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MORPHOLOGICAL CHANGES IN LEYDIG TM3 CELLS INDUCED BY NEONICOTINOID ACETAMIPRID

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ABSTRACT

Numerous studies demonstrate the vulnerability of the male reproductive system to various exogenous environmental factors, including agrochemicals such as pesticides, herbicides, and insecticides. Acetamiprid, a widely used chlornicotinyl insecticide of a new generation, was developed as a safer alternative to harmful organophosphates and carbamates for controlling insect pests. Our study aimed to investigate the impact of acetamiprid on Leydig TM3 cells cultivated as three-dimensional (3D) cultures after prolonged repeated exposure. The 3D model was treated with acetamiprid in a concentration range of 4 to 500 µM. Morphological parameters, specifically the size and shape of the cell spheroids, were evaluated for up to 7 days. Using a Zeiss Z1 Observer automated inverted microscope and TissueFAXS software, the development of spheroids was monitored. Three separate iterations yielded the experimental findings. Microsoft Office 365 and GraphPad Prism 8.01 were used to process the results. The obtained results showed no statistically significant changes (P>0.05) in morphological parameters at any of the tested acetamiprid concentrations.

Keywords: acetamiprid, Leydig cells, 3D- cultivation, morphology

INTRODUCTION

Despite increasing evidence of the detrimental impacts of agrochemicals, including pesticides, on ecosystems and human health, global agrochemical consumption continues to rise (**Devi *et al.*, 2022**; **Phogat *et al.*, 2022**). Fourth-generation pesticides, known as neonicotinoids (NEOs), are commonly used to control insect pests in vegetables, fruits, cotton, rice, and other industrial crops. NEOs are considered the best alternatives to highly harmful insecticides such as organophosphates, pyrethroids, and carbamates. However, some studies indicate that NEOs can negatively affect non-target organisms, including birds, mammals, aquatic life, and bees (**Zhang *et al.*, 2023**). Acetamiprid (ACE), a new-generation chloronicotinyl insecticide, targets nicotinic acetylcholine receptors in insects. Despite its intended use, ACE has negatively affected non-target organisms, including mammals (**Phogat *et al.*, 2022**). Male reproductive ability has been steadily decreasing with increasing levels of social industrialization. Infertility affects 13–18% of couples, and a growing body of clinical and epidemiological research indicates that male reproductive issues are becoming more common (**Durairajanayagam, 2018**). The widespread use of pesticides, along with other endocrine-disrupting compounds, is discussed as a possible contributor to the impairment of male fertility (**Costa *et al.*, 2015**). Over the past 20 years, there have been an increasing number of research investigating the casual relationship between insecticide exposure and sexual dysfunction (**Elnamaky *et al.*, 2018**; **Sharma *et al.*, 2014**). Exposure to insecticides *in vitro* or *in vivo* can cause a variety of issues with the function of Leydig or Sertoli cells, as well as deactivate any stage of the hormone regulatory process in endocrine mechanisms (**Mathur *et al.*, 2010**). A certain type of cells called Leydig cells (LCs) are found in the testis' interstitium (**Li *et al.*, 2020**). Leydig cells' (LC) primary function is to produce androgens for both the testis's internal paracrine control over spermatogenesis and the various androgenic and anabolic systemic endocrine actions that occur outside of it (**Teerds *et al.*, 2015**). Over the past few decades, possible pesticide-induced reproductive system disorders have received considerable attention (**Mehrpour *et al.*, 2014**). Neonicotinoid imidacloprid has been shown to cause reproductive abnormalities in adult male rats, including reduced plasma testosterone levels, increased formation of reactive oxygen species (ROS) in the testes, and escalated Leydig cell damage (**Bal *et al.*, 2012**). Administration of ACE in male guinea pigs resulted in decreased weights of the testes, epididymides, vas deferens, and supporting glands. Negative effects were also observed in testicular structure, testosterone concentration, testosterone response time, and epididymal sperm characteristics (**Kenfack *et al.*, 2018**). Additionally, there was a dose-dependent alteration in the morphology of seminiferous tubules in rats (**Kong *et al.*, 2017**). Compared to traditional 2D cultures, 3D cell cultures differ radically in terms of cellular dynamics, nutrition, and cell-cell contact (**Edmondson *et al.*, 2014**). 3D culture methods are getting more and more popular since they can accurately mimic the *in vivo* environment. Many epithelial systems, however, may be accurately represented in two dimensions, even when compared to *in vivo* systems. In two-dimensional surfaces, for example, lung airway epithelia will grow normally in air-liquid interface culture (**Kesimer *et al.*, 2009**; **Nalayanda *et al.*, 2009**). However, due to their simplicity, 2D monolayer cell culture methods may not always be able to replicate the *in vivo* cell development process seen in the physiological setting. The absence of a complex and biologically rich environment in these 2D systems could be the cause of this disparity. Both 2D and 3D cell culture techniques have differing effects on behaviours related to *in vivo*-like cell growth, such as cell motility, apoptosis, differentiation, and proliferation, compared to 2D planar cell cultures (**Baker & Chen, 2012**; **Friedl *et al.*, 2012**; **Bonnier *et al.*, 2015**). Unlike those in 3D cultures, gradients of cells in 2D cultivation do not affect the access to nutrients since only viable cells stay exposed on the culture surface while necrotic cells separate into the media. For instance, the surface of aggregated spheroids shows the highest levels of proliferation, whereas the interior of the 3D cell bodies has the highest concentration of necrotic or quiescent cells (**Edmondson *et al.*, 2014**). The complex relationships among cells in a three-dimensional substrate could be the cause of the variations in cell migration between dimensionalities. Since 3D cells are surrounded and attached to each other on all sides, migration is hindered, which alters cell motility and the processes that cells use (**Grinell, 2003**; **Yoshii *et al.*, 2011**; **Gjorevski *et al.*, 2015**). In this research we focused on the effect of acetamiprid on Leydig cells cultured as a scaffold-free 3D *in vitro* testicular model. Monitoring the changes in morphological parameters affected by ACE could help us understand the extent to which this substance negatively affects the reproductive system of non-target organisms.

MATERIAL AND METHODS

Cell culture and 3d in vitro setup

Leydig TM3 cell line was obtained from ATCC (American Collection of Cell and Tissue Culture, CRL-1714TM). Cells were cultured in DMEM/F12 (Sigma-Aldrich, Cat. No. D6434) medium supplemented with 5% horse serum (Sigma Aldrich), 2.5% fetal bovine serum (BiochromAG, Berlin, Germany), and 2.5 mM L-glutamine (Sigma-Aldrich) in 25 cm^2 tissue culture flask (TPP, Trasadingen, Switzerland, Cat. No. 90025) precoated with gelatine (Sigma-Aldrich, Cat. No. 9000-70-8) under defined culture conditions: 95% relative humidity, 37°C and 5% CO_2. TM3 cells were routinely screened for contamination. Cells were passages every 2 or 3 days after reaching 85-90% confluency.
A 3D Petri Dish™ (Sigma-Aldrich, Cat. No. type 24–35, Sigma-Aldrich) microforming system was used to prepare spheroids as reported previously (**Sychrová et al., 2022**). This system created agarose microgels, each consisting of 35 microwells with an inner diameter and depth of 800 µm. Each microwell formed one spheroid, arranged in a 5×7 grid, resulting in 35 spheroids per well of a 24-well plate. Initially, 2% (w/w) agarose (Sigma-Aldrich, Cat. No. 9012-36-6) in 0.9% (w/v) sodium chloride solution diluted in distilled water was prepared. The agarose solution was sterilized in an autoclave and stored at room temperature. Before use, the agarose was heated in a microwave until fully dissolved. Once cooled to approximately 65°C, 330 µL of the agarose onto pre-sterilized microforms, ensuring no bubbles formed. After solidification, the microgel was released from the microform and placed in a 24-well plate. This process was repeated until the required number of 3D Petri Dish™ microgels was prepared. The microgels were positioned in the lower right part of each well. To immobilize them, 150 µL of agarose at 40°C was pipetted into the well. The microgels were equilibrated by adding 1 mL of culture medium to each well, incubating for 1 h at 37°C. After equilibration, the culture medium was aspirated from the wells and the inner chamber of the 3D Petri Dish™ microgels, preparing them for cell seeding. The cell suspension was prepared by trypsinization with Trypsin-EDTA (Sigma-Aldrich), with cell concentration measured using a Cellometer Auto 1000 Automated Cell Counter (Nexcellom, Lawrence, MA, USA). For the experiment, the cell suspension was adjusted to a concentration of 4000 cells/spheroid. Then, 65 µL of the cell suspension was pipetted into the inner chambers of the microgels. After seeding, the microgels were left undisturbed for 15 min to allow cell stelling. Finally, 1 mL of culture medium was carefully added to each well to prevent washing out of the cells. The medium was regularly changed (day D1, D3, D7, and D10). On day 3, the prepared spheroids of Leydig TM3 cells were exposed to ACE.

Tested substance and exposure

Twenty-four hours before exposure, providing sufficient time for recovery and stabilisation, Leydig cells were inoculated in a culture medium in tissue culture plates with agarose microgels, facilitating optimal growth conditions and simplifying cell processing for the several parameters included in this study. The crystal form of acetamiprid (Acetamiprid-N-desmethyl) was purchased from Sigma-Aldrich (St. Louis, USA); molecular weight of 208.65 $g.mol^{-1}$ and purity of ≥ 99 % was used. The stock solution of ACE was prepared using DMSO (dimethylsulfoxide) and then added to the culture medium to achieve the following experimental concentrations for 3D cultivation: 4, 8, 16, 31, 63, 125, 250, and 500 µM. The final concentration of DMSO in the medium was 0.1% DMSO. The morphology of spheroids was monitored over a 7-day exposure period (from D3 to D10). On D3 we prepared various doses of acetamiprid 1.545×, because microgels absorb part of the exposing solution. Second treatment was provided on D7 with concentration of acetamiprid 1.000×, where we put away previous exposed medium and added a new one and cultivated for another 2 days. Cells were cultured in an incubator under defined conditions of 37°C, 95% humidity, and 5% CO_2.

Analysis of morphological parameters of spheroids

The formation of spheroids was documented using TissueFAXS software (Tissue Gnostics, Austria) using a Zeiss Z1 Observer automated inverted microscope (Zeiss, Germany) equipped with a 2.5× objective and a digital camera (Hamamatsu, C11440). Photomicrographs of spheroids from each well of a 24-well culture plate were taken at predetermined time intervals on the first, third, seventh, and tenth day (D1, D3, D7, D10). Size parameters (area and perimeter) and shape parameters (circularity and solidity) were assessed for each spheroid image using an in-house developed macro in ImageJ/Fiji60 software (**Basu *et al*., 2020; Basu *et al*., 2018**). The description of individual parameters is provided in **Table 2**. The median of each parameter was calculated for each duplicate (i.e., 70 spheroids) and then averaged from three independent experiments (n = 3) using MS Excel. Three independent experiments were conducted, each involving two replicate wells for each treatment.

Table 2 Characterization of geometric parameters evaluating size and shape of spheroids (2D projection of spheroids)

	Unit	Characteristic
Parameters defining the size of spheroids		
Area	μm^2	•Corresponds to the 2D projection of the spheroid onto the image plane (~its cross-sectional area) •The sum of all individual pixels within the contour of the monitored object (spheroid) •Often converted to calibrated square units (e.g., mm^2, μm^2)
Perimeter	µm	•Length of the outer limit of selection •The sum of the distances between consecutive pixels on particle outline, then converted to a unit length
Parameters defining the shape of spheroids		
Circularity	DP	$4\pi \times \frac{area}{perimeter^2}$ •Specifies an ideally circular object (= 1 – ideally circular particle, <1 – particle with elongated shape)
Solidity	DP	$\frac{area}{convex\ area}$ •Determines the ratio of the total area, the projection of the spheroid, to the imprinted convex area •The smoother and rounder the particle, the more the value the more the integrity approaches 1

DP – dimensionless parameter; convex area - the area of the convex hull

Statistic analysis

The experimental data were obtained from three independent repetitions. The results were processed using GraphPad Prism 8.01 (GraphPad Software Inc., San Diego, CA, USA) and Microsoft Office 365. Initially, descriptive statistics such as median and mean were calculated. For statistical evaluation, one-way ANOVA was performed, followed by Dunnett's multiple comparison test to determine significant differences at various levels of statistical significance (****P<0.0001; ***P<0.001; **P<0.01; *P≤0.05) or non-significance (P>0.05) between the experimental groups and the control group. The resulting values were expressed as the mean value from 3 independent experiments ± SD (standard deviation) and presented in graphs.

RESULTS

Morphological characterization of the size of spheroids cultured in static 3D Petri Dish™ system

Leydig TM3 cell spheroids, with an initial concentration of 4000 of cells/spheroid, were photographically documented via microscopy over a 10-day period. The size of the spheroids was characterized by parameters such as area and perimeter, while their shape was characterized by circularity and solidity. By the first day of culture (D1), TM3 cells had formed compact, round spheroids with relatively smooth and regular edges. On the third day, we performed ACE exposure in different concentrations (4-500 µM). In **Figure 1** (a) we displayed area of spheroids before treatment. On the other hand, **Figure 1** (b) and Figure 1 (c) determined fifth day of ACE exposure (D7) and eighth day (D10) of exposure. There have been no significant changes in both cases within the area parameter.
Another size parameter of spheroids that we observed was perimeter. Similar to previous results, there were no significant changes in the perimeter of spheroids treated with ACE doses (**Figure 2** a, b, c). The average values from three independent experiments were 49 311.21 μm^2 for area and 848.09 µm for perimeter on the third day of cells cultivation (D3).

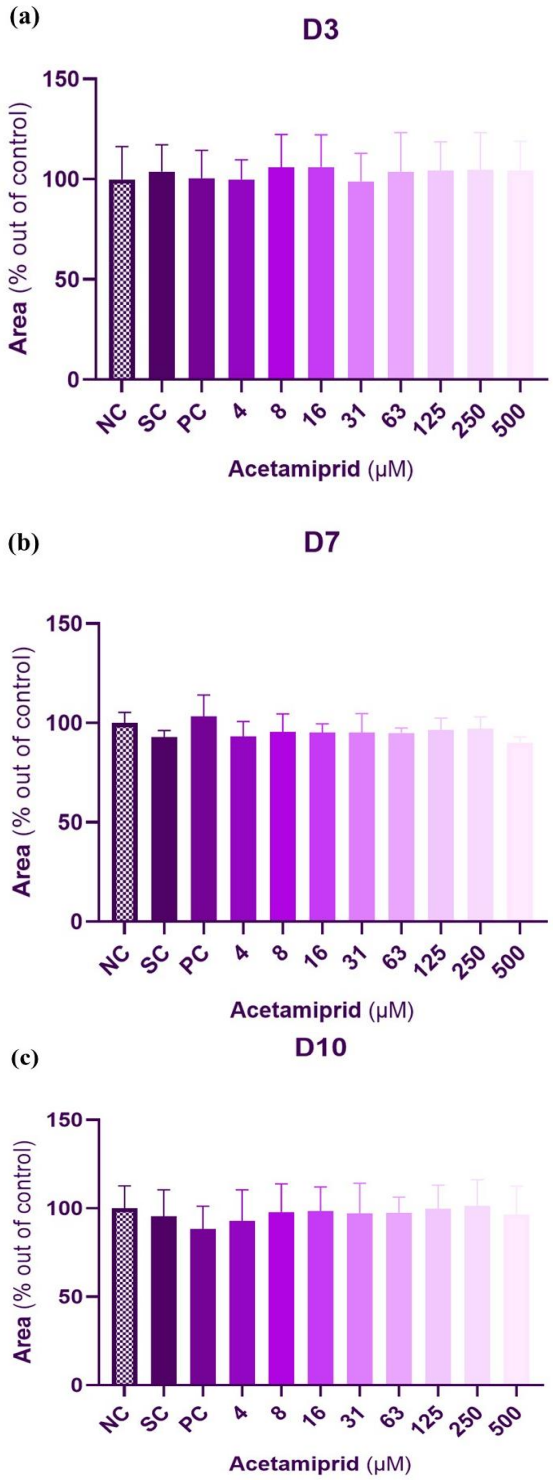

Figure 1 The relation between Leydig TM3 cell spheroid area and ACE concentrations in 3D *in vitro* culture. **(a)** graph showing the spheroid area before the ACE exposure on third day of cell cultivation; **(b)** Graph showing the spheroid area on the seventh day of culture (D7) after four days of exposure to acetamiprid. **(c)** Graph showing the spheroid area on the tenth day of culture (D10) after seven days of exposure to acetamiprid. Negative control group **(NC)** refers to the non-treated spheroids, while **SC** represents the solvent control (0.1% DMSO). The **PC** showed the results of positive control (10% DMSO). Photographic documentation was done on D1 and D3 without acetamiprid and on D7 and D10 with acetamiprid. Data points in represent individual values from independent experiments (n=3) for each time interval. The obtained results (a), (b) and (c) are expressed as mean values ± SD for each concentration and time interval.

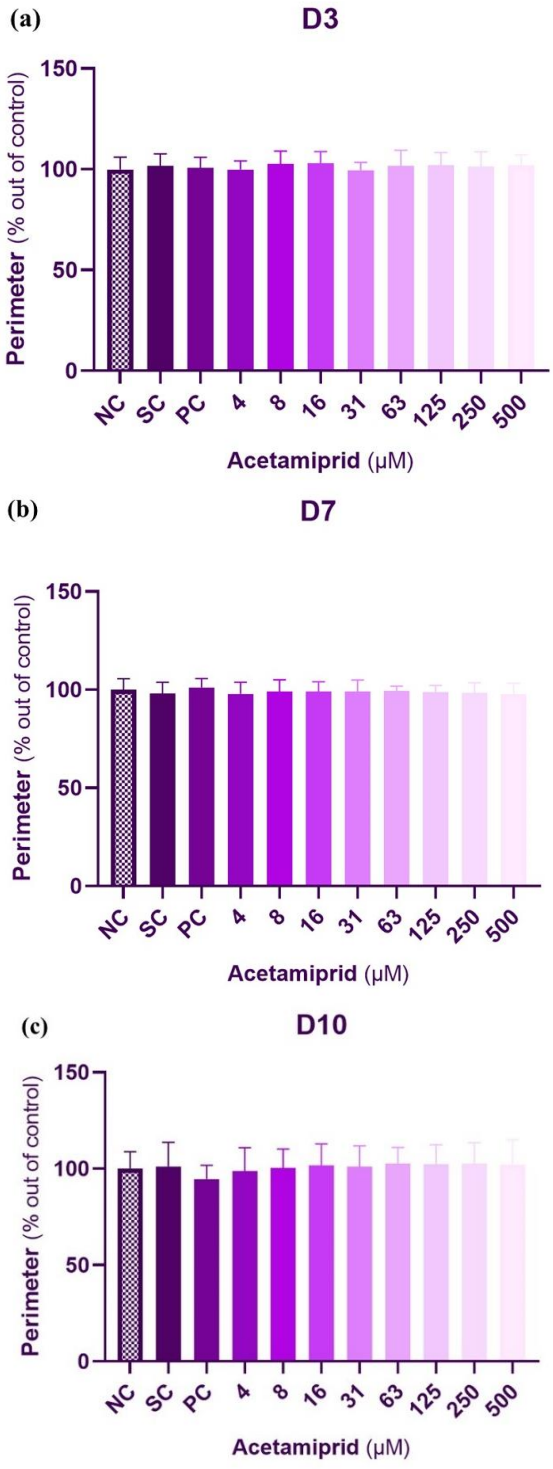

Figure 2 The relation between Leydig TM3 cell spheroid perimeter and acetamiprid concentrations in 3D *in vitro* culture. (a) graph pointing to spheroid perimeter before the ACE exposure on third day of cell cultivation; **(b)** Graph showing the spheroid perimeter on the seventh day of culture (D7) after four days of exposure to acetamiprid. **(c)** Graph showing the spheroid perimeter on the tenth day of culture (D10) after seven days of exposure to acetamiprid. Negative control group **(NC)** refers to the non-treated spheroids, while **SC** represents the solvent control (0.1% DMSO). The **PC** showed the results of positive control (10% DMSO). Photographic documentation was done on D1 and D3 without acetamiprid and on D7 and D10 with acetamiprid. Data points in represent individual values from independent experiments (n=3) for each time interval. The obtained results (a), (b) and (c) are expressed as mean values ± SD for each concentration and time interval.

Morphological characterization of shape of spheroids cultured in static 3D Petri Dish™ system

The shape parameters of the spheroids (circularity and solidity) were also monitored over seven days of acetamiprid exposure. On the first day (D1) of in vitro culture in a 3D static system, Leydig TM3 spheroids exhibited the most regular shape, resembling an almost perfectly smooth circle. The average values from three independent experiments were 0,87 for circularity and 0.96 for solidity on the third day of cells cultivation (D3). During the initial days of cultivation, the spheroids maintained their regular, spherical shape. The circularity and solidity of the spheroids was not significantly ($P>0.05$) affected depending on the effect of concentrations of acetamiprid (4-500 µM) in *in vitro* 3D static cultivation (**Fig. 3 a, b, c; Fig. 4 a, b, c**).

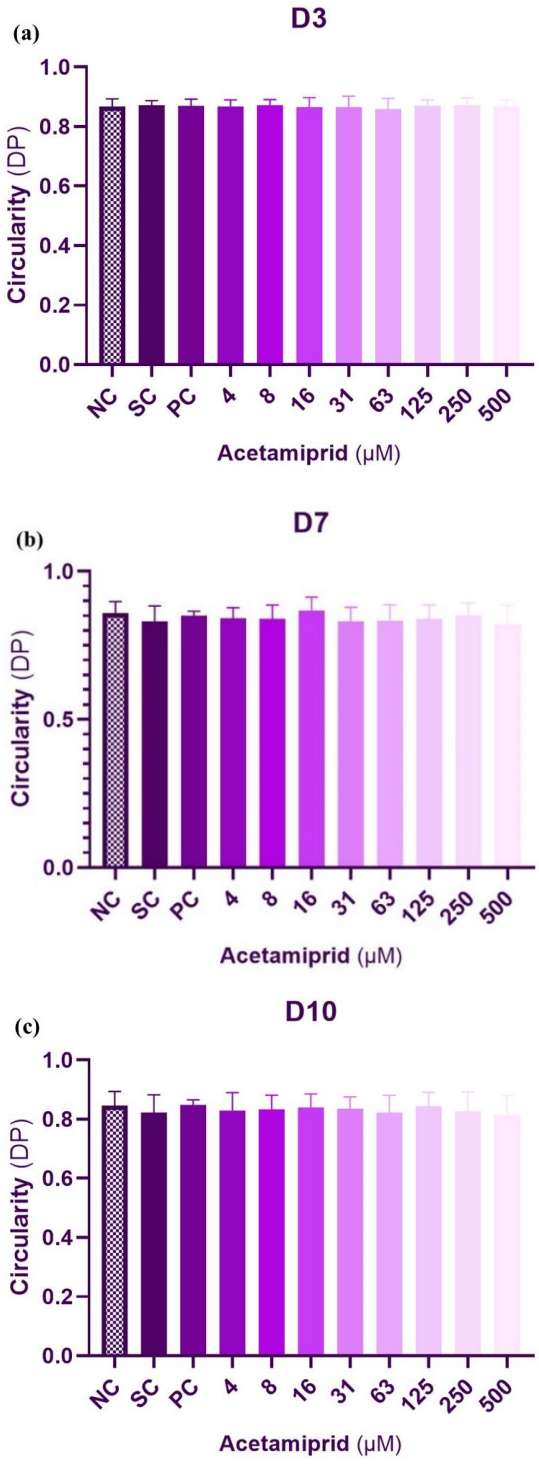

Figure 3 The relation between Leydig TM3 cell spheroid circularity and acetamiprid concentrations in 3D *in vitro* culture. (a) graph pointing to spheroid circularity before the ACE exposure on third day of cell cultivation; **(b)** Graph showing the spheroid circularity on the seventh day of culture (D7) after four days of exposure to acetamiprid. **(c)** Graph showing the spheroid circularity on the tenth day of culture (D10) after seven days of exposure to acetamiprid. Negative control group **(NC)** refers to the non-treated spheroids, while **SC** represents the solvent control (0.1% DMSO). The **PC** showed the results of positive control (10% DMSO). Photographic documentation was done on D1 and D3 without acetamiprid and on D7 and D10 with acetamiprid. Data points in represent individual values from independent experiments (n=3) for each time interval. The obtained results (a), (b) and (c) are expressed as mean values ± SD for each concentration and time interval.

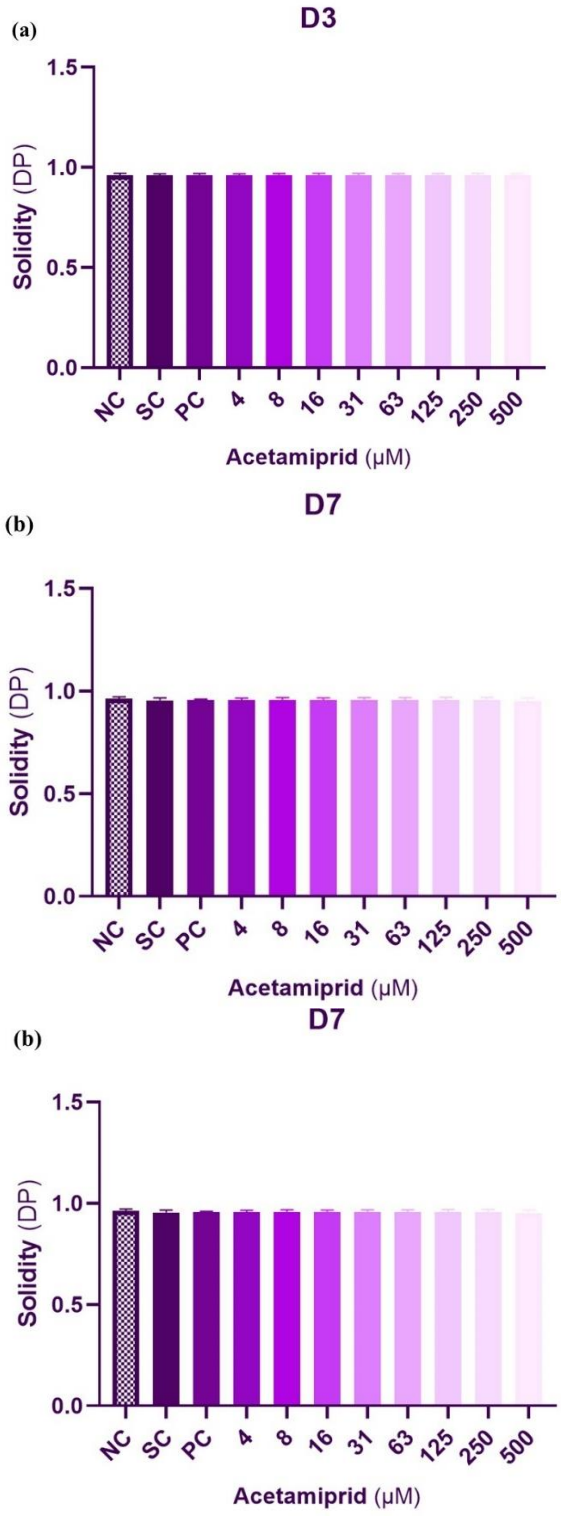

Figure 4 The relation between Leydig TM3 cell spheroid solidity and acetamiprid concentrations in 3D *in vitro* culture. (a) graph pointing to spheroid solidity before the ACE exposure on third day of cell cultivation; **(b)** Graph showing the spheroid solidity on the seventh day of culture (D7) after four days of exposure to acetamiprid. **(c)** Graph showing the spheroid solidity on the tenth day of culture (D10) after seven days of exposure to acetamiprid. Negative control group **(NC)** refers to the non-treated spheroids, while **SC** represents the solvent control (0.1% DMSO). The **PC** showed the results of positive control (10% DMSO). Photographic documentation was done on D1 and D3 without

acetamiprid and on D7 and D10 with acetamiprid. Data points in represent individual values from independent experiments (n=3) for each time interval. The obtained results (a), (b) and (c) are expressed as mean values ± SD for each concentration and time interval.

DISCUSSION

Currently, the occurrence of infertility in humans is becoming increasingly prevalent worldwide. A large number of xenobiotics represent potential threats to various animal species and humans. Among the most commonly used contaminants in agriculture are pesticides, which act as toxicants through several mechanisms during both acute and long-term exposure (**Kara *et al*., 2019**). Neonicotinoids are a relatively new group of insecticides widely used due to their high toxicity to various insect species, ease of application, long persistence, and systemic nature. These properties increase their hazard and risk, including potential toxicity to non-target organisms such as mammals (**Bonmatin *et al*., 2015**). While acetamiprid primarily targets the neurological systems of insect species, certain research indicates that it adversely affects mammalian reproductive systems (**Arıcan *et al*., 2020**). In our study, we utilized recently established *in vitro* testicular models of Leydig TM3 spheroids (**Sychrová *et al*., 2022**) to study the potential male reproductive toxicity of ACE.

In the presented work, we determined the possible influence of acetamiprid (4 – 500 µM) and the time interval (D1, D3, D7, D10) on the morphological parameters of spheroids (parameters for size and shape). However, the obtained results did not show significant changes ($P<0.05$) in both cases exogenous factors. There are not many studies looking at the effects of the insecticide and cultivation time in parallel. Considering this fact, we decided to compare ourselves achieved results with the results of analyzes where the same methodological procedure was used with the influence of a different endocrine disruptors (ED). In previous study, authors investigated the effect of selected EDs, including insecticide methoxychlor, on the TM3 line of prepubertal Leydig cells in a 3D culture system *in vitro*. During the experiment, authors evaluated several morphological parameters (depending on the concentration of cells/spheroid; 8000 vs. 4000 vs. 1000 cells/spheroid), along with those analyzed in our research. Micrographs for morphology assessment were taken over 14 days (D1, D3, D7, D11 and D14). Selected concentrations were in range of 0.02-100 µM. Within all the identical analyzed morphological characteristics (area, roundness, solidity), it can be stated that there was a significant decrease in values for each of the results (**Sychrová *et al*., 2022**). Using this advanced model, we investigated the impact of acetamiprid (4 – 500 µM) on the size and shape of Leydig TM3 spheroids over the prolonged 7 days repeated exposure. The obtained results did not show significant changes in any studied parameters even after 7 days. An earlier study examined the effect of orally given ACE in concentrations 26.67, 40 and 80 mg/kg body weight, to adult male guinea pigs during 90 days. Results showed that the administration of ACE led to a significant decrease in the testosterone concentration, reproductive organs weights, sperm count, sperm motility, plasma membrane integrity, reaction time and a significant increase of abnormal spermatozoa (**Kenfack *et al*., 2018**). **Mosbah *et al*. (2017)** investigated the toxic effect of ACE on the reproductive system in male rats. The male Wistar rats were treated with ACE in concentration 27 mg/kg b. w. for 45 consecutive days. ACE induced a significant decrease in total weights of the reproductive organs (testis, seminal vesicles and epididymis), while abnormal sperm level was significantly elevated. Acetamiprid was orally given to pregnant rats at a dose level of 31.4 mg/kg b.w. (equivalent to 1/10 LD50). Pregnant rats treated with ACE throughout the organogenesis phase resulted in morphological, soft tissue, and skeletal abnormalities, a developmental hazardous effect (**Abou-Zeid, 2018**). **Zhang *et al*., 2011** supplied Kunmin male mice with dose of ACE 30 mg/kg for 35 days. The weight of the testicles, epididymis, seminal vesicle, prostate, and other testosterone-responsive organs was shown to be greatly reduced by ACE. In addition, acetamiprid markedly decreased the level of serum testosterone as well as the number, viability, motility, and intactness of the sperm acrosome. The mice administered ACE indicated damaged seminiferous tubules and Leydig cells, as evidenced by the histological examination of the testes. Moreover, degradation of the mitochondria and endoplasmic reticulum in Leydig cells was observed.

Ibrahim *et al*. (2023) demonstrated impact of neonicotinoid imidacloprid (IMI) on a rat Leydig cell line (LC-540) in doses 75-800 µM incubated for 24, 48 and 72 hours. Morphological alterations were observed in all treatment groups, becoming more pronounced with higher doses. In the control groups, mitochondria were evenly dispersed throughout the cytoplasm. Among the alterations observed was cellular shrinkage linked to an aberrant distribution of mitochondria. Additionally, a significant decrease in the size (area and perimeter) and length (form factor and aspect ratio) of the mitochondria was found in all treatment groups compared to controls, as determined by quantitative analysis of mitochondrial morphology. A study by **Issa *et al*. (2016)** established the relationships between the IMI dosages and sperm morphology and histopathological findings in subchronically exposed mature male rats. IMI doses were selected based on its LD50 (450 mg/kg b.w.) IMI were given orally in various concentrations 45, 90 and 450 mg/kg for 15 days. The results showed vacuolar degeneration of germinal epithelium and loss of spermatids. Spermatogonial cell development appears to have stopped in seminiferous tubules, with spermatogonial cell development appearing to have stopped in the seminiferous tubules. Subsequent results showed various morphological changes in sperm.

There are many studies, that proved negative impact of ACE on male reproductive system, beside the morphology properties. The harmful effects of ACE on reproduction were examined in a prior study (**Arıcan *et al*., 2020**). Male rats were administered the ACE samples orally at doses of 12.5 mg/kg, 25 mg/kg and 35 mg/kg for 90 days. The findings demonstrated a dose-dependent decline in both sperm count and plasma testosterone levels.

CONCLUSION

In our study, there were no significant changes in the morphology of TM3 cells *in vitro* in used dosages of acetamiprid. These results can be caused due to acetamiprid physical and chemical properties, associated with the formation and accumulation period of individual metabolites of acetamiprid. However, further research is needed for better understanding of the acetamiprid mechanism of action in the 3D system *in vitro* of Leydig cells.

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