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EFFECTS OF NANOPLASTICS ON FEMALE REPRODUCTIVE SYSTEM

INVESTIGATING THE EFFECT OF POLYSTYRENE NANOPLASTICS ON FEMALE REPRODUCTIVE SYSTEM

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2023) Hamilton, Ontario (Medical Sciences)

TITLE: INVESTIGATING THE EFFECT OF POLYSTYRENE NANOPLASTICS ON FEMALE REPRODUCTIVE SYSTEM

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NUMBER OF PAGES: xv, 75

Lay Abstract

Plastics in the environment break down into smaller particles called micro- and nanoplastics. These plastic particles are pollutants present in the aquatic and terrestrial environments entering every level of the food chain and ultimately reaching humans, yet few studies have examined the effects of nanoplastics on human health. A recent World Health Organization (WHO) report on nanoplastics has stressed the urgent need for toxicological studies to assess potential human health effects. Therefore, this study examined the effect of nanoplastics on the female reproductive system. This study was carried out in female mice exposed orally to a solution containing a vehicle control (water) and two different concentrations of nanoplastics (100 and 1,000 μg/l). Exposure occurred daily for a period of 29 days. At the conclusion of the study the mice were humanly euthanized with their blood and reproductive tissues collected for laboratory analysis**.** Results showed that nanoplastics exposure resulted in irregular reproductive cycle in mice along with a decrease in antral follicle size and progesterone levels which are indicators of anovulation and can lead to disorders, such as infertility and polycystic ovary syndrome (PCOS) which should be further investigated in future studies.

Abstract

Introduction

The degradation of plastic waste into smaller micro- and nanoplastic (MNPs) molecules has led to widespread distribution of these particles and accumulation in the environment, making human exposure inevitable. This can result in, or exacerbate, pathological conditions leading to immune dysfunction, neurodegenerative diseases, and infertility. Yet few studies have examined the effects of nanoplastics (NPs) on human health, especially the reproductive system. Reproductive toxicity of plastic particles has been mostly studied in males with most studies investigating microplastics. Therefore, the present study aims to assess the reproductive health consequences of NPs exposure in females by quantifying serum estradiol and progesterone, examining estrous cyclicity, and assessing ovarian reserve (number and quality of follicles) which is a key indicator of female fertility.

Materials & Methods

The present study was carried out in female mice (C57BL/6) exposed orally to water (control) or one of two solutions containing different concentrations of Polystyrene nanoplastics (PS-NPs; 100 μg/l or 1000 μg/l in water. Exposure occurred daily for 29 days, and vaginal lavage samples were collected for the last 15 days of the exposure phase to check for change in estrous cyclicity. Mice were euthanized at the end of the study and their blood samples and reproductive tissues were collected. Ovaries were fixed in 10% formalin, embedded in paraffin wax, serially sectioned at 5 μm thickness, and stained with hematoxylin and eosin (H&E) for microscopy and follicle analysis. ELISA was also performed to quantify the progesterone and estradiol serum levels.

Results

There was a significant increase in the estrous cycle length in the high dose (1000 μg/l) PS-NPs exposure group compared to control (5.53±.25 days vs 4.7±0.23 days, P=0.02). Moreover, there was a significant decrease in serum progesterone levels in the high-dose exposure group compared to control (mean difference=1.64 pg/ml, standard error of difference (SED)=0.64, P=0.03). Additionally, it was shown that PS-NPs exposure significantly reduced antral follicles' diameter in both the low dose (238.61±19.01 μm vs 167.35±19.01 μm, P=0.03) and high dose exposure groups compared to the control group with the higher dose showing a more pronounced reduction in antral follicle' size (238.61±19.01 μm vs 131.95±19.01 μm, P=0.001).

Conclusion

Oral PS-NPs exposure in female mice appears to induce toxicity by reducing antral follicles size, increasing the estrous cycle length, and decreasing progesterone levels which may result in anovulation and different reproductive issues, such as infertility and

polycystic ovary syndrome (PCOS). The effect of PS-NPs on infertility along with NPs' mechanism of action in female reproductive system should be investigated in future studies.

Acknowledgements

I am deeply thankful and consider myself fortunate to embark on the journey of pursuing my Master's degree. The opportunity to pursue one's aspirations and transform them into reality is a privilege that merits recognition. I firmly believe that acknowledging this privilege is just as crucial as recognizing the challenges faced by those less fortunate. There are individuals, especially women, who are unable to pursue their ambitions. They are deprived of many fundamental rights, such as the right to dream and take action. I'd like to acknowledge them and hope for a more just world where borders are not killers of dreams and geographical locations don't define one's opportunities and future. With that being said, I would like to extend my gratitude to my supervisor, committee members, mentors, friends, family, and all the amazing people who helped me in this journey. Dr. Leonardi, thank you for your support, guidance, and all the opportunities throughout my Master's journey. Thank you for allowing me to explore the field of women's health and gynecology research beyond my main project which has helped me learn so much and improve my research skills in both basic and clinical science. Additionally, thanks for always being open-minded towards new ideas and novel approaches to science. Your encouragement of creativity and outside-the-box thinking is greatly appreciated. Your passion and commitment to science and improving patients' lives and clinical care through research are an inspiration to me and many. Dr. Inman, thank you for your support and guidance throughout my Master's journey. There's a Persian saying that vaguely translates to: "In order to build a straight wall, you have to put the first layer of bricks properly. Basically, for a proper and functional building, the foundation matters the most." So, thank you for teaching me and many

other graduate students the right research foundation, from ethics to methodology and analysis, as well as many other important lessons. It's been an absolute pleasure to be your student and a teaching assistant for MS 771, which has also contributed to my growth both academically and on an individual level.

Dr Foster, thank you for your help and guidance and for taking on this project despite the unusual circumstances. Your inspiring approach to science and your intriguing questions from the lab meetings back when I was an undergraduate student in your lab to my Master's project, has helped me develop my scientific thinking and approach to research problems. And for that I am grateful.

I also want to thank my mentor, Dr. Jocelyn Wessels, for all her help and ongoing support. Jocelyn, it's been an absolute pleasure to work with you and have you as my mentor for the past few years. From my thesis to my Master's project, I have learned so much from you both academically and on an individual level. Your love for science, persistence, and hardworking personality are an inspiration to people around you, including myself.

Next, I want to thank Ms. Victoria Turpin. Victoria, thank you for being a true friend and sharing your experience and perspective on matters. I appreciate the genuine care you have for your work and the people around you.

I would also like to acknowledge the amazing instructional assistants, Ryan Belowitz and Devon Jones, as well as the amazing lab technician, Chelsey Weatherup, at the School of Interdisciplinary Science, for allowing me to use their microscope room and capture my images. Ryan, your passion for science and improving the learning experience of students is truly impressive. I had an amazing experience serving as a

teaching assistant for LIFESCI 3L03. Thank you for creating such a safe and friendly space, not only for students but also for the instructional team. I have learned so much from you and this amazing experience, lessons, and memories that I will cherish forever. Also, a big thank you to Mr. Kyle McGowan the former research coordinator at the department of obstetrics and gynecology. Kyle, thank you for your support and help. Your caring soul and compassionate personality combined with your love for science makes you great at what you do. And a huge shout out and thank you to Mrs. Jennifer Laframboise the amazing administrative assistant to Dr Leonardi. Jen, thank you for your support, encouragement, and for organizing and scheduling basically everything, which is one challenging job given Dr Leonardi's busy schedule. Jen, your smile and positive energy are contagious. They light up any room you step into and are a remedy for difficult times as well. I also want to thank the amazing Dr. Jayesh Tigdi, assistant professor and gynecological surgeon at McMaster, for his support and mentorship throughout my journey. Jay, your kind and caring soul, combined with your passion to help patients and improve clinical care through research, is inspiring and deeply appreciated. Thank you for all that you do and for all the great lessons you have taught me.To my friends and colleagues in the Medical Sciences program, professors, and the administrative team, thank you for your support and encouragement through my Master's journey. The opportunity to learn within a collaborative environment has been phenomenal and I will strive to promote the same qualities within my future endeavors. Lastly, I'm deeply grateful to my parents for enabling my pursuit of science and to my sister for her unwavering support. Their sacrifices and encouragement have been

instrumental in bringing me to this moment. Without them, I wouldn't be here, let alone writing my thesis. So, thank you for making me feel privileged.

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List of Abbreviations and Definitions

Abbreviation	Definition
MNPs	Micro and nanoplastics
MPs	Microplastics
NPs	Nanoplastics
PS-MPs	Polystyrene microplastics
PS-NPs	Polystyrene nanoplastics
ROS	Reactive oxygen species
AMH	Anti-Müllerian hormone
AFC	Antral follicle count
RIRR	ROS-induced ROS-release
PCOS	Polycystic ovary syndrome
EDCs	Endocrine disrupting chemicals
SD	Standard deviation
SED	Standard error of difference
SEM	Standard error of the mean

Declaration of Academic Achievement

All the experiments, imaging, and subsequent analyses were performed by the author of this thesis. Ms. Victoria Turpin assisted with the ELISAs, and Dr Jocelyn Wessels acted as an additional reviewer for the estrous cycle stage identification and follicle type identification.

1. Introduction

1.1 Plastics toxicity

The impact of environmental contaminants on human health is a subject of ongoing concern. Among these concerns, the global production of plastics has garnered significant attention. This surge in plastic pollution coincides with the period of global industrialization and modernization, which has witnessed a substantial increase in the production and consumption of plastic items since the early 1950s (1,2). Additionally, the COVID-19 pandemic has contributed to the substantial rise in plastics production and pollution, amounting to approximately 700 million tons in the year 2020 alone (3). This includes the disposal of single-use face masks, gloves, gowns, COVID-19 testing kits, and eye protectors (4,5). Global plastics production has been estimated to exceed 1.1 billion metric tons by the year 2050, representing a surge of over 30% from current levels (6).

Plastics are generated by polymerization of different monomers or derived from combinations of a range of materials such as cellulose, starch and petrochemicals like crude oil, natural gas and coal (7). The most prevalent types of plastics produced globally include polyethylene (PE; 36%), polypropylene (PP; 21%), polyvinyl chloride (PVC; 12%), polystyrene (PS) and polyurethane, each comprising less than 10% of total global plastic production. These plastics are commonly found in different environmental compartments such as oceans, sludge, or soil (8).

Plastics in the environment originate from discarded consumer and manufacturing products with human activities playing a pivotal role in the escalating global plastic production. These activities include but are not limited to packaging and food waste, littering of single-use plastics, and insufficient waste management practices, such as open landfills (9–12). When plastics are not recycled or disposed of properly, they can end up in landfills where they may degrade and get released into the surrounding soil or water. Plastic debris on land can be transported into water bodies through runoff. Rainwater can carry plastic waste from streets, landfills, and other areas into storm drains, eventually leading to rivers, lakes, and oceans (13). Additionally, synthetic fibers from clothing, microbeads from personal care products, and other small plastic particles can pass through filtration systems and end up in rivers, lakes, and oceans (14). Over time, larger plastics can break down into smaller fragments called micro and nanoplastics (MNPs) via different factors, such as exposure to sunlight (photodegradation), physical forces (mechanical degradation), oxidative processes, biodegradation, and hydrolysis. MNPs are ubiquitous in the environment and can be transported over long distances by wind and water currents, infiltrating land, water bodies, and even the air (Figure 1) (15). Plastics will persist in the environment for hundreds of years due to their high ratio of aromatic compounds and consequent resistance to degradation, posing serious threats to ecosystems and wildlife (15) (Figure 1).

1.1.1 Micro & Nanoplastics (MNPs)

Microplastics (MPs) are typically defined as plastic particles that are less than 5 millimeters in size. However, they can vary greatly in size, ranging from microscopic to several millimeters in diameter (7,16). MPs are classified into primary and secondary sources. Primary MPs are manufactured in microscopic size and are designed for commercial use such as cosmetics and microfibers (17–19). Secondary MPs are from degradation and breakdown of larger plastics such as plastic bags and food packaging (19). Nanoplastics (NPs) are smaller, range between 1-1000 nm in size and are also divided into primary and secondary sources. Primary NPs are manufactured nano-sized plastic particles directly released into the environment for biomedical, industrial, and agricultural uses (17,20,21). Secondary NPs come from degradation of MPs and macro plastics, such as bulk plastics and plastic litter (22,23). NPs are used in the manufacturing of cosmetics, exfoliants, paints, toothpaste, medications, and abrasives making human exposure inevitable. Given their higher surface-to-volume ratio and surface reactivity, NPs likely represent a greater toxicity than MPs. Due to their small size, MNPs can be readily taken up by land or marine organisms and enter the food chain. This combined with their persistent non-biodegradable nature leads to chronic human exposure and intensified toxicity. Research has shown that the uptake and toxicity of MNPs are intricately linked to their size and duration of exposure (24).

In addition to their small size and persistent nature, MNPs contain different additives and can carry environmental pollutants which can further enhance their toxicity. Plastics additives are used during plastic processing to give them their desired characteristics,

such as the ability to tolerate extreme temperatures and pH levels. Additives can make up to 70% of the composition of some plastics (25). Additives can be heavy metals like chromium, lead, and cadmium (26–28) or organic compounds such as polybrominated diphenyl ethers [PBDEs], phthalates, organotins, perfluorinated compounds, and bisphenol A (BPA) (29,30). Some MNPs additives have been shown to be endocrine disruptors that can influence the expression of various hormone receptors and interfere with the synthesis, secretion, transport, or action of hormones, leading to endocrine and developmental abnormalities (31). Additionally, MNPs interact with the environment. Due to their high surface area MNPs act as vectors for microorganisms like bacteria and pollutants such as Polychlorinated biphenyls (PCBs) and dioxins. These pollutants are not only very resistant to degradation, but they also accumulate in animal fats and tissues and induce toxic effects (32,33).

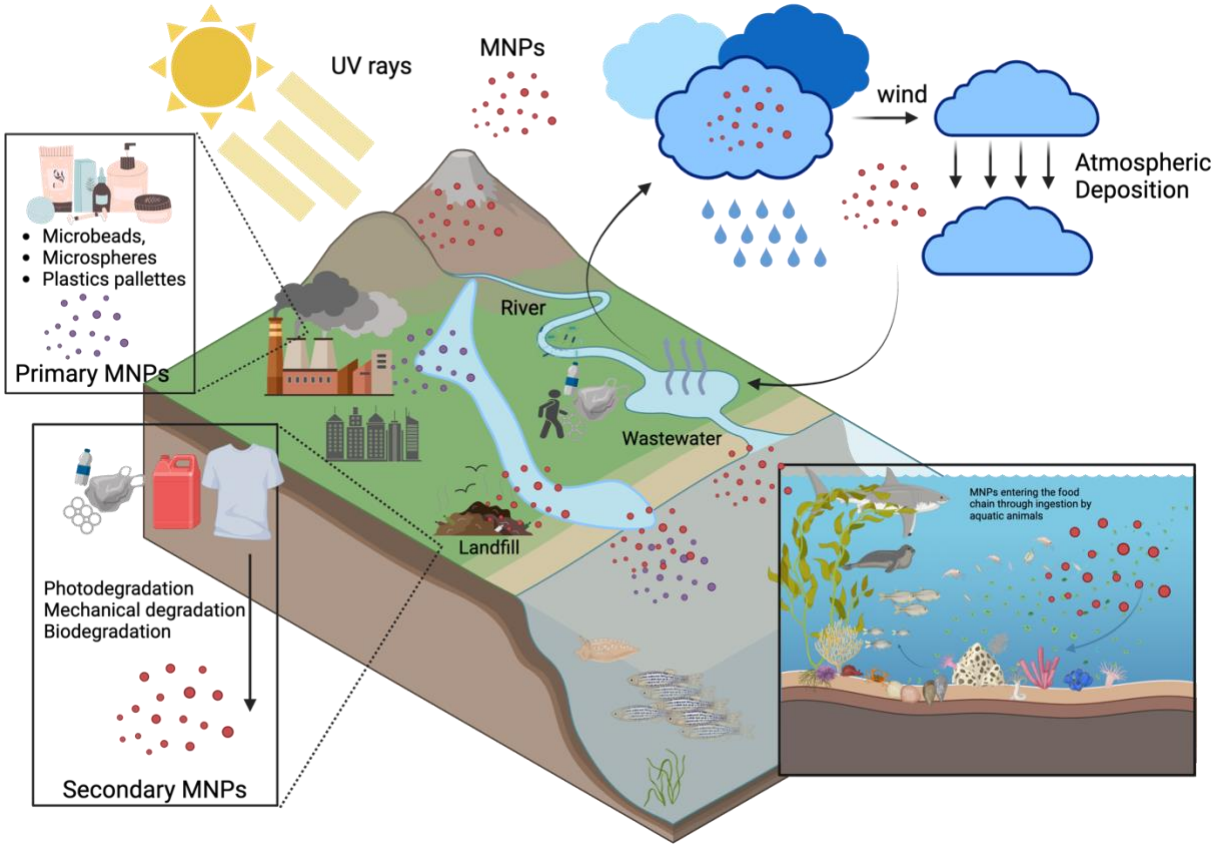


Figure 1. Plastics cycle in the environment.

Plastics predominantly stem from human activities, notably the inadequate management of industrial waste and disposal of single-use plastics. Over time, these macroplastics degrade into smaller micro and nano-sized particles through weathering processes such as biodegradation, mechanical breakdown, and exposure to sunlight (photodegradation), resulting in the formation of secondary MNPs. Additionally, MNPs can be directly produced and introduced into the environment such as microbeads in cosmetics (primary MNPs). These particles disperse into water, soil, and air, where they are ingested by aquatic and terrestrial organisms, eventually finding their way into the human food chain. Created with BioRender.com.

1.2 MNPs & aquatic toxicity

The degradation of plastic waste into smaller MNPs, as well as their synthesis by various industries, has led to widespread distribution of these particles and their accumulation in aquatic wildlife species. MNPs tend to float on water due to their size and density (34). Upon entering aquatic environments MNPs get colonized by algae and microorganisms. It has been reported that upon exposure to the environment, microbial biofilms rapidly colonize plastic surfaces. Estimates suggest that plastic marine debris may harbor between 1000 and 15,000 metric tons of microbial biomass (35). This biomass accumulation increases MNP density and causes them to sink (36). Alternative pathways of sedimentation include adhering to microalgae or ingestion by zooplankton, followed by deposition within fecal pellets (37,38).

MNPs impact every trophic level and microalgae are the first level in the food chain that are also indispensable for the marine ecosystem equilibrium. MNPs seem to affect the well-being and growth of microalgae, induce oxidative stress, and reduce chlorophyll and photosynthesis (39,40). Zooplankton is the second food chain level and a study by Cole et al. has highlighted that polystyrene beads are ingested by zooplankton which negatively affects their health and leads to reduction of algae consumption as well (41). The ingestion of hazardous substances and MNPs facilitates their transfer from one trophic level to the subsequent one, resulting in bioaccumulation within the food chain. Given that MNPs do not undergo degradation, they persist in the digestive systems of marine organisms throughout the entire food chain, causing adverse biological and physical impacts on marine life (42,43). While large fish may not immediately manifest the effects of chemically contaminated MNPs upon ingestion, the gradual accumulation

of these particles could potentially lead to fatal consequences. Limited studies have delved into the fate of these particles in freshwater environments due to lack of standardized and reliable methods for sampling, detecting, and characterizing MNPs. Consequently, the level of toxicity that NPs pose to freshwater ecosystems remains uncertain. The existing studies on this subject are primarily laboratory-based and may not accurately replicate the same biological toxicity observed in natural environments (44).

With more than 890 fish species (mostly marine species), fish are the most reported organisms to contain MNPs in their bodies (45–47). MPs have been reported to exist in various fish species' brains, guts, livers, and gills (48). Furthermore, among aquatic organisms, bivalves, including mussels, oysters, and clams, constitute the second most extensively researched group, as highlighted by Li et al (49,50). MNPs are frequently identified in crabs and shrimps as well (51,52). These species are globally significant in aquatic food sources and are commonly consumed by organisms at high trophic levels, including humans. The widespread prevalence of MNPs in these organisms has a crucial role in amplifying health risks for consumers.

1.2.1 MNPs reproductive toxicity in aquatic animals

Several studies have demonstrated the adverse effects of both MPs and NPs on aquatic organisms, with NPs being identified as more harmful (53–55). For example, in zebrafish, NPs have been found to diminish locomotor activity and reduce body length (54), induce oxidative stress and impede microalgal growth in freshwater biofilms (55), and result in tissue accumulation and embryonic developmental toxicity (53), whereas

MPs exhibit negligible effects. These studies suggested that owing to their smaller dimensions and increased surface area, NPs possess a greater capacity to adsorb additional contaminants, rendering them more hazardous than MPs (56). Consequently, inadvertent ingestion of NPs by organisms is likely to result in more severe damage (57). An increasing body of research indicates that NPs can increase the production of reactive oxygen species (ROS), elevate the activity of antioxidant enzymes, and modify gene expression patterns. These alterations can lead to adverse outcomes including oxidative stress, neurotoxicity, cytotoxicity, intestinal inflammation, and toxicity to the reproductive system (58–62). Given the widespread presence of NPs in the aquatic environment, there is limited information on the effect of NPs on reproductive system of aquatic vertebrates especially at environmentally realistic concentrations. Existing studies have shown that NPs can affect sperm mobility and velocity by binding to sperm membranes which results in reduction in fertilization rate in oysters (63,64). Moreover, PS-NPs exposure has been shown to affect the early development of zebrafish embryos in a size and dose-dependent manner (53). For example, exposure to 20 nm PS-NPs causes oxidative stress and DNA damage due to accumulation in the brain (65). 100 nm PS-NPs exposure has been shown to activate oxidative stress and base excision repair pathways which caused a reduction in heart rate and body length. Similarly, hatching and survival of zebrafish embryos was decreased as well (66). Moreover, 100 nm PS-NPs exposure at a higher dose exacerbated oxidative stress in oocytes, resulting in oocyte apoptosis and impaired reproductive function in zebrafish (67). Additionally, a recent study on zebrafish has revealed that NPs exposure resulted in higher testosterone levels and decreased fecundity in females along with a reduced

proportion of mature spermatocytes in the testis and developmental impairments in the F1 generation (68). NPs have also been shown to delay gonadal maturation by inhibiting oogenesis and spermatogenesis in medaka (69).

Overall, existing literature showed that exposure to MNPs can trigger a range of toxicological effects in aquatic animals including oxidative stress (70) and reproductive abnormalities (71,72). Zooplankton, planktivorous fish, and piscivorous fish directly ingest MNPs within the aquatic ecosystem. MNPs will then be transferred up the food chain eventually reaching terrestrial animals and humans (73).

1.3 MNPs in terrestrial environment

MNPs are also found in terrestrial environments, with MP contamination reported to be 4 to 23 times higher in land compared to oceans (74). Every year, between 44,000 and 300,000 tons of MPs are deposited into the agricultural soil of North America (75). MNPs are taken up by land animals, through ingestion, inhalation via lungs or gills (e.g., land crabs), and epidermal infiltration (76–78). Studies have reported that terrestrial animals such as rodents, birds, chicken, snails, earthworms, and humans are mostly exposed to MNPs through ingestion (79–81). For example, up to 2019 MNPs have been detected in the digestive system of approximately 87,000 individuals (46). Ingestion of MNPs can cause damage at the organ, tissue, and cellular level, such as inflammation, gut blockage, DNA damage, hepatotoxicity, neurotoxicity, mortality, and reproductive toxicity in terrestrial organisms (82–84).

1.4 MNPS in humans

The presence of plastic particles in food, drinking water and in the atmosphere indicates that human exposure is inevitable. Human exposure to MNPs can occur via ingestion, inhalation, and dermal contact. (85,86) (Figure 2).

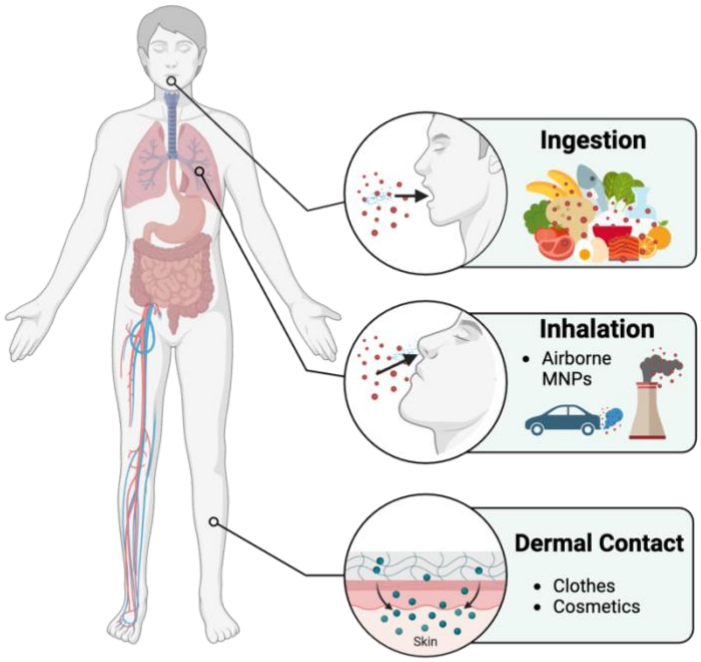


Figure 2. MNPs routes of uptake in humans. Humans can be exposed to MNPs through ingestion, inhalation, and skin contact. Created with BioRender.com.

1.4.1 Dietary intake

In 2018, plastic particles were found in the stool of eight people for the first time and researchers estimated that more than half of the world population may have MPs in their stool (87). This confirmed humans' exposure through ingestion which varies based on age, lifestyle, sex, and diet. MNPs can be found in food packaging, drinking water,

table salt (1–10 MPs/kg) and food (88,89). A Chinese study looked at different water samples within the country and found MNPs in 38 tap water samples from different cities with the proportion of smaller particles (<40 μm) being the greatest (90). Another study looked at different bottled water brands and found that 93% were contaminated with MNPs (91). MNPs originating from environmental pollution have infiltrated lower-level animals and plants, thus entering the human food chain and posing a significant threat to food safety (92). For example, it has been reported that consumption of the soft tissues of bivalves from Germany can expose humans to 0.36 particles/g of MPs and bivalves from Belgium, France, and Netherlands resulted in exposure of 0.2 particles/g MPs (93,94). Moreover, it has been shown that MNPs get released from plastic food packaging and containers under high temperatures leading to food contamination (95). The average mass of ingested plastics in adults has been estimated at 4.1 μg per week (96) corresponding to 50 plastic bags per year (97) (Figure 2).

1.4.2 Inhalation

Inhalation is another MNPs exposure route for humans. It has been estimated that on average an individual inhales up to 130 MPs per day (98). MPs has been reported to make up 4% of indoor air and sources of airborne MPs include construction materials, road-wear particles, landfills, sewage, synthetic textiles, abrasions of plastic materials, and waste incineration (86,99–102). These plastic particles can reach the respiratory system and cause adverse health effects in humans (103,104). The smaller the plastic particles, the further they can penetrate the airways, leading to more severe health consequences. Particles smaller than 2.5 μm are more prone to reaching alveolar sacs,

translocating from epithelial to endothelial cells, and permeating the capillaries (105). Consequently, NPs have the capacity to disperse throughout the human body by entering the circulatory system (106) (Figure 2).

1.4.3 Skin contact

Another route of MNPs exposure is through skin contact. Major sources of skin exposure are microbeads in personal care and cosmetic products and atmospheric fallout of synthetic fibers (107,108). Exposure through skin contact can be significant. For instance, a single laundry cycle can release millions of fibrous MNPs into the environment (109). Similarly, wearing a single synthetic fiber sweatshirt can emit tens of thousands of microplastic particles (110). It has been reported that NPs can pass through dermal barriers (85). Skin has four layers: the stratum corneum, viable dermis, dermis and the subcutaneous connective tissue (111). The stratum corneum is the outermost layer and forms a defensive barrier against injuries, chemicals and pathogens (112,113). Since MNPs are hydrophobic, it is predicted that absorption through the stratum corneum is unlikely; however, plastic particles can be transported through the skin barrier via the transappendageal pathway, which involves passage across hair follicles, sebaceous glands, and sweat glands (112). It is important to note that transport of MNPs across the skin is size dependent. For example, in a study by Vog et al, a notable concentration of Langerhans cells (dendritic cells) was observed surrounding hair follicles. These cells demonstrated an ability to internalize nanoparticles of assorted sizes. However, the transport across the epidermis was found to be limited to particles measuring 40 nm or less in their experimental model (114).

Comprehensive research is necessary to investigate the precise amount of MNPs that can penetrate the skin (Figure 2).

1.5 MNPs mechanism of toxicity

Presently, there is limited research on the mechanism of toxicity of MNPs, with most studies focusing on assessing morphological changes. The few mechanistic studies conducted have primarily used animal or *in vitro* models. To our knowledge, there are no *in vivo* studies involving humans to date. The current evidence indicates that the accumulation of MNPs in mammalian and human tissues may have adverse long-term effects. Although the exact nature of these consequences remains uncertain, current literature employing various test models indicates the translocation and distribution of MNPs from the primary exposure site to distant locations within the body (115,116).

1.5.1 Translocation and biodistribution

Upon ingestion and inhalation, MNPs encounter different host defense mechanisms. The first line is the mucus layer covering the epithelial barrier. Within the gut, the mucus layer lines the inner layer of the digestive tract, playing a vital role in preserving intestinal homeostasis (117). Similarly, the lungs contain goblet cells within the epithelial layer, which generate mucus to entrap inhaled particles (118). Following entrapment in the mucus layer, particles, including plastic particles, may reach the epithelial layer, encountering two potential pathways for crossing this barrier. Smaller particles (<100 nm) are transported transcellularly through the epithelium by endocytosis and larger particles are transported paracellularly (119–122). For example, within the lungs,

particles smaller than 10 μm typically become trapped in the nasopharyngeal area by hair and mucus, while those smaller than 2.5 μm can reach the bronchioles and alveoli. Particles smaller than 0.1 μm can directly translocate across the alveolar epithelium via the transcellular route (118,123).

Paracellular transport is primarily governed by junctional complexes, including tight junctions, adherence junctions, and desmosomes. Tight junctions, located at the apical-most position, serve as adhesive complexes that seal the intercellular space, presenting a challenge for the paracellular transport of particles (124). Nonetheless, goblet cells intervene by disrupting the network of tight junctions, thereby loosening the connections between epithelial cells and adjacent goblet cells. This facilitates the paracellular transport of MNPs (122,125). After MNPs cross the epithelium, they encounter another line of defense which are immune cells such as dendritic cells, macrophages, T and B lymphocytes, mast cells, and eosinophils. These immune cells reside underneath the interstitium of the lung, the dermis of the skin or within the lamina propria, deep to the dermis (126). The exact transport mechanism of MNPs that may trigger inflammatory responses has not been fully investigated. However, it has been shown that MNPs can be phagocytosed and internalized by macrophages (127-129) which can trigger an inflammatory response resulting in cytokine secretion (130) or MNPs will migrate into the mesenteric lymph nodes and trigger an immune response there. Following immune response activation, MNPs can travel through the lymph vessels, reaching the thoracic duct, entering the blood stream and distributing throughout the organism (131–135) (Figure 3).

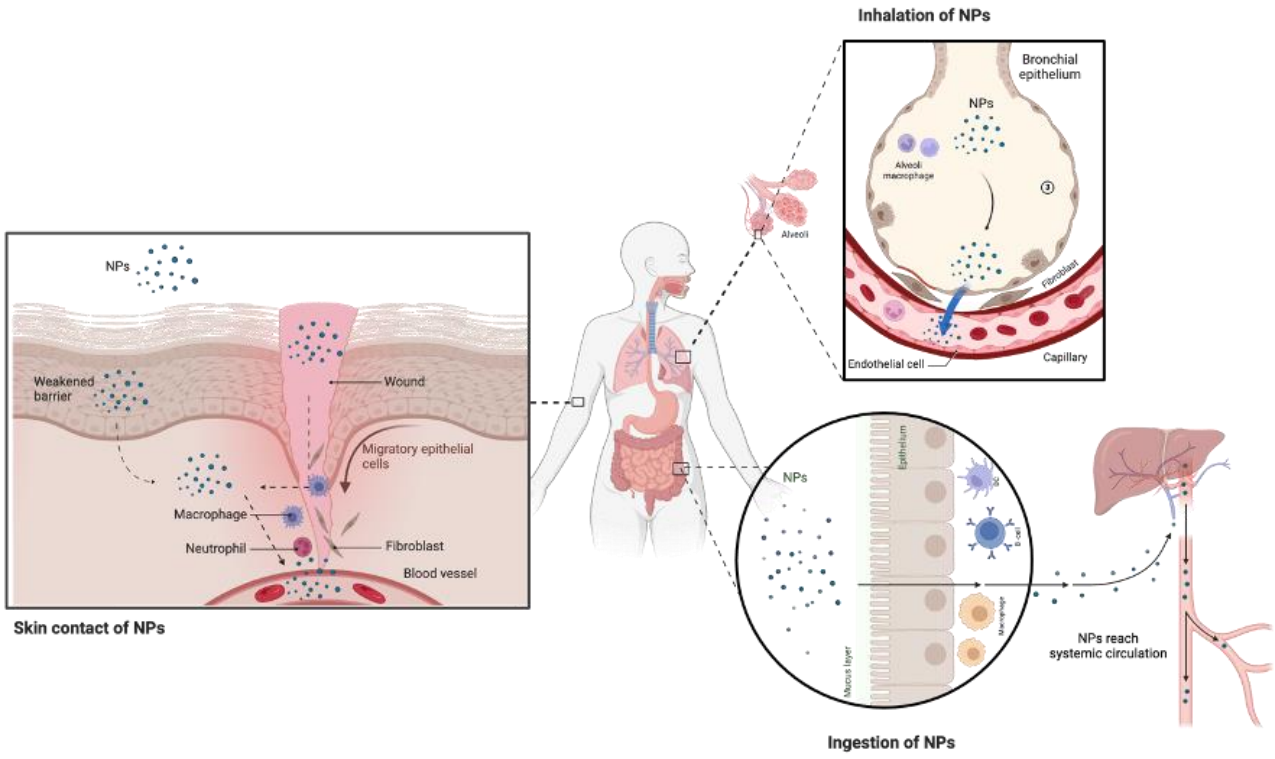


Figure 3. Translocation and biodistribution of NPs upon ingestion, inhalation, and skin contact. MNPs are ubiquitous in the environment resulting in constant exposure. Upon ingestion, inhalation, and skin contact, MNPs encounter different barriers, including mucosal surfaces, epithelial barriers, and immune cells. They can cross these barriers and enter the circulatory system, reaching various organs in the body. Created with BioRender.com.

1.5.2 Cellular uptake

After absorption into the body, MNPs interact with cells depending on size and surface properties and encounter different macromolecules, such as lipids, carbohydrates and proteins (136). Once NPs enter the biological milieu and encounter tissues, they are exposed to protein molecules and form a complex known as a “corona” (137).

Coronas have been shown to modulate transportation, internalization, biodistribution, and elimination of MNPs within biological systems (138,139). Walczyk and colleagues showed that protein coronas significantly increase NPs interactions with the environment (140). Cao et al demonstrated that protein coronas facilitate entry and translocation of NPs into the cell at higher rates (141). In vitro studies have also reported higher translocation rates of NPs due to protein coronas (142). Additionally, protein coronas trigger physiochemical changes affecting MNPs behaviour and toxicity (141). This has been reported in mice and zebrafish studies (143,144).

1.5.3 Internalization & toxicity

MNPs can get internalized into the cell via passive or active transport. Passive transport takes place when there is a difference in concentration of MNPs inside and outside of the cell. Active transport works against MNPs concentration and requires ATP (125). Under a normal physiological state only passive transport can take place given that MNPs can pass through the surface pores. This is called size-dependent internalization of MNPs. For example, it has been demonstrated that 50 and 500 nm PS-NPs showed significant penetration and distribution in lipid membranes (145) while no cellular uptake was reported for 3–5 μm MPs (146). This further suggests the size, corona compounds, shape, and surface modifications affect MNP mode of transport (125,147). In addition to passive transport, MNPs can penetrate cellular barriers through endocytosis which is an active internalization pathway and typically includes phagocytosis and pinocytosis (clathrin- and caveolae-mediated endocytosis, clathrin/caveolin-independent pathways, macropinocytosis) (148–150). Xu and colleagues identified macropinocytosis and

clathrin-mediated endocytosis as the primary mechanisms for NPs uptake in an immortalized intestinal epithelial cell line (Caco-2) (151) Internalization of MNPs starts with cellular membrane damage leading to intracellular biological effects, change in fluidity, and eventually leading to cell death and apoptosis (152–154). Following internalization, MNPs can permeabilize the endosomal membrane and be released into the cytosol. Once they enter the cytosol, they can interact with different organelles such as mitochondria and the nucleus and affect important cellular processes such as mitotic spindle formation and migration of chromosomes during cell division. MNPs could also interfere with the trafficking of transport carriers in the cell along the exocytotic pathway consequently leading to inhibition of the cell surface expression of important signaling receptors or membrane transports (155,156). Additionally, MNPs can disrupt endosomal membrane traffic that many cellular processes depend on, such as surface protein turnover and signaling attenuation as well as retrograde signaling from endosomal compartments. Furthermore, the buildup of MNPs within the lysosome or late endosome may hinder their ability to degrade substances and disrupt the essential cellular membrane turnover process known as macroautophagy (157). A disruption in autophagic clearance has the potential to initiate cascading processes that ultimately result in autophagic cell demise. Conversely, internalized MNPs can also activate autophagy. It is documented that metallic nanoparticles can influence autophagy thus raising the possibility that MNPs might have a similar effect (158).

These processes inherently induce cellular stress. Stressors affecting both the plasma membrane and endo-lysosomes prompt cellular stress responses. Research conducted on freshwater flea species *Daphnia* has revealed that exposure to PS-NPs impacts

growth and reproduction (159). Intriguingly, this exposure also led to an increase in AMP activated protein kinase (AMPK) levels, signaling a stress response occurred (160).

A broader aspect related to cellular stress response seems to involve the generation of reactive oxygen species (ROS), which has been recognized as the molecular initiating event in recent analyses of adverse outcome pathways within the field (161). Cells generate ROS through two primary mechanisms: either through the mitochondrial electron transport chain (ETC) during regular aerobic respiration or through oxidative bursts facilitated by nicotinamide adenine dinucleotide phosphate oxidases (NOXs) (162). Elevated ROS levels from impaired mitochondrial function may stem from the former, whereas the latter is often linked to bacterial invasion, as NOXs are triggered by bacterial byproducts and cytokines. Every cell possesses a conserved innate immune system, evolved to defend against pathogen intrusion or exposure to foreign substances (163). Nevertheless, elements of the innate immune system, like Toll-like receptors (TLRs), have the capability to react to a range of internally produced or secreted molecules referred to as damage-associated molecular patterns (DAMPs) as well (164,165). This can lead to sterile inflammation, characterized by inflammatory reactions in the absence of pathogenic infection (166). Within the organism, pro-inflammatory cytokines released during local inflammation can draw circulating immune cells, potentially exacerbating the inflammation and resulting in cell and tissue damage. Notably, NPs have been demonstrated to induce stress responses in the innate immune system of fish (167) (Figure 4).

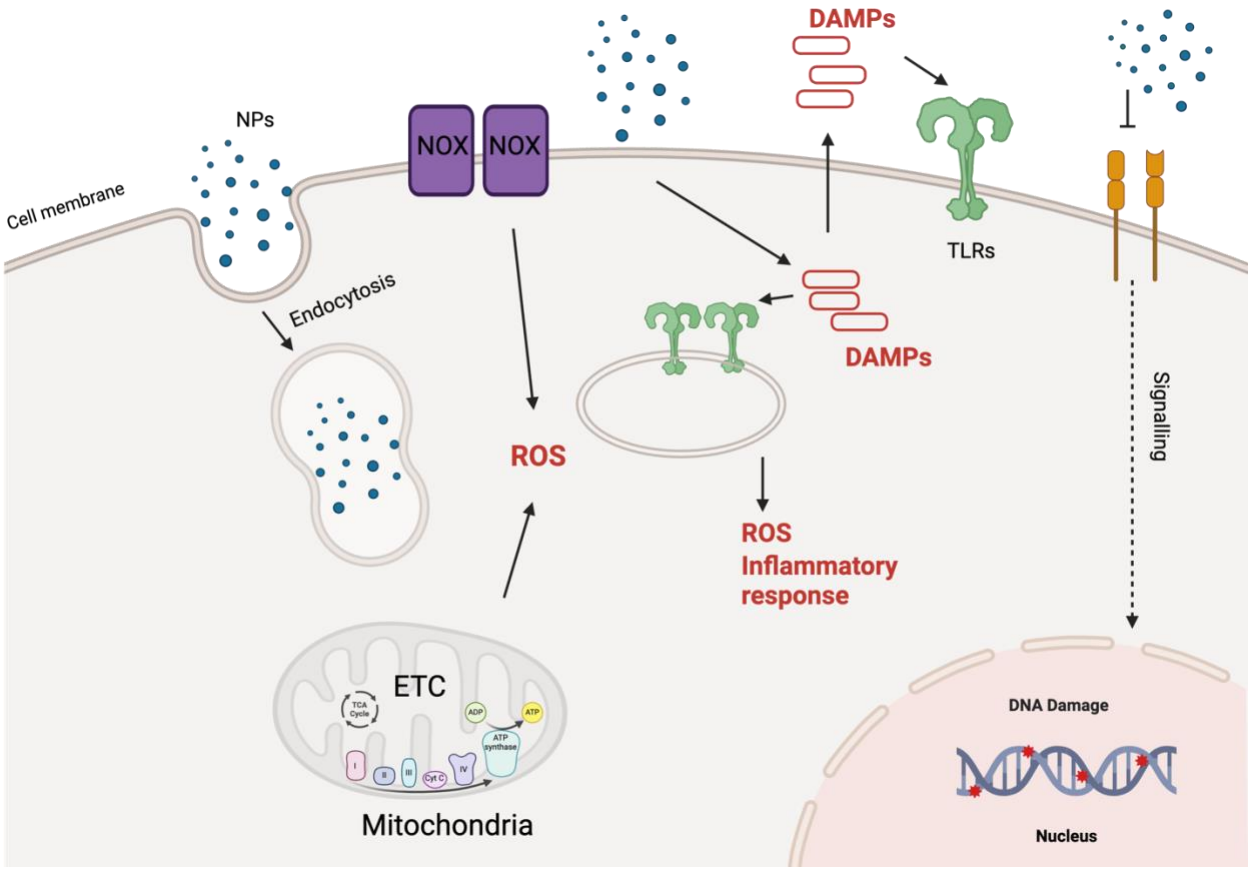


Figure 4. Cellular uptake and internalization of NPs.

MNPs can be taken up internally via endocytosis, breaching the plasma membrane and disrupting the signaling of cell surface receptors. They can also interfere with the endocytic pathway. This disturbance can trigger the activation of the immune system due to the emergence of stress, facilitated by both endogenous and secreted damage-associated molecular patterns (DAMPs), thereby activating toll-like receptors (TLRs) that mediate innate immunity. Furthermore, stress induced by MNPs can prompt the production of reactive oxygen species (ROS) from NADP oxidases (NOXs) or from impaired mitochondrial function in the electron transport chain (ETC), potentially resulting in DNA damage and cellular apoptosis. Created with BioRender.com.

1.6 Reproductive toxicity

MNPs pose a significant risk to human health due to their frequent and continuous presence in the human living environment. MNPs research is still in its early stages with MNPs reproductive toxicity in humans being the least investigated, especially in females. Studies have been increasing in recent years using animal models primarily focusing on phenotypic changes. While these findings may not directly apply to humans, they offer valuable insights into understanding the potential effects and mechanisms involved in the reproductive toxicity of MNPs.

1.6.1 Male reproductive toxicity

When it comes to reproductive toxicity, most studies have focused on males. In numerous animal studies MNPs have been shown to interfere with the blood-testis barrier, potentially causing detrimental effects on male reproductive function. For instance, recent research suggests that even at a minimum human equivalent dose, estimated to be 0.016 mg/kg/d, MNPs could lead to abnormalities in semen quality (168). Different studies have showed that PS-MNPs can disrupt the blood-testis barrier, leading to male reproductive toxicity, such as spermatogenesis disorders. For example, it was shown that following oral administration of PS-MNPs in mice, they were absorbed in the blood 30 minutes after and penetrated the blood-testis barrier within four hours (169). Similarly, in a study by Jin et al, PS-MNPs were found in mouse testes 24 hours after exposure, and testosterone concentration and sperm vitality and quality decreased 28 days after exposure (170). Furthermore, research indicated that the combined exposure to MNPs and plasticizers could exacerbate reproductive harm in male mice,

leading to reduced sperm count, decreased sperm motility, and lower testosterone levels (171,172). Most of the studies mentioned earlier have found that MNPs negatively impact male reproductive health, though the exact mechanisms remain unclear. Recent research suggests that the primary mechanisms of toxicity are likely inflammatory changes and oxidative stress damage (173,174).

1.6.2 Female reproductive toxicity

Female reproductive disorders, such as infertility and polycystic ovarian syndrome (PCOS) are global health issues, which may be closely related to environmental deterioration (175–177). The ovaries are vital in endocrine and reproductive functions. Ovaries are vulnerable to endocrine disrupting chemicals (EDCs) which are natural or human-made substances that mimic, interfere, or even block endogenous hormones and they have been reported to exist in MNPs as well (178). Studies have shown that exposure to EDCs can lead to reproductive health issues, such as premature ovarian insufficiency, sex hormone imbalance, and infertility (179).

Studies have also shown that MNPs exposure can lead to reproductive toxicity in the female reproductive system. A study by Wang et al showed that a 60-day PS-MPs exposure reduced 17β-estradiol (E2) and testosterone (T) concentrations in the plasma of female *Oryzias melastigma* (180). Moreover, Zhang and colleagues showed that mice exposed to PS-MPs through oral administration for 30 days (40 mg/kg/day) underwent oxidative stress, DNA damage, and mitochondrial dysfunction in their oocytes. This led to a reduction in their fertilization rate, oocyte maturation, and disturbances in embryonic development (181). This further was investigated by Liu et al

who showed that continued exposure (35 days) to PS-MPs led to polar body extrusion rate and a decreased survival rate of superovulated oocytes in mice ovaries (182). Overall, these studies showed that a 30 to 35-day PS-MPs exposure could lead to ovarian inflammation and a decrease in oocyte quality in mice. Nevertheless, these investigations remain constrained, primarily centered on MPs, and have solely examined morphological alterations without delving into the underlying mechanisms. Recent studies have shown MNPs exist in the human placenta and that placental translocation depends on MNPs physicochemical properties, such as size, corona formation, and charge (183). For example, Dusza and colleagues conducted an in vitro study with BeWo b30 choriocarcinoma cells and showed that MNPs uptake was size-dependent. They also showed that MNPs passing through the placental barrier could contribute to the disturbance in fetal development (184). Another study by Wick et al used an *ex vivo* human placental perfusion model and showed that PS NPs with diameters up to 240 nm can pass through the placental barrier and are capable of transplacental transfer (185). Remarkably, Ragusa and colleagues observed 12 MP fragments (5–10 μm in size) in 4 human placentas for the very first time. The presence of MPs in human placentas may trigger adverse pregnancy outcomes and transplacental passage and could potentially have intergenerational transfer (186). Over time plastic particles degrade and release low levels of chemicals resulting in potentially prolonged chronic exposure (187,188). All plastics contain reactive oxygen species due to their polymerization and synthesis. Given their persistence, NPs can induce oxidative stress, resulting in cell death (188,189). A concern associated with exposure to NPs is that they can act as vectors for microorganisms and other pollutants

and have been shown be more reactive and infiltrate deeper in the tissues due to their higher surface area and smaller size respectively, and thus inducing more toxic effects compared to MPs (190,191). NPs' persistent nature and induction of oxidative stress, especially those consisting of polystyrene, can cause inflammation in vivo and vitro (189,192,193). A World Health Organization (WHO) report on NPs in drinking water indicated that levels in drinking water are low but stressed the urgent need for toxicological studies to assess potential human health effects (194). While the mechanisms by which environmental factors impact human health vary, dysregulated inflammation represents a common mechanism associated with multiple environmental factors (195,196). Such effects can result in, or exacerbate, pathological conditions leading to immune dysfunction, neurodegenerative diseases, and infertility. MPs have been linked to PCOS and endometriosis and recent studies have shown that MNPs have lowered sperm count and quality, decreased male fertility, and overall are harmful to male reproductive system (173,197–199). However, there is limited understanding of the effect of NPs on the female reproductive system, which this study aims to investigate.

1.7 Summary & objectives

The emergence of plastics as global pollutants has received considerable attention. MNPs are reported to accumulate in the environment and their presence in food, drinking water and the atmosphere indicates that human exposure is inevitable, yet few studies have examined the effects of NPs on human health. NPs can enter the human body and translocate through its physical barriers to reach secondary organs, including the reproductive organs and tissues. Some studies demonstrate the impact of NPs on cultured immune cells including induction of ROS and genotoxicity in lymphoblasts (Tk6 cells), and lymphocyte (Raji B-cells) cell lines (200). Immune cells including natural killer (NK), monocytes, macrophages and dendritic cells are present throughout the female reproductive tract and are involved in intra-ovarian regulation and endometrial physiology (197,201). Cytokines, chemical messengers synthesized by cells including immune cells, like IL-6 and IL-12 are involved in folliculogenesis, cumulus-oocyte interactions, ovulation, corpus luteum formation and leuteolysis (201,202). A finely tuned balance of immune cells is important for reproduction. Dysregulation of immune cells and thus function adversely affects folliculogenesis, oocyte maturation, and ovulation, and is thought to be central to chronic inflammation characteristic of PCOS, endometrial angiogenesis, spiral artery remodeling and endometriosis (197,198,201). Therefore, this study aims to investigate the effects of chronic NPs exposure on female reproductive function in a mouse model. The study will examine the impact of NPs on ovarian function and structure by assessing circulating reproductive hormones, estrous cyclicity, and follicle development in female mice orally exposed to NPs.

2. Materials and Methods

2.1 Experimental design

To elucidate the reproductive effects of NPs exposure, sexually mature (40-60 days of age) female C57bl6j mice (n=30) were randomly assigned to one of the three exposure groups as follows: control (0 µg of polystyrene beads/l in tap water tap water), low dose (100 µg of polystyrene beads/l in tap water), and high dose (1000 µg of polystyrene beads/l in tap water). During the exposure phase mice were exposed to fluorescently labeled polystyrene beads (500 nm in diameter) in their drinking water for 29 days at a concentration of 0 (vehicle control), 100 or 1,000 µg/l (n=10/group) (203). Mice were weighed weekly and checked daily for signs of systemic toxicity including change in fur color and lacrimation. Vaginal smears were collected for the last 15 days to check for the estrous cycle stages, as per below. At the end of the exposure period (day 29) mice were euthanized by CO2 inhalation, and cervical dislocation. Blood was collected by cardiac puncture and reproductive tissues were collected for analysis (Figure 5).

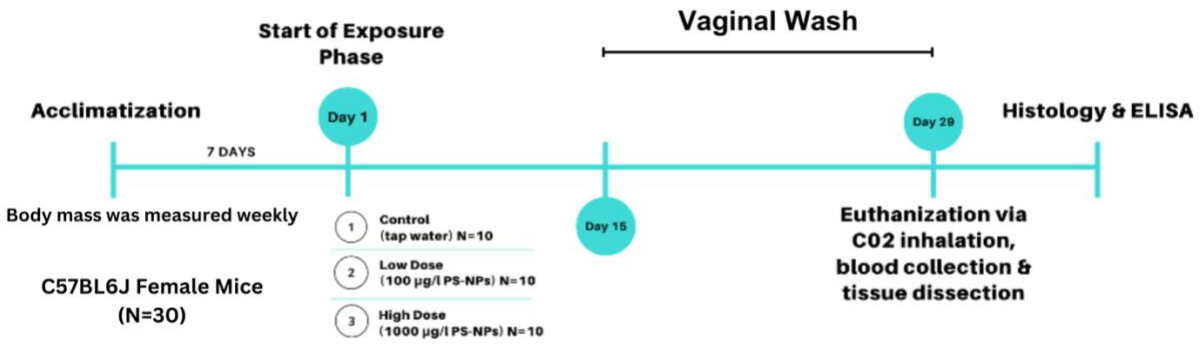


Figure 5. Methodology summary

Following acclimatization, mice were randomly assigned to one of three exposure groups: control, low dose, and high dose PS-NPs. Exposure phase lasted 29 days and estrous cycle stage was assessed by vaginal lavage during the last 15 days. At the end of the exposure phase, mice were euthanized, and peripheral blood and reproductive tissues were collected.

2.2. Vaginal smears collection

To assess the effect of PS-NPs exposure on the estrous cycle, vaginal lavage was collected from mice each day at 8 AM beginning on study day 15 and continuing through to the end of the study for a total of 15 days. To collect the vaginal lavage 0.5 mL of phosphate-buffered saline (PBS) was placed at the vaginal opening using a pipette and a pipette tip and was gently pushed into the vaginal canal and then

aspirated. Vaginal lavage was collected in Eppendorf tubes, placed on a clean glass histology slide, air dried and then placed under the microscope to identify the estrous cycle stages of the mice. Identification of estrous cycle stages was done by two investigators (MG & JMW). In case of disagreements, consensus was achieved by discussion between the two investigators (MG & JMW). The estrous cycle is the rodent reproductive cycle. Unlike the roughly 28-day reproductive cycle in women (e.g. the menstrual cycle), the mouse estrous cycle typically lasts 4-5 days and does not include menses (bleeding and shedding of the uterine lining). The estrous cycle consists of 4 stages: proestrus, estrus, metestrus, and diestrus. Proestrus lasts about 12 hours; indicated by round, nucleated epithelial cells and only few leukocytes in the vaginal lavage. During proestrus, around 80-90% of the epithelial cells are intact, active, and nucleated. Additionally, clumps of leukocytes are present (Figure 6A). Estrus is the next stage in the estrous cycle and is a period of sexual receptivity lasting about 12 hours (typically 12-8 AM in mice). Estrus can be identified by the dominance of large, cornified (degenerative cells that lose nuclei) epithelial cells in the vaginal lavage. Approximately 100% of epithelial cells are cornified during estrus (Figure 6B). Estrus is followed by metestrus and takes place shortly after ovulation lasting about 21 hours. Metestrus is indicated by large cornified epithelial cells mixed with polymorphonuclear leukocytes (about 1/3 the size of the epithelial cells) and a lot of cellular debris in the vaginal lavage. During metestrus, there is an approximate equal distribution of about 50% cornified cells and 50% leukocytes (Figure 6C). Diestrus is the final stage and is a period of inactivity lasting about 60-70 hours; indicated by mostly polymorphonuclear leukocytes and a few nucleated epithelial cells in the vaginal lavage. During diestrus,

approximately 80-95% of the cells present are leukocytes, with some epithelial cells also observed (204) (Figure 6D). To evaluate the impact of PS-NPs on the estrous cycle, both the overall length of the estrous cycle and the duration of each stage were tracked.

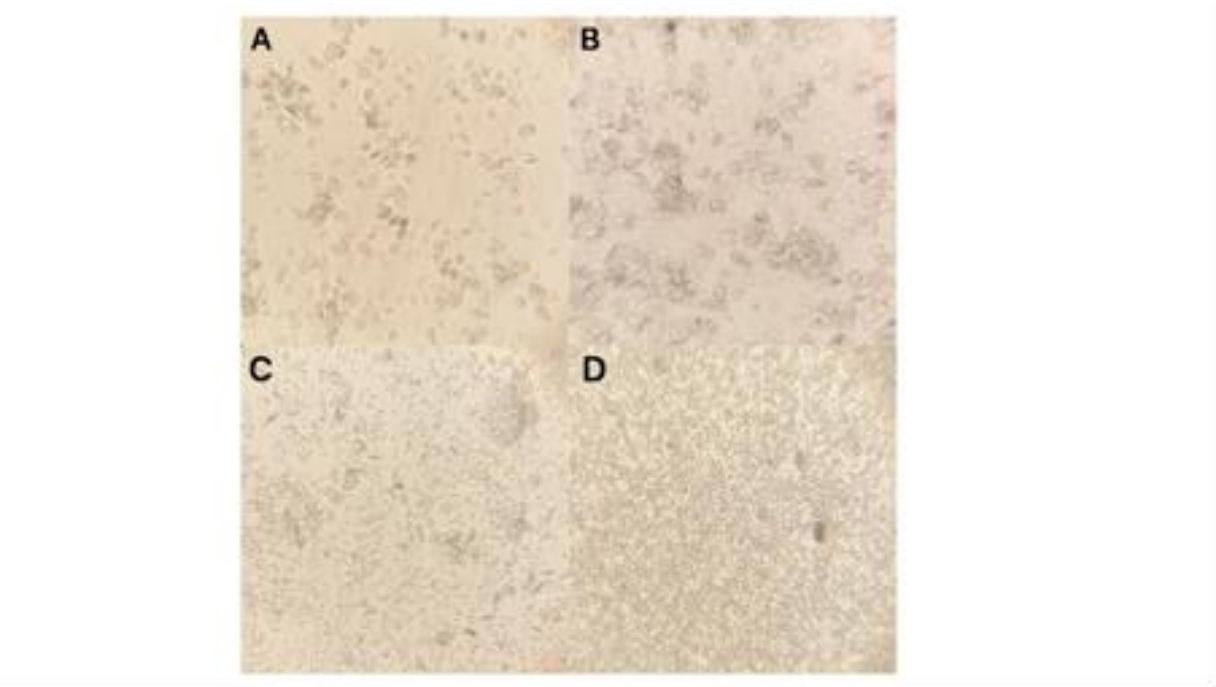


Figure 6. Mice vaginal cytology. The panels depict the estrous cycle stages; (A) proestrus, (B) estrus, (C) metestrus, and (D) diestrus.

2.3. Staining with hematoxylin and eosin (H&E) & histomorphometric analysis

Ovaries were fixed in 10% formalin, dehydrated in graded concentrations of ethanol and xylene and embedded in paraffin wax. The entire ovary was serially sectioned at 5 µm thickness and stained with hematoxylin and eosin (H&E) for microscopic examination and quantification of ovarian follicles. Five random sections per ovary were chosen for follicle counting and diameter measurement, based on the method by Smith et al (205).

Images of ovarian sections were captured at 10X, and the number of primordial, primary, secondary, and antral follicles was counted, and their diameter was measured. To prevent double counting the follicles, multiple images were taken per section in a row or column and then were stitched together using ImageJ (Figure 7). ZEISS ZEN Microscopy Software was used for imaging. Follicle types were identified using Myers et al approach which is based on morphological features including the number of granulosa cell layers surrounding the oocyte (206). Primordial follicles were identified as an oocyte surrounded by one layer of squamous (flattened) granulosa cells (Figure 8 A). Primary follicles were identified as an oocyte surrounded by one layer of cuboidal granulosa cells (Figure 8 B). Secondary follicles possessed more than one layer of cuboidal granulosa cells while antral follicles had more than one layer of cuboidal granulosa cells with a defined antrum (Figure 8 C & D respectively). To measure the diameter, three diameters per follicle were measured, and the average was calculated for a more accurate measurement.

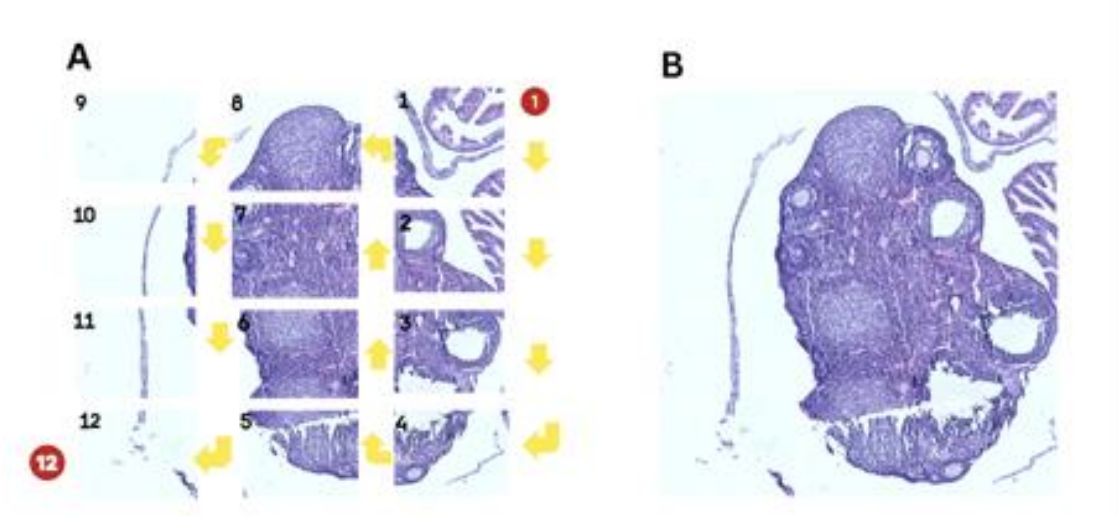


Figure 7. Imaging and stitching process of the ovary sections. (A) Imaging of ovarian slides was performed row or column-wise and ovarian sections were stitched together for a full section visualization (B).

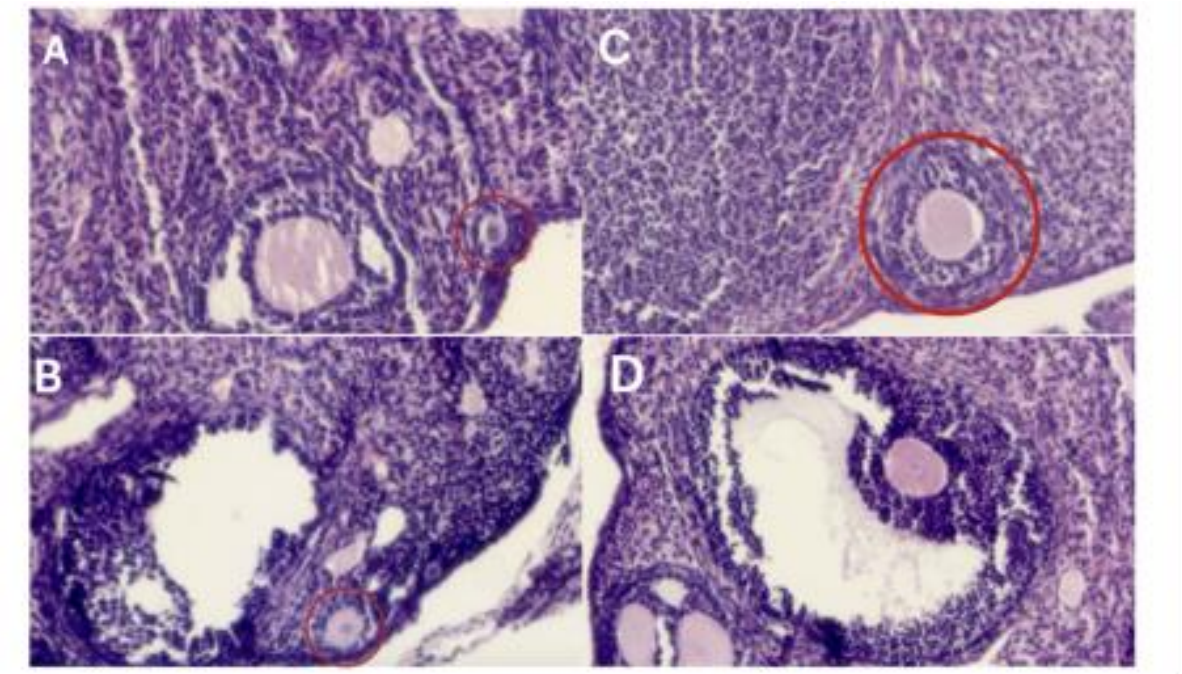


Figure 8. Morphological classification of ovarian follicles. (A) primordial follicle with one layer of squamous granulosa cells (red circle), (B) primary follicle with one layer of cuboidal granulosa cells (red circle), (C) secondary follicle with more than one layer of cuboidal granulosa cells and no visible antrum (red circle), and (D) antral follicle with a defined antral space.

2.4 ELISA Analyses of serum reproductive hormones

The levels of estradiol (E2) and progesterone (P4) were measured using commercially available ELISA kits (MyBioSource, San Diego, CA, USA), according to manufacturer's instructions.

2.5 Statistical Analysis

Data is presented as Mean ± Standard Deviation (SD) unless otherwise stated. All analyses were performed using SPSS 29.0 Software (SPSS Inc.Chicago, USA). Repeated measures analysis of variance (ANOVA) was performed to examine the effect of PS-NPs exposure on murine body mass. Welch's t-test (unequal variance assumed) was performed to examine the effect of PS-NPs on estradiol and progesterone levels in the control vs low dose exposure group and control vs high dose exposure group. Mixed model two-way ANOVA was performed to compare the estrous cycle stage lengths, and follicles count and diameters between the three exposure groups (control, low dose exposure, and high dose exposure). A $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 Effect of chronic oral exposure to PS-NPs on murine body mass

Mice were orally exposed to PS-NPs for 29 days, and mass was documented on a weekly basis for each mouse. There was no significant change in body mass over the period of PS-NPs exposure was observed in either group exposed to PS-NPs as compared to the control, unexposed, mice ($P>0.05$) (Figure 9).

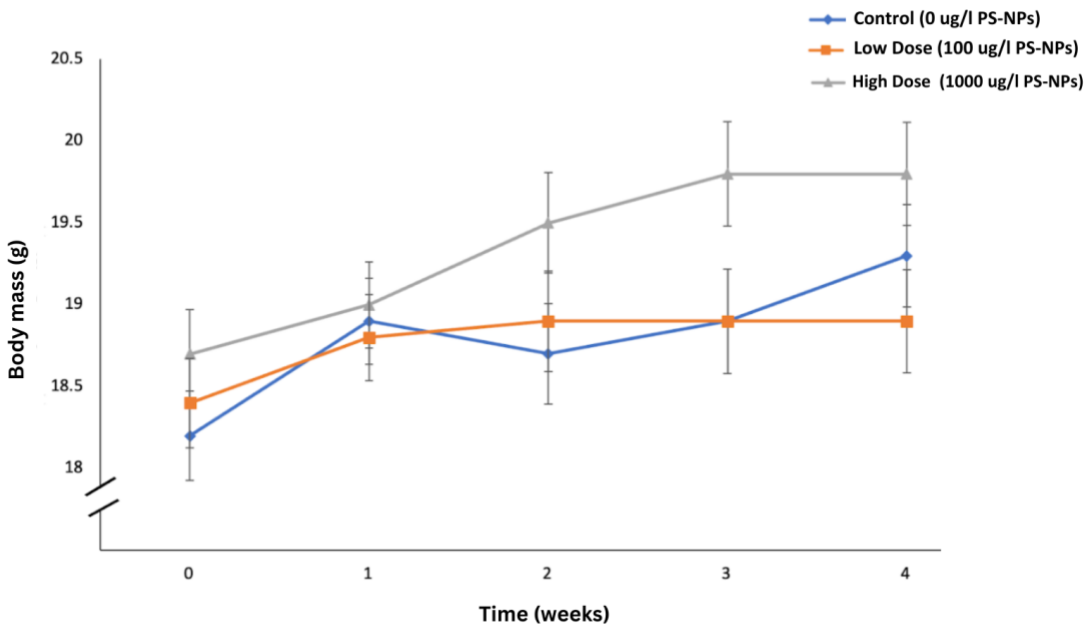


Figure 9. Murine body mass is unaffected by chronic PS-NPs exposure.

This figure shows mean and SEM of mice in the control group (no exposure to PS-NPs), low dose exposure group (100 ug/l PS-NP), and high dose exposure group (1000 ug/l PS-NPs) on weeks 0 (baseline body mass),1, 2, 3, and 4 of the experiment.

3.2 Effect of PS-NPs on murine estrous cycle

Estrous cycle stage was monitored using vaginal cytology for the final 15 days of the study period. Mice in the control group (N=10) spent an average of 2.30±2.00 days in proestrus, 5.50±1.84 days in estrus, 2.40±1.43 days in metestrus, and 4.80±2.20 days in diestrus during the 15 days of estrous cycle staging. Mice (N=10) in the low dose exposure group (100 μg/l PS-NPs) spent an average of 2.00±1.56 days in proestrus, 6.20±1.69 days in estrus, 2.80±1.69 days in metestrus, and 3.90±1.59 in diestrus. Mice (N=10) in the high dose exposure group (1000 μg/l PS-NPs) spent an average of

1.9±2.02 days in proestrus, 5.80±1.93 days in estrus, 2.40±1.71 days in metestrus, and 4.90±2.23 days in diestrus respectively. No significant change in the length of estrous cycle stage was observed as a result of PS-NPs exposure (P>0.05) (Figure 10). The effect of PS-NPs exposure on the overall length of the estrous cycle was also measured. There was a significant increase in overall estrous cycle length in the high dose PS-NPs exposure group compared to the unexposed controls (5.53±.25 days vs 4.70±0.23 days, P=0.02) (Figure 11).

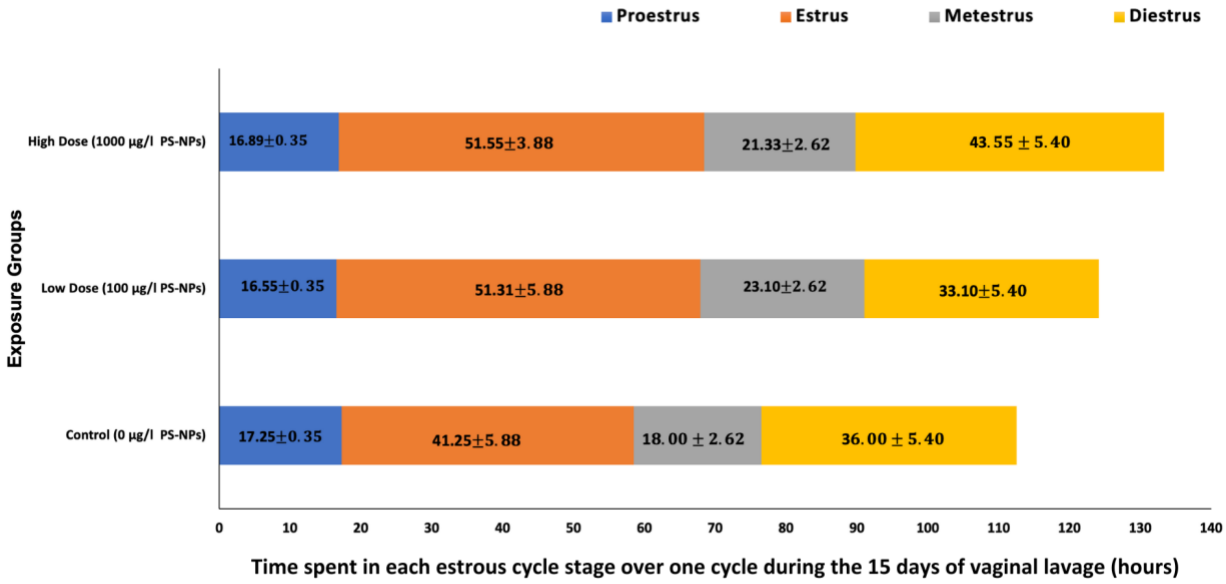


Figure 10. Effect of PS-NPs on the length of proestrus, estrus, metestrus, and diestrus in mice. This figure shows the average amount of time (hours) that mice in each exposure group spent in each estrous cycle stage over one estrous cycle during the final 15 days of exposure. Data is presented as mean ± SEM.

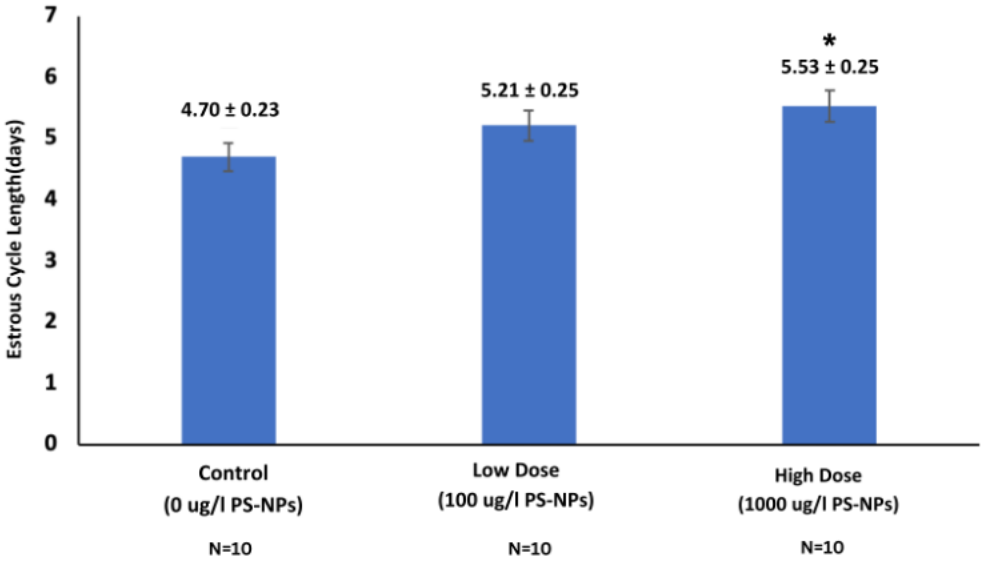


Figure 11. Effect of PS-NPs on overall estrous cycle length in mice. This figure shows the estrous cycle length in days of mice exposed to PS-NPs. Cycle stage was monitored by vaginal cytology for the last 15 days of the 29-day exposure period and is presented as mean ±SEM. *P<0.05

3.3 Effect of PS-NPs on serum estradiol and progesterone levels

Serum estradiol levels were measured by ELISA, and no significant change was detected in the PS-NPs exposed groups as compared to the controls (high exposure group vs control: mean difference=5.60 pg/ml, standard error of difference (SED)=2.95, P=0.09 and low exposure group vs control: mean difference=1.97 pg/ml, SED=2.92, P=0.52) (Figure 12). Serum progesterone levels were also quantified by ELISA, and there was a significant decrease in progesterone levels in the high dose exposure group

compared to control (mean difference=1.64 pg/ml, SED=0.64, P=0.03). However, chronic exposure to PS-NPs had no significant effect on progesterone levels in the low dose exposure group (mean difference=1.10 pg/ml, SED=0.65, P=0.12) (Figure 13).

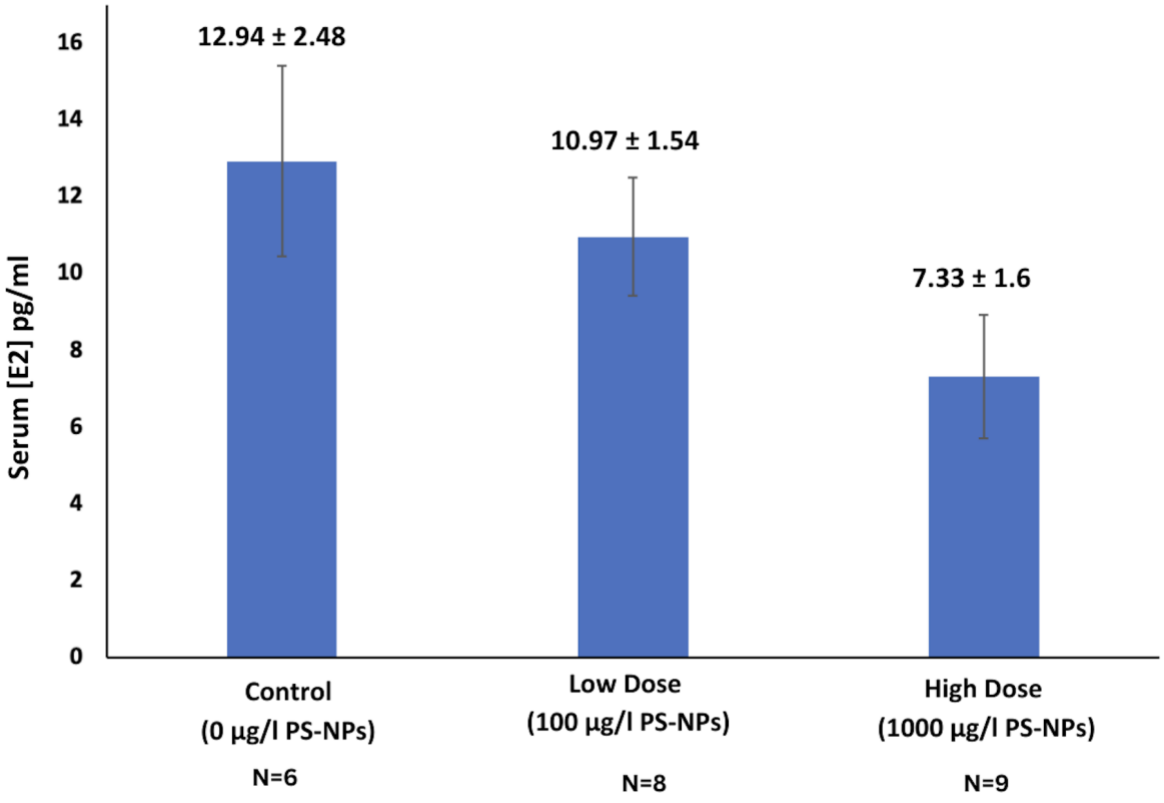


Figure 12. Effect of PS-NPs on serum estradiol level in female mice. This figure shows the change in the estradiol level as a result of PS-NPs exposure for 29 days.

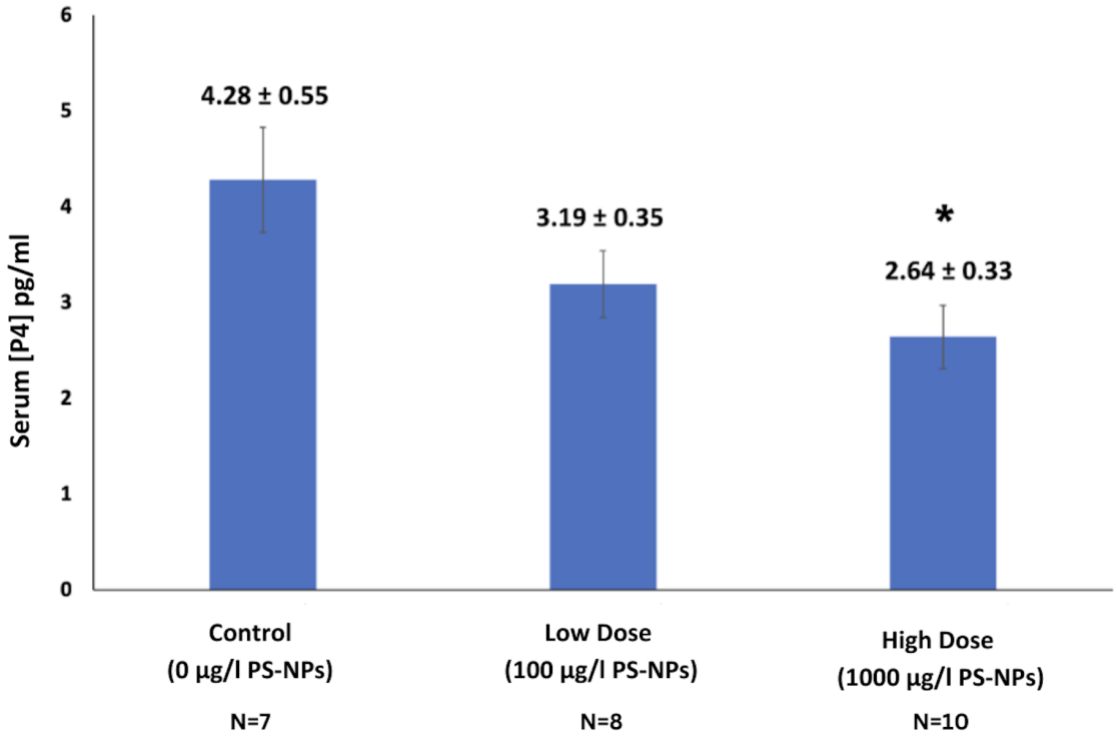


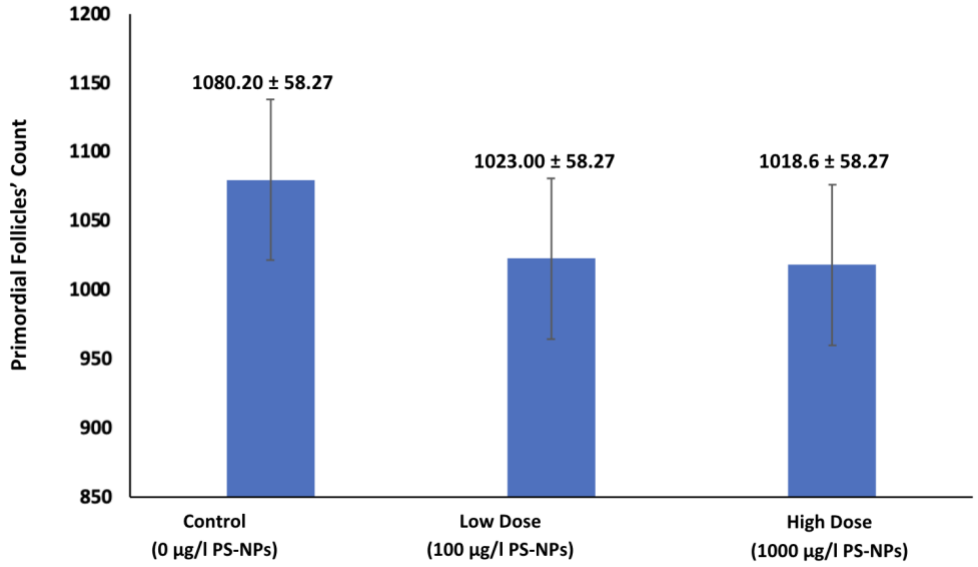
Figure 13. Effect of PS-NPs on serum progesterone level in female mice. This figure shows the change in serum progesterone level as a result of PS-NPs exposure for 29 days. *P<0.05

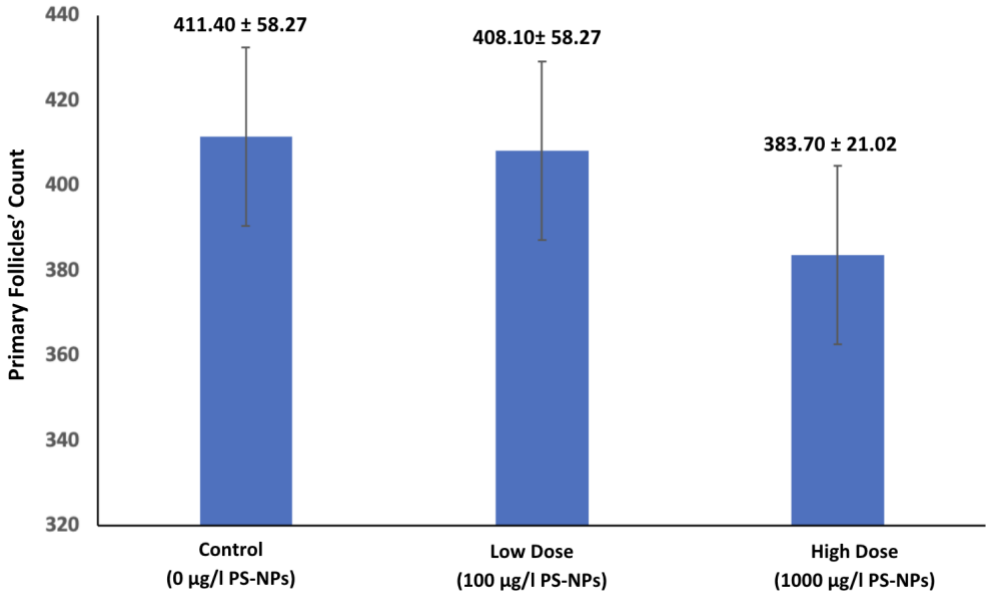
3.4 Effect of chronic oral exposure to PS-NPs on ovarian follicle count and diameter

The effect of PS-NPs on ovarian follicle count and diameter was investigated. On average there were 1080.20±58.27 primordial follicles, 411.40±21.02 primary, 298.40±15.43 secondary, and 216.90±16.02 antral follicles in the control group. The low-exposure PS-NPs group had an average of 1023.00±58.27 primordial, 408.10±21.02 primary, 284.50±15.44 secondary, and 187.50±16.02 antral follicles. Finally, the high-exposure PS-NPs group had an average of 1018.60±58.27 primordial,

383.70±21.02 primary, 271.00±15.44 secondary, and 177.10±16.02 antral follicles. When compared with controls, there was no statistically significant difference in the number of primordial follicles, primary, secondary, and antral follicles in the PS-NPs-treated ovaries (Figure 14).

Next, the diameter of follicles was measured in the three groups. Antral follicle diameter was significantly smaller in the PS-NPs exposure groups compared to the control group mean diameter (Figure 15). When compared to the controls, PS-NPs had a more pronounced effect on reducing antral follicle diameter in the high dose exposure group ($p=0.001$) compared to the low dose exposure group ($p=0.03$).


Primordial Follicles' Count
1200
1150
1100
1050
1000
950
900
850
1080.20 ± 58.27
1023.00 ± 58.27
1018.6 ± 58.27
Control
(0 µg/l PS-NPs)
Low Dose
(100 µg/l PS-NPs)
High Dose
(1000 µg/l PS-NPs)


Primary Follicles' Count
440
420
400
380
360
340
320
411.40 ± 58.27
408.10± 58.27
383.70 ± 21.02
Control
(0 µg/l PS-NPs)
Low Dose
(100 µg/l PS-NPs)
High Dose
(1000 µg/l PS-NPs)

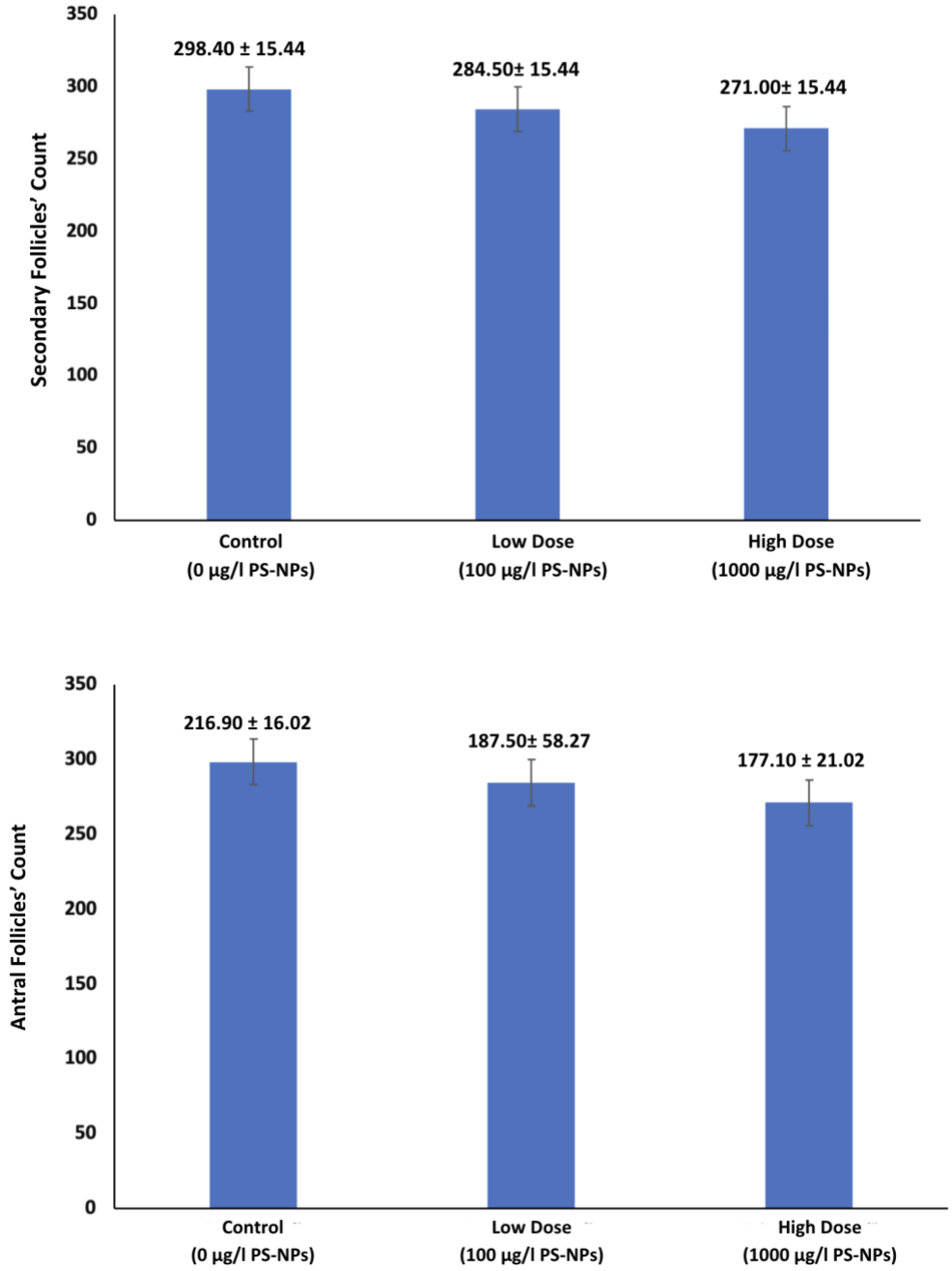
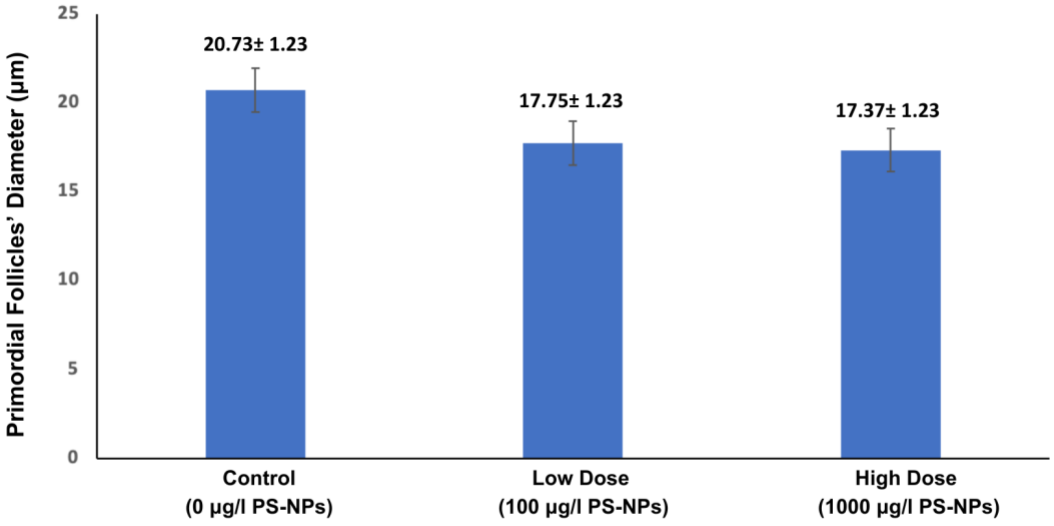
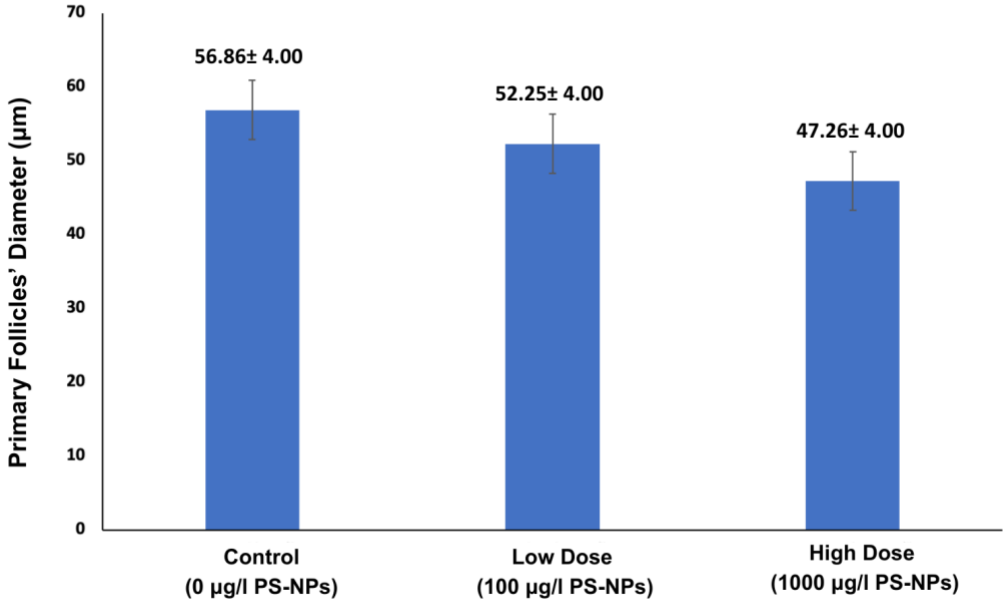


Figure 14. Effect of PS-NPs on Follicle Count. These figures show the primordial, primary, secondary, and antral follicles count of different PS-NPs exposure groups. Data is presented as mean and SEM.


Primordial Follicles' Diameter (µm)
25
20
15
10
5
0
20.73± 1.23
17.75± 1.23
17.37± 1.23
Control
(0 µg/l PS-NPs)
Low Dose
(100 µg/l PS-NPs)
High Dose
(1000 µg/l PS-NPs)


Primary Follicles' Diameter (µm)
70
60
50
40
30
20
10
0
56.86± 4.00
52.25± 4.00
47.26± 4.00
Control
(0 µg/l PS-NPs)
Low Dose
(100 µg/l PS-NPs)
High Dose
(1000 µg/l PS-NPs)

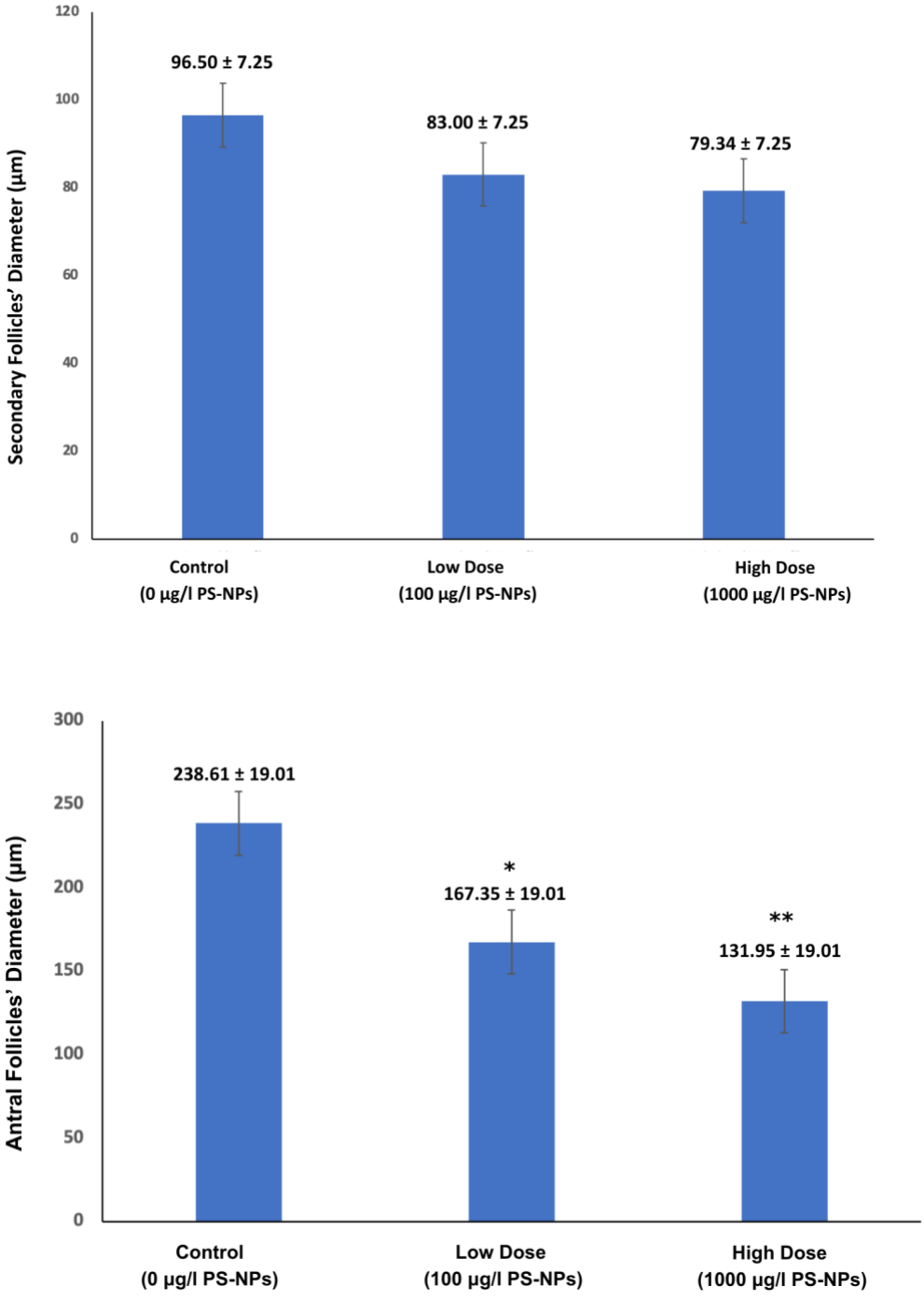


Figure 15. Effect of PS-NPs on follicles size These figures show the primordial, primary, secondary, and antral follicle size of different PS-NPs exposure groups. Data is presented as mean and SEM. *****P<0.05, **P<0.001

4. Discussion

4.1 Principal findings and implications

Industrial development and anthropogenic activities have led to a dramatic expansion of global production chains for plastic inputs for decades (207,208). Currently, 460 million tons of plastic materials are produced each year, a mass projected to reach 33 billion tons in the next 30 years (209,210). Due to accelerated production and limited reuse, transformation or recycling initiatives, the world's ability to deal with excess plastic products has been surpassed (211,212). These problems are even more evident in developing countries, where plastic waste collection and treatment systems are often inefficient or unavailable (207). As a result, the most common destination for industrial and domestic plastic waste is environmental disposal, determining extensive land and water pollution (213,214). It is undeniable that plastic materials have a notorious relevance and applicability in the food, pharmaceutical, agrochemical, transport, construction, and electronic industries (215,216). However, the irrational disposal of plastic products with very low shelf life (e.g., bags, packaging and bottles) often ignores the negative environmental impact of these pollutants, which can persist for decades or hundreds of years in nature causing ecological imbalances (207,217). Plastics break down into MNPs which can enter the human body and systems through digestion, inhalation, and dermal contact and cause inflammation and immune dysfunction. NPs can pose a serious risk to human health due to their smaller size and larger relative surface area which allows for an enhanced ability to adsorb toxic chemicals and pathogens and a higher penetration rate into tissues. It has been shown that NPs can accumulate in the human heart, liver, spleen, lung, kidney, brain, stomach, large and

small intestine (218) and can lead to neurotoxicity, developmental toxicity, oxidative stress, DNA damage, and compromise the immune and circulatory systems (16,219,220). However, there is little known about the accumulation and distribution of NPs in the female reproductive system. Therefore, this study experimentally investigated the effect of chronic oral exposure to NPs on the female reproductive system using a mouse model.

In this study we showed that PS-NPs exposure decreased antral follicle diameter, decreased serum progesterone levels, and increased estrous cycle length in female mice. Ovarian reserve, indicated by antral follicle count, is the capacity of the ovary to provide oocytes (egg cells) and is a key indicator for assessing female fertility. Poor ovarian reserve characterized by a reduction in the ovarian follicle pool is an important cause of infertility (221,222). It has been shown that poor ovarian reserve is associated with lower pregnancy rate and higher rates of pregnancy loss (223).

A follicle is the basic structural unit of the ovary which through the process of folliculogenesis undergoes different morphological and functional changes from a primordial to ovulatory follicle, ultimately releasing an oocyte (egg) through ovulation. Folliculogenesis can be divided into gonadotropin-independent stages which consist of preantral follicles (primordial, primary, and secondary follicles) and gonadotropin-dependent stages which include antral follicles (224). Preantral stages are characterized by growth and proliferation of granulosa cells. Luteinizing hormone (LH) stimulates internal cells to produce androgens which diffuse into the granulosa cell compartment via the basal lamina. Under the influence of follicle stimulating hormone

(FSH) granulosa cells turn androgens to estrogens, mainly estradiol, which causes granulosa cells to proliferate. During the antral phase, production of estradiol by granulosa cells increases under the combined effect of FSH and estradiol. This surge in estradiol is a crucial signal for the neuroendocrine system indicating that the antral follicle is ready for ovulation. The process of ovulation happens around day 14 in a 28-day human reproductive cycle. An increase in estradiol increases gonadotropin releasing hormone (GnRH) secretion onto the pituitary gland, leading to a surge of LH. The LH surge triggers a series of events in the ovary, including an increase in intrafollicular proteolytic enzymes. These enzymes weaken the follicular wall, facilitating the release of the mature follicle (ovulation). Additionally, the LH surge induces luteinization of thecal and granulosa cells, leading to the formation of the corpus luteum. This structure is pivotal in synthesizing progesterone, and is crucial for maintaining pregnancy if fertilization occurs. Following ovulation, the ovulated follicle is guided into the fallopian tubes by the fimbriae. Meanwhile, the oocyte within the follicle remains arrested in metaphase II of meiosis II until fertilization takes place (225).

In this study, we found chronic oral exposure to PS-NPs significantly decreased antral follicle diameter. A decrease in antral follicle diameter can disrupt folliculogenesis and increases follicle atresia. This can lead to anovulation (absence of ovulation) as antral follicles are the only ovarian functional unit capable of releasing an oocyte through ovulation. Anovulation can cause irregular menstrual cycles; without regular release of an oocyte, the hormonal signals that regulate the menstrual cycle can become disrupted. This can result in cycles that are shorter or longer than usual, or cycles without ovulation altogether (226). Interestingly, in addition to the decrease in antral

follicle diameter, a significant increase in estrous cycle length was observed for mice chronically orally exposed to high dose PS-NPs which further confirms the lack or decreased in ovulation. In humans, chronic anovulation may have implications for long-term health, as ovulatory cycles are important for maintaining hormonal balance and overall reproductive health. Women with persistent anovulation may be at increased risk for conditions such as polycystic ovary syndrome (PCOS), which can have various metabolic and reproductive implications (227). Moreover, ovulation is essential for fertility, as it is the process by which an oocyte is released and can be fertilized by sperm. Anovulation can make it difficult for women to conceive, as there is no oocyte available for fertilization (228). Additionally, a decrease in antral follicle diameter and follicle atresia can ultimately lead to a lower AFC which is a marker of ovarian reserve. AFC is proportionally related to the size of the ovarian reserve and has been reported to be lower in infertile women (229–231). Therefore, it is possible that PS-NPs exposure could lead to anovulation contributing to infertility, menstrual irregularities, and PCOS. Arrest in antral follicle development has been linked to an abnormal endocrine environment and insulin resistance as well. For example, in PCOS, an increase in LH levels relative to FSH can disrupt normal ovarian function and contribute to the arrest of antral follicle growth (232). Normally, FSH stimulates the growth and development of ovarian follicles, while LH is responsible for triggering ovulation. However, in PCOS, the elevated LH levels can disrupt this balance. High levels of LH can lead to premature luteinization of ovarian follicles, causing them to stop growing at the antral stage (232). There is very limited literature on the effect of NPs on LH or FSH levels in females and variability exists among the few studies. For example, a study by Wei and colleagues

reported LH surge in mice exposed to 0.1 mg/d of S-MPs for 30 days (233). In contrast, Liu et al reported a reduction in antral follicle count but no change in FSH or LH levels after chronic exposure for 35 days to 790 nm PS-NPs (182). Insulin resistance is another potential cause of antral follicle arrest that is also observed in PCOS and could be linked to NPs exposure as well. Insulin resistance in PCOS leads to elevated insulin levels in the blood (hyperinsulinemia) which can directly impact ovarian function by stimulating androgen production in the ovaries (234). Insulin stimulates thecal cells in the ovarian follicles to produce excess androgens, such as testosterone. Androgens can disrupt the normal development and maturation of ovarian follicles. For instance, the excess androgens produced as a result of insulin resistance can disrupt the normal selection and growth of dominant follicles (235). Instead of a single dominant follicle maturing and ovulating, multiple small follicles may continue to grow but fail to reach maturity. This can lead to the accumulation of small antral follicles in the ovaries, contributing to the characteristic appearance of "polycystic" ovaries seen on ultrasound. Elevated androgen levels can also lead to systemic effects, such as increased LH secretion from the pituitary gland (234). LH further stimulates androgen production by the ovaries, exacerbating the hyperandrogenic state and contributing to the arrest of antral follicle growth which we observed with NPs exposure as well.

Recent literature has demonstrated the potential of NPs to induce insulin resistance. For example, a study conducted by Wang and colleagues where mice were orally exposed to PS-NPs over an 8-week period, resulting in a notable elevation in blood glucose levels, glucose intolerance, and oxidative stress, ultimately culminating in insulin resistance (236). Furthermore, another recent study revealed that mice exposed to

airborne nanoparticles exhibited a systemic inflammatory phenotype and manifested complete insulin resistance, characterized by weight loss and elevated blood glucose levels (237).

Overall, our results show a reduction in antral follicle size and arrest of antral follicle growth. This phenomenon can potentially be attributed to NPs triggering a surge in LH levels and inducing insulin resistance and metabolic dysregulation. A decrease in follicle size and volume can lead to follicle atresia and a diminished antral follicle count over time or with a higher NPs exposure dose, resulting in a lower ovarian reserve and impaired fertility. Our study is the first to quantify follicle size and currently, there is no research addressing the impact of NPs on ovarian follicles. However, some literature has explored the effects of MPs on follicle count. For example, Liu et al showed a lower antral follicle count after exposing mice to 30 mg/kg body mass PS-MPs for 35 days (182). Wei et al reported a lower total follicle count in the 0.1 mg/d PS group compared to the control group (233). Additionally, a study by Haddadi et al showed that PS-MPs exposure for 0.1 mg/day (5 μm diameter) for 24–26 days by oral gavage perturbed folliculogenesis, disrupted follicles maturation, differentiation, and increased number of atretic and cyst follicles in Wistar rats (238).

Furthermore, in the present study estrous cycle length was measured, and it was found to be significantly longer in the high dose PS-NPs exposure group compared to control. The estrous cycle is the reproductive cycle in rodents and is 4-5 days long. The estrous cycle has 4 stages: proestrus, estrus, metestrus, and diestrus. During metestrus and diestrus, estradiol levels are low but gradually rise. In the late afternoon of proestrus, heightened estradiol levels prompt a surge in GnRH release from the hypothalamus.

This surge, in turn, triggers the LH and FSH surge during proestrus, typically occurring at the onset of the active (dark) period. Ovulation typically occurs 12 to 14 hours later during estrus, which spans approximately 15 hours (204). On the other hand, the ~28d human menstrual cycle has three phases of menstrual, proliferative (follicular), and secretory (luteal) and ovulation occurs around day 14 of the cycle.

Ovulation is essential for fertility because it releases an oocyte from the ovary, which is necessary for conception. If ovulation does not occur, the menstrual/reproductive cycle may become irregular which can result in infertility (239). Anovulation often occurs due to hormonal imbalances, such as elevated levels of androgens (male hormones) or disruptions in the balance of estrogen and progesterone. These imbalances can disrupt development and release of oocytes from the ovaries, leading to irregular menstrual cycles and longer cycles without ovulation (239).

This aligns with our previous finding as reduction in antral follicle size can lead to anovulation which in turn can result in a longer estrous cycle further confirming that PS-NPs could lead to irregular ovulation and contribute to infertility.

There is currently no literature on the effect of NPs on estrous cyclicity but there are a few MPs studies yielding varied results. For example, Wei et al showed no significant effect from 5-5.9 µm PS-MPs chronic exposure on the length of the estrous cycle of mice (233). On the other hand, Haddadi et al showed a significant decrease in metestrus stage of exposed rats to 5 µm PS-MPs compared to control (238). These findings suggest a potential size-related effect of MNPs, with smaller particles exhibiting toxicity towards estrous cyclicity.

Moreover, we showed that PS-NPs exposure caused a significant decrease in P4 levels. A drop in P4 has been linked to anovulation (240). In cases where a follicle fails to mature and release an oocyte, progesterone remains unreleased, causing the uterine lining to continuously thicken in response to estrogen. Over time, this thickening becomes unstable and eventually collapses, resulting in bleeding. This bleeding can be unpredictable, often characterized by heavy flow and prolonged duration. Without ovulation, the corpus luteum does not form, leading to low progesterone levels during the luteal phase (240). Anovulation can result from hormonal imbalances, or hypothalamic dysfunction, and can contribute to infertility and irregular periods and reproductive cycles. In the case of ovulation, P4 is primarily produced by the corpus luteum which is a structure formed in the ovary after ovulation. During the luteal phase of the menstrual cycle, progesterone levels rise to prepare the uterine lining for implantation of a fertilized egg. If there is insufficient progesterone production during this phase (known as a luteal phase defect), the endometrial lining may not develop adequately, making it difficult for a fertilized oocyte to implant and establish a pregnancy (241).

Overall, a decrease in P4 levels can be suggestive of anovulation which our results have suggested through longer estrous cycle length and a decrease in antral follicle size in the PS-NPs exposure groups.

Decrease in P4 has also been shown in previous studies; It has been suggested that MNPs' additives and pollutants act as endocrine disruptors causing hormonal changes (31). For example, Bisphenol A (BPA) which is used primarily in the production of polycarbonate plastics and resins has been shown to decrease progesterone and

estradiol levels in humans (242). Polychlorinated biphenyls (PCBs) are other plastics additives and have been shown to lower progesterone levels and cause follicle atresia (243). Reduction in progesterone levels can contribute to irregular menstruation, difficulty conceiving, and pregnancy loss (244).

Additionally, P4 has been shown to be a potent suppressor of several inflammatory pathways. For example, it has been shown that the withdrawal of P4 increases expressions of *IL8* and monocyte chemoattractant protein-1 (*MCP1*) transcripts in human endometrial explants, suggesting that P4 suppresses these cytokines in the uterine tissues (245). Therefore, P4 stimulates anti-inflammatory responses while suppressing pro-inflammatory responses and reduction of P4 by PS-NPs suggest that NPs can contribute to inflammation and immune dysfunction which needs to be investigated in future studies.

Lastly, we measured mice body mass at baseline and every week, however, no significant change was detected in PS-NPs exposure groups compared to the control. The effect of MNPs on body mass in the literature is variable. For example, Shen et al showed that treatment with 1 mg/L of PS-MPs had no effect on mice body mass (246) but Haddadi et al showed a significant decrease in body mass gain in rats exposed to PS-MPs (238). These suggest that the effect of NPs on body mass is size dependent. Further studies are needed to investigate the effect of long-term treatment of PS-NPs in mice.

4.2 Strengths, limitations & future directions

This study is the first to examine the effect of NPs on several female reproductive characteristics in mammals. It is also the first plastic toxicity study to have measured follicle size and overall estrous cycle length. In this study we showed that NPs exposure affected the estrous cycle length, progesterone levels and antral follicle size, suggesting the potential translocation of these particles into the ovaries. While current literature lacks definitive insights into the precise mechanisms governing NP transport and translocation within biological systems, emerging research provides intriguing insights. Notably, a recent ex vivo study demonstrated the transport of PS-NPs across the intestine of the European sea bass (fish species) (247). Additionally, aquatic studies have documented the translocation of microplastics from the gastrointestinal tract to systemic circulation and other tissues (248–250). Furthermore, Jin and colleagues have revealed the ability of PS-MPs to traverse the testis-blood barrier (170,251) and Hadadi et al and An et al have reported accumulation of PS-MPs in luteal cells of the corpus luteum and in the thecal cells of the follicles as well (238,252). Hence, it is plausible that the translocation of NPs into ovarian tissues may occur through crossing the intestinal barrier, entering the circulatory system, and even crossing the blood-follicular barrier. However, future studies should prioritize delving into the fundamental mechanisms governing the translocation of NPs and their subsequent effects on the female reproductive system.

This study is not without its limitations. One of the limitations of our study is that mice were euthanized at different estrous cycle stages which could potentially serve as a

confounding variable in our analysis. Therefore, in future studies euthanasia should take place during the same estrous cycle stage. Additionally, we did not have an adequate sample size for subgroup analysis including controlling for the difference in estrous cycle stages prior to euthanasia. Therefore, a larger sample size is required for future studies to allow for post hoc analysis. Finally, it is noteworthy that our study utilized manufactured PS-NPs, which may not fully replicate the characteristics of NPs in the environment with regard to their size, shape, and composition. This limitation is inherent in toxicological investigations involving synthetic particles.

5. Conclusion

Overall, this study suggests that PS-NPs exposure can induce reproductive toxicity by disrupting folliculogenesis through decreasing antral follicle diameter (arrest in antral follicle growth), increasing the estrous cycle length (irregular cycles), and decreasing progesterone levels. Disruptions in folliculogenesis and estrous cycle length serve as significant indicators of anovulation, a condition associated with infertility and PCOS. Furthermore, the reduction in progesterone levels not only signals the possibility of anovulation but also acts as an indicator of heightened inflammation. This observation suggests a potential link between the induction of inflammation and immune responses in the reproductive system by NPs. This should be further investigated in future studies. It is imperative to acknowledge that this study constitutes a repeated dose toxicity assessment, specifically designed to discern potential adverse effects of NPs on the female reproductive system. It is conceivable that certain effects may necessitate a prolonged duration to fully manifest or become apparent. Therefore, our study provides

a basis for further exploring the molecular mechanism of NPs exposure induced reproductive dysfunction in female mammals.

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