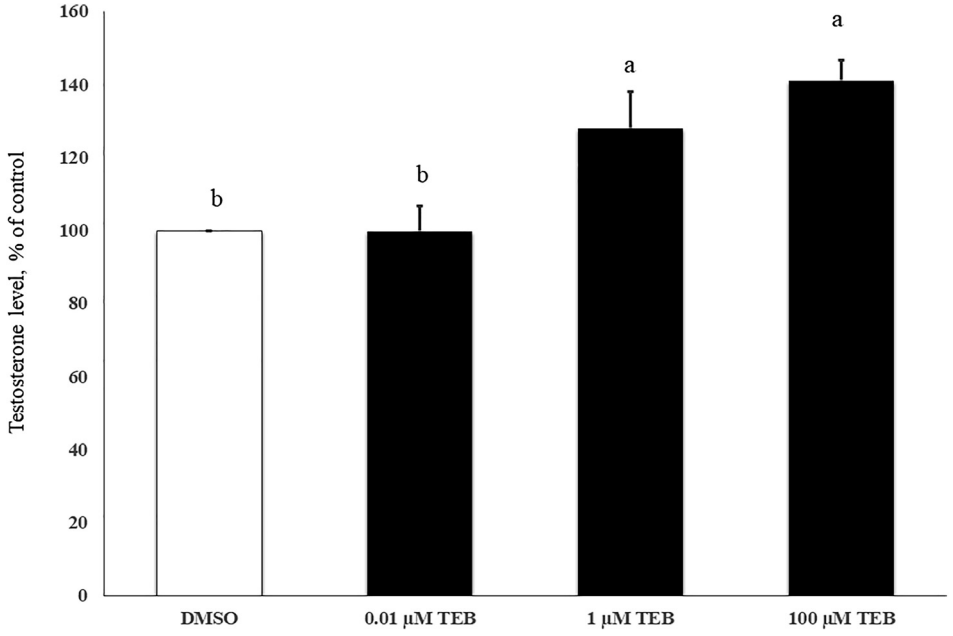

Fig. 4. Effects of different concentrations of tebuconazole on testosterone secretion by bovine testicular cells following a 48-h exposure period. The data obtained from % of control values were expressed as the mean ± standard error of the mean. The testosterone secretion in the groups treated with 1 and 100 µM TEB was significantly different from that obtained with 0.1% DMSO. Different letters above the bars indicate significant differences at $P < 0.05$. DMSO: dimethyl sulphoxide, TEB: tebuconazole

control group. Also, exposure of adult rats to 50 mg/kg TEB caused no statistically significant reductions in the number, motility, and velocity parameters of epididymal spermatozoa (Taxvig et al., 2007). In contrast, Joshi et al. (2016) demonstrated that sperm density and motility decreased in rats orally exposed to 250 mg/kg body weight TEB. The authors have correlated these effects with the endocrine disrupting and suppressive effects of TEB on the testis and

spermatogenesis, respectively. The differences in the results of these studies may be attributed to the different doses used. The probable reason why the results of our study are similar to some of these research results (Goetz et al., 2007; Taxvig et al., 2007) and different from others (Joshi et al., 2016) is that the studies were conducted *in vivo* and with different doses. It is well known that sperm motility is affected by many factors, particularly sex steroids in the body. That is why the results of *in vivo* and *in vitro* studies might be different. Therefore, TEB could not affect the motility because it only exerted an effect on sperm without the disruptive effects on steroidogenesis and spermatogenesis in this study. However, there was a time-dependent decrease in sperm motility in all groups in our study. The decline in the sperm motility of the treatment groups at 2, 4, and 6 h of incubation compared to 0 h (time control) was not meaningful as the decrement was also seen in the control group depending on the incubation time. A similar decrease was also reported in the motility of non-treated epididymal spermatozoa of goats (Ouennes et al., 2019), rams (Kaya et al., 2020), and bulls (Bertol et al., 2013) over time in *in vitro* studies, whereas the total motility of bull spermatozoa obtained with an artificial vagina was not reduced during a 4-h *in vitro* cultivation in our previous study (Kabakçı et al., 2019). Seminal plasma is produced by the accessory glands of the male reproductive system and has a critical role in sperm quality in terms of saving its motility from ejaculation to fertilisation. Although Cunha et al. (2016) assumed that ejaculated and epididymal spermatozoa have similar semen quality, Silva et al. (2003) reported that the time-dependent motility of epididymal spermatozoa was reduced due to the lack of seminal plasma. Furthermore, the decrease in the content of motility proteins, such as carnitine that is produced by the epididymal epithelium, in semen obtained from the cauda epididymidis without seminal plasma, resulted in a time-dependent reduction of epididymal sperm motility under *in vitro* conditions (Ghosh, 1993). Therefore, the lack of seminal plasma may be responsible for the time-dependent reduction of epididymal sperm motility in all groups including the control group.

Just like the motility results, in this study no significant differences in plasma membrane integrity indicative of the viability of epididymal spermatozoa were observed in the stained spermatozoa in the treatment groups compared to the control groups at any incubation time (0–6 h) Alternatively, higher concentrations of TEB (1–100 µM) used in this study caused significant cytotoxicity on spermatozoa in a dose-dependent manner during 24-h exposure. In this study, plasma membrane integrity, i.e. the viability of spermatozoa, was tested using the SYBR-14/PI method, in which live spermatozoa are stained green while dead ones are stained red due to membrane damage (Garner and Johnson, 1995). Cytotoxicity was also evaluated using the WST-1 assay. It relies on the capacity of mitochondrial succinate dehydrogenases in living cells to cleave stable tetrazolium salt WST-1 into soluble coloured formazan crystals which are measured spectrophotometrically. According to a previous study (Bulbul and Ozhan, 2012), the cytotoxic effects of several azole fungicides were more sensitively detected by the MTT assay, which has a principle similar to the WST-1 assay, compared to the lactate dehydrogenase (LDH) assay, which shows membrane damage. Also, it was reported that these cytotoxic effects increased over time. Thus, it was suggested that the effects of selected azole fungicides on mitochondrial activity and the respiratory chain could be more frequently observed than membrane damage (Bulbul and Ozhan, 2012). Furthermore, dose-dependent inhibitory effects of azole fungicides on succinate dehydrogenases have been reported on isolated rat liver mitochondria by Rodriguez and Acosta (1996). These reports are able to explain the dose- and time-dependent cytotoxic effects of TEB on epididymal spermatozoa detected by the WST-1 assay after 24 h rather than by SYBR-14/PI staining after 0–6 h. Cytotoxicity might result from the intrinsic toxicity of pesticides, which lead to reactive oxygen species (ROS) production (Pollákova et al., 2012). Therefore, another reason for the cytotoxic effects of TEB on sperm cells may be oxidative stress, since TEB may induce an ROS-dependent reduction in the viability of mammalian cells by activating lipid peroxidation, DNA damage or mitochondrial apoptotic pathway (Othmène et al., 2020, 2021).

Primary testicular cell cultures have great potential for screening the effects of environmental pollutants on reproduction. These cultures mainly contain Leydig cells, which produce testosterone, and peritubular myoid and Sertoli cells, which surround the seminiferous tubules to support spermatogenesis (Yurdakok-Dikmen et al., 2019). To evaluate the viability rate, bovine testicular cells were exposed to different TEB concentrations *in vitro*. Even if a slight non-significant reduction in viability was seen at low doses (0.001–0.1 µM), TEB doses between 0.001 and 100 µM did not affect the viability of bovine testicular cells compared to the vehicle control. Similarly, *in vitro* TEB exposure at concentrations of 10 nM–100 µM did not cause cytotoxicity on MA-10 cells but led to endocrine disrupting effects (Roelofs et al., 2014). In addition, Chen et al. (2019) also demonstrated that 100 mg/kg TEB exposure administered by gavage did not induce any proliferation of rat Leydig and Sertoli cells. Triticonazole, another triazole fungicide, showed agonistic activity on androgen receptors at doses of 25–50 µM without any signs of proliferative or cytotoxic effects (Draskau et al., 2019). On the other hand, in some previous studies it was reported that TEB at concentrations ranging from 5 to 60 µM significantly decreased the viability of bovine lymphocytes (Schwarzbacherova et al., 2017), human cardiac (Othmène et al., 2020) and intestinal (Othmène et al., 2021) cell lines, mainly as a consequence of ROS generation during 24-h cultivation. Similarly, TEB treatment at 20–80 µM concentrations for 24 or 48 h caused a reduction in the viability of the human placental trophoblast cell line HTR-8 (Zhou et al., 2016). It can be understood that there is a discrepancy about the effects of TEB on the viability of mammalian cells including testicular cells because of both differences of cell types and incubation periods. Even so, these evidences also suggest that TEB

exerts its adverse effects on male reproductive cells primarily as an endocrine disruptor.

Additionally, in this study the *in vitro* administration of 1 and 100 µM TEB to bovine testicular cells resulted in higher testosterone production than that induced by the vehicle control and the lowest doses of TEB (0.01 µM). Similarly, Goetz et al. (2007) showed that triazole fungicides caused an increase in serum testosterone levels of rats at doses higher than 500 ppm. Moreover, Chen et al. (2019) demonstrated that the blood testosterone concentrations were higher in rats exposed to TEB at doses of 100 mg/kg. In contrast to these reports, Roelofs et al. (2014) revealed that 0.3–10 µM TEB significantly reduced basal and LH-stimulated testosterone secretion by MA-10 Leydig cells *in vitro*. However, the expression levels of StAR, HSD3β and CYP11A1 enzymes, which are related to testosterone synthesis, were not changed by TEB treatment in that study. Besides, according to Strushkevich et al. (2010), TEB may disrupt the activity of alpha-lanosterol demethylase, also called CYP51, which is highly expressed in the testes and has a critical role in cholesterol synthesis and eventually in testosterone production. Some previous studies showed that TEB was able to directly stimulate the expression of CYP11A1, which converts pregnanolone to progesterone in rat Leydig cells (Chen et al., 2019) but inhibits human adrenal (Kjærstad et al., 2010) and placental (Cao et al., 2017) aromatase (CYP19A1) activity, which converts testosterone to oestradiol in many kinds of steroid cells. Therefore, the increase in testosterone release by bovine testicular cells following 1 and 100 µM TEB treatment may be related to its modulatory effects on steroidogenic enzymes.

In conclusion, here we describe for the first time that *in vitro* exposure to TEB did not affect the epididymal sperm quality parameters, such as motility and plasma membrane integrity; however, TEB caused a reduction in the viability of spermatozoa. Although testicular cell viability did not change, testosterone level was increased, especially at 1 and 100 µM TEB. It is probable that TEB might cause an increase in some steroidogenic enzymes involved in testosterone secretion at these doses. It is well known that high levels of testosterone may lead to an earlier onset of maturity and in this case, insufficient sperm count can lead to reproductive problems in males. It was concluded that TEB, a commonly used fungicide, may cause infertility in bulls at concentrations higher than 1 µM by impairing the physiological functions of testicular cells. The fertility problems of these animals are very essential in terms of both animal health and the economy.

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