Effect of Polystyrene Microplastics on Viability and Testosterone Production of TM3 Leydig Cells *in vitro*

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Abstract

The spread of microplastic particles in the human environment is ubiquitous and thus can be found in rivers, sediments, sand beaches, soil, and also in drinking water. MPs have the ability to transition through the food chain from lower to higher nutritional levels, endangering human health in the process. Altogether, increased concentrations of MPs have been shown to have negative impacts on mammals, including decreased feeding activity, body condition, oxidative damage, intestinal barrier malfunction, inflammation, neurotoxicity, energy disruption, and last but not least, reproductive toxicity. Industrialization, which leads to a considerable release of synthetic, frequently hazardous xenobiotics (such as microplastics and pesticides) into the environment, is linked to the notable decline in the reproductive rate, reflected by the decline in gamete quality. Male mice's testicles and sperm may be affected by polystyrene microplastics (PS-MP), which is apparent by a decline in the quantity of spermatogenic cells, a disruption in their structure, and a decrease in the activity of enzymes linked to sperm metabolism. In our study, we used polystyrene microplastics as a treatment addition for the TM3 Leydig cells. Methodology tests consisted of viability assays – Alamar Blue and MTT. Afterwards, we used the ELISA method to evaluate testosterone production.

Keywords: Polystyrene, microplastics, testosterone, Leydig cells

1. Introduction

One of the biggest worldwide concerns is environmental contamination brought on by waste made of plastic. Indirect degradation of plastic materials (secondary MPs) by light, UV radiation, embrittlement, and biological processes can be the source of microplastic environmental pollution [1]. The buildup of polluted plastic particles in the environment and in various organisms has drawn more attention in recent years. Indeed, despite the poor rate of biodegradation, the growing manufacturing and usage of plastics has raised serious concerns about this widespread and persistent environmental hazard [2]. Human health and marine ecosystems may be negatively impacted by plastic macroparticles, microparticles, and nanoparticles [3]. Microplastics (MPs) are particles smaller than 5 mm that accumulate in plastic litter and are released into the environment through a variety of methods. These include: i) the breakdown of larger plastic fragments; ii) the direct release of micro-particles used as abrasives; iii) unintentional industrial losses of raw materials; and iv) discharge in sewage sludge [4]. Their hydrophobicity and small size allow for easier cellular absorption, which may interfere with endocrine function and other physiological functions. At the moment, the three polymers that are most often found in environmental MPs are polyethylene, polypropylene, and polystyrene [5]. Due to its extensive usage in packaging, disposable cutlery, and insulating materials, polystyrene (PS) accounts for 10% of the world's plastic waste, out of an annual production of over

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350 million tons. Furthermore, MPs have the ability to advance up the food chain from lower to higher nutritional levels. The hydrophobic surfaces of PS-MPs easily absorb environmental contaminants including endocrine disruptors or heavy metals, producing hazardous effects that work in synergic way. According to a number of studies, small plastic fragments infiltrate cells and first cause oxidative stress by ROS. This sets off a series of biological reactions, including inflammation, apoptosis, and signaling pathways triggered by oxidative stress [6]. Oxidative stress can have negative impacts on development and reproduction in addition to its direct consequences on an individual's health [7].
The reproductive process is one of the most sensitive physiological mechanisms to the action of toxic substances from the environment. The disruptive effect of polystyrene microparticles can affect the reproductive system on several levels, as these are sufficiently diversified mechanisms. In this study, the reproductive toxicity of these chemical substances was investigated by use of TM3 Leydig cell line, that was isolated from a male mouse. The testis's Leydig cells are responsible for generating testosterone. The tm3 cells' high mitochondrial activity, which is necessary for steroidogenesis, makes them especially vulnerable to oxidative damage [8]. The multi-step process of testosterone production in Leydig cells is controlled by the binding of luteinizing hormone (LH) to its receptor (LHR), which triggers cAMP signaling. By upregulating the steroidogenic acute regulatory (StAR) protein, this cascade makes it easier for cholesterol to enter mitochondria [9]. Although we still don't fully understand how MPs affect methabolic ways of reproductive processes, their ecological implications are of particular concern currently [10].

2. Materials and methods

As we mentioned in the previous chapter, the main laboratory model for our study was TM3 Leydig mouse cell line, because of their steroidogenic properties. The Leydig cell lines - TM3 cells were obtained from the testes of 11–13-day-old mice (ATCC No CRL-1714, Manassas, VA, USA). In this in vitro investigation, different experimental exposure doses were compared to a control group. Cells were cultured according to standard protocols in TM3 Cell Complete Medium (Culture medium - Sigma-Aldrich, St. Louis, MO USA, Cat. No. D8437). After reaching the desired confluence, we passaged the cells gradually. After the end of the passage cycle, we exposed the cells on a 96-well plate to polystyrene microplastics. Cells were seeded into the 96-well plates at an initial concentration of 20,000 cells per well. Prior to exposure to microplastics, the cells were pre-cultivated for 24 hours to allow for proper adherence and stabilization. We used polystyrene microplastics from Sigma-Aldrich, St. Louis, MO USA (Cat. No. 90517, Source BCCL7448). The microplastic solution used in our experiments was commercially purchased from Sigma-Aldrich and is specifically intended for research purposes. The stock solution was diluted to the desired concentrations using the respective culture medium. For each well in the 96-well microplates, 150 µL of the prepared microplastic solution was added. Final Polystyrene particles concentrations were 5; 10; 20; 40; 80; 100; 200; 400 and 800 µM; mg/L. After a 24-hour exposure to microplastics, we tested cell viability using the AlamarBlue test and also using the MTT test.
After the completion of the viability tests, we used the ELISA method to determine the amount of Testosterone produced by Leydig cells after exposure to polystyrene microplastics. The Alamar Blue assay was used to assess cell viability according to the manufacturer's instructions. A volume of 100 µL of Alamar Blue reagent was added to each well of the 96-well plate. A sensitive oxidation-reduction indicator, alamar blue fluoresces and changes color as it is reduced by live cells. Enzymes in the mitochondria are thought to be responsible for the decrease of Alamar Blue. Alamar Blue monitors the live cell's decreasing environment. Resazurin, also known as diazo-resorcinol, azoresorcin, resazoin, or resazurine, is the active component. It is water soluble, stable in culture media, non-toxic, and permeable through cell membranes. Its IUPAC name is 7-hydroxy-10-oxidophenoxazin-10-ium-3-one. This allows cells in culture to be continuously monitored. Resorufin is a pink, extremely fluorescent dye that is derived from the blue, non-fluorescent dye resazurin [11]. In the MTT test, mitochondrial reductase transforms the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide] into an insoluble purple formazan. After that, formazan is dissolved, and the optical density at 570 nm is used to calculate the concentration. Similar to the alamarBlue test, when a cell is exposed to a hazardous substance, even in the absence of direct cell death, one can identify cell stress since little changes in metabolic activity can result in significant changes in MTT [12]. Testosterone levels were evaluated by comercially available Testosterone High Sensitivity Competitive ELISA Kit. All samples are read using a user-generated standard curve, which is created using a testosterone standard to create a standard curve for the assay. A transparent microtiter plate coated with donkey anti-sheep IgG antibodies is pipetted with standards or diluted samples. The wells are filled with a monoclonal sheep antibody and a compound of testosterone and peroxidase. The sheep testosterone antibodies will compete with the testosterone conjugate and any other testosterone present in the sample for binding. The plate is cleaned and substrate is added following incubation. The bound testosteron-peroxidase conjugate interacts with the substrate. The reaction is halted after 30 minutes of incubation, and a microtiter plate reader is used to measure the intensity of the color that is produced. The amount of testosterone in the sample is negatively correlated with the intensity of the color that is produced. Each experiment was performed in three independent replicates. Within each 96-well plate, a minimum of 8 technical replicates per experimental group were included.

Statistical analysis of outcomes

One-Way ANOVA (Dunett's test) was used to process the data using the software program GraphPad Prism 9.0.0 (GraphPad Software Inc., San Diego, CA, USA). Mean values and standard error of the mean are used to express the data. A significance value of *P<0.05, **P<0.01, ***P<0.001, and **** P<0.0001 was selected for the differences between the control and experimental groups.

3. Results and discussion

We obtained presented results by using polystyrene microplastics as a treatment addition for the murine TM3 Leydig cells in concentrations of 5; 10; 20; 40; 80; 100; 200; 400 and 800 mg/L. According to Figure 1., the metabolic activity of TM3 cells was significantly higher in experimental group treated with 5 mg/L. From this value, the concentration range showed a decreasing trend in metabolic activity. Another statistically significant results were shown in experimental groups treated with 400 mg/L (*P<0.05) and 800 mg/L (***P<0.001) where the metabolic activity decreased. In figure 2. the MTT assay showed very similar results to AlamarBlue. Higher mitochondrial metabolic activity was obtained in group treated with 5; 10 and 20 mg/L in the level of significance (***P<0.001). From that point the same pattern happened as in previous viability analyses, decreasing levels up to concentration of 400 mg/L (**P<0.01) and 800 mg/L (***P<0.001). The production of Testosterone after 24h of cultivation with MPs shown significantly reduced production in comparison between control and experimental groups. Groups treated with concentrations of 20; 40 and 100 mg/L shown decrease in the level of significance *P<0.05. Even bigger decrease (**P<0.01) was obtained in group treated with 200 mg/L. Experimental groups with treatment of 80; 400 and 800 mg/L shown decreased levels of testosterone in dose dependent manner. Considering the significant decrease in testosterone levels after 24 hours of cultivation, this condition can of course also be caused by reduced cell viability. In the AlamarBlue and MTT assays, we observed a decrease in cell metabolic activity and viability at concentrations of 400 and 800 mg/L MPs. Since the cells in the experimental groups are significantly less viable, their production and secretory function is clearly altered. At lower concentrations, we observed the opposite scenario in viability tests than at higher concentrations, an increase in cell viability at a statistically significant level. In the scientific literature, this is similar for toxic substances similar to MPs in this formula and a pronounced biphasic effect of these toxicants is described.

Various authors have suggested several mechanisms by which polystyrene microplastics could affect the physiological mechanisms of Leydig cells. Lou et al. (2023) studied effect of polystyrene microplastic on the apoptosis of male germ cells. The mRNA and protein levels of p53 and Bax in the p53 signaling pathway were up-

regulated (P<0.05), downstream molecules Caspase3 and Caspase9 were enhanced (P<0.05), and Bcl2 was inhibited at the mRNA and protein levels in TM3 cells (P<0.05). The growth of cells was significantly inhibited while cell apoptosis was enhanced after 48 hours of treatment with PS-MPs of 5 µm and 80 nm in size [13]. In the study of Sun et al., (2023), polystyrene nanoparticles were added to TM3 murine Leydig cells in vitro at doses of 50, 100, and 150 µg/mL to examine their cytotoxicity. Authors findings showed that TM3 Leydig cells may internalize PS-MPs, which leads in a concentration-dependent reduction in cell viability [14]. The findings of another study from Qu et al. (2024) showed that 50 µm PS-MPs are absorbed into the cytoplasm and accumulated in the mice testes. Authors suggest that this process decreased the viability of Leydig cells and the serum levels of GnRH, FSH, LH, and testosterone in addition to harming the testicular histomorphology and ultrastructure [15]. The authors attribute the adverse effects of polystyrene microplastics primarily to oxidative stress. Persistent oxidative stress and endoplasmic reticulum stress activate intrinsic apoptosis pathways, PS-MP induce structural and functional damage to organelles important for steroidogenesis, PS-MP directly inhibit testosterone synthesis by disrupting key pathways. Studies also indicate a direct correlation between the reduction in testosterone production and the duration of exposure to microplastics. The results obtained in this study correlate with the results of other authors. All measured parameters show a similar pattern with respect to the exposure time and selected concentrations.

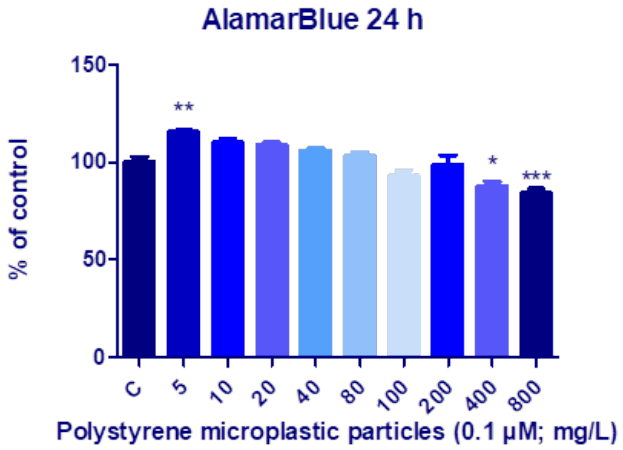

Figure 1. AlamarBlue assay after 24h incubation with distinct concentrations of MPs

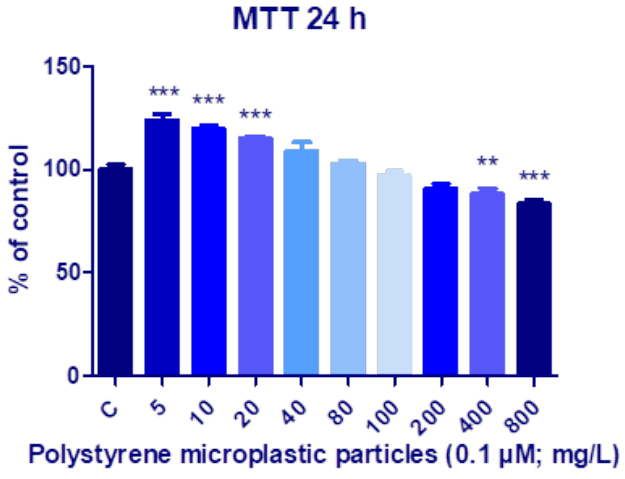

Figure 2. MTT assay after 24h incubation with distinct concentrations of MPs

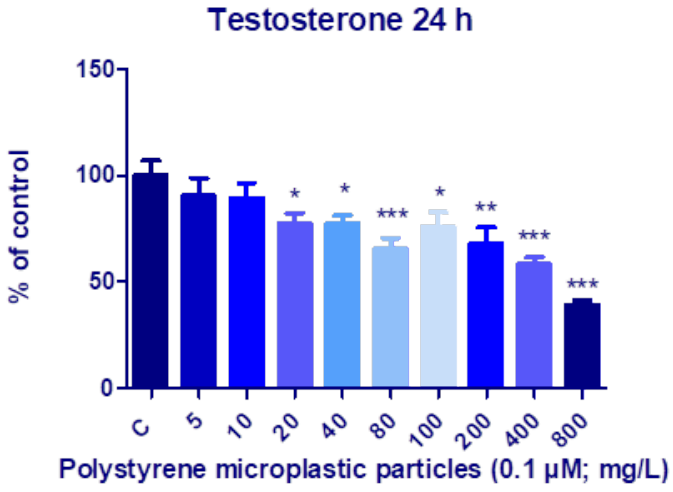

Figure 3. Production of testosterone after 24h incubation with distinct concentrations of MPs

4. Conclusions

The present study clearly demonstrates the toxic effect of MPs on TM3 Leydig cells. Changes were manifested in the mechanisms of cell viability and production activity. It is very important to investigate the impact of ubiquitous microplastics on physiological functions. In the last decade, this scientific field has received increased attention. However, concrete evidence is still lacking that clarifies the exact molecular physiological effect of these substances. The scientific literature agrees and describes the main adverse effect of these substances through oxidative stress processes. However, we still need more studies and evidence in this area.

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