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Impact of Tebuconazole Fungicide on Drone Semen Quality

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Research Article

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ABSTRACT

Tebuconazole (TEB) is a widely used fungicide of the triazole group, especially in fruit tree cultivation. It has adverse effects on the reproductive system by disrupting cellular and hormonal mechanisms in most species. Lots of studies are proven the presence of TEB's residues on fruit trees. However, no study has been found on its effect on the reproductive parameters of drones. Honey bees are considered at risk in terms of reproductive systems since they most probably transport this chemical to the colony through nectar and pollen. Therefore, it was aimed to investigate to potential toxic effects of TEB on drone semen quality, a crucial element in reproductive system of honey bee in dose and time dependent manner. Honey bee semen in five different tubes, each containing 1.0 – 1.5 x 108/ml spermatozoa, were exposed to 0, 1, 10, 100, and 1000 µM of TEB, respectively for 24 h. Afterward, semen were analyzed for motility (MOT), plasma membrane integrity (PMI), and mitochondrial membrane potential (MMP) at 0 and 24 hours. The findings of this study revealed that highest concentration of TEB (1000 µM) significantly reduced ($p<0.05$) MOT and PMI of semen compared to other concentrations even at 0 h. Following 24 h incubation, MOT, PMI and MMP values of groups exposed to 1000 µM TEB significantly lower ($p<0.05$) than other groups. On the other hand, lower concentrations of TEB between 0-100 µM did not significantly change any parameters evaluated in this study at both 0 and 24 h ($p>0.05$). In conclusion, although it is only observed at the highest dose of TEB, our results showed that TEB has a detrimental effect on drone semen. Furthermore, it would be useful to conduct more comprehensive studies supported by in vitro and in vivo research in the future.

Keywords: Honey bee, Drone semen, Spermatological parameters, Tebuconazole, Toxicity

Bir Fungusit Olan Tebukonazol'ün Arı Sperma Kalitesine Etkisi

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ÖZ

Tebukonazol (TEB) özellikle meyve ağacı yetiştiriciliğinde yaygın olarak kullanılan triazol grubu bir fungisittir. Birçok türde hücresel ve hormonal mekanizmaları bozarak üreme sistemi üzerinde olumsuz etkileri vardır. Meyve ağaçlarında TEB kalıntılarının varlığı birçok çalışma ile kanıtlanmıştır. Ancak, erkek arıların üreme parametreleri üzerindeki etkisine ilişkin bir çalışmaya rastlanılmamıştır. Bal arıları üreme sistemleri açısından risk altında kabul edilmektedir. Çünkü büyük olasılıkla bu kimyasalı nektar ve polen yoluyla koloniye taşımaktadırlar. Bundan dolayı, bu çalışmada TEB' in bal arısının üreme sisteminde önemli bir unsur olan erkek arı sperm kalitesi üzerindeki potansiyel toksik etkilerinin doza ve zamana bağlı olarak araştırılması amaçlanmıştır. Her biri 1.0- 1.5 x 108/ml spermatozoa içeren beş farklı tüpteki bal arısı sperması 24 saat boyunca sırasıyla 0, 1, 10, 100 ve 1000 µM TEB' e maruz bırakıldı. Daha sonra sperma 0 ve 24. saatlerde motilite (MOT), plazma membran bütünlüğü (PMB) ve mitokondriyal membran potansiyeli (MMP) açısından analiz edildi. Bu çalışmanın bulguları, en yüksek TEB konsantrasyonunun (1000 µM) arı spermasının MOT ve PMB değerlerini 0. saatte bile diğer konsantrasyonlara kıyasla önemli ölçüde azalttığını ($p<0,05$) ortaya koydu. 24 saatlik inkübasyonun ardından, 1000 µM TEB'e maruz kalan grupta MOT, PMB ve MMP değerleri diğer gruplara kıyasla önemli ölçüde daha düşüktü ($p<0,05$). Öte yandan, hem 0. hem de 24. saatte TEB' in 0-100 µM arasındaki düşük dozları çalışmada incelenen hiçbir parametreyi anlamlı bir şekilde değiştirmedi ($p>0,05$). Sonuç olarak, sadece en yüksek TEB dozunda gözlenmesine rağmen, sonuçlarımız TEB' in erkek arı sperması üzerinde zararlı etkisi olduğunu gösterdi. Ayrıca, gelecekte in vitro ve in vivo araştırmalarla desteklenen daha kapsamlı çalışmaların yapılması faydalı olacaktır.

Anahtar Kelimeler: Bal arısı, Erkek arı sperması, Spermatolojik parametreler, Tebukonazol, Toksisite

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Introduction

Honey bees are affected by declining natural habitats and negative aspects of modern agricultural production. It is well known that honey bee deaths are increasing worldwide. Most contributing factors include changing seasonal conditions, bee diseases, poor breeding practices and chemical pesticides (Gregorc, 2020). Among these factors, the impact of agricultural pesticide application on honey bees is attracting increasing attention. Honey bees ensure pollination in fruit orchards (Halder et al., 2019). Thus, agricultural fields are

important locations for beekeepers to place their beehives. However, inappropriate timing and excessive use of pesticides lead to honey bee mortality and disruption of biodiversity. In particular, the use of pesticides during the flowering season in orchards poses a potential threat to honey bees (Uhl & Brühl, 2019).

Tebuconazole (TEB) ((RS)-1-p-chlorophenyl-4,4-dimethyl-3-1(1H-1,2,4-triazol-1-yl-methyl) pentan-3-ol) is a fungicide, and effectively inhibits ergosterol biosynthesis in fungi, providing control against soil-borne and foliar fungal pathogens (Ito et al., 2013). However, previous studies have reported various harmful effects of TEB on body tissues, including reproductive organs and/or cells, and the relevant information is provided below. A study conducted with human placental trophoblast cell lines revealed that TEB at the concentration of 10 µM decreased cell proliferation, increased apoptosis, reduced cellular migration, and lowered intracellular mRNA levels by disrupting the function of proteases, hormones, angiogenic factors, growth factors, and cytokine expansion (Zhou et al., 2016). It has been shown that 0.1 µM TEB inhibited testosterone and estrogen levels in hamster ovarian tissue (Kjærstad et al., 2010), and 10 µM TEB inhibited progesterone synthesis of bovine luteal cells (Atmaca et al., 2018). However, 1 µM TEB has been reported to increase testosterone secretion in bull testicular cells while reducing spermatozoa viability (Kabakci et al., 2021). Although TEB is classified as slightly hazardous by the World Health Organization, it has been withdrawn from the market in some European countries (EUR-Lex, 2009) duo to its endocrine disrupting effects (TEDX, 2017). In this context, the concentration of 1700 mg/kg determined in rats as LD50 suggests low toxicity for mammals (Authority, 2015). For honey bees, this concentration was 2770 mg/L and 1840 mg/L for 2 and 4 days of exposure, respectively. However, authors have revealed that 20-320 fold diluted LD50 concentrations of TEB reduced enzymes related to biochemistry, digestion and detoxification and negatively affected genes related to immune response, growth or apoptosis in honey bees (Cang et al., 2023).

Numerous studies have shown that TEB residues on plants and fruits. The fact that this pesticide can be used during the flowering period of trees such as apples, pears, cherries, and peaches, which serve as nectar and pollen sources for honey bees, indicates a potential threat (Fang et al., 2020; Li et al., 2020; Lucini & Molinari, 2009; Mohapatra, 2015; Szarka & Ramanarayanan, 2021). It has been previously reported that honey bees landing on flowers of trees treated with pesticides to collect nectar can lead directly to acute toxicity or larval mortality (Fine et al., 2017; Yoder et al., 2017). Johnson et al. (2010) have detected high amounts of TEB residues in honey bees while low amounts of TEB residues were detected in pollen and honey (Johnson et al., 2010). The quality of drone semen is a critical factor in reproduction. Semen motility (MOT) is an essential criterion that is widely used to assess fertilization capacity. After mating, spermatozoa are initially stored in the oviduct of the queen bee. They migrate for about 40 hours to reach the spermatheca of the queen. Worker bee embryos are fertilized when the queen bee's egg cell is fertilized by spermatozoa from the spermatheca. The motility of semen plays a crucial role in both storing semen in the spermatheca and fertilizing the egg cell during the queen bee's oviposition (Cobey et al., 2013). Plasma Membrane Integrity (PMI) and Mitochondrial Membrane Potential (MMP) are another important criterion for semen quality. The former provides information on cell viability based on the selective permeability of the cell membrane while the latter gives information regarding mitochondrion, the ATP synthesis in the cells. Fluorescent staining can determine whether this potential is at between -140 and -180 mV which is the appropriate level (Kaya & Uysal, 2023). If drone semen is of poor quality, it will hurt the continuity of the honey bee colony. One of the factors influencing spermatological parameters is environmental pollutants. Although the negative effects of TEB on the reproductive system of various animals and humans, its impacts on drone semen quality are still unknown (Kabakci et al., 2021; Taxvig et al., 2007; Yan et al., 2023; Zhou et al., 2016). Therefore, this study investigated the impacts of TEB, a triazole fungicide, on spermatological parameters, including MOT, PMI and MMP of drones in a dose and time-dependent manner at in vitro conditions.

Materials and Methods

Collection of Semen From Drones

The drones used in this study were obtained from the Kırıkkale University Beekeeping Research Center. The honeybee is not classified as a laboratory animal according to Article 11(b) of the Regulation on Working Procedures and Principles of Local Ethics Committees for Animal Experiments, published by the Ministry of Agriculture and Forestry of the Republic of Turkey in the Official Gazette of 15 February 2014, No. 28914. Therefore, ethical committee approval was not required for this study. Semen was collected from drones during May and June. This period was preferred because it has the seasonal highest adult drone's population in this region with a continental climate. We used six different colonies with same ages queens and similar strength for all experiments. Approximately 100-150 drones were collected among all drones returning from afternoon flights and transported to the laboratory. Manual pressure was applied to the thorax and abdomen of the drones to induce eversion and ejaculation. A Total of 100 µl of semen was obtained by pooling from these drones using an artificial insemination instrument (Scheley, Germany), for each experiment. The concentration of collected semen was determined using a Thoma chamber and divided into five different tubes in 1 ml Phosphate-buffered saline (PBS) containing 1.0-1.5 ×108/ml spermatozoa for each one. The experimental groups were set up according to TEB (Sigma-Aldrich, Catalog no: 32013, USA) concentrations as 0, 1, 10, 100, and 1000 µM in these tubes. The semen were exposed to these TEB

concentrations for 24 h in an incubator at 33 °C. In the first (0 h) and last (24 h) time points of incubation, the semen were analyzed in terms of spermatological parameters (Cobey et al., 2013). All the experimental designs were repeated at least 6 times.

Motility

Semen motility assessed using aheated stage-phase contrast microscope, Leica DM1000 (Leica, Germany). Motility was determined as previously described by modified method of Kaftanoglu and Peng (Kaftanoglu & Peng, 1984). A 10 µl semen was taken from each sample, dropped onto a glass slide, and examined at 10 × magnification. Semen motility was subjectively scored on a scale of 0 to 5 (Kaya & Akyol, 2023)

Plasma Membrane Integrity

Hoechst 33342 (Sigma-Aldrich Catalog No: B2261) and PI (Sigma-Aldrich Catalog No: B4170) fluorescent dyes were used to determine the color difference in the head of the spermatozoa and the integrity of the plasma membrane during double staining, using a dye that can pass through the membranes of both living and dead cells. A 50 µl sample was taken from the semen, mixed with 10 µl Hoechst 33342 (5 µg/ml) and 5 µl PI (10 µg/ml) and incubated for 10 min. Then, 3 µl of this mixture was examined under a Leica DM3000b (Leica, Germany) fluorescent attachment inverted microscope at 40× magnification using the "A" filter. Plasma membrane integrity was determined as percentage (%) for 200 spermatozoon per sample using Cells Calculator (v. 2.2) software (Kaya & Akyol, 2023).

Mitochondrial Membrane Potential

In this study, JC-1 (M34152, Molecular Probes, Eugene, OR, USA) fluorescent dye was used for this purpose. A 50 µl sample was taken from the semen, mixed with 5 µl JC-1 (2 µM final concentration), and incubated for 30 minutes. Then, it was mixed with 3 µl of Hancock solution, and 3 µl of this mixture was examined under a Leica DM3000b fluorescent attachment inverted microscope at 40 × magnification using the "I" filter. Mitochondrial membrane potential was determined as a percentage (%) for 200 spermatozoon per sample using Cells Calculator (v. 2.2) software (Kaya & Akyol, 2023).

Statistical Analysis

The obtained data were analyzed using one-way ANOVA by SPSS (v. 15.6) software package. Post-hoc comparison between the groups performed by Tukey test. Statistical significance level was accepted as $p<0.05$ and results were expressed as mean ± standard error mean.

Results And Discussion

The motility of the drone semen exposed to different concentrations of TEB assessed by subjectively scoring while PMI and MMP were measured by counting cells. As shown is Figure 1, in the two patterns of sperm staining in order to evaluate viability, live spermatozoa with intact membranes were labelled only with Hoechst 33342 dye and their heads fluoresced blue; dead spermatozoa, were permeable for PI and their heads fluoresced red (Figure 1).

In the staining performed to determine MMP levels, cells were observed in two different colors. It was clearly understood that spermatozoa stained orange color had a high MMP level while spermatozoa stained green color had a low MMP level (Figure 2).

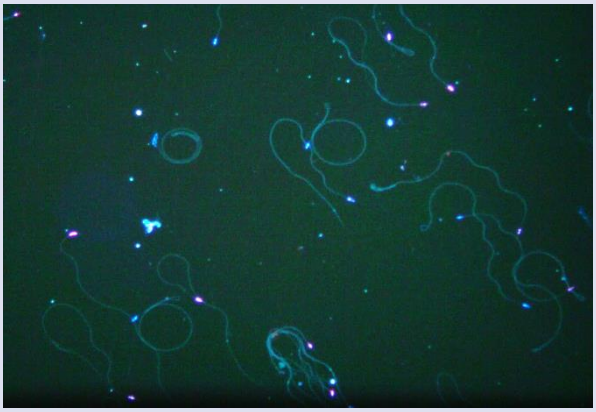

Figure 1: Assessment of PMI (Blue color: Intact, Red or Pink color: Damaged). PMI: Plasma Membrane Integrity

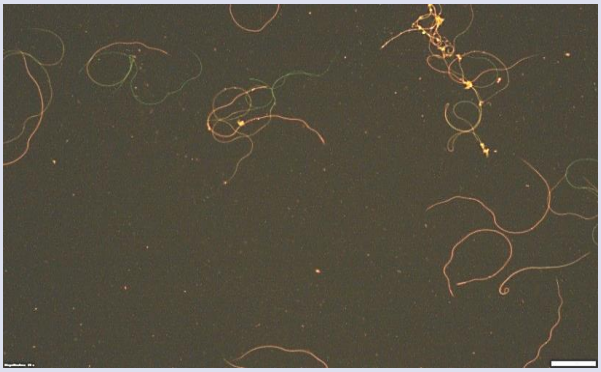

Figure 2: Assessment of MMP (Orange color: High MMP, Green color: Low MMP). MMP: Mitochondrial Membrane Potential

According to our results, a significant decrease in MOT and PMI levels of spermatozoa was observed at 0 h only in the group treated with 1000 µM TEB ($p<0.05$). However, when comparing the MMP level in the 1000 µM TEB group and the levels of MOT, PMI, and MMP in the 1, 10, and 100 µM TEB groups with the control group, no statistical difference was found (Figure 3). After 24 hours of incubation, the highest concentration of TEB (1000 µM) significantly reduced ($p<0.05$) MOT, PMI and MMP of spermatozoa compared to other concentrations (Figure 4). During the 24-hour incubation period, just like at 0 hours, there was no statistically significant difference in

the groups containing 0, 10, and 100 micromoles of TEB when compared to the control group (p>0.05).

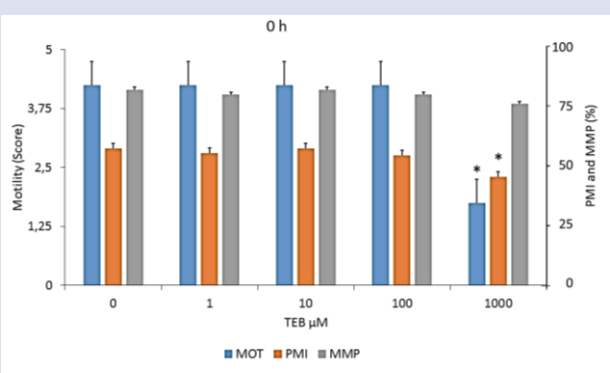

Figure 3: The effect of different concentrations of TEB (0-1000 µM) on spermatological parameters of drone semen at 0 h. Asterix (*) indicates statistical significance at p≤0.05 level
MOT: Motility, PMI: Plasma Membrane Integrity, MMP: Mitochondrial Membrane Potential TEB: Tebuconazole

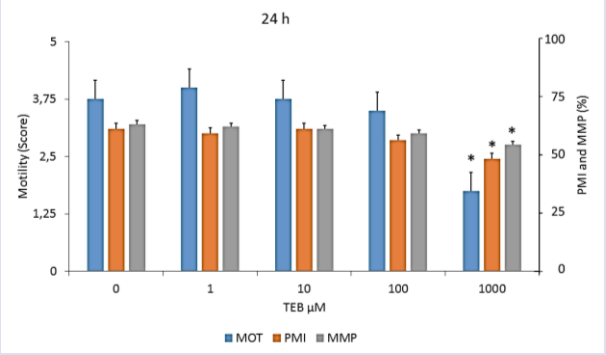

Figure 4: The effect of different concentrations of TEB (0-1000 µM) on spermatological parameters of drone semen at 24 h. Asterix (*) indicates statistical significance at p≤0.05 level
MOT: Motility, PMI: Plasma Membrane Integrity, MMP: Mitochondrial Membrane Potential TEB: Tebuconazole

This study investigated the effects of various concentrations of TEB on drone spermatological parameters under in vitro conditions, at different incubation times. The findings of this study revealed that TEB adversely impacts of drone semen in a dose and time dependent manner. Our results indicated that a dose of 1000 µM adversely affected semen MOT and PMI at 0 hours (Figure 1) and MMP was also negatively impacted after 24 hours of incubation (Figure 2). However, no adverse effects were observed in either incubation at levels of 1, 10, and 100 µM. Several studies have investigated the effects of triazole fungicides, including TEB, on reproduction in various species under both in vitro and in vivo conditions. For instance, a study on bovine testicular cells and semen revealed that 1 µM TEB increased testosterone production by Leydig cells but reduced semen viability (Kabakci et al., 2021). The study indicates that bull semen is adversely affected even at low doses of TEB exposure. while negative effects on drone semen were only observed at high doses. This difference may be attributed to the structural and lifespan differences between mammalian and insect spermatozoon. The ability of drone semen to maintain viability for a longer period at room temperature (approximately 6 weeks) compared to bull semen (approximately 2-3 days) supports this idea (Burley et al., 2008; De Pauw et al., 2003). In pregnant rats, oral administration of TEB led to an increase in female characteristics in male offsprings and alterations in the production of testosterone and progesterone (Taxvig et al., 2007). In a study investigating the effects of TEB exposure on zebrafish, it has been determined that TEB adversely affected testis development, and decreased semen motility (Yan et al., 2023). However, honey bees in the natural environment can be exposed to various pesticides, including TEB, there are highly limited studies on the effects of pesticides on honey bee colonies and reproductive performance. In a study conducted with drones, orally 200 ppb imidacloprid administration reduced semen motility and affected MMP but did not change PMI (Ciereszko et al., 2017). In another study, several pesticide residues (amitraz, fluvalinate, coumaphos) in beeswax, which is used to rearing honey bees, led to a 5-20% reduction in semen viability in drones (Fisher & Rangel, 2018). Kairo et al. (2016) showed that fipronil exposure led to lower PMI and ATP levels in spermatozoa of drones in comparison with the control (Kairo et al., 2016). Similarly, treatment of Neonicotinoid to the drones resulted in 20% lower spermatozoa PMI than that of control group (Straub et al., 2021).

In vitro exposure is an important part of toxicological studies and at the same time in vitro studies are sensitive and complex investigations (Kabakci et al., 2019). Limited research has been conducted on the impact of pesticides on in vitro spermatological attributes of drones. Although few previous studies have been reported that selected pesticides significantly changed MOT, PMI, and/or ATP content of drone spermatozoa (Abdelkader et al., 2015; Inouri-Iskounen et al., 2020). This is the first research investigating the effect of in vitro spermatological parameters on tebuconazole fungicide on drones. Our results provide evidence of the adverse effects of TEB on drone semen quality. As seen in our results, in contrast to low doses between 0-100 µM, highest concentration of TEB at 1000 µM decreased MOT, PMI, and MMP (at only 24 h).

Semen motility is the key parameter of reproductive quality. This parameter exhibited a decrease in both incubation periods in the group containing 1000 µM TEB. Disruption of the integrity of the plasma membrane leads to cellular damage and reduced semen motility. Numerous studies have shown a correlation between motility and PMI (Ahmad et al., 2014; Fraser et al., 2001;

Varisli et al., 2009). Spermatozoa are required continuous energy for their movement. The ATP transformation is the main processes of energy production to provide semen motility (Abdelkader et al., 2015).This required energy is supplied by mitochondria in the cells. Our results revealed that MMP significantly declined following 1000 µM TEB exposure for 24 h. This may be another reason of the reduction in semen motility observed at the end of the exposure period. Plasma membrane integrity is usually measured to evaluate viability in drone spermatozoa (Kaya & Uysal, 2023). We also found that in vitro 1000 µM TEB exposure to drone semen reduced PMI. This may be associated with the cell membrane being the target of TEB. It was well defined that TEB inhibits P450 enzyme on the cell membrane of fungi so affects their integrity (Ito et al., 2013). In accordance with this Hajer et al. (2020) reported that TEB inhibited testicular P450 and glutathione S-transferase activities, and decreased cauda epididymal spermatozoa count in rats (Hajer et al., 2020). Similarly, in vivo TEB treatment in rats (Hajer et al., 2020) and chicken (Serra et al., 2023) increased spermatozoa abnormalities by declining motility, velocity, counts and/or viability. Another reason of reduced PMI in spermatozoa may be increased TEB-related oxidative stress since TEB has been previously reported to cause lipid peroxidation, protein oxidation, and severe DNA degradation in male reproductive organs (Hajer et al., 2020). On the other hand, MMP is a vital parameter for evaluating the function of mitochondria. Previous studies have shown that environmental pesticides may affect spermatozoa ATP levels (Abdelkader et al., 2015; Kairo et al., 2016). Similarly, we observed that 1000 µM TEB reduced MMP levels of spermatozoa at 24 h. Whereas MOT and PMI, MMP did not affected by TEB at 0 h. This may be resulted from the location of mitochondria in the cell and the self-protective membrane of mitochondria. This may explain the time-dependent effects of TEB on spermatozoa MMP level.

In this study, lower concentrations of TEB between 0-100 µM had no negative effects on drone spermatological parameters. However, possible sublethal alterations in semen quality cannot be excluded due to potential bioaccumulation of TEB and cumulative effects of its low concentrations. It is well known that fungicidal residues including TEB have contaminated environmental components as well as honey bee and honey bee products. Even at low concentrations, their long-term exposure can cause endocrine, metabolic, or systemic disruption in cellular, mitochondrial and/or organisms such as fish, insects, bees, plants, mammalians, and/or humans (Johnson et al., 2010).

Conclusion

This study investigated the possible in vitro effects of TEB, a triazole fungicide, on drone semen quality. Our results revealed that over the 100 µM TEB may adversely affect some spermatological parameters of drones even at short-term exposure. Especially, 1000 µM TEB significantly reduced semen MOT, PMI and MMP parameters following 24 h incubation. In conclusion, considering the importance of semen viability and motility dependent on plasma membrane integrity and mitochondrial activity on reproductive health, adverse effects of TEB on drone semen quality may finally impact reproduction of honey bee and production of honey bee products. However, further research is expected to provide a more comprehensive results understanding the mechanism of the influence of TEB on honey bees.

Conflict of interest

The authors declare that there is no conflict of interest.

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