UNIVERSIDADE ESTADUAL PAULISTA

"Júlio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

LETHÍCIA VALENCISE

NANOPLÁSTICOS DE POLIESTIRENO PELA VIA ORAL:

EFEITOS NA REPRODUÇÃO E DESENVOLVIMENTO DE ROEDORES

BOTUCATU-SP
2025

Programa de Pós-graduação em Biologia Geral e Aplicada
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ORIENTADORA: DRA. WILMA DE GRAVA KEMPINAS

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, como requisito para Defesa de Doutorado no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia Celular Estrutural e Funcional.*

Dra. Wilma De Grava Kempinas

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Impacto potencial desta pesquisa

Título da tese: Nanoplásticos de poliestireno pela via oral: efeitos na reprodução e desenvolvimento de roedores.

Impacto científico: Este trabalho explora o impacto da exposição a nanoplásticos de poliestireno pela via oral em diferentes idades, sexos, concentrações e períodos de exposição em ratos e camundongos. A pesquisa contribui substancialmente para o campo da toxicologia de nanoplásticos ao elucidar seus efeitos na reprodução e no desenvolvimento, destacando os órgãos-alvo dessas partículas e os mecanismos responsáveis pelos danos causados.

Impacto econômico e social: Os seres humanos estão expostos diariamente a nanoplásticos e a principal via de exposição é a oral. No organismo, estas partículas podem causar efeitos tóxicos na saúde e fertilidade. Com o nosso estudo, trazemos atenção para como os nanoplásticos podem prejudicar o sistema genital, e que a idade e o sexo são fatores que influenciam na extensão dos danos causados pela exposição a essas partículas. Além disso, os resultados podem influenciar regulamentações e políticas públicas voltadas para o controle da produção, descarte e poluição causada por plásticos, a fim de reduzir os riscos ao meio ambiente e à saúde. No campo da saúde pública, são incentivadas iniciativas para reduzir a exposição a esses materiais. A pesquisa também pode aumentar a conscientização pública sobre os perigos dos nanoplásticos, incentivando práticas mais sustentáveis e o consumo consciente.

Potential impact of this research

Thesis Title: Oral Exposure To Polystyrene Nanoplastics: Effects On Reproduction And Development Of Rodents

Scientific impact: This study explores the impact of oral exposure to polystyrene nanoplastics across different ages, sexes, concentrations, and exposure periods in rats and mice. The research substantially contributes to the field of nanoplastic toxicology by elucidating their effects on reproduction and development, highlighting the target organs of these particles and the mechanisms responsible for the damage caused.

Economic and social impact: Humans are exposed daily to nanoplastics, with oral exposure being the main route. In the body, these particles can cause toxic effects on health and fertility. Our study draws attention to how nanoplastics can affect the genital system, and that age and sex are factors influencing the extent of damage caused by exposure to these particles. Additionally, the results may influence regulations and public policies aimed at controlling the production, disposal, and pollution caused by plastics, to reduce risks to the environment and health. In the field of public health, initiatives are encouraged to reduce exposure to these materials. Our research can also raise public awareness about the dangers of nanoplastics, promoting more sustainable practices and conscious consumption.

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Câmpus de Botucatu

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: NANOPLÁSTICOS DE POLIESTIRENO PELA VIA ORAL: EFEITOS NA REPRODUÇÃO E DESENVOLVIMENTO DE ROEDORES

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Dedicatória

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"Se vi mais longe, foi por estar de
pé sobre ombros de gigantes"
Isaac Newton

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Resumo

Nanoplásticos são pequenas partículas de plástico (<1µm) consideradas contaminantes emergentes. Os nanoplásticos têm distribuição ubíqua e capacidade de bioacumular, contaminando água, solo, ar e alimentos. Os humanos estão em contato direto com os nanoplásticos por diversas vias de exposição, sendo a ingestão uma das principais vias. Tendo estas partículas sido identificadas em diversas amostras de material biológico humano, é fundamental que os efeitos adversos da exposição a nanoplásticos sobre a saúde e fertilidade humanas sejam investigados. Por esta razão, o objetivo deste trabalho foi avaliar o impacto da exposição oral a concentrações próximas às ambientais de nanoplásticos de poliestireno (PS-NP; 500nm) sobre parâmetros de fertilidade e desenvolvimento de roedores – ratos e camundongos. A metodologia foi dividida em 3 capítulos que buscaram abordar diferentes aspectos desta exposição sobre os parâmetros reprodutivos e de desenvolvimento. No primeiro capítulo, investigamos os efeitos da exposição a 0,015mg/dia de PS-NP por 25 dias sobre a fertilidade de ratas Wistar adultas. O segundo capítulo explora o impacto de duas concentrações de PS-NP (0,15mg e 1,50mg/dia) administradas por gavagem durante 60 dias para ratos Wistar adultos sobre parâmetros de fertilidade. Também são abordados os potenciais efeitos intergeracionais no desenvolvimento da prole masculina e feminina (F1) de ratos Wistar adultos (F0) expostos a 1,50mg/dia de PS-NP durante 60 dias. O terceiro capítulo investiga o desenvolvimento pós-natal de camundongos (machos e fêmeas) híbridos C57BL/6xBALB/c que foram expostos do dia gestacional 5 ao 19 a 0,15mg/dia de PS-NP. Como resultados da exposição à PS-NP, observamos alterações hormonais, no ciclo estral e na morfologia uterina das ratas Wistar adultas; alterações na qualidade espermática de ratos Wistar adultos; consequências intergeracionais a longo-prazo na prole de ratos Wistar, sendo as fêmeas mais susceptíveis a essas alterações; e impacto no desenvolvimento sexual de camundongos expostos no período gestacional. Os PS-NP impactam diferentemente o sistema genital dependendo da concentração, tempo de exposição, idade, gênero e espécie. Desta forma, os PS-NP atuam como desreguladores endócrinos que impactam a saúde e fertilidade de roedores, o que traz ainda mais atenção aos potenciais efeitos negativos da exposição a nanoplásticos sobre a saúde e fertilidade humanas.

Abstract

Nanoplastics are small plastic fragments (< 1 μm) considered contaminants of emerging concern. Nanoplastics are ubiquitously distributed and have the capacity to bioaccumulate, contaminating water, soil, air, and food. Humans come into direct contact with nanoplastics through various exposure pathways, and ingestion is one of the main routes. Given that these particles have been identified in various samples of human biological material, it is essential to investigate the adverse effects of nanoplastic exposure on human health and fertility. For this reason, the objective of this study was to evaluate the impact of oral exposure to environmentally relevant concentrations of polystyrene nanoplastics (PS-NP; 500 nm) on fertility and developmental parameters in rodents – rats and mice. The methodology was divided into five chapters that aimed to address different aspects of this exposure on reproductive and developmental parameters. In the first chapter, we investigated the effects of exposure to 0.015 mg/day of PS-NP for 25 days on the fertility of adult female Wistar rats. The second chapter explores the impact of two concentrations of PS-NP (0.15 mg and 1.50 mg/day) administered by gavage for 60 days to adult male Wistar rats on fertility parameters. Additionally, this chapter addresses potential intergenerational effects on the development of male and female offspring (F1) from adult Wistar rats (F0) exposed to 1.50 mg/day of PS-NP for 60 days. The third chapter investigates the postnatal development of C57BL/6xBALB/c hybrid mice (males and females) that were exposed from gestational day 5 to 19 to 0.15 mg/day of PS-NP. As a result of PS-NP exposure, we observed hormonal changes, estrous cycle alterations, and uterine morphology changes in adult female Wistar rats; alterations in sperm quality of adult male Wistar rats; long-term intergenerational consequences in the offspring of Wistar rats, with females being more susceptible to these changes; and impact on the sexual development of mice exposed during the gestational period. PS-NPs differentially impact the genital system depending on concentration, exposure time, age, gender, and species. Thus, PS-NPs act as endocrine disruptors that affect the health and fertility of rodents, which further highlights the potential negative effects of nanoplastic exposure on human health and fertility.

LISTA DE ABREVIATURAS E SIGLAS EM PORTUGUÊS / INGLÊS

ANOVA: Análise de variância / Analysis of Variance

BSA: Albumina sérica bovina / Bovine Serum Albumin

CCh: Carbacol / Carbachol

CO_2: Dióxido de carbono / Carbon dioxide

Cyp19a1 (gene), Cyp19a1(proteína): Citocromo P450, Família 19, Subfamília A, Polipeptídeo 1 / Cytochrome P450, Family 19, Subfamily A, Polypeptide 1

DAB: Diaminobenzidina / Diaminobenzidine

DAG: Distância anogenital / Anogenital distance

DEHP: Bis(2-etilhexil)ftalato / Bis(2-ethylhexyl) phthalate

DG / GD: Dia gestacional / Gestational day

DiNP: Ftalato de diisononilo / Diisononyl phthalate

DL / LD: Dia lactacional / Lactation day

DPN / PND: Dia pós-natal / Postnatal day

DSP: Produção espermática diária / Daily Sperm Production

EDC: Químico desregulador endócrino / Endocrine-disrupting Chemical

ELISA: Ensaio Imunoenzimático / Enzyme-Linked Immunosorbent Assay

EPM / SEM: Erro Padrão da Média / Standard Error of Mean

HDL: Lipoproteína de Alta Densidade / High-Density Lipoprotein

HE: Hematoxilina e Eosina / Hematoxylin and Eosine

HPA: Eixo hipotalâmico-hipofisário-adrenal / Hypothalamus-pituitary-adrenal axis

HPG: Eixo hipotalâmico-hipofisário-gonadal / Hypothalamus-pituitary-gonadal axis

HPT: Eixo hipotalâmico-hipofisário-tireoidiano / Hypothalamus-pituitary-thyroid

IHC: Imunohistoquímica / Immunohistochemistry

IL1β: Interleucina 1-beta / Interleukin 1 beta

IL-6: Interleucina-6 / Interleukin 6

IL-8: Interleucina-8 / Interleukin 8

KCl: Cloreto de potássio / Potassium chloride

LDL: Lipoproteína de Baixa Densidade / Low-Density Lipoprotein

MAPK: Proteínas Quinases Ativadas por Mitógenos / Mitogen Activated Protein Kinases

mDF: Fluido de Davidson modificado / Modified Davidson's Fluid

MNP: Micro- e nanoplásticos / Micro- and nanoplastics

NaCl: Cloreto de sódio / Sodium chloride

NE: Norepinefrina / Norepinephrine

NLRP3: NOD-like receptor family, pyrin domain containing 3

PA: Poliamidas / Polyamides

PBS: Tampão fosfato-salina / Phosphate-Buffered Saline

PCNA: Antígeno nuclear de proliferação celular / Proliferating Cell Nuclear Antigen

PCOS: Síndrome do Ovário Policístico / Polycystic Ovary Syndrome

PE: Polietileno / Polyethylene

PET: Polietileno Tereftalato / Polyethylene Terephthalate

PP: Polipropileno / Polypropylene

PS: Poliestireno / Polystyrene

PS-MP: Microplásticos de Poliestireno / Polystyrene Microplastics

PS-NP: Nanoplásticos de Poliestireno / Polystyrene Nanoplastics

PVC: Policloreto de Vinila / Polyvinyl Chloride

RBL-2H3: Linhagem celular de ratos de célula de leucemia basofílica / Rat basophilic leucemia cells

RNA: Ácido ribonucleico / Ribonucleic acid

ROS: Espécies reativas de oxigênio / Reactive Oxigen Species

Star (gene), StAR (proteína): Proteína Reguladora Aguda da Esteroidogênese / Steroidogenic Acute Regulatory protein

T3: Triiodotironina / Triiodothyronine

T4: Tiroxina / Tiroxine

TM4: Linhagem celular de célula de Sertoli de camundongo / Mouse Sertoli cell

TSH: Hormônio Tireoestimulante / Thyroid-stimulating hormone

ULF: Fluido luminal uterino / Uterine Luminal Fluid

UV: Radiação Ultravioleta / Ultraviolet radiation

Sumário

Introdução

1.1. Os plásticos como contaminantes ambientais

A infertilidade é uma doença que afeta de 10 a 15% dos casais, e é definida como a incapacidade de conceber após um ano de coito regular e desprotegido (1). Esta condição afeta entre 50 e 80 milhões de pessoas no mundo e pode resultar tanto de fatores femininos, masculinos, ou ambos associados. Embora a infertilidade seja cada vez mais prevalente para ambos os sexos, o declínio na fertilidade tem se feito mais evidente nos homens. Até 70% dos casos de infertilidade estão relacionados a fatores masculinos (2). Diversos estudos sugerem que a concentração e a qualidade espermáticas de homens têm decaído. A análise da literatura referente ao período de 1938 a 1991 revelou a queda da concentração espermática de 113 x 10^6/ml (1940) para 66 x 10^6/ml (1990) (3). A concentração espermática diminuiu em 32,5% em homens europeus de 1965 a 2015 (4). Enquanto as causas da infertilidade são multifatoriais, e incluem fatores genéticos, distúrbios hormonais e físicos, os fatores ambientais são considerados contribuintes relevantes (2).

A poluição ambiental é um problema global que afeta a qualidade da água, solo, e ar. Isto pode impactar negativamente diferentes ecossistemas e espécies, incluindo seres humanos (5). Fatores ambientais podem prejudicar a saúde e fertilidade humanas por meio de diferentes vias de exposição. A inflamação desregulada representa um mecanismo comum associado a essas alterações (6,7). Dentre possíveis implicações de fatores ambientais na fertilidade estão a alteração da morfologia, motilidade, concentração e viabilidade espermáticas (8,9), concentração de hormônios reprodutivos, duração do ciclo menstrual (9), insuficiência ovariana prematura (10), redução da contagem e qualidade foliculares, redução na taxa de fertilização e qualidade embrionária (11).

Dos principais contaminantes ambientais, destacam-se os plásticos. Estima-se que cerca de 10 milhões de toneladas de lixo plástico atinjam o oceano anualmente, o que evidencia seu impacto no ecossistema. O plástico é um contaminante emergente, durável, resistente à degradação, e a sua produção cumulativa de 1950 a 2019 se aproxima de 10 bilhões de toneladas (12). Embora sejam resistentes à degradação, macroplásticos podem ser fragmentados a pedaços menores por raios UV, processos oxidativos, e pela degradação química devido à

salinidade de ambientes marinhos (13). Estes fragmentos são chamados de microplásticos, partículas menores que 5mm em diâmetro, ou nanoplásticos (<1μm em diâmetro) (14). Concomitantemente com a degradação de macroplásticos, os microplásticos também são manufaturados e usados em produtos farmacêuticos e cosméticos (15).

Além dos oceanos, os microplásticos foram detectados no ambiente atmosférico urbano, no solo, na comida que comemos, e até mesmo em fezes humanas (16,17,18,19,20). Em um organismo, estes microplásticos podem induzir estresse oxidativo que leva à inflamação e potenciais patologias (21). A exposição a micro- e nanoplásticos (MNP) é ainda maior em ambientes internos do que externos, por meio do ar e poeira. Nos anos de 2020 a 2022, devido à pandemia de COVID-19, a exposição indoor a MNP aumentou, e o uso frequente de máscaras faciais e luvas também é associado à liberação de microplásticos e maior taxa de inalação de fibras e microesferas. A real exposição humana a microplásticos por inalação, ingestão e contato dérmico ainda está sendo debatida. A ingestão é uma das vias principais de exposição aos plásticos, e um estudo recente relata a ingestão de até 0,15mg/kg de peso corpóreo por dia dependendo da idade e localização (22).

Nanoplásticos são considerados contaminantes emergentes. A maioria dos métodos analíticos e de detecção não são capazes de identificar com precisão a extensão da distribuição de nanoplásticos devido ao seu tamanho reduzido. Por esta razão, os perigos da exposição a nanoplásticos e a distribuição dessas partículas no ambiente permanece indefinida. Devido às propriedades físicas associadas com o tamanho da partícula (como a área de superfície/volume), as implicações e as vias de transporte relacionadas à exposição a nanoplásticos podem ser diferentes daquelas observadas em microplásticos (23).

Os nanoplásticos foram encontrados em água potável e bebidas engarrafadas (24). Nanopartículas liberadas por embalagens plásticas podem contaminar alimentos e água (19). Os processos de filtração comuns não são capazes de reter partículas tão pequenas, aumentando a preocupação em relação à exposição de humanos a micro- e nanoplásticos (25). Ademais, os próprios sistemas de tratamento de água podem ser responsáveis pela liberação de micro- e nanoplásticos na água (26). Além da água, os nanoplásticos podem bioacumular e são persistentes na cadeia alimentar (19).

1.3. O impacto de plásticos e seus componentes na saúde e fertilidade

Produtos químicos presentes nos plásticos como ftalatos e bisfenóis podem atuar como

desreguladores endócrinos (EDCs), os quais podem prejudicar o desenvolvimento sexual e a fertilidade de espécies de mamíferos (27,9,11). A exposição a certos metabólitos/componentes dos plásticos como bis(2-etilhexil)ftalato (DEHP) e ftalato de diisononilo (DiNP) aparecem negativamente correlacionados com a função reprodutiva de jovens na Suécia (28). Semelhantemente, um estudo conduzido na Austrália reportou uma correlação negativa entre as concentrações de ftalatos nas mulheres durante a gravidez e as concentrações de testosterona quando os filhos atingiram a vida adulta (29). Em mulheres adultas na China, a presença de bisfenóis A, F e S na urina, isolados ou em mistura, foi associada a prejuízo na reserva folicular por medição de folículos antrais (30). No Brasil, metabólitos de ftalatos foram encontrados em amostras de urina e positivamente associadas com um marcador de estresse oxidativo (31).

Estudos recentes evidenciam o impacto da exposição a MNP no desenvolvimento e fertilidade de fêmeas e machos. Em fêmeas, a exposição oral a microplásticos de poliestireno leva à redução no número de folículos ovarianos tanto em camundongas quanto em ratas por meio da apoptose de células da granulosa (32,33). Em zebrafish fêmea, foi observado que estes plásticos são transferidos para o ovo durante a vitelogênese, acumulam no saco vitelínico, e eventualmente são transferidos para a prole (34). Além disso, ressalta-se que nanopartículas menores que 150nm são capazes de atravessar barreiras biológicas e atingir células e tecidos protegidos por estas barreiras (35,36). Por exemplo, estudos reportaram que nanobeads de poliestireno podem atravessar a membrana placentária de ratas prenhes expostas por instilação intratraqueal (37). Nanopartículas de poliestireno (50nm) foram internalizadas por células por difusão passiva pela membrana plasmática ou por endocitose pelas vias mediadas por clatrina ou caveolina, assim como por micropinocitose (50 e 500nm) (38).

Em camundongos machos, a exposição oral a microplásticos de poliestireno resulta em redução na produção e qualidade espermáticas, concentração de testosterona, e aumento no número de espermatozoides anormais (39,40). A exposição a microplásticos de poliestireno por 90 dias prejudica a barreira hematotesticular em ratos machos, causa apoptose de células germinativas, redução na qualidade e concentração espermáticas. Estas alterações foram associadas ao estresse oxidativo e ativação da via p38 MAPK (41). Beads de poliestireno (50nm) podem se acoplar à membrana espermática durante a fertilização externa de ostras e causar a redução da velocidade e mobilidade geral do espermatozoide, afetando o sucesso reprodutivo (42). Camundongos machos e fêmeas tratados com microplásticos de polietileno tiveram uma prole reduzida (43). Estes dados indicam que a reprodução é um alvo primário da exposição a micro- e nanoplásticos.

Não apenas os próprios MNP podem induzir toxicidade, como também podem carregar patógenos (44), outros toxicantes, e gradualmente liberar componentes utilizados nos processos plastificantes durante a degradação. Estes plastificantes são considerados desreguladores endócrinos que prejudicam a fertilidade (45,46). Os desreguladores endócrinos podem alterar o balanço hormonal por interferir com a produção normal, liberação, metabolismo e eliminação dos hormônios, ou mesmo por mimetizar hormônios endógenos, levando a desordens reprodutivas (47). Concentrações mais elevadas de ftalatos e seus metabólitos em amostras de urina foram correlacionadas com redução da distância anogenital em meninos, e prejuízo na qualidade espermática em adultos (46). Ademais, a exposição a ftalatos reduz a concentração de hormônios esteroides (48).

Diferentes tamanhos e dosagens de MNP podem induzir a geração de espécies reativas de oxigênio (ROS) tanto no ambiente intracelular como extracelular. Uma vez que estas partículas alcançam o compartimento intracelular dentro de um fagócito, disparam mecanismos de defesa inatos (49). Partículas menores com a razão massa/superfície maiores possuem potencial de geração de ROS mais elevadas (50). As ROS podem induzir ou mediar a ativação da via p38 MAPK, e esta ativação é mais frequentemente observada quando ocasionada por partículas menores (49,41).

O sistema imune geralmente reconhece MNPs como xenobióticos e ativa a liberação de citocinas pró-inflamatórias (51,52). Em células de adenocarcinoma humanas, as nanopartículas de poliestireno (44nm) induziram up-regulation dos genes das citocinas pró-inflamatórias IL6, IL8 e IL1β. A resposta inflamatória e a disbiose da microbiota foram observadas no intestino de zebrafish adulto exposto a micro- e nanoplásticos de poliestireno, sendo o último responsável por alterações mais sérias (53,54). Estas respostas pró-inflamatórias podem ser devido a estresse oxidativo e desintegração da membrana lisossômica, e a inflamação desregulada pode danificar tecidos (6,49).

A inflamação desregulada está presente em diversas doenças crônicas, incluindo desordens reprodutivas como a disgenesia testicular e o câncer de próstata (6). Inflamação no sistema genital masculino pode causar infertilidade pela disrupção das barreiras hematotesticular e hematoepididimária (55,56,57). A barreira hematotesticular é comprometida em camundongos machos expostos a microplásticos de poliestireno (41). Além disso, a inflamação testicular pode ser causada por plastificantes e ocorre em várias gerações após a exposição (58). A inflamação da pré à peri-concepção, durante a gestação e parto, e nos períodos neonatal aumenta a susceptibilidade a doenças crônicas na infância (59). Assim, a inflamação

desregulada causada por micro- e nanoplásticos pode prejudicar o sistema reprodutivo e causar infertilidade.

Dessa forma, a saúde e a fertilidade humanas podem ser prejudicadas pela exposição ambiental a nanoplásticos. Não obstante, partículas de plástico foram encontradas no sangue humano (1.6 μg/ml), e poliestireno (PS) foi o segundo plástico mais quantificado nestas amostras (60). Microplásticos também foram encontrados em amostras humanas de urina, leite materno, placenta e testículo (61,62,63,64). Amostras coletadas da pele facial, cabelo e saliva de trabalhadores de uma fábrica no Irã evidenciaram que mulheres potencialmente estão mais expostas a microplásticos que homens no mesmo ambiente, o que pode ser devido às vestimentas e uso de produtos cosméticos (65).

Devido à grande relevância da exposição a nanoplásticos sobre a fertilidade, este trabalho se propôs a avaliar os efeitos da exposição a nanoplásticos de poliestireno (PS-NP; 500nm) sobre parâmetros reprodutivos de roedores.

Relevância do tema e hipótese de estudo

A presença crescente de nanoplásticos em diversos ecossistemas representa uma preocupação emergente para o meio ambiente e para a saúde humana, sendo fundamental compreender os possíveis efeitos adversos dessas partículas no organismo. A fertilidade e o desenvolvimento são tópicos de interesse, uma vez o sistema genital é um alvo da ação tóxica dos nanoplásticos. Os efeitos dessas partículas sobre o sistema genital completamente maduro ainda são pouco estudados e foram mais profundamente abordados nesta Tese.

Além disso, a identificação de riscos associados aos nanoplásticos pode embasar políticas públicas de regulação da produção e descarte de plásticos, contribuindo para a redução da poluição e promoção de práticas sustentáveis. A conscientização sobre os perigos dessas partículas pode ainda impulsionar mudanças no comportamento da população em relação ao consumo e descarte de materiais plásticos, refletindo diretamente na saúde pública e no bem-estar coletivo.

Diante desse contexto, a hipótese deste estudo é que a exposição oral a nanoplásticos de poliestireno provoca alterações na fertilidade de ratos adultos, com potenciais efeitos intergeracionais, e impacta o desenvolvimento pós-natal de camundongos.

Objetivos

Objetivos gerais

Avaliar parâmetros de fertilidade e desenvolvimento de roedores machos e fêmeas expostos oralmente a concentrações baixas de nanoplásticos de poliestireno (PS-NP) e seus potenciais efeitos intergeracionais.

Objetivos específicos

- Determinar se as concentrações baixas de nanoplásticos impactam o sistema genital, a fertilidade e o desenvolvimento de ratos Wistar machos e fêmeas;
- Avaliar os potenciais efeitos intergeracionais da exposição a PS-NP por meio da análise de prole masculina e feminina de ratos Wistar machos tratados com PS-NP;
- Determinar se as concentrações baixas de nanoplásticos impactam o sistema genital e o desenvolvimento de camundongos híbridos C57BL/6x BALB/c machos e fêmeas.

Capítulo 1

Exposure to Low-Dose Polystyrene Nanoplastics Impairs the Estrous Cycle by Decreasing Ovarian Levels of Steroidogenic Acute Regulatory Protein and Serum Progesterone Levels in Rats

O primeiro capítulo reúne os resultados obtidos a partir da exposição de ratas Wistar adultas a PS-NP e deu origem ao artigo intitulado *"**Exposure to Low-Dose Polystyrene Nanoplastics Impairs the Estrous Cycle by Decreasing Ovarian Levels of Steroidogenic Acute Regulatory Protein and Serum Progesterone Levels in Rats**."*. Este artigo foi publicado na revista "Reproductive Toxicology" (Fator de impacto: 2,8).

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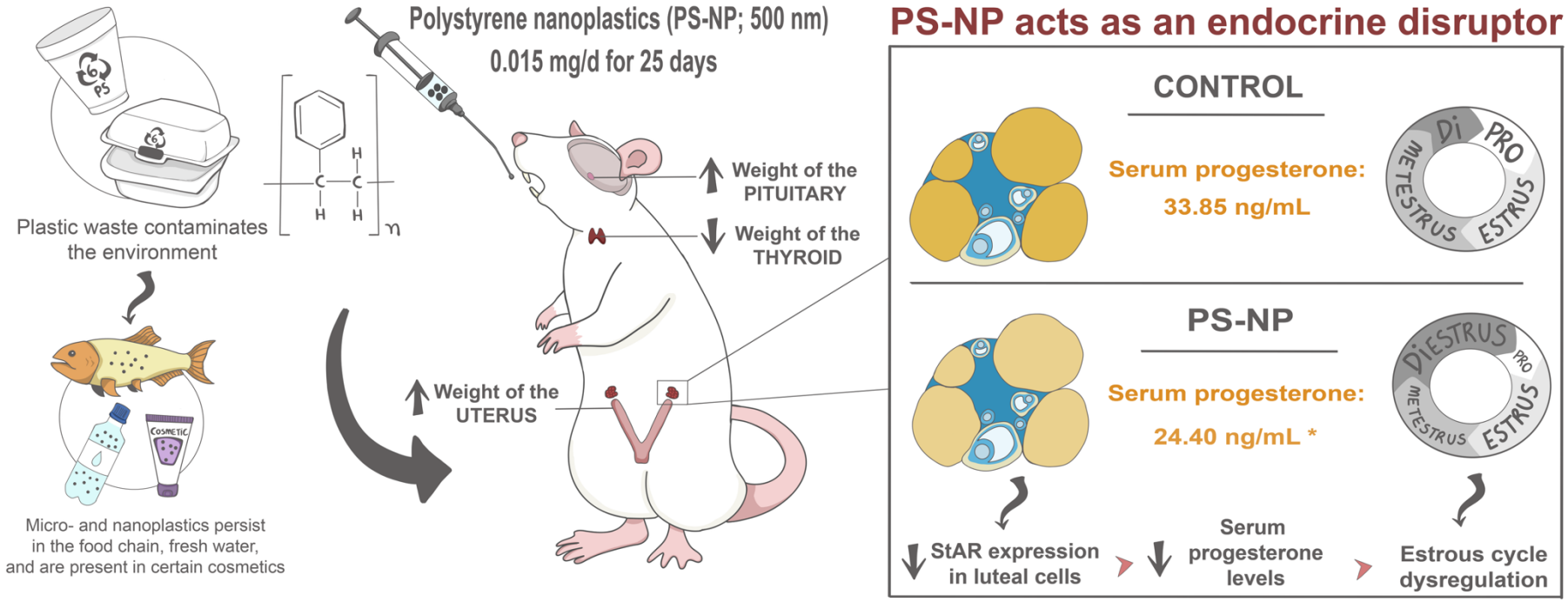
GRAPHICAL ABSTRACT



Exposure to Low-Dose Polystyrene Nanoplastics Impairs the Estrous Cycle by Decreasing Ovarian Levels of Steroidogenic Acute Regulatory Protein and Serum Progesterone Levels in Rats

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ABSTRACT

Plastic can be fragmented into smaller pieces referred to as microplastics (< 5 mm), or nanoplastics (< 1 μm). These particles have been reported to cross biological barriers and cause oxidative stress damage in several tissue types. Given that female reproductive tissues are considered a target for such particles, our study aimed to evaluate the effects of polystyrene nanoplastics (PS-NP, 500 nm) at a low concentration (0.015 mg/d), on reproductive parameters of adult female Wistar rats. Animals (n = 10/group) were treated by gavage for 25 days with PS-NP diluted in distilled water at a concentration of 0.015 mg/d. The Control group received only distilled water (vehicle). We assessed weight gain, estrous cyclicity, sexual behavior and fertility, morphology of ovaries and uteri, immunostaining for StAR in the ovaries, and serum levels of the steroid hormones: estradiol and progesterone. Data was evaluated by Student's t-test, Mann-Whitney test, or Fisher's Exact test. Results were considered significantly different when $P \leq 0.05$. The PS-NP group showed estrous cycle dysregulation, uterine inflammatory infiltration, increased uterus and pituitary weight, and decreased thyroid weight in the experimental conditions utilized. These findings are potentially due to the decrease in StAR expression in luteal cells, and consequent reduction of progesterone serum levels. These results indicate that nanoplastics act as endocrine disruptors impairing female endocrine and reproductive function, in a rodent model, and raise concern about outcomes after exposure to nanoplastics in other females and in adult women's reproductive health.

Keywords: nanoplastic, polystyrene, endocrine disruptor, female, reproductive toxicology, StAR.

1. INTRODUCTION

The causes of infertility are multifactorial, and include genetic factors, hormonal defects, physical disorders, and environmental factors. Among possible implications of environmental factors in female fertility are alterations in circulating levels of reproductive hormones, menstrual cycle length, premature ovarian insufficiency, reduced number and quality of follicles, decreased fertilization rate and embryo quality. In addition, reproductive disorders such as polycystic ovary syndrome (PCOS), endometriosis, and breast cancer have been associated with environmental toxins (Canipari et al., 2020; Green et al., 2021; Interdonato et al., 2023; Karwacka et al., 2019; Rutkowska and Diamanti-Kandarakis, 2016; Vabre et al., 2017).

Plastics are global contaminants which can be fragmented into smaller pieces referred to as microplastics (< 5 mm), or nanoplastics (< 1 μm) (Barnes et al., 2009). The most prevalent microplastic pollutants are polyethylene (PE), polystyrene (PS), polyamides (PA), polypropylene (PP) and polyvinyl chloride (PVC) (Li et al., 2016).

In addition to plastic degradation, microplastics are also manufactured and used in pharmaceutical and cosmetic products (Napper et al., 2015). Micro- and nanoplastics have been detected in oceans, atmospheric urban environment, soil, food, dog testis, and in human samples such as blood, urine, stool, placenta, breast milk, and testis, indicating human exposure (de Souza Machado et al., 2018; Garcia et al., 2024; Guo and Wang, 2019; Hu et al., 2024; Leslie et al., 2022; Pironti et al., 2022; Ragusa et al., 2022, 2021; Schwabl et al., 2019; Shruti et al., 2020; Toussaint et al., 2019; Wright et al., 2020). Regarding smaller nanoplastics, most detection and analytical methods are unable to precisely identify the extent of distribution given their reduced size. For that reason, the hazards of nanoplastic exposure and the distribution of these particles in the environment is not well defined (Mitrano et al., 2021). Currently available

environmental studies indicate an estimative of concentrations from 1 ng/L – 1 µg/L for particles smaller than 1 µm in aquatic systems (Lenz et al., 2016).

Exposure to micro- and nanoplastics causes a wide variety of damage in organisms. In aquatic species, this exposure compromises feeding behavior, induces oxidative stress, neurotoxicity and immunotoxicity, reduces the quality of gametes and fertility, and affects the development of embryos (Z. Liu et al., 2021; Martínez-Gómez et al., 2017; Sökmen et al., 2020; Wegner et al., 2012). Nanoparticles smaller than 150 nm can cross biological barriers and reach cells and tissues protected by these barriers (Mayor and Pagano, 2007; Keller et al., 2020). Polystyrene nanoplastic particles were found to be internalized into cells by passive diffusion across the plasma membrane or by endocytosis via either clathrin- or caveolin-mediated pathways, as well as by micropinocytosis (50 and 500 nm) (L. Liu et al., 2021). These molecules can induce oxidative stress, leading to inflammation and resulting in potential pathologies (Wright and Kelly, 2017).

The impact of micro- and nanoplastics on female reproduction has been reported. Samples collected from facial skin, hair, and saliva from workers from a plastic factory in Iran showed that women may be more exposed to microplastics than men in the same environment, which could be due to clothing and the use of cosmetic products (Shahsavaripour et al., 2023). Besides that, when subjected to the same experimental conditions, female mice have greater microplastic accumulation in the gonads than male mice (Wei et al., 2022). This indicates that females are potentially more sensitive to microplastics exposure.

Exposure to PS by direct drinking of 500 nm particles (PS-NP) dispersed in deionized water results in a reduction in the number of growing follicles via the induction of apoptosis of granulosa cells in female rats exposed to 1.5 mg/d for 90 days (An et al., 2021). In another study, females exposed to PS-NP for 90 days had a decrease in the number of growing follicles, and in the activity of antioxidant enzymes in 0.15 and 1.5 mg/kg/d groups. Pyroptosis and

apoptosis of granulosa cells were also observed via the NLRP3/caspase-1 signaling pathway, and associated with oxidative stress (Hou et al., 2021). Mammalian females are born with a limited follicle pool (Rimon-Dahari et al., 2016), and environmental exposure to toxicants may cause irreversible impairment of fertility.

The actual human exposure to microplastics through inhalation, ingestion and dermal contact remains controversial. Ingestion is one of the main routes of plastic exposure, and a recent study conducted in Australia reports the ingestion of 0.5 mg/kg/year of microplastics by adults in their household (Soltani et al., 2021). Another study estimated that humans ingest up to 5 g of microplastics weekly (Senathirajah et al., 2021).

Therefore, our study aimed to investigate the effects of low-dose PS-NP exposure, at an estimated human exposure concentration (Ageel et al., 2021; Senathirajah et al., 2021; Soltani et al., 2021), on reproductive parameters of adult female Wistar rats.

2. MATERIAL AND METHODS

2.1. Polystyrene nanoplastics

Polystyrene nanoplastic spheres (PS-NP) with a diameter of 500 nm obtained from Alpha Nanotech Inc. (Vancouver, BC) were diluted in distilled water to obtain the experimental dose of 0.015 mg/d. The volume of distilled water with or without PS-NP administered to the rats was 0.3 mL. The supplier provided a document with specification/characterization of PS-NP stating the following characteristics: Size: 500 nm; CV< 3%; Roundness > 0.980; Surface: Non-Functionalized.

2.2. Choice of dose

The concentration of nanoplastics chosen for this study was determined based on the estimated human exposure levels. A study in Australia reported that adults ingest approximately 0.0014 mg/kg/d of microplastics through indoor air (Soltani et al., 2021). Senathirajah et al. estimates that humans ingest between 0.1 and 5 grams of micro- and nanoplastics per week (Senathirajah et al., 2021), which equates to 0.014 - 0.71 g/d. This translates to approximately 0.23 to 12 mg/kg/d, considering an average human weight of 60 kg. From both of these studies, we decided to consider values of human exposure from 0.0014 to 0.23 mg/kg/d. To account for the metabolic differences between humans and rats, these values were adjusted using a conversion factor of 6.2 (Nair and Jacob, 2016). This adjustment resulted in a range of 0.01 to 1.426 mg/kg/d for a 150 g rat. Based on these calculations, we chose the concentration for treatment: 0.015 mg/d (approximately 0.1 mg/kg/d). This concentration closely aligns with the human daily exposure levels by ingestion, providing a relevant basis for our experimental design.

2.3. Animals

Female Wistar rats (45-60 days old; 150-200 g) were obtained for the experimental procedures. They were acclimated and housed under the controlled conditions described below until the beginning of treatment on day 110. Male Wistar rats (70 days old; 250 – 350 g) were used exclusively for the sexual behavior and fertility tests. Animals were obtained from the Central Biotherium of Botucatu and housed in pairs in polypropylene cages bedded with wood shavings under controlled conditions (22 °C, 30% air humidity, 12/12 h light/dark cycle). Food and water were available *ad libitum*. All experimental procedures in this study were approved by the local Ethics Committee for the Use of Experimental Animals of São Paulo State University (4936170322-CEUA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), and following ARRIVE guidelines (Percie du Sert et al., 2020). Euthanasia was performed by decapitation following CO_2 sedation.

2.4. Experimental design

Females were assigned to Control or PS-NP groups ensuring no differences in body weight between the groups before the beginning of the treatment. Adult female Wistar rats (110 days; 236.9 ± 5.6 g) were treated by gavage with either 0.015 mg/d of PS-NP diluted in 0.3 mL of distilled water, or 0.3 mL of distilled water (vehicle; Control group) for 25 consecutive days. This exposure period was chosen according to OECD TG 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents) with a slight modification to ensure the exposure during five complete estrous cycles (OECD, 2008). This test guide also requires at least 5 females/group for each endpoint analyzed, which is the sample number we are using, and has proven to be sufficient in detecting female reproductive impairment (Barros et al., 2020; Wang et al., 2023). Animals were weighed weekly from the beginning of the treatment (postnatal day – PND - 110). The estrous cycle was evaluated from PND 135 to 150, then the females were either destined to sexual behavior and fertility assessment (n = 5/group) or euthanized for sample collection (n = 5/group). The experimental timeline is shown in Figure 1. Additionally, all animals were daily monitored for signs of general toxicity or stress (mortality and morbidity, excessive weight gain/loss, weakness, lethargy, presence of bristly hair, and abnormal behavior) during the experimental period.

2.5. Body and organ weights

Females were weighed and euthanized (n = 5/group) at the first estrus detected from PND 150. Brain, pituitary, thyroid, adrenals, ovaries, and uterus (with fluid) were dissected and weighed. The ovaries and uteri were fixed in modified Davidson's fluid (mDF) for histology (Latendresse et al., 2002).

2.6. Histological evaluation

147 The right ovary and horn of the uterus were fixed by immersion in mDF, dehydrated and
148 embedded in Paraplast Plus® (P3683, Sigma-Aldrich). Tissues were sectioned (5 μm), mounted
149 on glass slides and stained with hematoxylin and eosin (HE). Histopathology was analyzed in
150 a blind assay using a Leica DMLB microscope mounted with a digital camera and analysis
151 software (LAS V4.12). Ovarian follicles in distinct stages of follicular development and corpora
152 lutea were counted, and the morphological aspect of this organ was observed as described by
153 Talsness et al. (Talsness et al., 2005), and Barros et al. (Barros et al., 2020). The morphometry
154 of the uterus was also evaluated. For that, the height of the luminal epithelium, endometrium,
155 and myometrium were measured on the uterine sections as previously described by E Silva et
156 al. (E Silva et al., 2016) with modifications. Photomicrographs of the entire uterine section were
157 acquired with a 4x objective, and the total number of endometrial glands was subsequently
158 counted (Cassiani et al., 2024).
159
160 2.7. Immunohistochemistry (IHC)
161 In the ovaries, immunostaining for StAR is positive in theca and luteal cells, and it's mainly
162 expressed in the corpus luteum (Tu et al., 2018; Zhang et al., 2024). We qualitatively evaluated
163 immunostaining for StAR on the theca cells of secondary and tertiary follicles and luteal cells.
164 Then, the intensity of staining for StAR in theca and luteal cells was measured in percentage of
165 staining using the ImageJ 1.52a software. For this analysis, images were captured of all corpora
166 lutea and ovarian follicles (secondary and tertiary) from a single ovarian section of each animal.
167 These images were converted to 8-bit, and a corpus luteum and an ovarian follicle from a single
168 control female were used to calibrate the ImageJ threshold to visualize the stained cells. Once
169 calibrated, the staining intensity was measured using the established threshold.
170 IHC assay for steroidogenic acute regulatory protein (StAR) in the ovary was performed based
171 on a previously described protocol (Barros et al., 2020) with modifications, as follows: ovaries

were sectioned at a thickness of 5 μm and placed on silanized slides. Then, the sections were dewaxed with xylol, hydrated with decreasing concentrations of alcohol, and washed with phosphate-buffered saline (PBS, pH 7.4). Antigenic recovery was performed with citrate buffer (pH 6.0) for 10 min in a microwave. After this step, the sections were incubated for 20 min with hydrogen peroxide (3.5%) and PBS, for blocking the endogenous peroxidase. In the next step, the sections were incubated for 30 min with Bovine Serum Albumin (BSA 3%) diluted in PBS and then incubated overnight with the anti-StAR antibody (STAR/2077 conjugated with horseradish peroxidase, 4 μL/mL, mouse monoclonal, Lot D150262, Novus Biologicals, Centennial, USA). On the next day, after further washing with PBS, the cuts were submitted, for 7 min, to diaminobenzidine (DAB; Sigma-Aldrich, São Paulo, Brazil) associated with hydrogen peroxide (Dinâmica, Indaiatuba, Brazil). After the reaction, the sections were washed with water and counterstained with hematoxylin. At the end of the procedure, the sections were dehydrated with increasing alcohol concentrations and then immersed in xylol. The sections were covered with coverslips and analyzed under a light microscope to identify the immunostaining pattern of StAR in the theca and luteal cells cytoplasm.

2.8. Hormonal assays

Blood samples were collected on PND 150, and the serum obtained by centrifugation (2400 rpm) for 20 min at 4 ºC. Serum samples were subsequently stored at -20 ºC. Progesterone (ER1255; sensitivity of 0.188 ng/mL) and estradiol (ER1507; sensitivity of 7.5 pg/mL) levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits and following the manufacturer's instructions (Fine Test, Wuhan, China). Optical density value was detected using an ELISA microplate reader (Kasuaki, purchased from IONLAB, Araucaria, Brazil), at 450 nm wavelength for both estradiol and progesterone. All samples were measured in the same assay to avoid inter-assay errors.

2.9. Estrous cyclicity

Normal estrous cycle lasts 4-5 days in rats, and around 3 cycles are expected to be observed within 15 continuous days of evaluation (E Silva et al., 2016; Figueiredo et al., 2023; Leite et al., 2018). From PND 136 to 150, 10 µL of saline solution was gently introduced into the rat's vagina with an automatic pipettor and the fluid aspired was placed on clean histological slides for analysis under a light microscope. The estrous cycle is divided into four phases in the rat and each phase can be identified by the observation of the proportions of cellular types on vaginal lavages, proestrus (predominance of nucleated epithelial cells), estrus (predominance of anucleated cornified cells), metestrus (the same proportion of leukocytes, cornified, and nucleated epithelial cells) and diestrus (predominance of leukocytes). The phase of the estrous cycle observed was recorded and used to calculate the frequencies of the phases (number of days each phase was recorded divided by the number of days evaluated), cycle length (number of days from the first day of a cycle phase to the first day of the next same phase), and number of cycles (number of complete cycles during evaluation) (E Silva et al., 2016).

2.10. Sexual behavior

Females at the first estrus phase after PND 150 (n = 5/group) were placed with a sexually competent male rat on the dark cycle of the photoperiod, then allowed 10 mounts on the female while registering lordosis behavior. Results were expressed as the lordosis quotient (lordosis number / 10 mounts × 100) (Beach, 1976). After the test, females were left overnight in the cage of the male and vaginal smears were collected on the next morning to confirm insemination. Inseminated females were kept for the fertility test. If no lordosis behavior was detected on two subsequent estruses, the female was considered sexually inactive and excluded from the test.

222 2.11. Fertility test

223 Females from the sexual behavior test were euthanized on gestational day (GD) 20 to evaluate
224 fertility. After collection of the uterus and ovaries the numbers of corpora lutea, implants and
225 reabsorptions were recorded, and the following endpoints determined: pregnancy rate: pregnant
226 females / sperm-positive females × 100; fertility potential (efficiency of implantation):
227 implantation sites / corpora lutea × 100; rate of pre-implantation loss: (number of corpora lutea
228 - number of implantations / number of corpora lutea) × 100; rate of post-implantation loss:
229 (number of implantations - number of live fetuses) / number of implantations × 100 (Borges et
230 al., 2017).

231

232 2.11. Statistical analyses

233 Data are presented as mean ± standard error of mean (SEM), median and interquartile range, or
234 percentage. Shapiro-Wilk's test was used to determine if data were normally distributed.
235 Student's t-test was used for comparison of parametric variables. Non-parametric variables
236 were compared using Mann-Whitney's test. Proportion data was compared using the Fisher's
237 Exact test. Results were considered significantly different when $P \leq 0.05$. Statistical analyses
238 were performed using the GraphPad InStat software (version 8; La Jolla, CA).

239

240 3. RESULTS

241

242 3.1. General toxicity

243 No evident signs of general toxicity or stress (mortality and morbidity, excessive weight
244 gain/loss, weakness, lethargy, presence of bristly hair, and abnormal behavior) derived from the
245 treatment were observed during the experimental period. Females treated with 0.015 mg/d of
246 PS-NP had no alterations in body weight (Control: 252.40 ± 6.13 g; PS-NP: 264.00 ± 6.25 g; *P*

247 > *0.05*) nor weight gain (Control: 18.86 ± 2.30 g; PS-NP: 23.08 ± 2.66 g; *P > 0.05*) during the
248 experiment (Figure 2).
249
250 3.2. Organ weights
251 Organ wet weights were measured, and the relative organ weight was calculated by dividing
252 the absolute organ weight by the body weight and multiplying by 100. As shown in Table 1, the
253 absolute and relative weights of the pituitary and uterus were significantly increased in the PS-
254 NP-treated group. Conversely, thyroid absolute and relative weights were decreased in the PS-
255 NP group (Table 1). A trend toward an increase in the absolute weight of the adrenals was
256 observed (P = 0.09), but the relative weight was not affected.
257
258 3.3. Histological evaluation
259 Histopathological evaluation of the uteri showed enlarged lumen in PS-NP group (4/5 animals).
260 In the endometrial region, enlarged blood vessels (5/5 animals) surrounded by inflammatory
261 infiltrate (5/5 animals) and a larger number of endometrial glands (3/5 animals) were observed.
262 Quantification of the endometrial glands showed a trend toward an increase in the total number
263 of glands in the PS-NP group (Control: 37.75 ± 3.01; PS-NP: 70.75 ± 15.92; *P = 0.0879*). The
264 morphometry of the uterus was not altered in neither luminal epithelium (Control: 24.84 ± 3.33
265 μm; PS-NP: 26.43 ± 1.11 μm; *P > 0.05*), endometrium (Control: 835.70 ± 69.46 μm; PS-NP:
266 700.40 ± 98.11 μm; *P > 0.05*), nor myometrium (Control: 463.10 ± 18.50 μm; PS-NP: 442.50
267 ± 19.79 μm; *P > 0.05*). Ovaries from animals in both experimental groups showed a normal
268 morphology. Follicles were categorized and quantified by type (primordial, primary, secondary,
269 and tertiary) when the oocyte was visible. Atretic follicles and corpora lutea were also counted
270 to determine the total ovarian structures. Only one cystic follicle was observed, which was

found in a single female from the control group. The ovaries had no overt visible alterations (Figure 3).

3.5. Immunohistochemistry (IHC)

There was a decrease in the immunostaining pattern for StAR on luteal cells in the PS-NP group evidenced by the qualitative analysis (4/5 animals), that was not observed in theca cells of secondary and tertiary follicles (Figure 4). This observation was confirmed by a quantitative analysis of the intensity of staining for StAR in luteal cells and theca cells as shown in Figure 4.

3.6. Hormonal assays

Progesterone levels (Control: 33.85 ± 3.28 ng/mL; PS-NP: 24.40 ± 1.43 ng/mL; $*P < 0.05$) were decreased in PS-NP females, whereas estradiol levels (Control: 22.82 ± 0.97 pg/mL; PS-NP: 22.20 ± 0.22 pg/mL; $P > 0.05$) remained unaltered (Figure 4).

3.7. Estrous cyclicity

The frequencies of the phases in Control animals were comparable to previous studies conducted by our research group (Moura et al., 2023). All females exhibited a cyclic pattern and were therefore included in the analysis. Proestrus was decreased, whereas diestrus was increased in PS-NP group. Estrous cycle length and number of cycles were not altered, as shown in Table 2.

3.8. Sexual behavior and fertility tests

The results of sexual behavior and fertility tests in the Control group followed the same pattern as previously shown by our research group (Barros et al., 2020). In the present work, the

lordosis coefficient and the parameters related to the fertility performance of the animals were comparable between the Control and treated groups (Table 3). All animals were sexually active and exhibited normal sexual behavior. Although there were fewer pregnant females in the PS-NP group, this difference was not statistically significant. The mean number of live pups per litter was similar between experimental groups, as was the sex ratio. The data from one female in the control group was excluded from the sex ratio analysis because her litter of seven male and one female pup was considered a significant outlier ($P < 0.05$). Body weight evolution during pregnancy was similar in both experimental groups (Figure 2).

4. DISCUSSION

Plastics are a global contaminant that may be degraded to smaller particles that persist in the food, water, and soil, and affect the health and fertility of different species (de Souza Machado et al., 2018; Guo and Wang, 2019; Toussaint et al., 2019; Wright et al., 2020). In this study adult female Wistar rats were orally treated with 0.015 mg/d of PS-NP for 25 consecutive days. This concentration was chosen based on the estimated human exposure through ingestion (Senathirajah et al., 2021; Soltani et al., 2021). We observed an increase in pituitary and uterus weights, a decrease in thyroid weight, uterine inflammatory infiltrate, and a decrease in immunostaining for StAR in luteal cells, associated with decreased progesterone serum levels, and estrous cycle dysregulation. Therefore, although in our study the decrease in StAR expression in luteal cells and the consequent reduction in serum progesterone levels seems to play a central explanation for the changes in the monitored parameters, multiple mechanisms were involved in the observed changes.

Endocrine disruption promoted by plastics and plasticizers is widely studied in several models (Amereh et al., 2020; Karwacka et al., 2019; Ullah et al., 2023). Micro- and nanoplastics can act as endocrine disruptors by interfering with hormonal receptors, thereby disrupting different

hormonal axes such as the hypothalamus-pituitary-gonadal axis (HPG), the hypothalamus-pituitary-thyroid axis (HPT), and the hypothalamus-pituitary-adrenal axis (HPA) (Ullah et al., 2023).

The hypothalamic-pituitary axis seems to be affected by exposure to micro- and nanoplastics (Hong et al., 2023). In the present work, we observed a decrease in thyroid weight, accompanied by an increase in pituitary gland weight. Even though our study lacks thyroid and pituitary hormone level measurements, which would better assess the endocrine status of these organs, we suggest that these alterations are related to a compromise in thyroid function and consequent compensation by the pituitary gland to restore the activity of the HPT axis. PS-NP of 25 and 50 nm disrupts thyroid endocrine function and causes a metabolic deficit in male rats. There is evidence that the levels of free active T3 and T4 in the serum decrease, whereas TSH increases after exposure to PS-NP (Amereh et al., 2019). In the same study, cholesterol and LDL levels were increased, and HDL levels were decreased in rats exposed to PS-NP (Amereh et al., 2019). The same was observed in adult female rats, besides the occurrence of oxidative stress in the liver, an increase in serum insulin, and glucose intolerance in these animals (Saeed et al., 2023). We also observed a non-significant increase in the weight of the adrenal gland of female rats treated with a low dose of polystyrene nanoplastics (approximately 0.1 mg/kg/d) for 25 days. The adrenals produce and release hormones such as mineralocorticoids, epinephrine, and small amounts of sexual steroids. Not much has been studied about the impacts of micro- and nanoplastics on adrenal glands, except for a study that reported an increase in adrenal relative weight in male rats after a single exposure to a high dose (125 mg/kg) of polyethylene terephthalate (PET) microplastics (Stojanović et al., 2021). The mechanistic reason behind our findings is yet unknown, but we suggest this could be related to an imbalance in the HPA axis. Further studies are encouraged to elucidate the specific effects of micro- and nanoplastics

exposure on hypothalamus-pituitary axes and pituitary function, as this seems to be a common factor in the results reported herein.

In this regard, studies by Amran et al. (Amran et al., 2023) and Wang et al. (Wang et al., 2025) provide direct examples, as they quantified the gonadotropins FSH and LH in female rats exposed to polystyrene microplastics (PS-MPs). In both studies, FSH was reduced following PS-MP exposure. On the other hand, LH was reduced in the study by Amran et al. (2023) but increased in the study by Wang et al. (2025). Therefore, gonadotropin levels are sensitive to microplastic exposure and may be responsible for some of the negative reproductive effects of this treatment.

Besides endocrine disruption, another factor that usually impairs the organs exposed to micro- and nanoplastics is inflammation. The immune system generally recognizes micro- and nanoplastics as xenobiotics and activates pro-inflammatory cytokines (Brun et al., 2018). An increase in inflammation and inflammatory markers has been a common finding in several studies with micro- and nanoplastics both *in vivo* and *in vitro* (Brun et al., 2018; Forte et al., 2016; Hu and Palić, 2020). More specifically on the female reproductive system, it has been shown that polystyrene microplastics induce inflammation and an increase in IL-6 in the ovaries of female mice (Liu et al., 2022). Congested blood vessels and inflammatory cell infiltration were found in the uteri of female rats exposed to PS-NP in our study. This shows that inflammation can be induced in different organs following micro- and nanoplastics exposure. Polyamide micro- and nanoplastics cause uterine artery dysfunction in rats within 24 h of exposure by inhalation, and systemic inflammation was detected by the increase in the circulating IL-6 levels (Cary et al., 2023). Furthermore, we observed an increase in uterine weight and enlarged lumen in rats from PS-NP group. This could be due to an increase in uterine fluid, since we also observed more endometrial glands in the exposed animals, whereas there were no changes in the thickness of uterine layers.

Damage to ovarian tissue following PS exposure has been recently reported in both mice (5 μm, 0.1 mg/d) (Wei et al., 2022) and rats (500 nm, 0.15 and 1.5 mg/d) (An et al., 2021). PS-MP induced oxidative stress and weight reduction in the ovaries of female mice (Wei et al., 2022). In rats, exposure to PS-NP (500 nm) reduced the number of growing ovarian follicles due to oxidative stress and apoptosis of granulosa cells (An et al., 2021). Our findings suggest that lower concentrations of PS-NP such as 0.015 mg/d may not trigger cell death in ovarian tissue, since we have not observed any morphological alterations in the ovaries of rats treated for 25 days. An et al. also observed no alterations in the ovary of females treated with 0.015 mg/d, even though higher doses affected ovarian tissue under the same experimental conditions (An et al., 2021). Another important consideration is that we have exposed adult rats to PS-NP, whereas the studies mentioned have exposed younger animals (prepuberty/puberty). Most studies have focused on the impact of nanoplastics on granulosa cells, yet the luteal cells are of great importance given their production of progesterone (Hong et al., 2023).

In a recent study (Wang et al., 2023) even though no alterations were found in the ovarian morphology, its function was damaged by PS-NP exposure, as evidenced by the decreased levels of estradiol and progesterone in rats exposed to 2 mg PS-NP/kg/d. This finding was related to the reduction in the expression of the steroidogenesis-related genes *Star* and *Cyp19a1*. The delivery of cholesterol to the inner mitochondrial membrane by the protein StAR is a rate-limiting stage of steroidogenesis (Tugaeva and Sluchanko, 2019). Theca and luteal cells express StAR, and the luteal cell population expresses the highest levels of this protein in the ovaries (Zhang et al., 2024).

Ovarian expression of StAR is intimately related to steroidogenic activity and reproductive function (Fan et al., 2022; Kahsar-Miller et al., 2001). When *Star* is up-regulated, serum levels of progesterone increase (Ding et al., 2024; Maurya et al., 2024); when *Star* is down-regulated, serum levels of progesterone decrease (Fiedler et al., 1999; Lu et al., 2024). Also, *Star* down-

395 regulation is related to luteal endocrine disorder, which negatively affects embryo implantation
396 success in pregnant mice exposed to bisphenol AF (Lu et al., 2024).
397 Similarly, in our study, immunostaining for StAR was less intense in the ovaries of animals
398 from PS-NP groups, suggesting a reduction in the expression of this protein specifically in luteal
399 cells. This culminates in the observed reduction of serum progesterone levels in animals
400 exposed to PS-NP, even though estradiol levels were not altered in this experiment. Contrarily,
401 estradiol levels seem to be increased in adult female rats treated with 10 mg PS-MP/kg for 45
402 days (Saeed et al., 2023). We recognize that not measuring other enzymes involved in
403 steroidogenesis in the ovarian cells, nor determining the Star gene expression are limitations of
404 the present work, that still deserves attention in future studies.
405 In our study, we observed a dysregulation of the estrous cycle in female rats exposed to PS-NP,
406 which also had an increase in the weight of the pituitary gland. Estrous cycles are regulated by
407 the HPG axis (Vidal, 2017). An imbalance in hormonal levels may lead to a dysregulation of
408 the estrous cycle and a potential decrease in fertility. One of the causes of this imbalance can
409 be exposure to endocrine-disrupting chemicals (Hamid et al., 2021), like plastics and their
410 derivates. Serum levels of progesterone change along the estrous cycle phases: peaks during
411 proestrus, decreases during estrus, and rises again during late metestrus and early diestrus. This
412 fluctuation is essential for a well-regulated estrous cycle (Jänne, 1981; Watanabe et al., 1990).
413 We suggest that the progesterone reduction caused a prolonged diestrus and shortened proestrus
414 in females of the PS-NP group. Metestrus and diestrus frequencies were also increased in mice
415 treated with 50 nm PS-NP, while estrus was decreased as reported by Huang et al. (Huang et
416 al., 2023). In another study, exposure to PS-MP (5 μm) reduced the duration of the estrous cycle
417 and the frequency of metestrus in rats (Haddadi et al., 2022). Other negative conditions that
418 trigger prolonged diestrus in rats, such as heat and stress exposures, were associated with lower
419 levels of serum estradiol and progesterone (Han et al., 2021). These findings reinforce an

imbalance in the HPG axis caused by PS-NP exposure. Our results also suggest a mechanistic understanding behind this imbalance, which is the reduction of serum progesterone levels by a reduction of ovarian StAR protein.

Steroid hormones also regulate uterine luminal fluid (ULF): estradiol stimulates its secretion, whereas progesterone causes its absorption. This regulation is important especially in the early stages of pregnancy, given that the ULF provides a support medium for sperm and unimplanted embryos, while its absorption promotes the closeness of the blastocyst to the uterine epithelium and the implantation (Salleh et al., 2005). Therefore, excessive fluid in the uterine lumen may lead to abnormal implantation (Lu et al., 2013). Progesterone reduction could have impaired ULF resorption, which led to an enlarged lumen and increased wet weight of the uterus of PS-NP females. Even with a potential increase in ULF in the rats treated with PS-NPs, there was no impairment of implantation rates. Besides, sexual behavior was also not altered in PS-NP group, neither were other analyzed fertility parameters.

However, we should not discard the possibility of fertility impairment in chronic exposure to micro- and nanoplastics (Song et al., 2023). For examples, oral exposure to polyethylene microplastics (10–150 μm; 40 mg/kg) for 30 days reduces oocyte maturation and fertilization rate, embryo development, and fertility in female mice (Zhang et al., 2023). Mice early embryonic development is also impaired upon exposure to polymethylmethacrylate nanoplastics during pre-implantation (You et al., 2024). In female zebrafish, exposure to 500 nm PS-NP resembles clinical PCOS, associated with lipid metabolism disorder (Zheng et al., 2024). Reproductive behavior was shown to be altered in amphipod species (Ramírez-Olivares et al., 2024) and zebrafish (Rojoni et al., 2024) after exposure to microplastics. Nonetheless, there is a gap in the knowledge of mammal sexual behavior impairment after exposure to these particles.

5. CONCLUSIONS

This study shows that a low concentration of PS-NP leads to estrous cycle dysregulation, uterine inflammation, increased uterus and pituitary weight, and decreased thyroid weight in rats. These effects are linked to reduced StAR expression and lower progesterone levels. The findings suggest that PS-NP act as endocrine disruptors, potentially harming female reproductive health and raising concerns about nanoplastic exposure in humans.

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Tables

Table 1. Body and organ weights on PND 150. Adult female Wistar rats were treated with 0 (Control) or 0.015 mg/d of PS-NP for 25 days.

Parameters	Experimental groups (n = 5)	
	Control	PS-NP
[1]Body weight (g)	252.40 ± 6.13	264.00 ± 6.25
[1]Brain (g)	1.91 ± 0.03	1.93 ± 0.03
[1]Brain (g/100g bw)	0.76 ± 0.02	0.73 ± 0.02
[1]Pituitary (mg)	11.88 ± 0.97	16.96 ± 0.64**
[1]Pituitary (mg/100g bw)	4.95 ± 0.38	6.42 ± 0.11**
[1]Thyroid (mg)	19.18 ± 0.77	15.65 ± 0.49**
[1]Thyroid (mg/100g bw)	7.44 ± 0.22	5.82 ± 0.13***
[1]Adrenal glands (mg)	77.72 ± 5.65	89.66 ± 2.30 (*P=0.09*)
[1]Adrenal glands (mg/100g bw)	30.99 ± 2.76	34.00 ± 0.82
[2]Ovaries (mg)	79.30 ± 2.39	73.40 ± 7.72
[2]Ovaries (mg/100g bw)	31.24 ± 0.91	29.59 ± 2.70
[2]Uterus (g)	0.40 ± 0.04	0.65 ± 0.08*
[2]Uterus (g/100g bw)	0.16 ± 0.02	0.25 ± 0.03*

Values expressed as mean ± SEM. [1]Student's t-test. [2]Mann-Whitney's test. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

Table 2. Estrous cyclicity of adult female Wistar rats from PND 135 to 150.

Parameters	Experimental groups	
	Control (n = 10)	**PS-NP (n = 9)**
[1]*Frequency of estrous phases (%)*		
Proestrus	23.50 (18.25 - 27.00)	13.00 (8.50 - 20.00)*
Estrus	27.00 (20.00 - 30.25)	27.00 (20.00 - 33.00)
Metestrus	33.00 (16.75 - 34.75)	20.00 (16.50 - 33.50)
Diestrus	20.00 (18.25 - 28.50)	33.00 (33.00 - 45.25)**
[2]Estrous cycle length (days)	3.91 ± 0.28	4.01 ± 0.14
[2]Number of estrous cycles	3.00 ± 0.21	3.00 ± 0.17

[1]Values expressed as median and interquartile intervals. Mann Whitney's test. [2]Values expressed as mean ± SEM. Student's t-test. *$P < 0.05$. **$P < 0.01$.

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740 Table 3. Fertility parameters of adult female Wistar rats treated with 0 (Control) or 0.015 mg/d of PS-NP for 25 days.

Parameters	Experimental groups (n = 5)	
	Control	PS-NP
[1]Lordosis coefficient	100.00 (95.00 - 100.00)	100.00 (50.00 - 100.00)
[2]Pregnancy rate (%)	80.00 (4/5)	60.00 (3/5)
[1]Fertility potential (%)	100.00 (94.65 - 100.00) (4)	100.00 (93.33 - 100.00) (3)
[1]Pre-implantation loss (%)	0.00 (0.00 - 5.36) (4)	0.00 (0.00 - 6.67) (3)
[1]Post-implantation loss (%)	6.97 (1.56 - 11.92) (4)	0.00 (0.00 - 27.78) (3)
[3]Body weight of the dams (g)	382.70 ± 32.46 (4)	387.70 ± 3.69 (3)
[3]Uterus weight with fetuses (g)	81.46 ± 10.74 (4)	87.53 ± 4.23 (3)
[1]Number of live fetuses per litter	12.00 (9.00 - 14.25) (4)	14.00 (13.00 - 15.00) (3)
[1]Sex ratio (male/female)	1.14 (1.00 - 1.40) (3)	0.63 (0.50 - 1.33) (3)
[3]Male fetus weight (g)	4.67 ± 0.12 (4)	4.48 ± 0.14 (3)
[3]Female fetus weight (g)	4.49 ± 0.07 (4)	4.26 ± 0.28 (3)
[3]Placental weight - males (g)	0.61 ± 0.03 (4)	0.61 ± 0.02 (3)
[3]Placental weight - females (g)	0.57 ± 0.01 (4)	0.58 ± 0.02 (3)

[1]Values expressed as median and interquartile intervals (Mann-Whitney's test). [2]Values expressed as percentages (Fisher's exact test). [3]Values expressed as mean ± SEM (Student's t-test). $P > 0.05$.

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743 **Figures**

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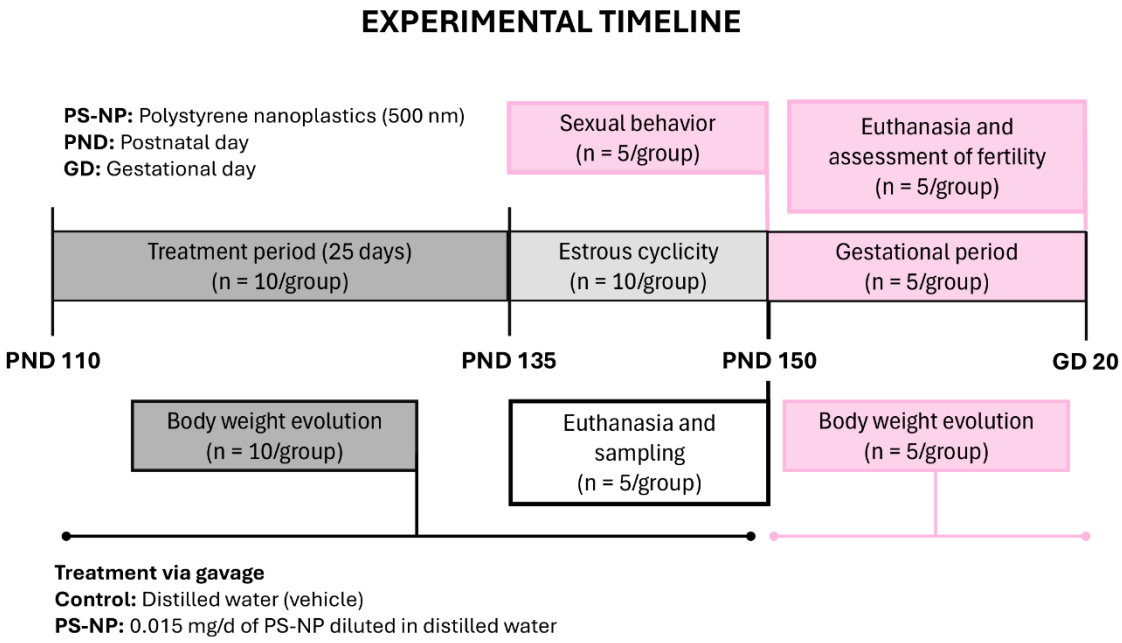
745 **1.**



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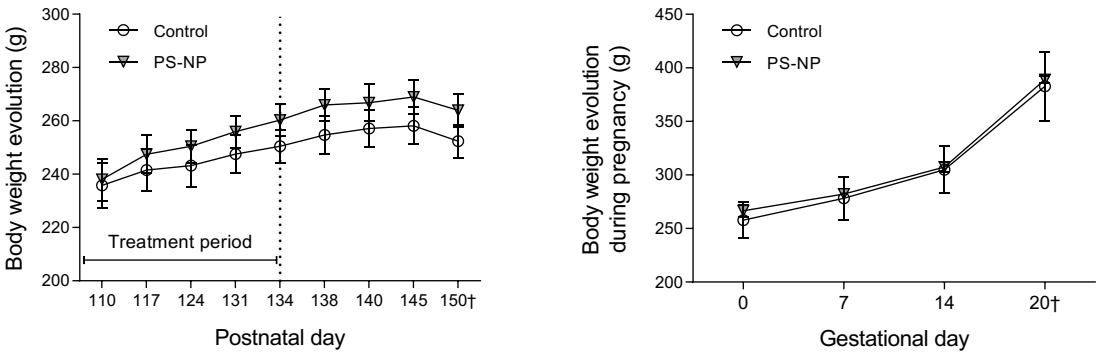
747 **2.**



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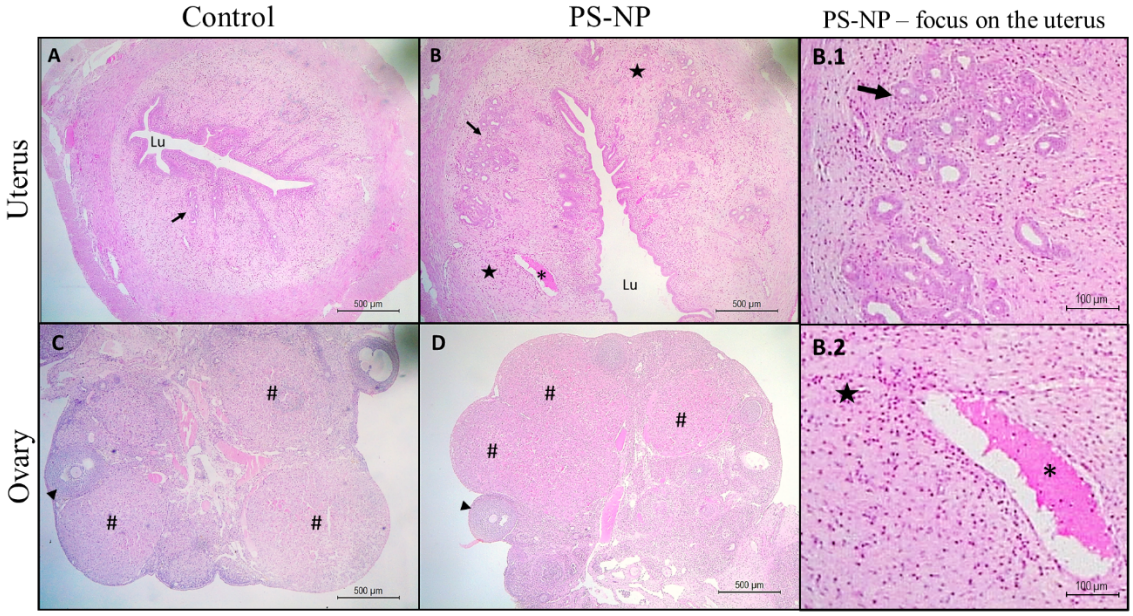
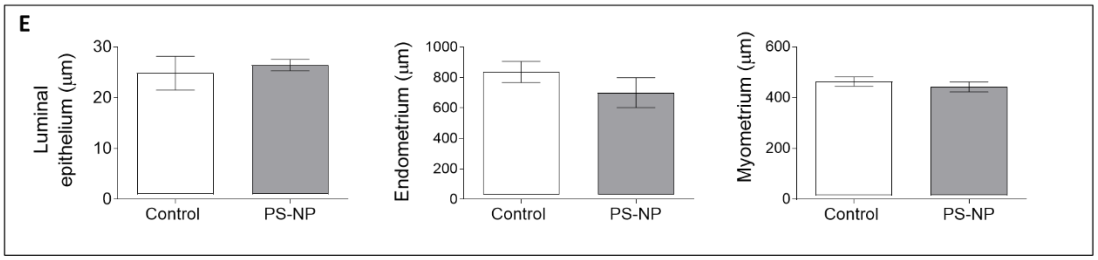
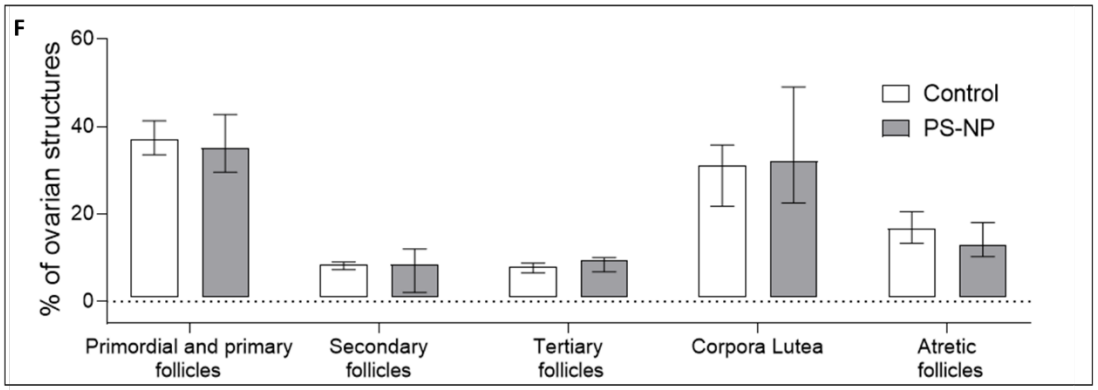
750 **3.**



Uterine morphometry



Ovarian Structures



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753 **4.**

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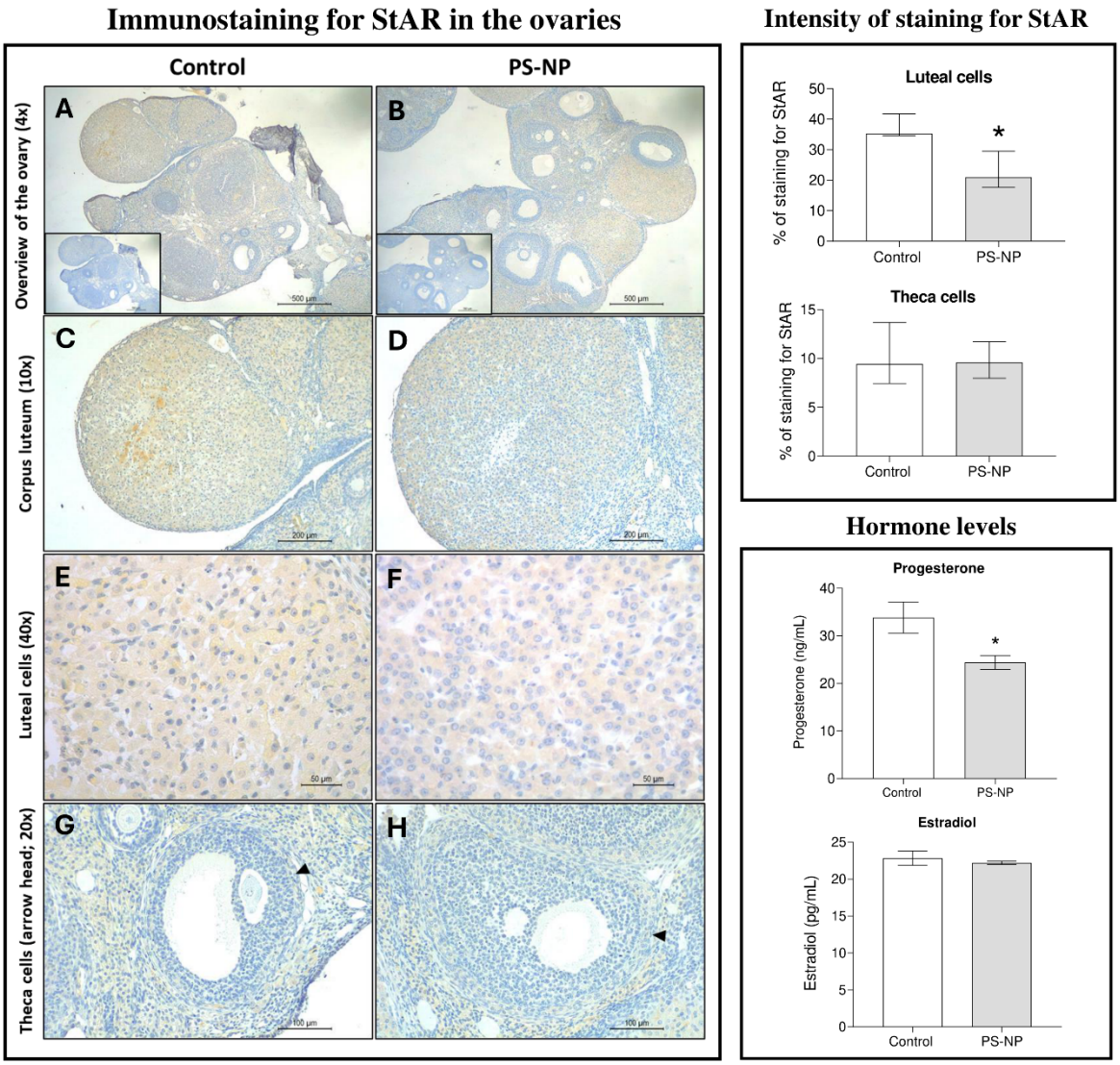
Immunostaining for StAR in the ovaries
Control
PS-NP
Overview of the ovary (4x)
Corpus luteum (10x)
Luteal cells (40x)
Theca cells (arrow head; 20x)
A
B
C
D
E
F
G
H
Intensity of staining for StAR
Luteal cells
% of staining for StAR
Control
PS-NP
Theca cells
% of staining for StAR
Control
PS-NP
Hormone levels
Progesterone
Progesterone (ng/mL)
Control
PS-NP
Estradiol
Estradiol (pg/mL)
Control
PS-NP

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Figure captions

Figure 1. Experimental timeline.

Figure 2. Body weight evolution and weight gain of adult female Wistar rats from Control (0 mg/d) and PS-NP (0.015 mg/d) groups (n = 10/group) during the experimental period (PND 110 - 150), and body weight evolution during pregnancy from GD 0 – 20 (Control: n = 4; PS-NP: n = 3). Data expressed as mean ± SEM. Student's t-test. $P > 0.05$.

Figure 3. Histological evaluation of ovary and uterus of female rats on PND 150. (A-B) A representative histological aspect of uteri. B.1 = Focus on endometrial glands. B.2 = Focus on enlarged blood vessel and inflammatory infiltrate. Lu = lumen. Arrow = endometrial gland. Star = inflammatory infiltrate. Asterisk (*) = enlarged blood vessel. (C-D) Representative histological aspect of ovaries. Arrowhead = tertiary follicle. # = corpus luteum. (E) Histomorphometric measurements of uterine layers. Data expressed as mean ± SEM. Student's t-test. $P > 0.05$. (F) Follicle count. Data expressed as median and interquartile intervals. Mann Whitney's test. $P > 0.05$.

Figure 4. Representative aspects of immunostaining for StAR on the ovary of female rats on PND 150. (A-B) General view of the ovary and their respective negative controls. (C-D) Corpus luteum. (E-F) Focus on luteal cells. Observe the less intense staining in luteal cells of PS-NP group. (G-H) Tertiary follicle. Arrowhead = theca cells. Intensity of staining for StAR in luteal and theca cells. Data expressed as median and interquartile intervals. Mann-Whitney's test. $*P < 0.05$. Serum progesterone and estradiol levels. Data expressed as mean ± SEM. Student's t-test. $*P < 0.05$.

Capítulo 2

Direct and Intergenerational Effects in Reproductive Parameters of Adult Male Wistar Rats and Their Offspring After Subchronic Exposure to Polystyrene Nanoplastics

O segundo capítulo reúne os resultados obtidos a partir da exposição de ratos Wistar adultos a PS-NP e potenciais efeitos intergeracionais sobre a prole e gerou o artigo intitulado *"Direct and Intergenerational Effects in Reproductive Parameters of Adult Male Wistar Rats and Their Offspring After Subchronic Exposure to Polystyrene Nanoplastics"*. Este artigo foi publicado na revista "Reproductive Toxicology" (Fator de impacto: 2,8).

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Direct and Intergenerational Effects in Reproductive Parameters of Adult Male Wistar Rats and Their Offspring After Subchronic Exposure to Polystyrene Nanoplastics

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ABSTRACT

Polystyrene is among the most prevalent types of plastic debris. Polystyrene nanoplastics (PS-NP) cause several alterations in young rodent reproductive tissue and fertility. Here, we investigated if the exposure to PS-NP (500 nm) in adult (90 days-old) male Wistar rats affects reproductive parameters and causes intergenerational effects on the offspring. Study 1: animals (n = 10/group) were exposed by gavage to either distilled water (vehicle; Control group), 0.15 mg/d of PS-NP (Low Dose) or 1.50 mg/d of PS-NP (High Dose) for 60 days. Sperm quality and testosterone serum levels were measured. Study 2: the exposure protocol was repeated using only Control (n = 10) and High Dose (n = 9) groups, then blood leukocytes, histopathology of the testis and the epididymis, and fertility parameters were evaluated. At the end of treatment males (F0) were mated with untreated females (70 – 90 days-old) to produce the first generation (F1) evaluated on Study 3 (Control: n = 7; High Dose: n = 8). Study 3: intergenerational damage was assessed in the male and female offspring (F1). The presence of sperm cytoplasmic droplets and the relative number of sperm in the cauda epididymis increased in the High Dose group (Study 1), as well as the relative number of monocytes in the blood stream (Study 2). Intergenerational effects were observed such as the dysregulation of the estrous cycle of F1-females (Study 3). Given that rats exhibit significantly higher fertility rates than humans, these results could imply that long-term environmental exposure to different types of plastics might have potential consequences for human reproductive health.

Keywords: nanoplastics, fertility, male, rat, adult, intergenerational.

1. INTRODUCTION

Polystyrene (PS) is one of the most prevalent types of plastic debris (Li et al., 2016). Micro- (< 5 mm) and nanoplastics (< 1 μm) are contaminants of emerging concern which are either manufactured for cosmetic and pharmaceutical products or are resultant of degradation processes of larger plastics (Barnes et al., 2009; Mitrano et al., 2021; Napper et al., 2015; Thushari and Senevirathna, 2020). They are found in foods, drinking water, bottled beverages, and even the air is contaminated with these particles (Ageel et al., 2021; Shruti et al., 2020; Toussaint et al., 2019). Inhalation and ingestion are considered the main routes of human exposure to micro- and nanoplastics; it is estimated that humans consume up to 5 g of microplastics per week (Ageel et al., 2021; Senathirajah et al., 2021). After consumption, these particles are absorbed by the intestines. Polystyrene nanoplastics (PS-NP) up to 500 nm can cross cellular membranes in RBL-2H3 rat basophilic leukemia cells, and in a tri-culture model of the human intestine (Choi et al., 2024; Liu et al., 2021). Microplastics are distributed through the body and reach several organs, and are quantified in human samples of blood (1.6 μg/mL), placenta (128.8 μg/g), and testis (328.44 μg/g) (Bai et al., 2024; Garcia et al., 2024; Hu et al., 2024; Leslie et al., 2022).

This widespread distribution raises concerns about potential health impacts, particularly on male reproductive health. Exposure to 500 nm PS-NP from prepuberty causes disruption of blood-testis-barrier, apoptosis of spermatogenic cells and reduction of overall sperm quality in rats (Li et al., 2021). Another study conducted in peripubertal rats showed that exposure to PS-NP (100 nm; 75 mg/kg/d) causes erectile dysfunction, endothelial dysfunction and reduces testosterone levels (Wang et al., 2024). Furthermore, polystyrene micro- and nanoplastics administered by intratracheal instillation leads to a reduction in the thickness of the seminiferous epithelium, quantity and motility of sperm, and an increase in sperm

malformations in mice. These alterations were associated with mitochondrial damage, and induction of pyroptosis in the testis by increasing the expression of inflammatory markers (Zhao et al., 2024). Increase in inflammatory markers and oxidative stress were also observed by Wu et al. in a study where they demonstrated the internalization of PS microplastics (1 μm) by Sertoli TM4 cells. Their findings further indicate premature senescence of those exposed TM4 cells (Wu et al., 2023).

Recent studies have investigated the effects of the exposure to micro- and nanoplastics in animals starting in peripuberty, which is a critical period in sexual development (Ojeda and Skinner, 2006). There is a lack of research regarding the impact of the exposure to these particles in the fully formed reproductive system, when animals are at peak reproduction (Arellano et al., 2024). Furthermore, exposure to PS-NP (60 nm) alters the genetic expression in a spermatocyte cell culture (Jiang et al., 2024), which indicates that nanoplastics can affect germ cells. We aimed to investigate the effects of the exposure to PS-NP (500 nm) in the mature reproductive system of 90 days-old male rats and their offspring.

2. MATERIAL AND METHODS

2.1. Polystyrene nanoplastics

Polystyrene nanoplastic spheres (PS-NP) with a diameter of 500 nm obtained from Alpha Nanotech Inc. (Vancouver, BC) were diluted in distilled water for the preparation of the two experimental doses: 0.15 mg/d (Low Dose) and 1.50 mg/d (High Dose). The volume of distilled water with or without PS-NP administered to the rats was 0.3 mL. The supplier provided a document with specification/characterization of PS-NP stating the following characteristics: Size: 500 nm; CV< 3%; Roundness > 0.980; Surface: Non-Functionalized.

2.2. Choice of dose

The concentrations of nanoplastics chosen for this study were determined based on the estimated human exposure levels. Estimates of human exposure through ingestion range from approximately 0.23 to 12 mg/kg/day, based on an average human weight of 60 kg (Senathirajah et al., 2021). To account for the metabolic differences between humans and rats, these values were adjusted using a conversion factor (K_m ratio). The K_m of species is calculated by dividing the average body weight (60 kg for humans; 0.4 kg for adult male rats from our study) to its body surface area (1.62 m^2 for humans; 0.025 m^2 for rats). Thus, Human K_m equals to 37 and Rat K_m equals to 16; K_m ratio (Human K_m/Rat K_m) is 2.3125 (Nair and Jacob, 2016). This adjustment resulted in a range of 0.53 to 27.75 mg/kg/d for a 400 g rat. Based on these calculations, we selected two concentrations for treatment: 0.15 mg/d (approximately 0.375 mg/kg/d) and 1.50 mg/d (approximately 3.75 mg/kg/d). These concentrations closely align with the lower end of human daily exposure levels, providing a relevant basis for our experimental design.

2.3. Animals

Wistar rats (postnatal day – PND – 45; 130 – 200 g) were obtained from the Central Biotherium of UNESP Botucatu and housed in polypropylene cages bedded with wood shavings under controlled conditions (22 °C, 30% air humidity, 12/12 h light/dark cycle), with food and water available *ad libitum*. All experimental procedures in this study were approved by the local Ethics Committee for the Use of Experimental Animals of São Paulo State University (1848200721-CEUA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), and following ARRIVE guidelines (Percie du Sert et al., 2020). Euthanasia was performed by decapitation following CO_2 narcosis.

122 2.3. Experimental design

123 This work was divided into three studies to address different aspects of the exposure to PS-NP
124 on male reproductive system, fertility, and offspring (Figure 1). During the experimental period
125 animals were daily monitored for signs of general toxicity or stress (mortality and morbidity,
126 excessive weight gain/loss, weakness, lethargy, presence of bristly hair, and abnormal
127 behavior).

128

129 *2.4. Study 1 – Sperm Quality*

130 Adult male Wistar rats (PND 90; 350 – 450 g) were divided into 3 experimental groups (n =
131 10/group) which were treated by gavage for 60 days with different concentrations of PS-NP: 0
132 (Control), treated with only the vehicle distilled water; 0.15 mg/d of PS-NP (Low Dose) diluted
133 in distilled water; 1.50 mg/d of PS-NP (High Dose). On PND 150 the animals were euthanized
134 for the evaluation of the following parameters: organ weights, sperm motility, sperm
135 morphology, sperm counts, and serum levels of testosterone.

136

137 2.4.1. Body and organ weights

138 On PND 150, animals were weighed and euthanized by decapitation following CO_2 narcosis.
139 Blood samples (n = 5/group) from the severed cervical veins were collected for testosterone
140 assays. Potential target organs were carefully dissected with surgical instruments and weighed
141 on a precision scale. The following organs were sampled: brain, pituitary, thyroid, adrenals,
142 testis, epididymis, ventral prostate, and seminal gland.

143

144 2.4.2 Sperm quality

145

146 *Sperm motility*

147 Sperm was released from the cauda epididymis and transferred to 1 mL of 10% Bovine Serum
148 Albumin (BSA) in Phosphate-Buffered-Saline (PBS) solution at 35 ºC. One hundred sperm
149 were counted in an aliquot of 10 µL of sperm suspension pipetted to a Makler chamber. Using
150 a phase-contrast microscope (400× magnification), 100 sperm were counted and classified as
151 Type A (mobile with progressive movement), Type B (mobile without progressive movement),
152 and Type C (immobile). The number of cells on the sperm suspension was counted and
153 posteriorly added to cauda epididymis sperm count (Quiarato Lozano et al., 2022).
154
155 *Sperm morphology*
156 An aliquot of 100 µL of sperm was added to 900 µL of formol saline (810 µL saline + 90 µL
157 formol). Smears were prepared on histological slides and left to settle. At least 100 sperm per
158 animal were analyzed under a phase-contrast microscope (400× magnification). Morphological
159 parameters were grouped as head abnormalities (isolated head and head without characteristic
160 curvature), tail abnormalities (broken and twisted into a loop), and presence or absence of sperm
161 cytoplasmatic droplet.
162
163 *Sperm counts*
164 Testes (n = 6/group) were decapsulated, weighted, and homogenized in 5 mL of NaCl 0.9%,
165 followed by sonication for 30 s. Samples were diluted (10-fold), transferred to Neubauer
166 chambers (4 fields per animal), and mature spermatids (step 19 of spermiogenesis) were
167 counted (Borges et al., 2017). The total number of spermatids was divided by 6.1 to calculate
168 the daily sperm production (DSP) (Robb et al., 1978). The relative DSP was obtained by
169 dividing the daily sperm production by testis weight.
170 The epididymides were sectioned in caput/corpus and cauda regions for sperm counts and
171 transit time estimation. The caput/corpus and cauda epididymis were cut into small fragments

with scissors, homogenized, sonicated, and sperm was counted as described for the testis. The number of cells counted in the sperm suspension (motility assay) was added to the sperm counted in the homogenized tissue to obtain the total number of sperm in the cauda epididymis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each region of the epididymis by the DSP.

2.4.4. Hormonal assays

Blood samples (n = 5/group) were centrifuged at 2400 rpm for 20 minutes at 4 ºC to separate and collect the serum in Eppendorf tubes, which were then frozen at -20 ºC for posterior analysis. Testosterone (NBP3-23568) was dosed by competitive enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Novus Biologicals, Centennial - USA). All samples were analyzed in the same assay to avoid interassay variability.

2.5. Study 2 – Sexual behavior and Fertility

Adult male Wistar rats (PND 90; 350 – 450 g) were divided into 2 experimental groups: Control (n = 10), which was treated with only the vehicle distilled water, and High Dose (n = 9), which was treated with 1.50 mg/d of PS-NP by gavage for 60 days. We then evaluated body and organ weights (for methodology, refer to section 2.4.1), histopathology of the testis and epididymis, sexual behavior, fertility parameters, contractility of the vas deferens and leukocyte counts. At the end of the treatment (PND 150), these animals (F0; Control: n = 7; High Dose: n = 8) were mated with untreated adult females (PND 70 – 90) to produce the first generation (F1) evaluated in Study 3. The offspring from each mating constituted an independent litter. The selection of F1 animals for subsequent evaluations was conducted to ensure that each litter was equitably represented.

197 2.5.1. Histological evaluation

198 Testes and epididymides samples previously fixed by immersion in modified Davidson's fluid
199 (mDF) (Latendresse et al., 2002) were dehydrated and embedded in Paraplast Plus® (P3683,
200 Sigma-Aldrich), sectioned (5 μm), mounted on glass slides, and stained with hematoxylin and
201 eosin (HE). Histopathology was analyzed in a blind assay using a Leica DMLB microscope
202 mounted with a digital camera and analysis software (LAS V4.12 and ImageJ v1.48).

203

204 *Histopathology and morphometry of testis*

205 Morphological alterations were classified according to specific guidelines for toxicological
206 studies in 100 cross-sections of seminiferous tubules: acidophilic cells, multinucleated cells,
207 retained spermatid, degeneration of cell types, vacuolization of the epithelium, or exfoliation
208 of cells in the lumen (Creasy et al., 2012; Foley, 2001; Picut and Remick, 2017). The aspect of
209 the testicular interstitium was assessed qualitatively. Morphometrical analysis included
210 measuring the diameter of ten sections of seminiferous tubules in stage IX of spermatogenesis
211 and performing the karyometry of Leydig cells in 50 random nuclei that had a circular or
212 elliptical shape. The nuclear volume of Leydig cells, which is an indicator of its secretory
213 activity, was obtained using the image analyzer software ImageJ v1.48 (Fichna and
214 Malendowicz, 1975).

215

216 *Frequency of stages of the spermatogenic cycle*

217 The relative frequency of the spermatogenic stages were estimated: I-VI (presence of two
218 generations of spermatids), VII-VIII (presence of step 19 mature spermatids), IX-XIII (presence
219 of a single generation of spermatids), XIV (presence of secondary spermatocyte/meiotic figures
220 at metaphase or anaphase) in 100 cross-sections of seminiferous tubules per animal (Clermont
221 and Harvey, 1965).

Histopathology of epididymis

The entire length of the longitudinal section of the organ (initial segment, caput, corpus, and cauda) was evaluated for the structure and appearance of the epithelium, presence of sperm in the ductular lumen, and interstitial aspect (Creasy et al., 2012; Kempinas and Klinefelter, 2014).

2.5.2. Sexual behavior

At the end of the treatment males (PND 150; Control: n = 10; High Dose: n = 9) were placed in a cage alone for 10 min before the introduction of a sexually receptive female (70 - 90 days) in the dark period of the light/dark cycle. In the sexual behavior test, the following parameters were counted for a period of 20 min after the first mount: latency until the first mount (maximum of 10 min) and the number of mounts, latency until the first intromission and number of intromissions, latency until the first ejaculation and number of ejaculations. The sexually active animals were kept in cages with the females during the rest of the night. The females were removed in the subsequent morning, and vaginal smears were collected to confirm if they were inseminated. Inseminated females were kept in individual cages until euthanasia (Control: n = 10; High Dose: n = 9).

2.5.3. Fertility test

The females used in the male's sexual behavior test were euthanized on gestational day (GD) 20 (Control: n = 10; High Dose: n = 9) and the uterus, ovaries, placentas, and fetuses were evaluated. The numbers of corpora lutea, implants and resorptions were determined. Fetuses were separated from the uterus and fetal membranes, and the fetus and placenta were weighed separately. From these results the following parameters were calculated: fertility potential (implantation efficiency): implantation sites/corpora lutea × 100, rate of pre-implantation loss: (number of corpora lutea - number of implantation sites)/number of corpora lutea × 100, and

247 rate of post-implantation loss: (number of implantation sites - number of living fetuses)/number
248 of implants × 100 (Borges et al., 2017). The fetal testis (F1) was collected from one male per
249 litter (n = 6/group) and immersed in mDF for histology.
250
251 2.5.4. Leukocyte counts
252 Animals were euthanized one week after the sexual behavior test (PND 157). Blood samples
253 (Control: n = 7; High Dose: n = 8) were collected in EDTA tubes. Total and differential
254 leukocyte counts were obtained in a blind assay using a Leica DMLB light microscope. Blood
255 smears were prepared in Neubauer chambers for total leukocyte count. Differential leukocyte
256 count was done in blood smears on clean histological slides stained with Panotic dye.
257 Leukocytes were classified into eosinophils, monocytes, neutrophils and lymphocytes.
258
259 2.5.5. Contractility of the vas deferens
260 The vas deferens (PND 157; n = 6/group) was isolated and prepared for the tension recording
261 (Borges et al., 2017). Samples were placed in organ baths in 10 mL of a modified Tyrode's
262 solution at 30 ºC under 9.8 mN resting tension, and allowed to equilibrate for 30 min. Then,
263 tissues were repeatedly challenged with 80 mM KCl every 30 min until two reproducible
264 contractions were recorded. Cumulative concentration–response curves to norepinephrine (NE,
265 10^{-9}M – 10^{-4}M) and carbachol (CCh, 10^{-9}M – 10^{-4}M) were obtained at 30 min interval. The
266 maximal tension developed (Emax, in milliNewtons, mN) by the vas deferens stimulated by
267 NE and CCh was evaluated.
268
269 *2.6. Study 3 – Intergenerational Aspects*
270 Each independent litter (F1; Control: n = 7 litters; High Dose: n = 8 litters) of males treated in
271 Study 2 (F0; section 2.5) was evaluated for intergeneration aspects after paternal exposure to

PS-NP. Therefore, the test unit for analysis was either one animal per litter or the mean of animals from the same litter, depending on the specific endpoint being evaluated and as specified for each analysis. F1-males (1 male per litter) were euthanized for sampling of testis on GD 20, and testis and epididymis on PND 21, 42 and 90. F1-females (1 female per litter) were euthanized for sampling of ovaries and uterus on the first estrus between PND 75 – 80. Both male and female rats were evaluated for body and organ weights (for methodology, refer to section 2.4.1), developmental milestones, and histopathology of sexual organs. Sperm morphology and counts were evaluated on F1-males (for methodology, refer to section 2.4.2), and estrous cycle was evaluated on F1-females. For puberty onset and estrous cycle parameters, two animals from each litter were evaluated (Control: n = 14; High Dose: n = 16). The data were analyzed using the mean of each litter as the experimental unit to avoid pseudoreplication. To ensure the robustness of our findings, we also analyzed the data from individual animals. The results showed no significant differences between the experimental groups, confirming the consistency of the findings obtained using litter means.

2.6.1. Developmental milestones

Physical and sensorimotor development were evaluated on all male and female F1 pups (n = 7 litters/group), as described previously in the literature (Gallavan et al., 1999; Heyser, 2004).

Physical development

Pinnae detachment and fur development were observed daily from PND 1; upper and lower incisor eruption was observed daily from PND 6; complete eye opening was observed from PND 12.

Sensorimotor development

297 The following parameters were evaluated daily from PND 1 until three identical consecutive
298 results were observed. Surface rightening reflex: pups were given 30 seconds to turn completely
299 from their back on their belly (positive result). Grasping reflex: the paw of the rat was gently
300 stroked with a paper clip, and the day this reflex disappeared was recorded. Negative geotaxis:
301 pups were placed on a 40º inclined platform facing downwards, and they were given 60 seconds
302 to turn upwards. Cliff avoidance: pups were placed on the edge of a platform with nose and
303 forepaws over the edge; the pups were given 60 seconds to turn away.
304
305 *Sexual development*
306 Animals were weighed and their anogenital distance (AGD) was measured on PND 1. AGD
307 was corrected by body weight as described by Gallavan et al. (Gallavan et al., 1999). Puberty
308 onset was determined by total manual retraction of the preputial skin in two males per litter
309 (Control: n = 7 litters; PS-NP: n = 8 litters) (PND 40 – 50); for female puberty onset, two
310 females per litter (Control: n = 7 litters; PS-NP: n = 8 litters) were evaluated for vaginal opening
311 and day of the first estrus (PND 30 – 40). The first estrus was determined by histological
312 inspection of the vaginal lavage. Animals were weighted on the day of determined puberty onset
313 (Control: n = 7; High Dose: n = 8).
314
315 2.6.2. Estrous cyclicity
316 From PND 60 to 75, 10 µL of saline solution was gently introduced into the rat vagina with an
317 automatic pipettor and the fluid aspired was placed on clean histological slides for analysis
318 under a light microscope. The estrous cycle is divided into four phases in the rat, which can be
319 identified by the observation of the most predominant cellular type on vaginal lavages:
320 proestrus (nucleated epithelial cells), estrus (anucleated cornified cells), metestrus (same
321 proportion of leukocytes, nucleated and cornified cells) and diestrus (leukocytes). The phase of

the estrous cycle observed was recorded and used to calculate the frequencies of the phases (number of days each phase was recorded/number of days evaluated), cycle length (number of days from the first day of a cycle phase to the first day of the next same phase) and number of cycles (number of complete cycles during evaluation) (E Silva et al., 2016).

2.6.3. Histological evaluation

Histopathology of the testis and epididymis of F1-males was done as described (section 2.5.1). We also evaluated the testicular maturation of F1-males, and the histopathology of ovaries and uteri of adult F1-females.

Fetal testis (GD 20)

A whole section of the fetal testis (n = 6/group) was evaluated regarding the general aspect of the seminiferous cords, and the following parameters were recorded: presence of acidophilic cells, vacuoles, and multinucleated germ cells. Sertoli cells and gonocytes were counted, and the Sertoli cell/gonocyte ratio was determined. The Leydig cells in the interstitium were evaluated for their general aspect and if they formed any clusters (Barlow and Foster, 2003; Borch et al., 2006).

Degree of maturation of the seminiferous epithelium (PND 42)

The degree of maturation on 100 cross-sections of seminiferous tubules was evaluated on the testis of 42-day old F1-male rats. Seminiferous tubules were assigned values (V) according to the abundance of mature germ cells in the epithelium (Toledo et al., 2011), with modifications, as follows: degree 1 (value = 1): spermatocytes I or II; degree 2 (value = 2): young spermatids with rounded nucleus; degree 3 (value = 4): spermatids in maturation phase, with ovoid or elongated nucleus; degree 4 (value = 6): few mature spermatids (step 19); degree 5 (value = 8):

347 average amount of mature spermatids (step 19); degree 6 (value = 10): abundance of mature
348 spermatids (step 19). The values assigned for each tubule were used to calculate an average
349 degree of maturation of the seminiferous epithelium: Average degree = (V1 + V2 +… + V99 +
350 V100)/100.
351
352 *Histopathology of the ovary and the uterus*
353 Ovarian follicles in distinct stages of follicular development and corpora lutea were counted,
354 and the morphological aspect of this organ was observed as described previously in the
355 literature (Barros et al., 2020; Dixon et al., 2014). The morphometry of the uterus was also
356 evaluated. For that, the height of the luminal epithelium, endometrium, and myometrium were
357 measured on the uterine sections as previously described by E Silva et al. (E Silva et al., 2016)
358 with modifications.
359
360 2.7. Statistical analysis
361 Shapiro-Wilk's test was used to determine if data were normally distributed.
362 Study 1: the data was analyzed by the parametric ANOVA test, followed by Tukey test, or by
363 the non-parametric Kruskal-Wallis' test, followed by Dunn test. Categorical variables derived
364 from the qualitative analysis were evaluated by the frequency/proportion of
365 occurrence/observation of the alterations in the experimental group. Then, the proportions were
366 analyzed by the Fisher Exact test.
367 Studies 2 and 3: the data was analyzed by the parametric Student's t test, or by the non-
368 parametric Mann-Whitney's test. Proportions were analyzed by the Fisher Exact test.
369 Differences were considered significant when $p \leq 0.05$. Statistical analyses were done using
370 GraphPad Prism program (version 8).
371

3. RESULTS

No general signs of toxicity or stress were observed. Body weight evolution, total weight gain, and organ weights (Table 1) were similar between experimental groups in both the first and the second studies, except for a trend in the decrease of the weight of the spleen of animals treated with the high dose in Study 2. Given this trend in the increase of the weight of the spleen in animals treated with the high dose, we decided to evaluate leukocyte counts. Although total leukocyte counts were not different between the groups, differential leukocyte counts evidenced a higher percentage of monocytes in the High Dose group than in the Control group as shown in Figure 2B.

Evaluation of testis (Table 2; Figure 3) and epididymis (Figure 3) in the second study showed no differences between experimental groups. Testes had well-arranged germ cells, and an intact interstitium in samples of both groups. Epididymis had intact ductular epithelium, with the expected cellular distributions along the duct, and intact interstitium. Though sperm motility was similar between experimental groups, sperm morphology and sperm counts were altered in animals treated with the high dose of PS-NP when compared to the Control group in Study 1 (Table 3). The presence of sperm cytoplasmic droplets, as well as the relative number of sperm in the cauda epididymis. Both parameters are influenced by sperm transit time in the epididymis, which is regulated by the contractility of smooth muscles present in male sexual organs (Kempinas et al., 1998; Trajano et al., 2023). Nevertheless, the contractile response of the vas deferens to norepinephrine and carbachol was similar between experimental groups (Figure 2C) in Study 2. All animals were sexually active, and there were no differences between the experimental groups on sexual behavior nor on the fertility test (Table 4). Finally, serum testosterone levels were similar between experimental groups (Figure 2A). In Study 3 we evaluated the intergenerational aspects of paternal PS-NP exposure. There were no differences in early developmental milestones (Table 5), as well as puberty onset (Table 6). Estrous

cyclicity, however, was altered in F1-females of the High Dose group: the frequency of proestrus decreased whereas estrus increased. This was not accompanied by morphological effects in the ovaries nor in the uterus (Table 6). Male testis development was also similar between experimental groups as observed in GD 20 (data not shown), PND 21, 42 and 90 (Table 2; Figure 3). Organ weights were not altered, except for the weight of the spleen which was reduced in adult females (Table 1).

4. DISCUSSION

Male factor infertility can be responsible for up to 70 percent of infertility cases, and environmental factors are important contributors to male infertility (Babakhanzadeh et al., 2020; Saradha and Mathur, 2006; Zegers-Hochschild et al., 2017). Exposure to microplastics may negatively affect sperm quality in men in China, showing a correlation between higher levels of microplastic exposure and reduced semen quality. (Zhang et al., 2024). Our work evaluated the effects of polystyrene nanoplastics on mature male Wistar rats' reproductive function and their offspring. Although no signs of general toxicity were observed, nor significant changes in body weight or most collected organ weights, there was a trend of decreased spleen weight, accompanied by an increase in monocyte percentage in blood samples. The weights and histology of the testis and epididymis remained consistent across experimental groups; however, the presence of sperm cytoplasmic droplets increased, along with a higher relative number of sperm in the cauda epididymis. The contractility of the vas deferens, sexual behavior, and fertility parameters were unaffected by the treatment. Regarding intergenerational effects, estrous cycle dysregulation was observed, along with an increase in spleen weight in adult females.

The weight of the spleen was reduced in adult males (Study 2; not significantly) and F1-females (Study 3), though the other sampled organs - brain, pituitary, thyroid, adrenals, testis,

epididymis, ovaries, uterus, ventral prostate, and seminal gland – had similar weights in experimental groups. Except for the weight of the ventral prostate (not collected), Lim et al. also observed no differences in the weight of the same organs we sampled in male rats (Lim et al., 2021). In addition, a study in male mice revealed that the weight of the spleen is reduced after exposure to PS-NP, indicating immune system damage (Jing et al., 2022). Spleen is essential to the normal functioning of the immune system by filtering blood and storing lymphocytes (Kuper et al., 2016). This organ is sensitive to polyethylene microplastics (PE-MP): organelles accumulate near the nucleus and mitochondria abnormally in the spleen of mice (Park et al., 2020). This is one evidence of the impact of micro- and nanoplastics on the immune system. Another one is the effect on white blood cells; the total count of leukocytes in the blood stream reduces, as well as the relative number of lymphocytes, whereas the relative number of neutrophils increases in the same study by Park et al. (Park et al., 2020). Our results differ from these; we observed no significant alteration in total leukocyte counts, and only the relative number of monocytes increased in blood samples of the PS-NP group. It is known that micro- and nanoplastics trigger immune response (Brun et al., 2018). The immune response can be divided into innate and adaptative; the first relies on the recruitment of phagocytes. Among phagocytes, monocytes have the highest internalization rate of nanoplastics (Arribas Arranz et al., 2024). We hypothesize that the exposure to polystyrene nanoplastics leads to an increase in the number of monocytes which phagocyte the foreign particles in the blood stream. Monocytes can also be drawn by proinflammatory cytokines to specific tissues, where they became macrophages. Testicular macrophages phagocyte microplastics of 1 and 5 μm; these microplastics were even found in Leydig cells (Lin et al., 2024).

Several studies have reported testicular damage upon exposure to micro- and nanoplastics: depletion and exfoliation of germ cells, vacuolization, oxidative stress, reduction in the expression of blood-testis-barrier related proteins, multinucleated gonocytes, and reduction in

testosterone levels are some of them (Ebrahim et al., 2024; Ilechukwu et al., 2022; Jin et al., 2021; Li et al., 2020). All these studies have begun the exposure period during puberty, which is a critical period in sexual development. On the other hand, our study proposed to investigate the effects of the exposure to PS-NP in the fully mature male reproductive system (Arellano et al., 2024) and in the testicular development of the male offspring. We did not observe any morphological damage in the testicular tissue of the treated animals, nor did we observe changes in testicular sperm counts. Age of exposure seems to be a critical factor for the number and extent of damage caused in the reproductive system by micro- and nanoplastics. The epididymis has been less studied than the testis in this matter. We show that the histology of the adult epididymis was not altered after the treatment. However, we did observe some changes that could be related to epididymal function in Study 1.

The presence of cytoplasmic droplets and the relative number of sperm in the cauda epididymis increased. Sperm cytoplasmic droplets are formed in the neck of the sperm during spermiogenesis, and they migrate along the midpiece of the flagellum during epididymal transit (Hermo et al., 2019). Sperm transit time in the epididymis is regulated by the contractility of smooth muscles which are modulated by molecules such as neurotransmitters (noradrenaline, acetylcholine) (Trajano et al., 2023). Altered sperm transit time can lead to altered sperm quality and sperm counts (Kempinas et al., 1998). Because of the increase in the presence of sperm cytoplasmic droplets and in the relative number of sperm in the cauda epididymis, we decided to investigate potential deleterious effects of PS-NP exposure on sympathetic response in the male reproductive system. The epididymal vas deferens was chosen for the analysis of the contractility of the smooth muscle present in male sexual organs (de Almeida Kiguti et al., 2020). Nevertheless, the contractile response of the vas deferens to norepinephrine and carbachol was similar between experimental groups. Finally, the increase in the relative number

of sperm in the cauda epididymis in the High Dose group could have also been influenced by the lower weight of the cauda epididymis in this group ($p = 0.085$).

Sperm acquire motility and fertilizing ability in the epididymis. Since sperm was ultimately not affected by PS-NP exposure in our work, it was expected that fertilizing rates would not be altered. No sexual behavior nor fertility parameters were impaired in animals treated with PS-NP, which is consistent with our other results. Additionally, serum testosterone levels remained unchanged. These results go against what has been recently observed by other research groups. Li et al. treated male Wistar rats (6 weeks-old) with 0.015, 0.15 and 1.50 mg/d of PS-NP (low, medium, and high dose, respectively; 500 nm) for 90 days and observed reduced sperm quality and counts in the high dose group (Li et al., 2021). The test particles and the concentrations used are similar in our work, but the age of the animals and exposure period differ. Sperm counts, sperm quality, and testosterone levels all reduce in male Wistar rats exposed to PS-NP (3 and 10 mg/kg/d; 25 nm) for 60 days. This leads to fertility impairment: less implantation sites and more gestational losses, and reduced size and weight of the fetuses are observed (Ebrahim et al., 2024). This time, our work relates to this in the exposure period but differs in other aspects. The first difference is the particle size; we used larger 500 nm PS-NP particles. Secondly, our work exposed the rats to around 0.375 and 3.75 mg/kg/d of PS-NP (Low Dose: 0.15 mg/d, High Dose: 1.50 mg/d). Lastly, the age of exposure. Though they mention that the animals are sexually mature at the beginning of exposure, the age of the animals is unknown. The informed weight (150 – 180 g) suggests that the rats are around 6 weeks-old (Ebrahim et al., 2024). A study in *Daphnia pulex* evidenced age-dependent impact of polystyrene nanoplastics (Liu et al., 2018). The same relation was observed in mice exposed to pristine polystyrene microplastics (Gaspar et al., 2023), which further corroborates the importance of age of exposure. Thus, we hypothesize that larger polystyrene nanoplastics cause less damage

in the male fully developed reproductive system, with milder consequences for health and fertility in adult animals.

Even though we have not observed any evident damage to male germ cells in our work, other studies have shown that microplastics may interfere with spermatocyte's gene expression (Jiang et al., 2024). This could lead to intergenerational effects. One example is the study published by Sun et al., in which male mice were treated with 2 mg/L of polyethylene nanoplastics (200 nm) for 35 days. The male offspring had impaired growth, along with alterations in sex hormone concentrations and histological changes in the testes (Sun et al., 2024). One of the potential mechanisms for transmitting damage across generations is epigenetic regulation. The association between nanoplastics and ocean acidification causes transgenerational reproductive impairment in copepods by differential methylation of specific genes, indicating epigenetic modifications (Lee et al., 2023). Although most studies on genetic and epigenetic inheritance focus on maternal transmission, the significance of paternal inheritance should not be underestimated, especially following nanoplastic exposure (Sun et al., 2023).

Early development is also affected when animals are directly exposed to nanoplastics during gestation and lactation, leading to deficits in neurobehavioral development (Chen et al., 2024). In the case of exclusively paternal exposure, we did not observe this impairment in the offspring. Puberty onset, another developmental parameter, was also similar between experimental groups. Although there were no differences in the onset of puberty, the estrous cycle of females in the High Dose group was altered between postnatal days 60 and 75. The frequency of the estrus phase increased, while the proestrus phase decreased. However, cycle duration and number remained unchanged. The cycle length was similar to the estimated 4.5-day duration for adult Wistar rats (Paccola et al., 2013), but the shift in phase frequencies indicates estrous cycle dysregulation. Recently, studies have investigated estrous cycle dysregulation caused by PS-NP exposure (Haddadi et al., 2022; Huang et al., 2023). In mice

520 treated with PS-NP (5 mg and 25 mg/kg) for eight weeks, the frequency of metestrus/diestrus
521 increased while estrus frequency decreased (Huang et al., 2023). This dysregulation may be
522 driven by changes in pituitary and gonadal hormones. For instance, estradiol, testosterone, and
523 luteinizing hormone (LH) levels are elevated in rats exposed to micro- and nanoplastics (Saeed
524 et al., 2023). Since an LH surge is required for ovulation and the onset of estrus (Chaffin and
525 Vandevoort, 2013), it is possible that an increase in this hormone led to early ovulation in the
526 High Dose group, causing a shortened proestrus phase and prolonged estrus. These findings
527 evidence intergenerational consequences of PS-NP exposure, especially in females.
528
529 5. CONCLUSIONS
530 Plastic contamination is an important issue, with nanoplastics being contaminants of emerging
531 concern. Reproductive impairment is one of the consequences of exposure to nanoplastics, but
532 the extent and severity of this impairment relays on multiple factors. Our findings provide
533 evidence of reproductive consequences, such as an increased number of sperm with cytoplasmic
534 droplets and a higher relative sperm count in the cauda epididymis, along with health impacts,
535 including a rise in the relative number of monocytes. Furthermore, we observed
536 intergenerational effects, notably estrous cycle dysregulation in F1 females. These results
537 underscore the importance of considering the potential hazardous and intergenerational effects
538 of prolonged environmental exposure to various types of plastics and their additives on health
539 and fertility. This concern is particularly relevant given that rats exhibit significantly higher
540 fertility rates than humans, meaning that even seemingly minor disruptions in rats could
541 translate into substantial consequences for human reproductive health. It is important to
542 acknowledge that the absence of direct data on PS-NP presence in tissues represents a limitation
543 of this study. The absence of this critical endpoint inherently impacts on the overall robustness
544 and mechanistic relevance of our study, suggesting an area for future investigation.

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Tables

Table 1. Body and organ weights of male Wistar rats treated for 60 days with PS-NP (500 nm) at different concentrations and their adult offspring.

Parameters	[1]Study 1 (n = 10; PND 150)			[2]Study 2 (PND 150)	
	Control	Low Dose	High Dose	Control (n = 10)	High Dose (n = 9)
Body weight (g)	442.5 ± 19.04	439.6 ± 16.05	446.5 ± 14.14	436.6 ± 10.03	445.7 ± 18.16
Brain (g)	1.97 ± 0.02	1.99 ± 0.10	1.94 ± 0.04	2.05 ± 0.02	2.09 ± 0.03
Pituitary (mg)	10.47 ± 0.45	10.06 ± 0.41	10.45 ± 0.60	11.23 ± 0.79	11.93 ± 0.49
Thyroid (mg)	12.31 ± 1.39	11.05 ± 0.91	13.49 ± 1.22	19.26 ± 1.80	20.26 ± 1.56
Adrenals (mg)	67.94 ± 7.21	66.69 ± 5.96	68.65 ± 5.65	68.26 ± 2.78	63.04 ± 2.33
Testis (g)	1.70 ± 0.04	1.72 ± 0.04	1.74 ± 0.05	1.80 ± 0.05	1.75 ± 0.06
Epididymis (g)	0.69 ± 0.02	0.68 ± 0.02	0.70 ± 0.01	0.71 ± 0.05	0.67 ± 0.02
Ventral prostate (g)	0.57 ± 0.03	0.66 ± 0.02	0.62 ± 0.04	0.46 ± 0.02	0.43 ± 0.02
Seminal gland (g)	1.45 ± 0.07	1.38 ± 0.07	1.40 ± 0.07	1.36 ± 0.11	1.41 ± 0.11
Seminal gland: parenchyma (g)	0.55 ± 0.02	0.55 ± 0.02	0.49 ± 0.01	0.62 ± 0.04	0.70 ± 0.04
Spleen (g)	N.C.	N.C.	N.C.	0.73 ± 0.04	0.62 ± 0.04 (p = 0.07)

	[2]Study 3: Adult offspring (F1)			
Parameters	Males (PND 90)		Females (PND 75 - 80)	
	Control (n = 7)	High Dose (n = 8)	Control (n = 6)	High Dose (n = 6)
Body weight (g)	432.60 ± 23.86	395.30 ± 9.97	220.20 ± 4.74	230.30 ± 9.54
Brain (g)	2.08 ± 0.03	1.99 ± 0.03	1.83 ± 0.02	1.79 ± 0.02
Pituitary (mg)	11.25 ± 0.76	10.19 ± 0.87	14.32 ± 0.49	13.36 ± 1.34
Thyroid (mg)	15.23 ± 1.29	17.49 ± 1.78	17.03 ± 1.84	16.50 ± 1.84
Adrenals (mg)	71.00 ± 3.41	74.49 ± 4.26	89.10 ± 4.60	87.64 ± 6.76
Testis (g) | Ovaries (mg)	1.75 ± 0.04	1.77 ± 0.06	83.87 ± 6.13	82.65 ± 2.01
Epididymis (g) | Uterus (mg)	0.67 ± 0.02	0.67 ± 0.02	424.20 ± 41.88	429.20 ± 39.07
Ventral prostate (g)	0.41 ± 0.05	0.40 ± 0.03		
Seminal gland (g)	1.03 ± 0.07	1.10 ± 0.06		
Seminal gland: parenchyma (g)	0.49 ± 0.05	0.49 ± 0.05		
Spleen (g)	0.75 ± 0.04	0.68 ± 0.03	0.53 ± 0.01	0.48 ± 0.02*

Values expressed as mean ± SEM. [1]One-way ANOVA. [2]Student's t test. *p<0.05. N.C. = Not collected.

Table 2. Histopathology and morphometry of the testis, and spermatogenesis dynamics.

Parameters	Study 2		Study 3					
	PND 150		PND 21		PND 42		PND 90	
	Control (n = 10)	High Dose (n = 9)	Control (n = 7)	High Dose (n = 6)	Control (n = 5)	High Dose (n = 6)	Control (n = 6/7)	High Dose (n = 8)
[1]Normal seminiferous tubules (%)	94.50 (91.75 - 95.00)	92.00 (91.50 - 94.00)	79.00 (78.00 - 81.00)	78.50 (72.50 - 83.50)	72.00 (70.00 - 78.00)	75.50 (71.50 - 80.75)	93.00 (90.50 - 93.25)	94.00 (93.00 - 95.00)
[1]Abnormal seminiferous tubules								
Acidophilic cells (%)	1.00 (1.00 - 2.25)	2.00 (0.00 - 2.50)	15.00 (13.00 - 16.00)	15.50 (12.25 - 24.75)	6.00 (3.00 - 8.50)	6.00 (3.50 - 12.25)	0.50 (0.00 - 1.25)	0.00 (0.00 - 1.00)
Vacuoles (%)	4.00 (3.50 - 5.50)	3.00 (2.50 - 5.50)	5.00 (4.00 - 5.00)	2.50 (1.00 - 8.00)	20.00 (18.00 - 21.50)	16.50 (13.25 - 18.75)	6.00 (5.00 - 7.00)	4.50 (3.25 - 6.00)
Exfoliation (%)	0.00 (0.00 - 0.00)	1.00 (0.00 - 1.00)	1.00 (0.00 - 1.00)	0.00 (0.00 - 3.00)	1.00 (0.00 - 1.50)	0.50 (0.00 - 1.25)	0.00 (0.00 - 1.00)	0.00 (0.00 - 0.00)
Degenerated seminiferous tubules (%)	0.00 (0.00 - 0.25)	0.00 (0.00 - 3.00)	0	0	0	0	0.50 (0.00 - 1.50)	0.00 (0.00 - 1.00)
[1]Maturation degree of the SE	NA	NA	NA	NA	5.70 ± 0.17	5.78 ± 0.11	NA	NA
[2]Morphometry								
Seminiferous tubule diameter (µm)	286.10 ± 6.72	292.30 ± 6.53	NA	NA	NA	NA	371.30 ± 6.78	369.50 ± 4.59
Epithelium thickness (µm)	93.77 ± 3.13	92.13 ± 3.01	NA	NA	NA	NA	118.20 ± 3.19	114.70 ± 2.28
Volume of Leydig cell nucleus (µm³)	67.38 ± 2.49	70.85 ± 4.15	NA	NA	NA	NA	80.70 ± 1.94	83.39 ± 3.11
[1]Spermatogenesis dynamics								
I-VI (%)	41.00 (40.00 - 45.25)	42.00 (41.50 - 45.50)	NA	NA	NA	NA	43.00 (41.75 - 45.25)	42.00 (40.25 - 43.00)
VII-VIII (%)	27.50 (25.50 - 30.00)	27.00 (25.50 - 29.50)	NA	NA	NA	NA	25.50 (24.75 - 26.25)	25.00 (24.25 - 28.75)
IX-XIII (%)	28.00 (26.25 - 30.25)	27.00 (24.00 - 29.00)	NA	NA	NA	NA	27.50 (25.75 - 30.50)	29.00 (26.50 - 30.00)
XIV (%)	2.00 (1.00 - 3.25)	4.00 (2.00 - 4.50)	NA	NA	NA	NA	3.50 (2.50 - 4.50)	3.50 (2.25 - 4.75)

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2]Values expressed as mean ± SEM. Student's test $p > 0.05$. SE = Seminiferous Epithelium. NA = Not Analyzed.

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Table 3. Sperm quality of male Wistar rats treated for 60 days with PS-NP (500 nm) at different concentrations (Study 1) and their offspring (Study 3).

Parameters	Study 1 (PND 150; n = 6)			Study 3 (PND 90)	
	Control	Low Dose	High Dose	Control (n = 7)	High Dose (n = 8)
[1]*Sperm motility (%)*				NA	NA
Type A	62.00 (59.00 - 70.00)	72.00 (52.00 - 77.00)	68.00 (59.00 - 73.00)		
Type B	22.00 (13.00 - 23.00)	17.00 (9.30 - 23.00)	22.00 (12.00 - 23.00)		
Type C	14.00 (6.00 - 17.00)	15.00 (11.00 - 22.00)	15.00 (9.80 - 18.00)		
[1]*Sperm morphology (%)*					
Normal sperm	98.00 (96.75 - 99.00)	97.50 (96.50 - 98.25)	97 (95.25 - 98.25)	97.00 (96.50 - 97.50)	95.00 (94.50 - 97.50)
Head abnormalities	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.25)	0.00 (0.00 - 0.25)	1.00 (0.00 -1.50)	0.00 (0.00 - 1.00)
Tail abnormalities	2.00 (1.00 - 3.25)	2.50 (1.00 - 3.50)	2.50 (1.75 - 4.75)	2.00 (1.50 - 3.00)	4.00 (2.50 - 4.00)
Sperm cytoplasmic droplet	58.00 (52.00 - 67.50) a	65.50 (60.75 - 66.25) a	74.00 (68.75 - 77.75) b	56.00 (53.00 - 66.00)	56.00 (49.00 - 65.50)
[2]*Spermatid counts - Testis*					
Spermatid number ($x10^6$)	216.95 ± 10.41	234.98 ± 8.04	214.5 ± 11.91	208.50 ± 23.20	218.40 ± 15.21
Relative spermatid number ($x10^6$/g)	144.08 ± 4.72	146.66 ± 4.87	138.58 ± 6.08	123.00 ± 13.42	128.30 ± 7.01
Daily sperm production ($x10^6$/d)	35.57 ± 1.71	38.52 ± 1.32	35.16 ± 1.95	34.19 ± 3.81	35.80 ± 2.49
Relative daily sperm production ($x10^6$/g/d)	23.62 ± 0.78	24.04 ± 0.80	22.72 ± 0.99	20.17 ± 2.20	21.03 ± 1.15
[2]*Sperm counts - Epididymis*					
Sperm number in caput / corpus ($x10^6$)	99.26 ± 4.59	107.50 ± 8.78	98.35 ± 5.40	112.10 ± 10.81	110.50 ± 4.54
Relative sperm number in caput / corpus ($x10^6$/g)	270.00 ± 15.20	270.00 ± 13.17	259.20 ± 7.59	313.20 ± 21.56	304.00 ± 8.35
Sperm number in cauda ($x10^6$)	246.10 ± 11.91	249.90 ± 11.98	264.20 ± 14.20	299.10 ± 8.51	292.00 ± 10.94
Cauda epididymis (mg)	384.60 ± 18.31	378.30 ± 14.94	328.00 ± 20.81 (p = 0.085)	313.50 ± 6.13	311.50 ± 13.27
Relative sperm number in cauda ($x10^6$/g)	642.20 ± 26.70 a	661.50 ± 25.20 a	811.90 ± 33.26 b	954.30 ± 20.72	943.40 ± 31.74
Sperm transit time in caput / corpus (d)	2.81 ± 0.15	2.78 ± 0.18	2.81 ± 0.12	3.29 ± 0.09	3.20 ± 0.16
Sperm transit time in cauda (d)	6.99 ± 0.47	6.51 ± 0.33	7.61 ± 0.51	9.34 ± 0.80	8.42 ± 0.62
Total sperm transit time (d)	9.81 ± 0.53	9.29 ± 0.31	10.42 ± 0.55	12.61 ± 0.80	11.90 ± 0.92

[1]Study 1: Values expressed as median and interquartile intervals. Kruskal-Wallis's test followed by Dunn's test. Different letters represent statistical difference ($p < 0.05$). Study 3: Values expressed as median and interquartile intervals. Mann Whitney's test ($p > 0.05$). [2]Study 1: Values expressed as mean ± SEM. One-way ANOVA followed by Dunnett's test. Different letters represent statistical difference ($p < 0.05$). Study 3: Values expressed as mean ± SEM. Student's t test ($p > 0.05$). NA = Not Analyzed.

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Table 4. Sexual behavior and fertility parameters of adult male Wistar rats treated for 60 days with 0 (control) or 1.50 mg/d of PS-NP (High Dose).

Parameters	Study 2	
	Control (n = 10)	**High Dose (n = 9)**
Sexual behavior (PND 150)		
Sexually active animals	10	9
[1]Latency to the first mount (s)	55.00 (19.25 - 355.80) (10)	47.00 (33.50 - 128.00) (9)
[1]Number of mounts until the first ejaculation	2.50 (0.00 - 9.75) (10)	1.00 (0.00 - 3.00) (9)
[1]Latency to the first intromission (s)	60.00 (29.50 - 437.50) (8)	130.00 (47.00 - 465.00) (7)
[1]Number of intromissions until the first ejaculation	8.00 (6.25 - 27.25) (8)	11.00 (7.00 - 15.00) (7)
[2]Frequency of ejaculations (%)	20.00 (2)	22.22 (2)
Fertility (GD 20)		
[2]Pregnancy rate (%)	100.00 (10/10)	88.89 (8/9)
[1]Fertility potential (%)	100.00 (90.42 - 100.00)	100.00 (85.95 - 100.00)
[1]Pre-implantation loss (%)	0.00 (0.00 - 9.58)	0.00 (0.00 - 14.05)
[1]Post-implantation loss (%)	10.10 (0.00 - 18.32)	7.18 (0.00 - 11.46)
[3]Body weight of the dams (g)	372.60 ± 11.42	358.60 ± 12.79
[3]Uterus weight with fetuses (g)	83.36 ± 4.24	83.08 ± 4.10
[3]Male fetus weight (g)	4.84 ± 0.14	4.79 ± 0.28
[3]Female fetus weight (g)	4.34 ± 0.20	4.46 ± 0.24
[3]Placental weight; males (g)	0.63 ± 0.01	0.61 ± 0.01
[3]Placental weight; females (g)	0.58 ± 0.02	0.63 ± 0.05

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2]Values expressed as percentage (Fisher's exact test). [3]Values expressed as mean ± SEM (Student's t-test). $p > 0.05$.

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Table 5. Developmental milestones of the offspring (F1) of male Wistar rats (F0) exposed to 1.50mg/d of PS-NP (500nm) for 60 days.

Parameters (days)	Study 3 (n = 7)	
	Control	**High Dose**
Males		
Weight (PND 1; g)	6.56 ± 0.11	7.29 ± 0.36 (p = 0.07)
Anogenital distance (PND 1; mm/g$^{1/3}$)	1.24 ± 0.07	1.27 ± 0.04
Pinnae detachment	3.11 ± 0.07	2.96 ± 0.30
Fur development	7.86 ± 0.26	7.43 ± 0.20
Incisor eruption	8.86 ± 0.26	8.67 ± 0.36
Eye opening	15.00 ± 0.18	14.61 ± 0.20
Surface righting reflex	2.46 ± 0.17	2.54 ± 0.19
Palmar grasping reflex	6.17 ± 0.30	6.00 ± 0.82
Cliff avoidance	4.94 ± 0.34	4.87 ± 0.29
Negative geotaxis	4.27 ± 0.31	4.01 ± 0.31
Females		
Weight (PND 1; g)	6.20 ± 0.09	6.76 ± 0.29 (p = 0.09)
Anogenital distance (PND 1; mm/g$^{1/3}$)	0.53 ± 0.05	0.51 ± 0.03
Pinnae detachment	3.11 ± 0.07	2.89 ± 0.29
Fur development	7.86 ± 0.26	7.43 ± 0.20
Incisor eruption	8.67 ± 0.36	8.71 ± 0.36
Eye opening	14.61 ± 0.20	14.57 ± 0.25
Surface righting reflex	2.88 ± 0.31	2.68 ± 0.18
Palmar grasping reflex	5.28 ± 0.60	5.12 ± 0.38
Cliff avoidance	5.00 ± 0.41	5.07 ± 0.25
Negative geotaxis	4.29 ± 0.47	4.63 ± 0.27

Values expressed as mean ± SEM (Student's t-test). $p > 0.05$.

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Table 6. Puberty onset of F1-offspring, estrous cyclicity of adult female Wistar rats from PND 60 to 75, ovarian structures and morphometry of the uterus on PND 75 - 80.

Parameters	Study 3	
	Control (n = 7)	**High Dose (n = 8)**
[1]*Puberty onset from PND 30 to 50*		
Males: preputial separation (d)	45.81 ± 0.51	45.69 ± 0.67
Females: vaginal opening (d)	35.21 ± 0.58	34.56 ± 0.71
Females: first estrus (d)	35.43 ± 0.57	34.63 ± 0.72
[2]*Frequency of estrous phases (%) from PND 60 - 75*		
Proestrus	30.00 (20.00 - 36.50)	20.00 (13.00 - 25.25)**
Estrus	27.00 (20.00 - 28.50)	30.00 (27.00 - 40.00)*
Metestrus	23.50 (13.00 - 27.00)	27.00 (13.00 - 33.00)
Diestrus	20.00 (18.25 - 28.50)	27.00 (14.75 - 31.50)
[1]Estrous cycle length (d)	4.16 ± 0.24	4.29 ± 0.23
[1]Number of estrous cycles	2.93 ± 0.20	2.75 ± 0.11
	Control (n = 5)	**High Dose (n = 5)**
[2]*Ovarian structures on PND 75 - 80 (%)*		
Primordial and primary follicles	19.00 (17.50 - 20.50)	25.00 (19.50 - 29.50)
Secondary follicles	20.00 (6.50 - 22.00)	10.00 (6.00 - 18.50)
Tertiary follicles	8.00 (2.50 - 12.50)	4.50 (0.00 - 11.50)
Atretic follicles	32.00 (29.50 - 33.50)	31.00 (19.25 - 39.00)
Corpora lutea	21.00 (17.00 - 38.00)	(25.00 - 19.75 - 32.75)
[1]*Morphometry of the uterus on PND 75 - 80 (µm)*		
Luminal epithelium	39.42 ± 2.59	43.33 ± 2.43
Endometrium	618.30 ± 34.27	584.70 ± 40.23
Myometrium	420.00 ± 23.94	395.00 ± 7.51

[1]Values expressed as mean ± SEM. Student's t-test. [2]Values expressed as median and interquartile intervals. Mann Whitney's test. *$p < 0.05$. **$p < 0.01$.

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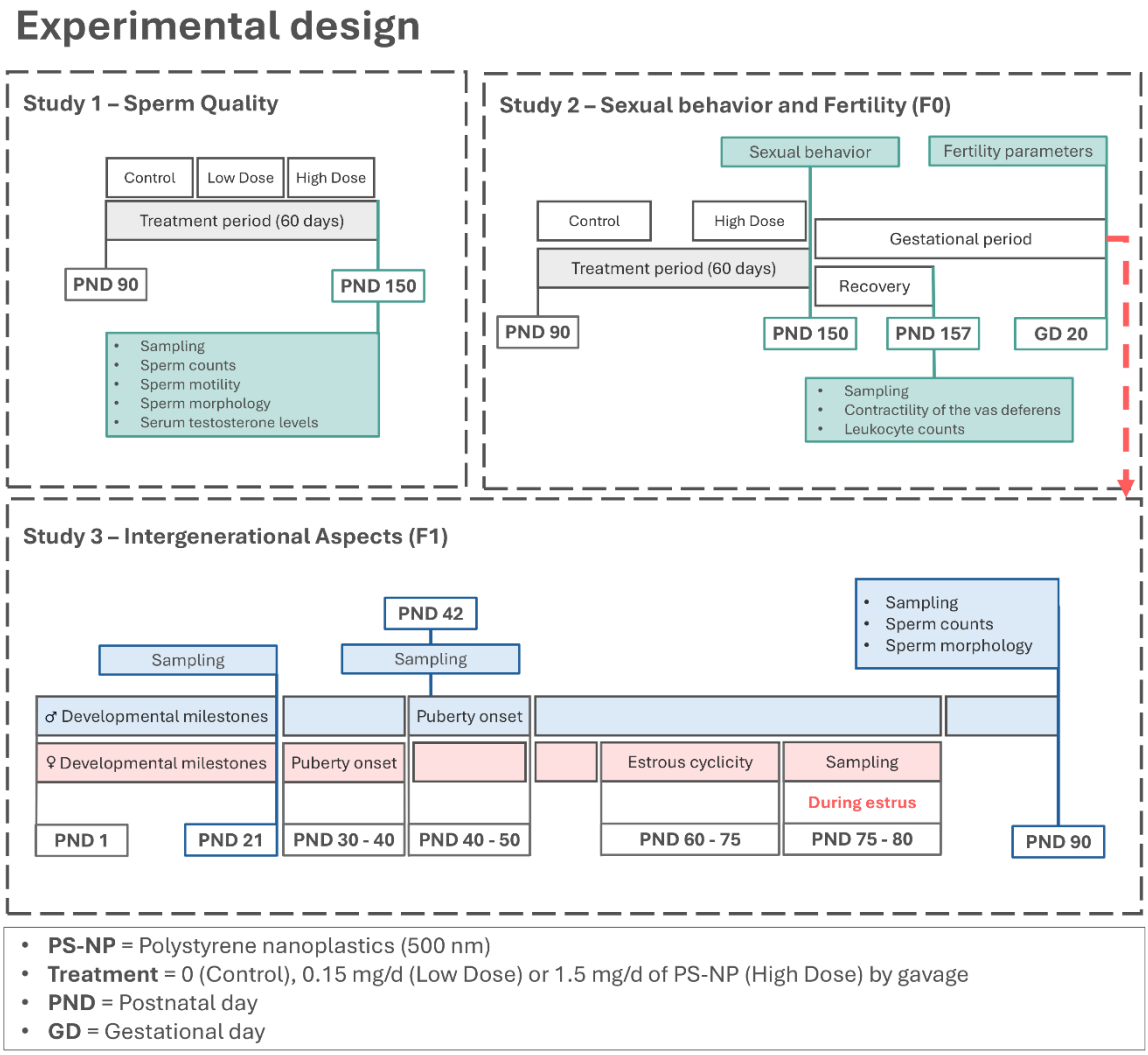
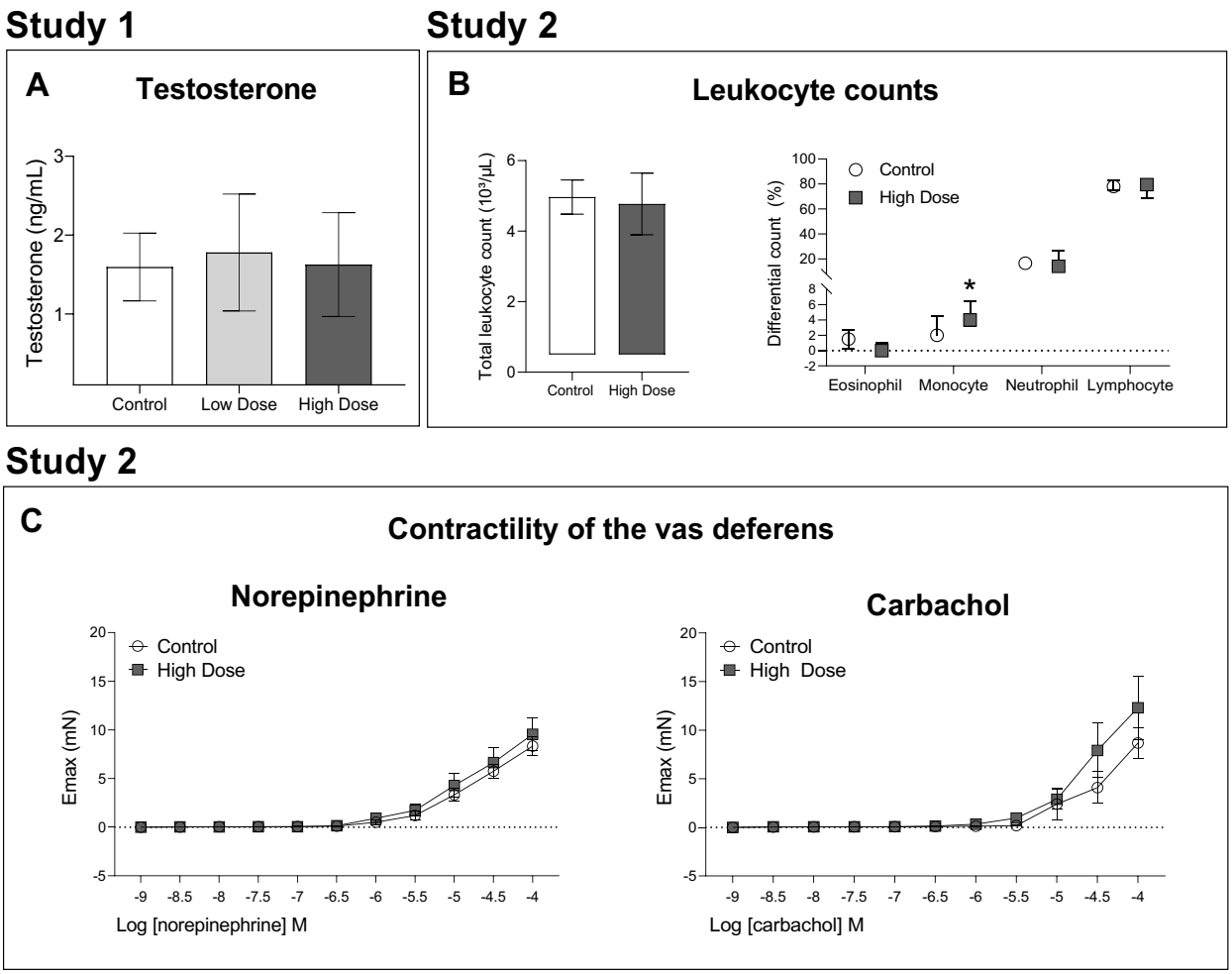
Figures

1.


Experimental design
Study 1 – Sperm Quality
Control
Low Dose
High Dose
Treatment period (60 days)
PND 90
PND 150
Sampling
Sperm counts
Sperm motility
Sperm morphology
Serum testosterone levels
Study 2 – Sexual behavior and Fertility (F0)
Sexual behavior
Fertility parameters
Control
High Dose
Gestational period
Treatment period (60 days)
Recovery
PND 90
PND 150
PND 157
GD 20
Sampling
Contractility of the vas deferens
Leukocyte counts
Study 3 – Intergenerational Aspects (F1)
PND 42
Sampling
Sampling
Sperm counts
Sperm morphology
♂ Developmental milestones
Puberty onset
♀ Developmental milestones
Puberty onset
Estrous cyclicity
Sampling
During estrus
PND 1
PND 21
PND 30 - 40
PND 40 - 50
PND 60 - 75
PND 75 - 80
PND 90
PS-NP = Polystyrene nanoplastics (500 nm)
Treatment = 0 (Control), 0.15 mg/d (Low Dose) or 1.5 mg/d of PS-NP (High Dose) by gavage
PND = Postnatal day
GD = Gestational day

781 2.


Study 1
Study 2
A
Testosterone
Testosterone (ng/mL)
Control
Low Dose
High Dose
B
Leukocyte counts
Total leukocyte count (10³/µL)
Control
High Dose
Differential count (%)
Control
High Dose
Eosinophil
Monocyte
Neutrophil
Lymphocyte
Study 2
C
Contractility of the vas deferens
Norepinephrine
Emax (mN)
Control
High Dose
Log [norepinephrine] M
Carbachol
Emax (mN)
Control
High Dose
Log [carbachol] M

782

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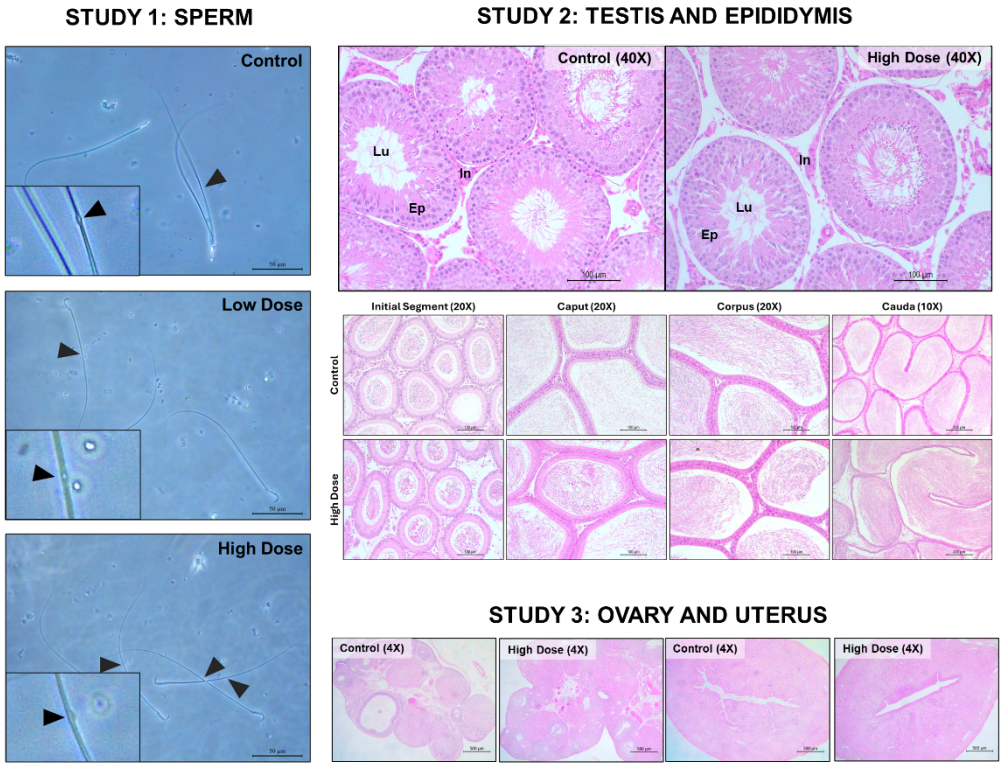
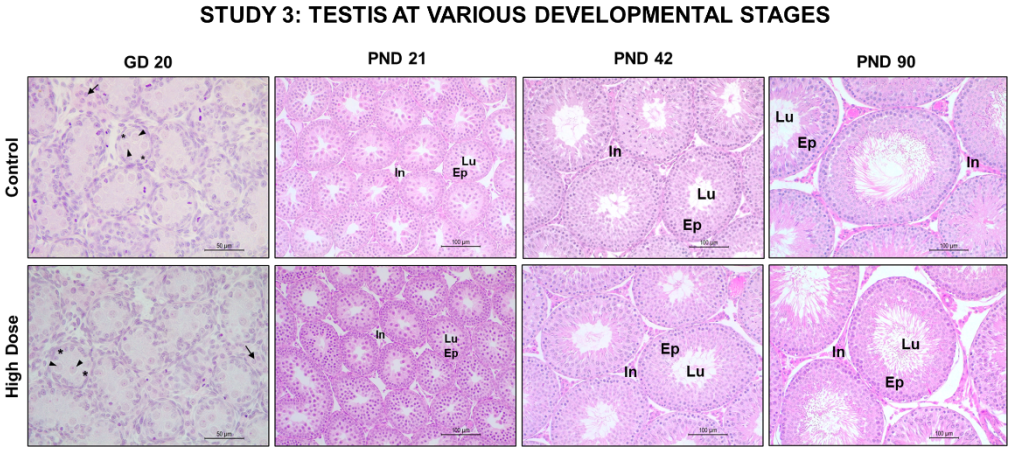

STUDY 1: SPERM
Control
Low Dose
High Dose
STUDY 2: TESTIS AND EPIDIDYMIS
Control (40X)
High Dose (40X)
Lu
In
Ep
Initial Segment (20X)
Caput (20X)
Corpus (20X)
Cauda (10X)
Control
High Dose
STUDY 3: OVARY AND UTERUS
Control (4X)
High Dose (4X)
Control (4X)
High Dose (4X)


STUDY 3: TESTIS AT VARIOUS DEVELOPMENTAL STAGES
GD 20
PND 21
PND 42
PND 90
Control
High Dose
In
Lu
Ep

784 3.

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Figure captions

Figure 1. Experimental design.

Figure 2. A: Serum testosterone levels (PND 150) of male Wistar rats treated for 60 days with PS-NP (500 nm) at different concentrations (n = 5/group). Values expressed as mean ± SEM. One-way ANOVA. $p > 0.05$. B: Total and differential leukocyte counts. Values expressed as mean ± SEM. Mann-Whitney's test. *$p < 0.05$. C: Vas deferens' *ex vivo* pharmacological response to norepinephrine and carbachol at different concentrations (10^{-9}M – 10^{-4}M). Vas deferens samples were collected 7 days after the end of treatment (PND 157). Mann-Whitney's test (n = 6/group).Values expressed as mean ± SEM. $p > 0.05$.

Figure 3. Study 1: Representative aspect of sperm samples of Control, Low Dose and High Dose groups on PND 150. Arrow head = sperm cytoplasmic droplet. Study 2: Representative aspect of the testis and epididymis of Control and High Dose groups on PND 157. Ep = seminiferous epithelium. In = Interstitium. Lu = lumen. Study 3: Representative aspect of the ovary and the uterus of F1-females of Control and High Dose groups on PND 75 – 80, and of the testis of F1-males at various developmental stages. Arrow = Leydig cell. Arrow head = gonocyte. Asterisk (*) = Sertoli cell. Ep = seminiferous epithelium. In = Interstitium. Lu = lumen.

Capítulo 3

Gestational Exposure to Polystyrene Nanoplastics: Mice Postnatal Development Outcomes

O terceiro capítulo teve como foco investigar o impacto de PS-NP (500nm) sobre o desenvolvimento pós-natal de camundongos híbridos C57BL/6xBALB/c. A partir dos resultados deste trabalho, um manuscrito foi elaborado: *"Gestational Exposure To Polystyrene Nanoplastics: Mice Postnatal Development Outcomes".* Este manuscrito foi submetido para a revista "Scientific Reports" (Fator de impacto: 3,9).

Gestational Exposure to Polystyrene Nanoplastics: Mice Postnatal Development Outcomes

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ABSTRACT

Nanoplastics (< 1 μm) are contaminants of emerging concern which are ubiquitously distributed. Humans are daily exposed to these particles, and they have even been found in placental tissue, raising concern regarding gestational exposure. Thus, the present work aimed to investigate the effects of gestational exposure to polystyrene nanoplastics (PS-NP) and postnatal development outcomes. Female C57BL/6 mice (n = 9) where mated with male BALB/c mice (n = 9) to obtain pregnant females. Dams were separated into two experimental groups: PS-NP (n = 4), which was treated by gavage with 0.15 mg/d of PS-NP (500 nm) diluted in distilled water (vehicle), and Control (n = 5), which was treated by gavage with distilled water between gestational days 5 and 19. The C57BL/6x BALB/c mice offspring was evaluated for developmental milestones between postnatal days (PND) 1 and 21. Males were euthanized on PND 42 and 70 to assess organ weights, testes and epididymides development, and sperm quality. In the PS-NP group, females had increased anogenital distance (AGD), while males showed a tendency toward reduced AGD and delayed cliff-avoidance reflex. On PND 42, male offspring had significantly lower brain and kidney weights. These findings highlight specific developmental and organ-weight changes following PS-NP exposure. These findings highlight that even low doses of PS-NP during pregnancy may affect early development and raises concerns regarding human health and fertility.

Keywords: nanoplastics, polystyrene, gestation, development, offspring, reproductive.

41 1. INTRODUCTION

42 Nanoplastics are small fragments of plastic (< 1μm) which are contaminants of emerging
43 concern [1,2]. These particles have recently drawn attention given their worldwide distribution
44 in innumerous ecosystems, though ocean pollution has been more extensively studied [3].
45 Micro- and nanoplastics (MNP) are persistent in the environment and can bioaccumulate [4],
46 damaging different living beings. For these reasons, the effects of micro- and nanoplastics on
47 health and fertility are being investigated in mammal animal models.

48 While bot rats and mice are experimental models which have advantages towards the study of
49 reproductive toxicology, mice seem to be more sensitive to MNP-exposure [5]. In male mice,
50 oral exposure to polystyrene (PS) microplastics results in reduced sperm production and quality,
51 decreased testosterone concentration, and an increase in the number of abnormal sperm [6,7].
52 These adverse effects are often related to blood-testis-barrier disruption [8]. In female mice,
53 MNP-exposure leads to a decrease in the number of growing ovarian follicles and an overall
54 reduction of the ovarian coefficient, findings which were related to oxidative stress and death
55 of granulosa cells [9,10]. Furthermore, exposure to polyethylene (PE) microplastics results in a
56 reduction of the number of live births in mice [11].

57 Although studies involving adult animals are crucial for understanding toxicological effects, in
58 the context of widespread environmental exposure to nanoplastics, it is imperative to assess
59 susceptibility during various stages of development. Pregnant women are routinely exposed
60 MNP, and studies have detected diverse microplastic particles in human placental tissue,
61 including both maternal and fetal compartments [12]. These particles may translocate across
62 the placental barrier, yet the potential risks to fetal development remain insufficiently
63 characterized [13]. Gestational exposure to nanoplastics in murine models has been associated
64 with alterations in the brain, liver, and testes [14,15]. Therefore, the present study aimed to

investigate potential postnatal developmental effects in mice prenatally exposed to polystyrene nanoplastics (PS-NP; 500 nm; 0.15 mg/d) during gestation.

2. MATERIAL AND METHODS

2.1. Polystyrene nanoplastics

Polystyrene nanoplastics (PS-NP; 500 nm) purchased from Alpha Nanotech Inc. (Vancouver, BC) were diluted in distilled water to obtain the experimental dose of 0.15 mg/d.

2.2. Choice of dose

The nanoplastic concentration selected for this study was based on estimated human exposure levels. Ingestion-related exposure estimates range from approximately 0.23 to 12 mg/kg/d for an average 60 kg individual [16]. To account for metabolic differences between humans and mice, a conversion factor of 12.3 [17] was applied, yielding an equivalent range of 2.83 to 147.6 mg/kg/d for a 20 g mouse. Thus, we opted for the dose of 0.15 mg/d (7.5 mg/kg/d), ensuring alignment with the lower end of daily levels of human estimated exposure through ingestion. This approach provides a relevant foundation for our experimental design.

2.3. Animals

Female C57BL/6 mice (n = 9) and male BALB/c mice (n = 9) (120 – 140 days old), obtained from CPPA-IBTEC/UNESP and previously used for mating in a prior project (CEUA 3597170322), were utilized in this study to obtain pregnant females. Hybrid C57BL/6xBALB/c offspring was evaluated for postnatal development. All animals were housed in polypropylene cages lined with wood shavings under controlled conditions (22°C, 30% humidity, 12/12h light/dark cycle). Experimental procedures were approved by the Ethics Committee on Animal

Use of Sao Paulo State University (CEUA 2930180822), conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), and following ARRIVE guidelines [18].

2.4. Experimental Design

Female C57BL/6 mice were mated with BALB/c males, and gestational day (GD) 1 was determined by the presence of a vaginal plug. Pregnant females were assigned to two experimental groups, ensuring no differences before the beginning of the experiment: a Control group, which received only distilled water (vehicle), and a group which received a daily dose of 0.15 mg of PS-NP diluted in distilled water by gavage from GD 5 to GD 19. In mice, implantation occurs at the end of GD 4 [19]. Whenever possible, hybrid C57BL/6xBALB/c litters were maintained with 8 pups per dam (4 males and 4 females) to ensure an equal distribution of food. Developmental milestones were evaluated in all pups from PND 1 to 21. Male offspring was euthanized on PND 42 and 70 for sampling.

2.4. Body and organ weights

Pregnant females were weighed weekly throughout gestation, and hybrid offspring were weighed on PND 1 and on sampling days. For organ collection, male C57BL/6x BALB/c mice were euthanized at 42 and 70 days of age (n = 1/litter; Control: n = 4/5; PS-NP: n = 4). Target organs for toxicological analysis were collected and weighed, including the brain, pituitary gland, heart, lungs, kidneys, adrenal glands, testes and epididymides. Testes and epididymides were collected for sperm quality assessment (PND 70) and histopathological analysis (PND 42 and 70; n = 1/litter; 4/group).

2.5. Developmental milestones

115 The developmental milestones of hybrid male and female offspring was assessed from PND 1
116 to 21, as previously described by other authors and briefly explained below [20,21].
117
118 *Physical Landmarks*
119 Pinnae detachment and fur development were monitored daily starting from PND 1; incisor
120 eruption was observed daily from PND 4; and eye opening was recorded from PND 10.
121
122 *Reflexes*
123 The following parameters were evaluated daily from PND 1 until three consecutive successful
124 results were observed.
125 Surface righting: Pups were given 30 s to turn over and place all four paws on the surface
126 (positive result).
127 Grasp reflex: The forepaw was gently touched with a paper clip, and a positive response was
128 recorded when the paw closed around it. The day this reflex disappeared was noted.
129 Negative geotaxis: Pups were placed on a 40° inclined platform facing downward and given 60
130 s to reposition themselves facing upward.
131 Cliff avoidance: Animals were positioned with their forepaws and nose extending over the edge
132 of a platform and given 60 s to retreat from the ledge.
133
134 *Anogenital distance*
135 Pups were weighed, and their anogenital distance (AGD) was measured on PND 1. AGD was
136 corrected for body weight as described by Gallavan et al. [22].
137
138 *Nipple number*

On PND 10, areolae were counted in both male and female pups. Females typically have five pairs of nipples, whereas males have none [23].

2.6. Sperm Quality

Sperm motility

The cauda epididymis was gently punctured to release sperm in 1 ml of PBS with BSA (10% BSA, g/ml PBS) at 34°C. After a 3-minute incubation, 10 µl were pipetted into a Makler chamber and 100 sperm were counted under a light microscope (400x magnification). Sperm were classified into three categories: sperm with progressive movement (Type A), sperm with non-progressive movement (Type B) and immotile sperm (Type C). Sperm concentration was determined and later incorporated into the epididymal cauda count.

Sperm morphology

A 100 µl sperm aliquot was fixed in 900 µl of formalin saline, and smears were prepared on histological slides. One hundred sperm per animal were analyzed under a phase contrast microscope (400x magnification) and classified as normal or abnormal, with defects categorized into head and tail abnormalities, as well as the presence or absence of a cytoplasmic droplet [24].

Sperm counts and transit time

Homogenization-resistant spermatids (stage 16 of spermiogenesis) were quantified as previously described by da Silva et al. [25]. Testes (n = 4/group) were decapsulated, weighed, and homogenized in 5 ml of 0.9% NaCl containing 0.5% Triton X-100, followed by sonication for 30 seconds. After a 10-fold dilution, samples were transferred to Neubauer chambers (four

fields per animal) for sperm counts. Daily sperm production (DSP) was calculated by dividing the total number of spermatids by 4.84, which represents their time in the seminiferous epithelium [26]. Relative DSP was obtained by normalizing daily sperm production to testis weight.

The epididymal caput/corpus and caudal regions were dissected, homogenized, and sonicated. Sperm quantification followed the same methodology as for testes. The caudal epididymal sperm reservoir was calculated by summing values from tissue homogenates and the suspension used for motility and morphology assessments. Sperm transit time was determined by dividing the sperm count in each region by DSP.

2.7. Histology

The testes and epididymis (n = 4/group) were fixed by immersion in mDF [27]. These samples were embedded in Paraplast, sectioned (5 μm), and stained with Hematoxylin and Eosin (HE).

Seminiferous epithelium maturation degree

Maturation was assessed in 100 transverse sections of seminiferous tubules from 42-day-old animals. Tubules were assigned a score (V) based on the abundance of the most mature germ cells present in the epithelium:

- Grade 1 (V = 1): Primary spermatocytes
- Grade 2 (V = 2): Secondary spermatocytes
- Grade 3 (V = 4): Round spermatids
- Grade 4 (V = 6): Oval or elongated spermatids
- Grade 5 (V = 8): Elongated spermatids with tuft-like epithelium
- Grade 6 (V = 10): Few mature spermatids (stage 16)
- Grade 7 (V = 12): Moderate number of mature spermatids (stage 16)

- Grade 8 (V = 14): Numerous mature spermatids (stage 16)

The designated values for each tubule were used to calculate the seminiferous epithelium maturation degree as follows: mean maturation degree = (V1 + V2 + … + V99 + V100) / 100 [28].

Adult testis

One hundred transverse sections of seminiferous tubules were analyzed by light microscopy, evaluating epithelial appearance, luminal content, interstitium, and potential morphological lesions classified according to specific toxicological study protocols [29,30]. Tubules were considered abnormal if the following features were observed: acidophilic cells, multinucleated cells, retained spermatids, degeneration of cell types, epithelial vacuolization, or exfoliation of cells into the lumen.

Testicular morphometry

Ten seminiferous tubule sections at stage IX of spermatogenesis (per animal) were used to measure seminiferous tubule diameter and epithelium thickness. Image analysis was performed using a Leica DMLB 12 microscope at 200x magnification, along with LAS V4.12 and ImageJ v1.48 software.

Spermatogenesis dynamics

This parameter was evaluated by recording the frequency of spermatogenesis stages:

- Stages I-VI: Presence of two generations of spermatids
- Stages VII-VIII: Presence of mature spermatids near the lumen
- Stages IX-XI: Presence of a single generation of spermatids
- Stage XII: Presence of secondary spermatocytes and meiotic figures

A total of 100 tubule sections per animal were analyzed [26].

Karyometry of Leydig cells

The nuclear volume of Leydig cells serves as an indicator of their secretory activity [31]. Therefore, karyometry of Leydig cells was performed on 50 nuclei, identified as circular or elliptical. Nuclear diameter was measured using ImageJ v1.48, and nuclear volume was determined accordingly.

Epididymis

The adult epididymis was qualitatively assessed using an optical microscope. The entire longitudinal section of the organ, including its segments (initial segment, head, body, and tail), was evaluated for epithelial structure and appearance, presence of spermatozoa in the ductal lumen, and interstitial characteristics [29,32].

2.8. Statistical Analysis

The Shapiro-Wilk test was used to determine whether the data followed a normal distribution. Student's t-test was used for comparisons of parametric variables. Non-parametric variables were compared using the Mann-Whitney test. Proportions were analyzed using Fisher's exact test. Results were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed using GraphPad Prism InStat software (version 8).

3. RESULTS

Daily monitoring revealed no signs of stress or discomfort in the animals. Female body weight remained consistent across groups during pregnancy and lactation, though a slight increase was noted in PS-NP–exposed females on gestational day 15 ($p = 0.088$; Figure 1).

Nine hybrid C57BL/6xBALB/c litters were produced. In the control group, one female delivered only a single male pup, and another produced three male pups and no females. All litters in the PS-NP group contained both males and females. Early developmental assessments showed increased anogenital distance (AGD) in PS-NP females and a tendency toward reduced AGD and delayed cliff-avoidance reflex in PS-NP males (Table 1). Body weights of male offspring (PND 42 and 70) were similar between groups. However, on PND 42, PS-NP–exposed males had significantly lower brain and kidney weights (Table 2). The pituitary gland, heart, lungs, and adrenals were not weighed in PND 42 males due to collection limitations at that age. Despite these findings, no differences were observed in sperm quality parameters (motility, morphology, counts; Table 3). Histological analysis of testes on PND 42 and 70 revealed no significant alterations in seminiferous epithelium maturation, morphology, or spermatogenesis dynamics between groups (Table 4; Figure 2).

4. DISCUSSION

The widespread presence of nanoplastics demands that we consider exposure across different life stages. Pregnant women are regularly exposed to MNPs, and various microplastics have already been identified in human placentas both in maternal and fetal compartments, and these particles have been reported to cross the placental barrier [12,13]. Given this context, the present work aimed to investigate potential postnatal developmental effects in mice exposed to PS-NP (500 nm; 0.15 mg/d) during gestation. There were no significant differences in body weight between females in both groups during pregnancy and lactation. However, on gestational day 15, a trend toward increased body weight was observed in the PS-NP group. This may be related to the impact of nanoplastics on metabolic parameters. It is theorized that micro- and nanoplastics (MNPs) may have obesogenic effects [33,34]. PS-NP exposure has

been associated with increased fat mass [35] and disrupted cholesterol metabolism [36], all suggesting metabolic disturbances following treatment.

The offspring's body weight, on the other hand, was not altered, though other parameters of early development were different. Anogenital distance (AGD) was increased in PS-NP females while a trend toward reduced AGD was observed in PS-NP males compared to controls. AGD is a sensitive marker of fetal androgenization; in males, it is typically twice that of females [37]. AGD may decrease in males and increase in females after exposure to anti-androgenic plasticizers [38]. MNPs exhibit anti-androgenic effects through reduced testosterone levels [39], which could explain the AGD alterations observed in our study. However, AGD measured on PND 35 and 70 was not affected in animals exposed to PS-NP pre- and postnatally, despite reduced serum testosterone levels at both time points [40].

Additionally, there was a trend in the delay of the development of the cliff-avoidance reflex in PS-NP males. Other developmental milestones were comparable across groups. This delay suggests potential neurobehavioral impairment. The brain is a known target of nanoplastics: maternal exposure to PS nanoplastics (50 nm) during gestation alters the metabolic profile of CD1 mouse offspring brains reducing GABA, creatine, and glucose levels on GD 17.5 [41]. Furthermore, exposure during gestation and lactation can cause hippocampal neuron apoptosis by PND 21 and long-term cognitive deficits (e.g., impaired memory and spatial learning at PND 45) [42]. Our findings support the hypothesis that PS-NP directly damages brain development.

On PND 42, brain and kidney weights were significantly reduced in PS-NP males compared to controls. Reduced brain weight may indicate impaired neurodevelopment, especially since only males showed delayed cliff-avoidance reflexes. Although the trend of reduced brain weight persisted by PND 70, it was not statistically significant. MNPs are known to bioaccumulate and damage the kidneys, causing reduced weight, necrosis, and epithelial detachment [43].

Similarly, kidney weight remained slightly lower in adult PS-NP–exposed mice, albeit without statistical significance.

Sperm motility, morphology, and counts were analyzed as indicators of sperm quality. None of these parameters were altered in PS-NP–exposed males compared to controls. In mice, sperm quality can be impaired by reduced epididymal viability, motility, and counts after prenatal and postnatal exposure to polystyrene MNPs [40,44]. The absence of significant findings in our study may be attributed to the lower daily dose used [40] and the smaller particle size (500 nm vs. 2 μm in previous studies) [44].

Testes from male mice were evaluated on PND 42 for seminiferous epithelium maturation and histopathology, and no significant differences were observed between groups. Although we did not detect histological alterations, we cannot rule out molecular-level changes. Pre- and postnatal PS-NP exposure is known to downregulate genes critical to testicular development, including those related to urogenital formation, histone methylation, hormone biosynthesis regulation, and hormone-responsive pathways. This can lead to reduced testis weight and disorganization of the seminiferous epithelium [14,40]. However, gestational-only exposure may cause milder effects in male offspring.

Similarly, no differences were found in testicular histopathology, morphometry, or spermatogenesis dynamics in adult males at PND 70. This aligns with our observations at PND 42 and with the absence of sperm quality impairments at PND 70. Despite this, the literature highlights the testis as a target organ for nanoplastics. The disruption of the blood-testis barrier appears to be the main mechanism of MNP-induced testicular damage, reducing junction protein expression. This exposes germ cells, leading to seminiferous epithelium disorganization, increased apoptosis, vacuolization, and germ cell exfoliation [45]. In our experimental conditions, no structural damage to the blood-testis barrier or seminiferous epithelium was observed.

5. CONCLUSIONS

Gestational exposure to polystyrene nanoplastics (PS-NP; 500 nm; 0.15 mg/d) caused developmental effects in mice such as changes in anogenital distance, reduction of brain and kidney weights, and a trend in delayed development of cliff avoidance reflex. These findings highlight that even low doses of PS-NP during pregnancy may affect early development and raises concerns regarding human health and fertility.

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459 **Tables**

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Table 1. Developmental milestones of mice exposed to 0.15 mg/d of PS-NP from gestational day 5 to 19.

Parameters	Experimental groups	
	Control (n = 5)	PS-NP (n = 4)
Males		
Body weight (PND 1; g)	1.63 ± 0.09	1.61 ± 0.02
Anogenital distance (PND 1; mm/g$^{1/3}$)	1.34 ± 0.07	1.15 ± 0.05 (p = 0.07)
Nipple number	0,00	0,00
Pinnae detachment (d)	3.97 ± 0.32	4.00 ± 0.00
Fur development (d)	7.20 ± 0.45	7.00 ± 0.00
Incisor eruption (d)	10.80 ± 0.20	11.00 ± 0.00
Eye opening (d)	13.87 ± 0.14	14.00 ± 0.00
Surface righting (d)	4.21 ± 0.18	4.57 ± 0.64
Cliff avoidance (d)	4.10 ± 0.41	5.17 ± 0.13 (p = 0.06)
Grasp reflex (d)	15.53 ± 0.31	15.66 ± 0.15
Negative geotaxis (d)	2.83 ± 0.38	2.38 ± 0.32
Females		
Body weight (PND 1; g)	1.66 ± 0.16 (3)	1.52 ± 0.02
Anogenital distance (PND 1; mm/g$^{1/3}$)	0.80 ± 0.03 (3)	0.94 ± 0.04*
Nipple number	10,00	10,00
Pinnae detachment (d)	4.00 ± 0.00 (3)	4.04 ± 0.17
Fur development (d)	7.00 (3)	7,00
Incisor eruption (d)	11.00 ± 0.00 (3)	10.94 ± 0.06
Eye opening (d)	13.83 ± 0.17 (3)	14.06 ± 0.06
Surface righting (d)	4.75 ± 0.12 (3)	4.92 ± 0.55
Cliff avoidance (d)	4.08 ± 0.74 (3)	4.73 ± 0.51
Grasp reflex (d)	15.33 ± 0.33	15.52 ± 0.26
Negative geotaxis (d)	2.75 ± 0.63 (3)	2.98 ± 0.19

Values expressed as mean ± SEM (Student's t test). *p < 0.05.

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Table 2. Body and organ weights of male mice (PND 42 and 70) exposed to 0.15 mg/d of PS-NP from GD 5 to 19.

Parameters	PND 42		PND 70	
	Control (n = 5)	PS-NP (n = 4)	Control (n = 4)	PS-NP (n = 4)
Body weight (g)	23.58 ± 0.67	22.74 ± 0.51	26.16 ± 1.17	26.48 ± 0.33
Brain (mg)	430.40 ± 4.49	408.70 ± 6.08*	450.30 ± 2.29	438.00 ± 3.53
Pituitary (mg)			1.05 ± 0.17	1.63 ± 0.31
Heart (mg)			178.20 ± 6.14	177.10 ± 8.03
Lungs (mg)			157.50 ± 2.52	161.10 ± 7.38
Liver (mg)	1561.00 ± 51.05	1423.00 ± 52.09	1453.00 ± 55.37	1419.00 ± 18.04
Kidneys (mg)	397.40 ± 12.15	348.70 ± 5.31*	464.00 ± 19.30	449.30 ± 5.92
Adrenals (mg)			5.83 ± 0.27	6.95 ± 1.20
Testis (mg)	77.92 ± 2.33	75.88 ± 2.19	100.60 ± 1.62	99.85 ± 1.16
Epididymis (mg)	21.20 ± 1.21	21.00 ± 0.69	37.47 ± 2.92	32.83 ± 1.55

Values expressed as mean ± SEM. Student's t test. *$p < 0.05$.

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Table 3. Sperm quality.

Parameters	Experimental groups (n = 4)	
	Control	PS-NP
[1]*Sperm motility*		
Type A	60.00 (51.50 - 74.50)	68.00 (63.00 - 75.00)
Type B	7.00 (1.25 - 10.50)	5.00 (1.00 - 11.25)
Type C	32.00 (23.25 - 40.00)	21.00 (24.00 - 26.00)
[1]*Sperm morphology*		
Normal sperm	68.00 (62.00 - 78.00)	70.00 (66.50 - 78.00)
Head abnormalities	6.00 (2.00 - 10.00)	3.00 (2.00 - 5.50)
Tail abnormalities	28.00 (16.00 - 30.00)	26.00 (17.50 - 31.50)
Sperm cytoplasmic droplet	46.00 (46.00 - 62.00)	53.00 (45.00 - 59.50)
[2]*Sperm counts*		
Testis		
Spermatid number ($x10^6$)	12.42 ± 1.19	13.70 ± 1.10
Relative spermatid number ($x10^6$/g)	123.10 ± 10.41	137.60 ± 12.11
Daily sperm production ($x10^6$/d)	2.57 ± 0.24	2.83 ± 0.23
Relative daily sperm production ($x10^6$/g/d)	25.42 ± 2.15	28.43 ± 2.50
Epididymis		
Sperm number in caput / corpus ($x10^6$)	5.41 ± 1.36	5.69 ± 0.99
Relative sperm number in caput / corpus ($x10^6$/g)	248.40 ± 69.44	317.50 ± 83.56
Sperm number in cauda ($x10^6$)	9.04 ± 1.96	10.19 ± 1.64
Relative sperm number in cauda ($x10^6$/g)	685.60 ± 134.80	761.50 ± 164.30
Sperm transit time in caput / corpus (d)	2.33 ± 0.83	2.07 ± 0.45
Sperm transit time in cauda (d)	3.20 ± 0.50	3.66 ± 0.68
Total sperm transit time (d)	4.73 ± 0.29	5.73 ± 1.09

[1]Values expressed as median and interquartile intervals. Mann-Whitney's test. [2]Values expressed as mean ± SEM. Student's t test. $p > 0.05$.

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Table 4. Histology, maturation degree and spermatogenesis dynamics.

Parameters	PND 42		PND 70	
	Control (n = 5)	**PS-NP (n = 4)**	**Control (n = 4)**	**PS-NP (n = 4)**
[1]Normal seminiferous tubules (%)	71.00 (66.00 - 77.50)	73.00 (67.25 - 75.75)	78.50 (76.00 - 83.25)	80.00 (71.75 - 81.50)
Abnormal seminiferous tubules				
[1]Acidophilic cells (%)	12.00 (9.00 - 20.50)	14.50 (12.50 - 15.75)	7.50 (5.50 - 8.00)	6.00 (5.25 - 13.50)
[1]Vacuoles (%)	12.00 (11.00 - 15.50)	13.50 (9.50 - 16.75)	12.50 (9.50 - 14.00)	13.50 (10.00 - 14.00)
[1]Exfoliation (%)	1.00 (0.00 - 2.00)	0.00 (0.00 - 0.75)	0.50 (0.00 - 1.75)	1.00 (0.25 - 1.00)
[1]Degenerated seminiferous tubules (%)	0	0	0.00 (0.00 - 3.75)	0.50 (0.00 - 1.75)
[2]*Maturation degree of SE*	7.64 ± 0.15	7.80 ± 0.11		
[2]*Morphometry*				
Seminiferous tubule diameter (µm)			266.50 ± 5.82	260.80 ± 11.28
Epithelium height (µm)			84.41 ± 2.31	78.90 ± 2.82
Volume of Leydig cell nucleus (µm³)			89.46 ± 3.89	87.96 ± 4.86
Spermatogenesis dynamics				
[1]I-VI (%)			45.00 (43.00 - 47.75)	38.50 (38.00 - 42.00)
[1]VII-VIII (%)			25.50 (24.25 - 28.25)	29.50 (26.75 - 30.00)
[1]IX-XI (%)			26.00 (23.50 - 29.25)	29.00 (27.50 - 27.75)
[1]XII (%)			2.50 (1.25 - 3.75)	3.00 (1.25 - 4.75)

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2]Values expressed as mean ± SEM. Student's test $p > 0.05$. SE = seminiferous epithelium.

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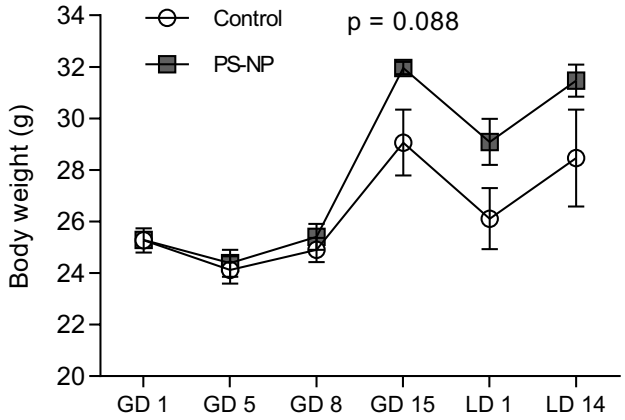
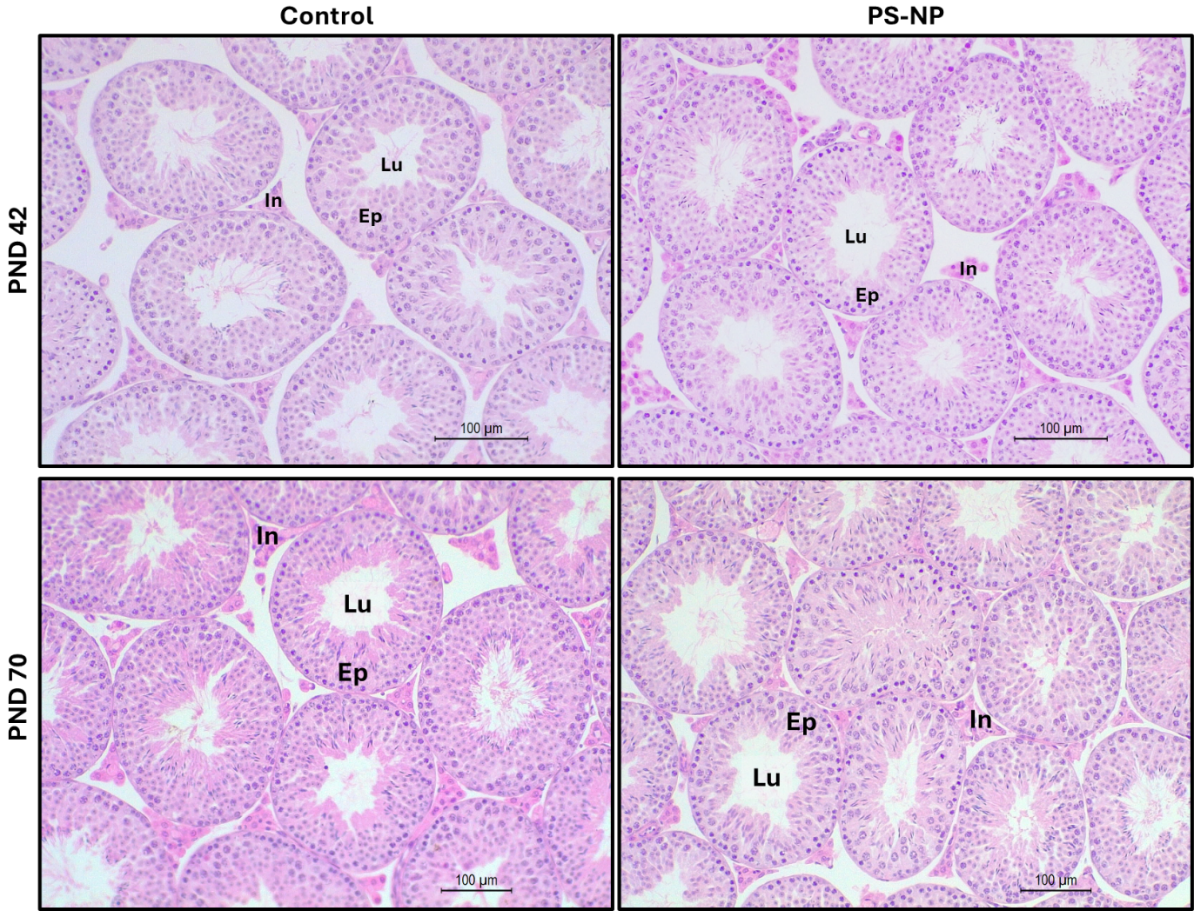
Figures

1.


Control
PS-NP
p = 0.088
Body weight (g)
34
32
30
28
26
24
22
20
GD 1
GD 5
GD 8
GD 15
LD 1
LD 14

2.


Control
PS-NP
PND 42
PND 70
Lu
In
Ep
100 µm

478 3.

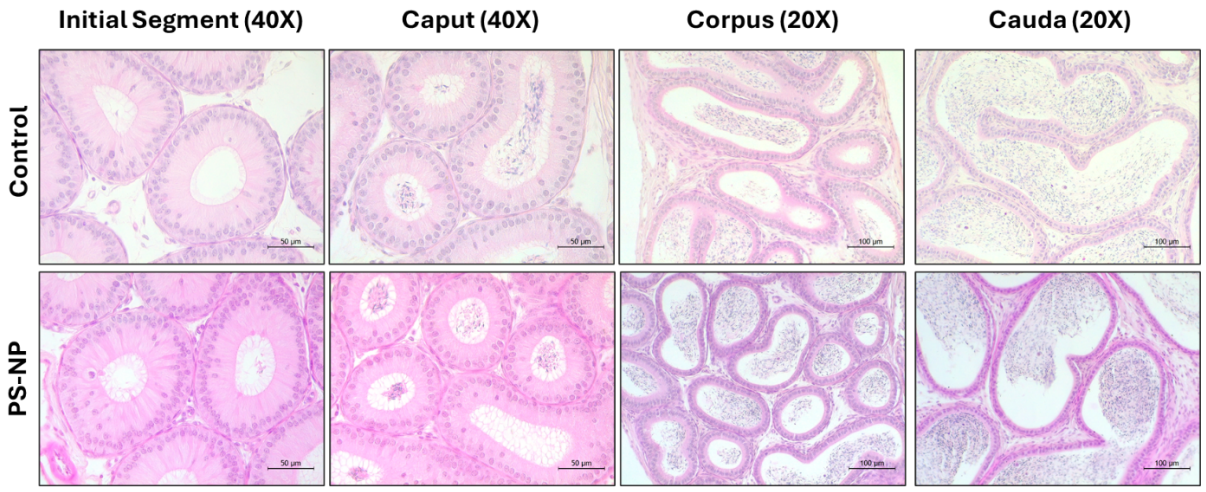

Initial Segment (40X)
Caput (40X)
Corpus (20X)
Cauda (20X)
Control
PS-NP

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Figure captions

1. Body weight of C57BL/6 female mice during gestation (GD) and lactation (LD). A trend toward increased body weight was observed in PS-NP–exposed mice on GD 15 ($p = 0.088$). No significant differences were found on the other evaluated days. Data presented as mean ± EPM. Student's t-test. $p > 0.05$.

2. Representative aspect of the testes of male mice on PND 42 and 70. In = interstitium. Lu = lumen. Ep = Seminiferous epithelium.

3. Representative aspect of the epididymides of male mice on PND 42.

Considerações finais

Os resultados obtidos nas condições experimentais avaliadas demonstram que a exposição a nanoplásticos de poliestireno (PS-NP) pode gerar consequências intergeracionais significativas em roedores, com as fêmeas apresentando maior susceptibilidade aos efeitos observados. Foi evidenciado que os PS-NP promovem alterações no sistema reprodutivo de ambos os sexos, afetando parâmetros hormonais, morfológicos e funcionais. A exposição gestacional resultou em modificações no desenvolvimento sexual da prole, indicando potencial para efeitos intergeracionais.

Os achados revelam que os impactos dos nanoplásticos no sistema genital são dependentes de múltiplos fatores, incluindo concentração, tempo de exposição, idade, gênero e espécie. Adicionalmente, foi constatado que diversos órgãos além do sistema reprodutivo podem ser afetados pela exposição aos nanoplásticos.

Em conjunto, os dados suportam a classificação dos nanoplásticos de poliestireno como desreguladores endócrinos capazes de comprometer a saúde e fertilidade em modelos animais. Considerando que os roedores apresentam eficiência reprodutiva superior à humana, estes achados reforçam a necessidade de atenção aos potenciais riscos que a exposição a nanoplásticos pode representar para a saúde e fertilidade humanas.

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Apêndices

Nos apêndices a seguir constam os certificados de aprovação pelo Comitê de Ética no Uso de Animais da Universidade Estadual Paulista para a realização dos experimentos descritos neste trabalho. Os apêndices correspondem:

1. CEUA 4936170322: Capítulo 1, correspondente aos experimentos com ratas Wistar fêmeas adultas.
2. CEUA 1848200721: Capítulo 2, correspondente aos experimentos com ratos Wistar machos adultos e aspectos intergeracionais.
3. CEUA 2930180822: Capítulo 3, correspondente aos experimentos com camundongas C57BL/6 e desenvolvimento pós-natal da prole híbrida C57BL/6x BALB/c.

689 **APÊNDICE 1**

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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Comissão de Ética no Uso de Animais
Instituto de Biociências de Botucatu

CERTIFICADO

Certificamos que a proposta intitulada "Efeitos da exposição a nanoesferas de poliestireno em doses ambientalmente relevantes sobre os aspectos reprodutivos de ratas Wistar", protocolada sob o CEUA nº 4936170322 (ID 000352), sob a responsabilidade de **Wilma de Grava Kempinas** *e equipe; Luan Reis Calixto; Lethícia Valencise* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais do Universidade Estadual Paulista (IBB/UNESP) na reunião de 29/04/2022.

We certify that the proposal "Effects of environmentally relevant doses of polystyrene nanospheres on female reproduction in Wistar rats", utilizing 23 Heterogenics rats (males or females), protocol number CEUA 4936170322 (ID 000352), under the responsibility of **Wilma de Grava Kempinas** *and team; Luan Reis Calixto; Lethícia Valencise* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the São Paulo State University (IBB/UNESP) in the meeting of 04/29/2022.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 05/2022 a 12/2022 Área: Ciências Biológicas

Origem:	Biotério Central da UNESP						
Espécie:	Ratos heterogênicos	sexo:	Machos ou Fêmeas	idade:	75 a 180 dias	N:	6
Linhagem:	Wistar			Peso:	140 a 400 g		
Origem:	Animais provenientes de outros projetos						
Espécie:	Ratos heterogênicos	sexo:	Fêmeas	idade:	75 a 180 dias	N:	17
Linhagem:	Wistar			Peso:	140 a 400 g		

Local do experimento: Os procedimentos e análises serão realizados no Depto. de Biologia Estrutural e Funcional (Setor de Morfologia), do Instituto de Biociências de Botucatu (IBB - UNESP).

Botucatu, 01 de maio de 2022

Profa. Dra. Ana Carolina Inhasz Kiss
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista

Prof. Assoc. Luis Fernando Barbisan
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista

Rua Prof. Dr. Antônio Celso Wagner Zanin, 250 - Distrito de Rubião Junior - Botucatu/SP - CEP 18618-689 - tel: 55 (14) 3880-0851
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CEUA N 4936170322

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692 **APÊNDICE 2**

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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Comissão de Ética no Uso de Animais
Instituto de Biociências de Botucatu

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação in vivo, in vitro e in silico de parâmetros de fertilidade de ratos Wistar machos expostos a nanopartículas de poliestireno", protocolada sob o CEUA nº 1848200721 (ID 000259), sob a responsabilidade de **Wilma de Grava Kempinas** *e equipe; Lethícia Valencise* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais do Universidade Estadual Paulista (IBB/UNESP) na reunião de 27/08/2021.

We certify that the proposal "In vivo, in vitro and in silico evaluation of fertility parameters of male Wistar rats exposed to polystyrene nanoparticles", utilizing 200 Heterogenics rats (males or females), protocol number CEUA 1848200721 (ID 000259), under the responsibility of **Wilma de Grava Kempinas** *and team; Lethícia Valencise* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the São Paulo State University (IBB/UNESP) in the meeting of 08/27/2021.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 09/2021 a 09/2023 Área: Ciências Biológicas

Origem:	Biotério Central da UNESP						
Espécie:	Ratos heterogênicos	sexo:	Machos ou Fêmeas	idade:	1 a 160 dias	N:	200
Linhagem:	Wistar			Peso:	5 a 500 g		

Local do experimento: Os procedimentos e análises serão realizados no Depto. de Biologia Estrutural e Funcional (Setor de Morfologia), do Instituto de Biociências de Botucatu (IBB - UNESP).

Botucatu, 13 de outubro de 2021

Profa. Dra. Ana Carolina Inhasz Kiss
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista

Prof. Assoc. Luis Fernando Barbisan
Vice-Coordenador da Comissão de Ética no Uso de Animais
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694 **APÊNDICE 3**

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Universidade Estadual Paulista/Instituto de Biociências

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Exposição gestacional a nanoesferas de poliestireno: impacto no desenvolvimento pós-natal de camundongos", protocolada sob o CEUA nº 2930180822 (ID 000442), sob a responsabilidade de **Wilma de Grava Kempinas** *e equipe; Lethícia Valencise* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Estadual Paulista/Instituto de Biociências (IBB/UNESP) na reunião de 04/10/2022.

We certify that the proposal "Gestational exposure to polystyrene nanospheres: outcomes in postnatal development in mice", utilizing 40 Isogenics mice (20 males and 20 females), 160 Heterogenics mice (males or females), protocol number CEUA 2930180822 (ID 000442), under the responsibility of **Wilma de Grava Kempinas** *and team; Lethícia Valencise* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the São Paulo State University/Biosciences Institute (IBB/UNESP) in the meeting of 10/04/2022.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 10/2022 a 10/2023 **Área:** Ciências Biológicas

Origem:	Animais provenientes de outros projetos (Provenientes do CPPA - IBTEC/UNESP e remanescentes de outro experimento aprovado pelo CEUA 3597170322)						
Espécie:	Camundongos isogênicos	**sexo:**	Fêmeas	**idade:**	120 a 200 dias	**N:**	20
Linhagem:	C57BL/6			**Peso:**	20 a 40 g		
Origem:	Animais provenientes de outros projetos (Provenientes do CPPA - IBTEC/UNESP e remanescentes de outro experimento aprovado pelo CEUA 3597170322)						
Espécie:	Camundongos isogênicos	**sexo:**	Machos	**idade:**	120 a 130 dias	**N:**	20
Linhagem:	BALB/c			**Peso:**	20 a 40 g		
Origem:	Animais provenientes de outros projetos (Geração F1 obtida após acasalamento entre os animais provenientes de outro projeto)						
Espécie:	Camundongos heterogênicos	**sexo:**	Machos ou Fêmeas	**idade:**	1 a 80 dias	**N:**	160
Linhagem:	Híbridos C57BL/6 / BALB/c			**Peso:**	3 a 40 g		

Botucatu, 04 de outubro de 2022

Profa. Dra. Ana Carolina Inhasz Kiss
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista/Instituto de Biociências

Prof. Assoc. Luis Fernando Barbisan
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