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IN VITRO ASSESSMENT OF CARBOXYLATED POLYSTYRENE NANOPLASTICS TOXICITY IN TM3 LEYDIG CELLS

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ABSTRACT

Pollution by plastic microparticles is a growing concern for both the environment and biological systems, including reproductive health. Nanoplastics can accumulate in the testis tissue and result in testosterone insufficiency and reproductive dysfunction in males, according to many *in vivo* studies. In the natural environment, plastic particles are rarely found in their original form because physical, chemical and biological processes lead to surface modifications that can alter their biological interactions. The present study aimed to evaluate the potential *in vitro* cytotoxicity of carboxylated polystyrene nanoplastics (PS-COOH NPs) at a size of 0.03 µm and dosages of 1, 10, 50, 100 and 200 µg/mL, representing environmentally weathered nanoplastics, on TM3 Leydig cells, with particular focus on cell viability, metabolism, oxidative stress, and steroidogenic function. TM3 Leydig cells were exposed to PS-COOH NPs for 24 h, followed by assessment of cell viability, proliferation and metabolic activity using MTT and Alamar Blue assays. Intracellular superoxide production as a marker of oxidative stress was measured by the nitroblue tetrazolium assay, and testosterone levels were determined using ELISA analysis. Our results demonstrated a concentration-dependent inhibition in cellular metabolic activity, with significant decline observed at concentrations 100 and 200 µg/mL. Increased superoxide radical production was detected at lower concentrations (1–50 µg/mL), indicating the induction of oxidative stress in metabolically active cells. Testosterone production was significantly suppressed at all tested concentrations, including those that did not markedly affect cell viability. The most significant reduction in testosterone levels was observed at the highest tested concentration of PS-COOH NPs. Overall, findings of this study suggest that carboxylated polystyrene nanoplastics may disrupt Leydig cell function by inducing oxidative stress and impairing steroidogenesis. These results highlight the potential endocrine-disrupting effects of environmentally relevant nanoplastics and emphasize the need for further studies.

Keywords: carboxylated polystyrene nanoplastics; Leydig cells; cell viability, oxidative stress; steroidogenesis; testosterone; endocrine disruption

INTRODUCTION

The contamination of ecosystems with micro- and nanoplastics (MNPs) poses a significant environmental and public health threat that is increasingly becoming more widespread due to growing awareness of the harm they pose to living organisms, including the human population. These plastic particles originate from different sources and undergo complex transformation processes in the environment, eventually accumulating in biological systems involving all forms of life. Microplastics (1 µm - 5 mm) and nanoplastics (<1 µm) are categorized as either primary (manufactured on purpose) or secondary (resulting from environmental degradation of larger plastic objects). Secondary MNPs form through various environmental weathering processes, including photodegradation by UV radiation, thermooxidation, mechanical abrasion, and limited microbial biodegradation (**Gigault *et al*., 2018; Liu *et al*., 2020**).

The most prevalent environmental microplastics and nanoplastics include polystyrene particles from food containers, polyethylene from packaging, polypropylene from textiles, polyethylene terephthalate from bottles, and polyvinyl chloride from construction materials (**Campanale *et al*., 2020**). These microparticles have been identified in all environmental compartments, including agricultural soils, atmospheric deposition, aquatic systems, and polar ice cores, illustrating their global spread (**Brahney *et al*., 2020; Allen *et al*., 2019**). In biological systems, MNPs with various surface modifications have been observed in marine organisms such as zooplankton and bivalves, terrestrial species including earthworms and birds, and alarmingly, in human tissues including placenta, blood, and lung samples (**Ragusa *et al*., 2021; Leslie *et al*., 2022**).

Environmental samples have been found to include a variety of surface-modified MNPs. Aminated (NH_2-modified) MNPs, which are produced by interactions with nitrogen-containing substances, are frequently observed close to wastewater treatment facilities (**Schirinzi *et al*., 2017**), sulfonated particles (SO_3H-modified) frequently occur in marine environments due to reactions with sulfur oxides in seawater (**Ter Halle *et al*., 2017**), particularly common in atmospheric samples are hydroxylated (OH-modified) MNPs, which are produced by interactions with hydroxyl radicals (**Brahney *et al*., 2020**). Environmental weathering of plastics, which includes oxidative processes (UV photodegradation, interactions with reactive oxygen species), chemical breakdown in acidic conditions, and microbiological transformation, results in carboxylated micro and nanoplastics. These procedures increase the hydrophilicity and bioavailability of particles by producing carboxyl groups (-COOH) on their surfaces. Consequently, carboxylated plastics are more frequently detected in ecosystems and pose higher risks to biological systems due to their enhanced ability to penetrate cell membranes and disrupt cellular processes (**Gewert *et al*., 2015; Auta *et al*., 2017**). Unaltered hydrophobic surfaces dominate early-stage plastic breakdown, however, prolonged contact with the environment leads to the accumulation of more polar functional groups (**Gewert *et al*., 2015**).

As mentioned, the small size and variable surface chemistry of MNPs allows their penetration of biological barriers and interaction with cellular structures. Due to research showing testicular accumulation, blood-testis barrier penetration, and germ cell DNA damage, it has raised significant concerns regarding male reproductive health (**Wu *et al*., 2020; Stabnikova *et al*., 2022**). According to recent findings, different surface-modified MNPs can reduce testosterone production by 30-40% at environmentally relevant doses, alter Sertoli cell function, and induce abnormal sperm morphology (**He and Yin, 2023**). The mechanisms appear to involve oxidative stress generation, disruption of steroidogenic enzymes like StAR and CYP11A1, and alteration of hormonal signalling pathways (**Pivonello *et al*., 2019**).

Another crucial aspect of MNPs' toxicity is their "Trojan horse" effect. They can absorb and concentrate a variety of environmental contaminants, such as heavy metals, endocrine disruptors, and persistent organic pollutants (POPs), thanks to their high surface area-to-volume ratio and hydrophobic qualities (**Brennecke *et al*., 2016**). Once entering the organism, MNPs can facilitate the transport of these toxic substances across biological barriers that they might not otherwise penetrate (**González-Acedo *et al*., 2021**). Additionally, research shows that MNPs can increase the bioavailability of chemicals that have been adsorbed by extending their retention in the gut, encourage cellular uptake of contaminants by internalizing the particles, and possibly synergize with carried pollutants to produce more toxic effects (**Barboza *et al*., 2018**).

The endocrine-disrupting potential of MNPs manifests through various mechanisms. Firstly, plastic polymers and their additives, such as phthalates and

bisphenols, can directly mimic or interfere with natural hormones (**Pivonello *et al*., 2019**). Studies have shown that in this manner, MNPs can bind to estrogen and androgen receptors, potentially altering normal hormonal signalling pathways (**Chen *et al*., 2021**). Second, by altering the hypothalamic-pituitary-gonadal axis, the inflammatory response brought on by MNPs accumulation in tissues may indirectly impair endocrine functions. Particularly concerning are data that indicate micro and nanoplastics can build up in endocrine glands, including the testes, thyroid, and adrenal glands, where they may locally disrupt hormone production (**Ragusa *et al*., 2022**). Because of their dual threat, functioning as both inherent endocrine disruptors and transporters of other dangerous substances, MNPs are especially detrimental to reproductive health. The observed effects on steroidogenic cells at lower doses than would be expected from the toxicities of individual compounds could be explained by the combination of chemical co-transport and direct endocrine disruption **(He and Yin, 2023)**. Furthermore, male fertility is particularly at risk due to MNPs' capacity to pass across the blood-testis barrier while carrying adsorbed pollutants, which could expose growing sperm to a variety of harmful substances (**Xi *et al*., 2020**).

In this study, the model for environmentally weathered nanoplastics, which represents modifications commonly occurring in nature, is carboxylated polystyrene nanoparticles (PS-COOH NPs). By employing *in vitro* approaches, we aim to elucidate the interactions between PS-COOH NPs and Leydig cell physiology, with findings that may extend to understanding the effects of environmentally relevant nanoplastics on male reproductive health.

MATERIAL AND METHODS

Cell culture and treatment

The experiment was conducted using the cell line TM3 (Leydig cells, ATCC CRL-1714; Washington, USA). The cells were cultivated in a 37°C humidified atmosphere with 5% CO_2 and 95% saturated atmospheric humidity in complete culture media containing Dulbecco's modified Eagle's Medium F/12 (DMEM F/12, Sigma-Aldrich, St. Louis, MO USA), supplemented with 5% horse serum (Sigma-Aldrich, St. Louis, MO, USA), 2.5 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 2.5% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotics solution (penicillin/streptomycin, Sigma-Aldrich, St. Louis, MO, USA). After achieving 85-90% confluence, TM3 cells were passaged and subcultured in standard 75 cm^2 culture flasks at a 1:50 ratio, cell culture was regularly checked for morphology and microbiological contamination. To enable appropriate cell adhesion and stability, the cells were plated at concentration 10 000 cells per well and pre-cultivated for 24 hours in a 96-well plates before being exposed to polystyrene nanoplastics.

In this study, 0.03 µm carboxylate-modified polystyrene latex beads were used, provided as a sterile 2.5% (w/v) aqueous suspension (Sigma-Aldrich, St. Louis, MO, USA), corresponding to concentration 25 mg/mL. Several limitations of this study regarding the characterization of nanoplastics must be acknowledged: The physicochemical properties of polystyrene nanoplastics in culture media, including hydrodynamic size, aggregation behavior, and zeta potential, have not been experimentally characterized under the specific conditions used in this experiment. According to the manufacturer's specifications, the particles are supplied as monodisperse carboxylated polystyrene nanoparticles with a nominal diameter of 0.03 µm. However, it should be considered that their behavior in biological media may vary due to aggregation and interactions with media components. Before application, the particle suspension was briefly sonicated (2 min, 40 kHz) and vortexed to ensure homogeneous dispersion. A working solution of nanoplastics in culture medium was prepared from this stock suspension. Dilutions were subsequently prepared from the working solution to achieve the desired final concentrations (1, 10, 50, 100 and 200 µg/mL). Although particle suspensions were sonicated prior to exposure to ensure homogeneous dispersion, their stability during the 24 h incubation period was not directly monitored in this study.

TM3 Leydig cells were exposed to nanoplastic particles at the above concentrations. Cells were treated after 24 h of adherence to the surface of the culture wells. Before each experiment, the stock suspension of carboxylated polystyrene particles was briefly sonicated (2 min, 40 kHz) to prevent aggregation and ensure uniform dispersion. Exposure was performed in standard culture medium at 37 °C, 5% CO_2 for 24h. As a negative control, cells were cultured without the presence of polystyrene particles. Since polystyrene particles can absorb or scatter light in the range used in colorimetric assays, so-called blank assays were performed. A solution of nanoplastics at appropriate concentrations without cells was added to selected wells and an identical staining procedure (MTT, NBT, alamarBlue) was subsequently performed. The measured optical density values were subtracted as background from the values obtained in the experimental groups to eliminate interferences. The post-exposure medium was collected from the wells, centrifuged and used to analyze steroidogenesis in Leydig cells by monitoring testosterone concentration in the experimental groups. All analyses were performed in three independent experiments with different cell passages to obtain objective results.

Cell viability and proliferation assays

Cell viability was determined using the MTT assay and the Alamar Blue assay. Cells were seeded at sub-confluent density to allow sufficient space for proliferation during the 24-hour exposure period. Therefore, changes in metabolic activity (MTT, Alamar Blue) reflect not only cell viability but also the extent of cell proliferation. As mentioned, plastic microparticles can interfere with colorimetric measurements, so the use of two different metabolic assays allowed for cross-validation of the results. The MTT assay assesses mitochondrial activity via formazan formation, while the Alamar Blue measures the overall redox metabolism of the cells, the combination of these methods increases the reliability of data interpretation.

The MTT assay as colorimetric assay for assessing TM3 Leydig cell metabolic activity was performed after 24 h of exposure to PS-COOH NPs (1, 10, 50, 100, 200 µg/mL). After exposure, culture medium with tested concentrations of PS-COOH NPs was collected and frozen for later hormone quantification. Then, the MTT (Sigma-Aldrich, St. Louis, MO, USA) solution prepared with DMEM/F12 medium without phenol red (100 µL; 1 mg/mL) was added to each well and incubated for 3 h in a 37°C humidified atmosphere with 5% CO_2 and 95% saturated atmospheric humidity. Subsequently, culture medium with MTT was removed from the wells of the culture plate, cells were washed with Dulbecco′s Phosphate Buffered Saline without calcium chloride and magnesium chloride (Sigma-Aldrich, St. Louis, MO, USA). Formed formazan crystals in the cells were dissolved using DMSO (Sigma-Aldrich, St. Louis, MO, USA), and the absorbance was read at 570 nm against 620 nm as a reference using a microplate reader Multiscan FC (Thermo Fisher Scientific, Waltham, MA, USA) ($n = 3$ independent experiments; 8 technical replicates / concentration).

The AlamarBlue assay was employed to assess dehydrogenase activity in exposed cells by measuring the enzymatic reduction of resazurin, a commercially available redox indicator. Briefly, after the exposure period, wells were aspirated, cells were washed with DPBS, and AlamarBlue reagent (5% (v/v); Sigma-Aldrich, St. Louis, MO, USA 5%) was added to the wells. Following a 1 hour incubation at 37 °C in a humidified atmosphere (95% humidity, 5% CO_2), absorbance was measured at 570 nm with 600 nm as a reference wavelength using a microplate reader Multiscan FC (Thermo Fisher Scientific, Waltham, MA, USA) ($n = 4$ independent experiments; 8 technical replicates / concentration).

Intracellular production of reactive oxygen species

The intracellular superoxide radical level was measured using the nitroblue-tetrazolium (NBT; Sigma-Aldrich, St. Louis, MO, USA) test. This colorimetric technique evaluates blue formazan deposits, which are formed when superoxide radicals reduce the membrane-permeable, yellow-colored nitroblue-tetrazolium chloride (2,2'bis(4-nitro-phenyl)-5,5'-diphenyl-3,3'-dimethoxy-4,4'-diphenylene) diterazolium chloride; Sigma-Aldrich, St. Louis, MO, USA). In 96-well plates, mouse TM3 Leydig cells were pre-cultured for 24 hours at a density of 10 000 cells per well. The cell culture medium was then changed to DMEM/F12 supplemented with PS-COOH NPs at appropriate dosages (1, 10, 50, 100, 200 µg/mL) for 24 of exposure. The NBT salt was prepared in DMEM/F12 medium without phenol red supplemented with 1.5% DMSO to a final concentration of 1 mg/mL and subsequently added to the Leydig cells. Following a 3-hour incubation at 37 °C (humidified atmosphere with 5% CO_2) the resulting blue formazan deposits were released from the exposed cells using 2M potassium hydroxide (KOH; p.a. CentralChem, Bratislava, Slovakia) and subsequently dissolved by adding DMSO. Absorbance was then measured at 620 nm, with 570 nm used as a reference wavelength, using a Multiscan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) ($n = 4$ independent experiments; 8 technical replicates / concentration).

Assessment of steroid hormone production

After a 24-hour exposure of Leydig cells to selected concentrations of PS-COOH NPs, culture medium was collected and centrifuged at 300× g for 10 min at 4 °C. Collected supernatant was stored at -80 °C until steroid hormone quantification. The analysis of the presence and level of testosterone in the samples was performed using ELISA analysis. Enzyme-linked immunosorbent assay (ELISA) is a method for detecting the presence of antigens/antibodies through highly specific antibody-antigen interactions in biological samples. Commercially available ELISA kits for testosterone were purchased from Dialab (Cat. #K00234, Dialab, Austria). The procedure was performed according to the manufacturer's instructions. Briefly, sample aliquots and calibration standards were added to wells pre-coated with specific capture antibodies and incubated to allow binding. Subsequently, enzyme-labeled detection antibodies were added to form a sandwich complex. After washing to remove unbound components, a chromogenic substrate was applied. Following a 15-minute incubation in the dark, the enzymatic reaction was stopped, and absorbance was measured at 450 nm using an ELISA microplate reader (Multiscan FC, Thermo Fisher Scientific Inc.), with the final coloration of the samples being inversely proportional to the amount of testosterone detected, as it is a competitive ELISA. The data obtained from the measurements were expressed as % of the control.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Descriptive statistics, including minimum, maximum, and standard error of the mean (SEM), were first assessed. Differences between PS-COOH NPs -treated groups and the control group were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons. Prior to statistical analysis, data were tested for normality using the Shapiro–Wilk test, and parametric tests were applied accordingly. Statistical significance was considered at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Data was obtained from 4 independent experiments (n = 4) using cells from different passages. Results are presented as means ± SEM and expressed as a percentage relative to the control group (100%) for all parameters.

RESULTS

Cell viability and proliferation

MTT assay was used to measure the viability of TM3 Leydig cells after exposure to different concentration of PS-COOH NPs. As shown in Figure 1, the cell viability was influenced by nanoplastic in a dose-dependent response. The PS-COOH NPs -treated cells showed significantly declined viability to 81.5 % and 86.1 % for the 200 ($p < 0.001$) and 100 ($p < 0.05$) µg/mL concentrations, respectively. Similarly, the other experimental groups showed a tendency of decreasing viability with increasing nanoplastic concentration compared to the control group, but without statistically significant differences (98.4 % for 1 µg/mL; 93.1 % for 10 µg/mL; 88.6 % for 50 µg/mL).

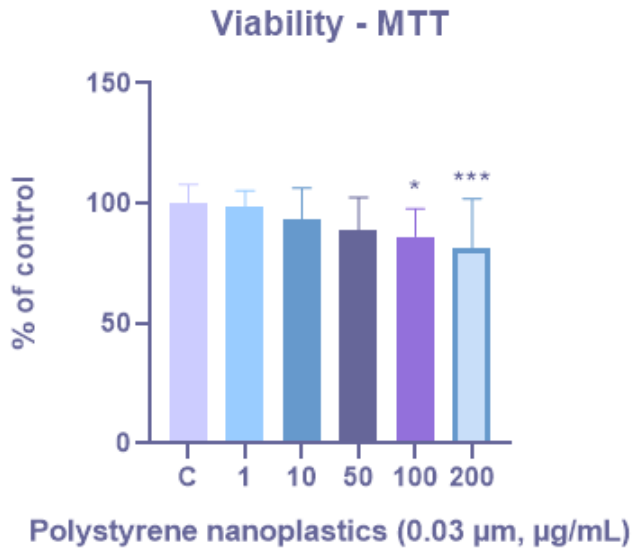

Figure 1 Viability of TM3 Leydig cells exposed to carboxylated polystyrene nanoplastics after 24 hours according to MTT reduction assay. Results are expressed as mean ± SEM, n = 4 for each group (control and experimental). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

For cross-validation of the viability results due to the possibility that plastic nanoparticles may interfere with colorimetric analyses, the metabolic activity of treated Leydig cells was also determined by the AlamarBlue assay. Based on this test, the proliferation status of Leydig cells decreased significantly to 93.6 % in the presence of 200 µg/mL PS-COOH NPs ($p < 0.01$); to 93.9 % in Leydig cells exposed to a concentration of 100 µg/mL PS-COOH NPs ($p < 0.01$); and to 94.1 % in the experimental group treated with 50 µg/mL PS-COOH NPs ($p < 0.05$) after 24 hours of cultivation compared to the control group. Slight decline in viability was observed also at lower concentrations of PS-COOH NPs, with cells exposed to 10 µg/mL showed values 96.7 % and 99.1 % for 1 µg/mL PS-COOH NPs, respectively, but without statistically significant differences compared to the control group (Fig. 2).

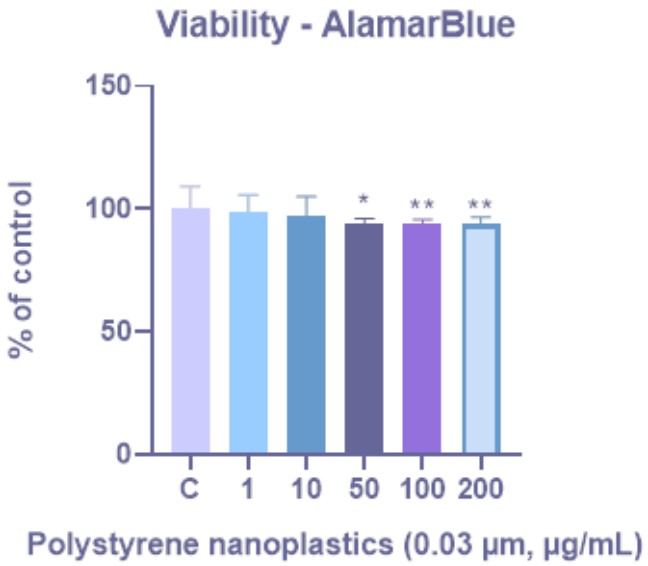

Figure 2 Viability of TM3 Leydig cells exposed to carboxylated polystyrene nanoplastics after 24 hours according to AlamarBlue assay. Results are expressed as mean ± SEM, n = 4 for each group (control and experimental). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Intracellular production of reactive oxygen species

The effects of carboxylated polystyrene nanoplastics on TM3 Leydig cells' superoxide radical production are shown in Figure 3. Exposure to low concentrations of nanoplastics resulted in a statistically significant increase in superoxide levels: 112.1 % at 1 µg/mL, 110.9 % at 10 µg/mL, and 111.1 % at 50 µg/mL ($p < 0.01$). These findings indicate an early oxidative stress response induced by nanoplastics even at environmentally relevant doses. At higher concentrations, a decrease in ROS production was observed: 97.0 % at 100 µg/mL and 94.3 % at 200 µg/mL. This decline is likely associated with the reduced number of viable and metabolically active cells at these doses, as confirmed by viability assays. Despite possible intracellular oxidative damage, the overall superoxide output is reduced due to decreased cellular density and metabolic capacity following cytotoxic exposure.

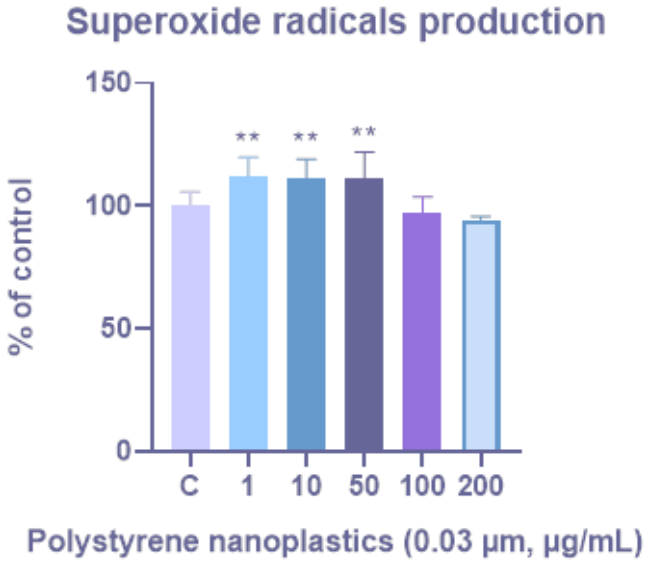

Figure 3 Production of superoxide radicals by TM3 Leydig cells exposed to carboxylated polystyrene nanoplastics after 24 hours according to NBT assay. Results are expressed as mean ± SEM, n = 4 for each group (control and experimental). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Testosterone production

Testosterone levels in TM3 Leydig cells exposed to carboxylated polystyrene nanoplastics were quantified using ELISA and expressed as a percentage relative to the untreated control, which represents 100%. Dose-dependent suppression of testosterone synthesis was observed across all tested concentrations. At the lowest concentration of 1 µg/mL, testosterone levels were already significantly reduced to 72.9% of control ($p < 0.05$), and further declined to 65.3% at 10 µg/mL ($p < 0.01$). Although slightly less affected, cells treated with 50 and 100 µg/mL also exhibited significantly reduced testosterone production, reaching 72.1% and 72.7%, respectively ($p < 0.01$). The most substantial decline was observed at 200 µg/mL, where testosterone levels dropped to just 62.1% of control values ($p < 0.001$) (Fig. 4). While reduced hormone production at higher concentrations (100 and 200 µg/mL) may, at least in part, result from decreased cell viability and metabolic activity, the decline observed already at 1 and 10 µg/mL, where cell viability remained largely preserved, strongly suggests a direct disruption of steroidogenic pathways. These findings indicate that even environmentally relevant concentrations of PS-COOH nanoplastics can impair testosterone biosynthesis independently of cytotoxicity.

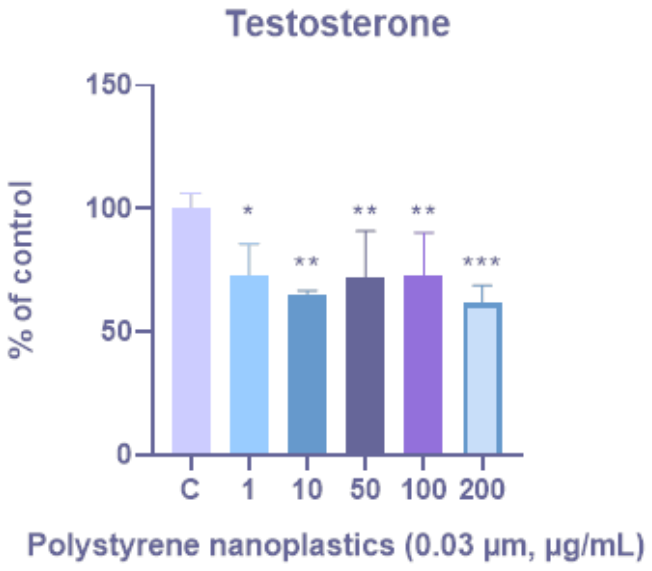

Figure 4 Testosterone production by TM3 Leydig cells exposed to carboxylated polystyrene nanoplastics after 24 hours according to ELISA assay. Results are expressed as mean ± SEM, n = 4 for each group (control and experimental). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

DISCUSSION

Microplastics and nanoplastics (MNPs) have garnered significant attention among the myriad of detrimental compounds impacting animal and plant health, due to the constant exposure of organisms to these particles (**Alimba and Faggio, 2019**). The growing presence of MNPs in ecosystems and their potential to infiltrate mammalian tissues highlight the need for thorough investigation into their biological effects (**Gao *et al*., 2024**). MNPs in the environment gradually develop new surface properties due to physical, chemical, and biological influences. Their surfaces can be modified by bacteria, other microorganisms, or natural organic matter. At the same time, exposure to UV light and oxygen produces reactive oxygen species that break down the plastic structure, forming new functional groups and changing the stability and behavior of MPs in organisms (**Wang *et al*., 2021**). Carboxylated polystyrene micro- and nanoplastics have potential to exhibit biological toxicity due to their high surface reactivity and enhanced cellular uptake. Their negatively charged carboxyl groups increase colloidal stability in biological fluids and facilitate interactions with cellular membranes and proteins. Several studies have shown that PS-COOH particles can induce oxidative stress, mitochondrial dysfunction, and inflammation in mammalian systems (**Schultz *et al*., 2021; Tomonaga *et al*., 2025**).

In this study, the effects of carboxylated polystyrene nanoparticles of 0.03 µm in size on TM3 Leydig cells were evaluated due to their central role in testicular steroidogenesis, enabling assessment of both cell viability and functional impairment upon exposure. The selection of concentration range (1 – 200 µg/ml) reflects both environmentally relevant levels and higher experimental doses commonly used in *in vitro* toxicology studies. Current data suggest that the actual concentrations of micro- and nanoplastics in the environment and biological systems are generally low, and their quantification remains difficult due to methodological limitations. However, localized exposure scenarios and bioaccumulation may lead to higher effective concentrations at the tissue or cell level (**González-Acedo *et al*., 2021; Rubio *et al*., 2020**). *In vitro* experiments often use a wider range of concentrations, including levels above environmental levels, to establish dose-response relationships and identify potential mechanisms of toxicity (**Paul *et al*., 2023**). In this context, the lower concentrations used in this study (1–10 µg/ml) can be considered more environmentally relevant, while the higher concentrations provide insight into potential cellular responses under conditions of increased exposure.

Our findings demonstrate a dose-dependent reduction in cell viability, elevated ROS production and significant suppression of testosterone synthesis even at sub-toxic concentrations. The MTT assay revealed a significant reduction in mitochondrial activity at 100 and 200 µg/mL, suggesting impairment of mitochondrial function at elevated nanoplastic concentrations. Leydig cell viability was assessed using two complementary metabolic assays, MTT and AlamarBlue, which reflect mitochondrial function, overall cellular redox activity and proliferation, respectively. Both assays demonstrated a concentration-dependent decline in metabolic activity, indicating cytotoxic effects at higher exposure levels after 24 hours of exposure. As previous studies have shown, mitochondria represent the primary intracellular target for the action of polystyrene MNPs, where accumulation and disruption of electron transport and the respiratory chain (**Xia *et al*., 2008**), as well as ATP synthesis and mitochondrial membrane potential, occur (**Ma *et al*., 2024**). *In vitro* experiments of **Shi *et al*.** (**2021**) and **Kik *et al*.** (**2021**) with different cell lines also confirmed that carboxylated polystyrene nanoparticles induce disruption of mitochondrial function and overgeneration of reactive oxygen species even in the absence of acute cell death, indicating the hypothesis that declining MTT values reflects early mitochondrial injury rather than nonspecific cytotoxicity.

Resazurin reduction within the Alamar Blue assay unveiled a milder but statistically significant inhibition in overall metabolic activity beginning at 50 µg/mL, which continued at higher doses. As the Alamar Blue test indicates global cellular redox homeostasis rather than mitochondrial activity alone, these findings suggest that PS-COOH NPs may initially interfere with cellular metabolism prior to the onset of more pronounced cellular dysfunction and potential involvement of mitochondrial processes cannot be excluded. Similar results associated with differences in assays have been reported in other *in vitro* nanoplastic studies, where early metabolic alterations precede detectable reductions in mitochondrial activity or membrane integrity in various hematopoietic cell lines (**Rubio *et al*., 2020**) and in a gastric cell line (**Banerjee *et al*., 2021**).

As cells were seeded before exposure into wells at sub-confluent density to allow proliferation, both assays also reflect the proliferative capacity of Lezdig cells. Therefore, the observed decline in metabolic activity at higher concentrations may reflect not only reduced cell viability but also impaired proliferation, consistent with study of **Park *et al*.** (**2025**), which linked *in vitro* cytotoxicity of carboxy polystyrene nanoplastics (50 nm; 2 µg/mL) to induction of chromosomal instability by disrupting mitotic fidelity, while subacute exposure to non-lethal doses disrupted mitotic progression, impairing cell proliferation and viability in HCT116 cells.

It has been demonstrated that polystyrene microplastics, particularly surface-modified, are capable of inducing an imbalance of redox reactions and the development of oxidative stress in biological systems, primarily through mitochondrial damage (**Hernández *et al*., 2025**) which in our case was already demonstrated by measurements of the metabolic activity of the tested cells. In aerobic cells, mitochondria are the main source of reactive oxygen species (ROS) and a primary target of oxidative stress brought on by ROS. The vulnerability of mitochondria to ROS assault is very high, while excessive ROS buildup can cause oxidative damage and mitochondrial degradation. The damaged mitochondria then exacerbate the propagation of ROS and ultimately disrupt the physiology of the cell. Thus, oxidative stress and mitochondrial dysfunction are inextricably linked (**Zorov *et al*., 2014**). Our measurements of intracellular superoxide radical production in TM3 Leydig cells exhibited a non-linear, concentration-dependent response following exposure to carboxylated polystyrene nanoplastics. At low and intermediate concentrations (1, 10, and 50 µg/mL), PS-COOH NPs significantly increased superoxide levels compared to the control group, suggesting the induction of oxidative stress in viable, metabolically active cells. Although the NBT assay provided data on the intracellular production of superoxide, which is one of the primary reactive oxygen species generated in cells, the assessment of oxidative stress in this study was limited to a single biomarker. The NBT assay is widely used to detect superoxide radicals and offers a reliable indication of oxidative processes; however, a more comprehensive assessment, including other oxidative stress parameters, such as antioxidant enzyme activity or total oxidant status, would provide a more detailed understanding of the overall redox state. Oxidative stress is among the most commonly reported adverse effects associated with MNPs exposure. MNPs can induce oxidative stress through two primary mechanisms. By directly increasing the production of reactive oxygen species in cells and also by impairing antioxidant defense systems of cells associated with disrupting the activity of key antioxidant enzymes such as superoxide dismutase, catalase, and glutathione, MNPs reduce the efficiency of ROS scavenging (**Kadac-Czapska *et al*., 2024; Das, 2023**). **Grillo *et al*. (2023)** evaluated oxidative stress in testicular cell lines (TM3 Leydig cells, TM4 Sertoli cells) by a quantitative analysis of malondialdehyde (MDA) levels, the main by-product formed during lipid peroxidation, which is considered an indirect indicator of cell oxidative

damage; and mitochondrial antioxidant enzyme superoxide dismutase levels after 24 h polystyrene microplastics (10 and 50 µg/mL) treatment with significantly increased MDA concentration and decline of superoxide dismutase, which directly refers to the development of oxidative stress in cells. Another *in vitro* study tracking the impact of polystyrene nanoplastics (20 nm; 50-150 µg/mL) on TM3 Leydig cells experienced remarkable overgeneration of reactive oxygen species along with significantly reduced antioxidant capacity of cells with a decline of SOD activity and GSH concentration after 24 h exposure (**Sun *et al*., 2023**). Although studies that monitor and compare the toxicity of polystyrene MNPs with carboxylated polystyrene microplastics *in vitro* are limited, experiments suggest that carboxylated polystyrene microplastic particles can induce a more toxic response in biological systems, particularly in terms of oxidative stress development. **Li *et al*. (2026)** evaluated the toxicity of environmentally relevant concentrations (0.1-100 µg/L) of carboxylated polystyrene microplastics in zebrafish (Danio rerio) larvae. Results revealed ROS overproduction, ATP/NAD+ depletion, biphasic SOD and CAT responses, and inhibition of important respiratory chain genes (mt-nd1, cox4i1, atp5a1) were all indicators of mitochondrial disruption, suggesting oxidative stress-mediated mitochondrial malfunction as a primary damage mechanism. The *in vitro* potential to induce redox and mitochondrial stress responses after 24 h of exposure of polystyrene beads functionalized with carboxylic surface groups (spherical - 200 nm and 2 µm; and fibre-/fragment-shaped - 8.9 ± 10.1 µm by 1.14 ± 0.97 µm) in a human epithelial colorectal (Caco-2) cell line was investigated by **Saenen *et al.* (2023).** Obtained data confirmed decreased oxidative stress response in cells by inducing changes in the transcriptional expression of oxidative- and anti-oxidative genes including HMOX1, CAT, and GPX1.
The initial location of testosterone biosynthesis is mitochondria. For Leydig cell steroidogenesis, mitochondrial ATP generation and mitochondrial membrane potential polarization are essential, while mitochondrial dysfunction can significantly inhibit the synthesis of steroid hormones (**Hales *et al*., 2006**). In accordance with these facts, in our experiment, dose-dependent suppression of testosterone synthesis was observed across all tested concentrations of PS-COOH nanoplastics. However, testosterone levels in this study were expressed relative to the control group, which may affect interpretation at higher concentrations of nanoplastics, where reduced cell viability was observed. Testosterone data should therefore be interpreted in relation to metabolic activity results, as changes in steroid biosynthesis are closely related to changes in cell viability and metabolic status. Decreased testosterone production through mitochondrial oxidative stress was associated with exposure to polystyrene microplastics both *in vivo* and *in vitro* in a study of **Liu *et al*. (2024)** with C57bl/6 mice and TM3 mouse Leydig cells. Data revealed that PS-MPs entered Leydig cells *in vivo*, resulting in a significant decrease in testosterone levels and steroid synthesis function. TM3 mouse Leydig cells were treated with polystyrene microplastics (0, 0.05, 0.1, 0.2, 0.4, and 0.8 mg/mL) to explore their mechanisms of damage. Similarly, *in vitro* experiments exhibited a dose-dependent reduction in steroid synthesis in Leydig cells exposed to PS-MPs. Endocrine-disrupting properties of PS-MPs (50–10,000 nm, 0.01–100 µg/mL) were also confirmed *in vitro* in a steroidogenic cell line H295R (adrenocortical cells) after 48 h exposure, which was demonstrated by statistically significant changes in hormone levels, though these showed no apparent concentration or size-dependent patterns. Estriol concentrations evaluated in the exposure media were decreased 25–37.5 % by various doses of 50 and 1000 nm PS-MNPs compared to controls. Interestingly, exposure to PS-MNPs resulted in a statistically significant increase in dihydrotestosterone, progesterone, and 17α-OH-progesterone levels (**Boxel *et al*., 2024**). Disruption of steroidogenesis in extracted primary granulosa cells from mouse ovaries was observed after 24 h treatment with polystyrene nanoplastics in concentrations of 80 µg/mL. The exposure led to a notable reduction in estradiol levels within the culture supernatant, which was also associated with downregulation of steroidogenic enzymes expression (**Xue *et al*., 2024**).

CONCLUSION

In summary, our data demonstrate that carboxylated polystyrene nanoplastics exert dose-dependent cytotoxic and antiproliferative effects on TM3 Leydig cells. The observed impact on testosterone production may be associated with mitochondrial alterations, as mitochondria play a central role in steroidogenesis and are a major source of reactive oxygen species. Increased superoxide production detected in this study may indicate mitochondrial involvement; however, mitochondrial function was not directly assessed. Increased superoxide radical production observed at lower sub-cytotoxic concentrations (1–50 µg/mL) indicates that oxidative stress represents an early cellular response to PS-COOH exposure. Notably, testosterone production was significantly suppressed across all tested concentrations, including doses that did not markedly impair cell viability and metabolism. This finding indicates that polystyrene nanoplastics can disrupt steroidogenic function independently of overt cytotoxicity, most likely through redox-mediated mitochondrial impairment. Overall, our findings indicate that testicular Leydig cells represent potential targets of polystyrene nanoplastic-induced reproductive toxicity. At the same time, it should be noted that acute *in vitro* exposure to nanoplastics cannot fully reflect the complexity of real environmental exposures *in vivo*. Therefore, further studies focusing on long-term exposure and *in vivo* models are necessary to better understand the potential risks of nanoplastics to male reproductive health.

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