Theriogenology 244 (2025) 117482

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

Effects of polystyrene nanoparticles on bovine oocyte in vitro maturation

Merlo Barbara [a,b,*], Alessandro Marino Volsa [c], Laura Tovar [a], Margherita Gaiani [a], Penelope Maria Gugole [a], Emilia Attolini [a], Iacono Eleonora [a,b]

[a] Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, Ozzano Emilia, 40064, Bologna, Italy
[b] Health Science and Technologies Interdepartmental Center for Industrial Research (CIRI-SDV), University of Bologna, Bologna, Italy
[c] Department of Physiology, International Excellence Campus for Higher Education and Research (Campus Mare Nostrum), University of Murcia, C. Campus Universitario 7, 30100, Murcia, Spain

ARTICLE INFO

Keywords:
Nanoplastic
Polystyrene
Bovine
Oocyte
Maturation
Fertilization

ABSTRACT

Polystyrene nanoparticles (PS-NPs) have emerged as a significant environmental concern due to their widespread presence and potential toxicity. This study investigates the effects of PS-NPs on bovine oocyte in vitro maturation (IVM) to assess their impact on reproductive health. During IVM, oocytes were exposed to varying concentrations of 100 nm PS-NPs (0, 5, 50, 100, and 200 μg/mL) or of fluorescent 30 nm PS-NPs (0, 5, 35, and 70 μg/mL). Higher PS-NPs concentrations (100 and 200 μg/mL for 100 nm PS-NPs and 70 μg/mL for 30 nm PS-NPs) significantly reduced nuclear maturation rates and increased degeneration. Similarly, cytoplasmic maturation was negatively affected at higher concentrations ($P < 0.05$). PS-NPs exposure also elevated reactive oxygen species (ROS) levels and reduced glutathione (GSH) content within the oocyte. Notably, PS-NPs were internalized by both oocytes and cumulus cells in a concentration-dependent manner. These findings confirm the reproductive toxicity of PS-NPs, emphasizing their potential to compromise mammalian fertility and raising concerns regarding their environmental and health implications.

1. Introduction

Plastic pollution is a global concern, first recognized in marine environments [1]. Microplastics are defined as synthetic polymer particles ≤5 mm, while nanoplastics range from 1 nm to 1 μm. Micro- and nanoplastics (MNPs) originates primarily from two sources: primary plastic waste and secondary derivatives. Primary MNPs are released into the environment in micro- or nanoscale forms from sources such as personal care products, nanoimaging, nanomedicine and nanosensors. Secondary MNPs derive from plastics degradation due to physical, chemical or biological factors [2].

Nanoplastics pose several ecological risks and exhibit toxicity to both humans and animals [2–4]. One widely studied type is polystyrene nanoparticles (PS-NPs), commonly used in the manufacturing of various products, including packaging materials and cosmetics, and in biomedical applications [5]. Their environmental presence has raised concerns about their impact on living organisms and ecosystems, particularly their potential adverse effects on reproductive health have gained significant attention [6].

Oocyte in vitro maturation (IVM) is a pivotal step in the context of assisted reproduction techniques in both human and veterinary medicine. During IVM, immature oocytes are cultured in a controlled environment to attain full developmental competence, facilitating fertilization and embryo production. The process is regulated by a complex interplay of biochemical and biophysical factors, and any disruption to this delicate balance can compromise oocyte quality and developmental potential [7]. For this reason, oocyte IVM can be considered a valuable tool for testing reproductive toxicology. A significant portion of the data in reproductive toxicology comes from studies conducted in rodent models, whether in vivo or in vitro. However, rodent models require animal sacrifice to retrieve oocytes, embryos or other biologically relevant materials for these studies. By replacing in vitro assays in these laboratory animal models with the bovine model, no animal sacrifice is required since cow ovaries can be easily collected from slaughterhouses [8]. Several studies [9–11] have validated the reliability and predictability of the bovine model, particularly in assessing oocyte nuclear maturation, for screening reproductive toxicity. Additionally, beyond adhering to the 3R principle in

* Correspond Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, Ozzano, 40064, Emilia (BO), Italy.
E-mail addresses: barbara.merlo@unibo.it (M. Barbara), amarino.volsa@um.es (A.M. Volsa), laura.tovar2@unibo.it (L. Tovar), margherita.gaiani@studio.unibo.it (M. Gaiani), penelopemaria.gugol2@unibo.it (P.M. Gugole), emilia.attolini2@unibo.it (E. Attolini), eleonora.iacono2@unibo.it (I. Eleonora).

https://doi.org/10.1016/j.theriogenology.2025.117482
Received 25 February 2025; Received in revised form 11 May 2025; Accepted 12 May 2025
Available online 14 May 2025

animal testing, human and bovine species share certain similarities, such as mono-ovulation, oocyte diameter, oocyte transcriptome, time to the 2-cell stage embryo after fertilization, and the timing of oocyte maturation and early embryo development [12]. Finally, assessing reproductive toxicity using the bovine model offers an additional advantage, as the reproductive health of cows is crucial for productivity in the livestock industry. Indeed, reproductive performance plays a fundamental role in determining the efficiency and long-term sustainability of cattle production systems.

While various studies provide new insights into the risks that MNPs pose to mammalian fertility [13], the specific effects of PS-NPs on oocyte IVM remains largely unexplored [14], particularly within the bovine model. This study aims to investigate the impact of PS-NPs on oocyte in vitro maturation, utilizing the bovine model as a representative system.

2. Materials and methods

All chemicals were obtained from Sigma-Aldrich (Merck, Italy) unless otherwise stated.

2.1. Oocyte collection, in vitro maturation (IVM), and experimental design

Cumulus-oocyte complexes (COCs) were retrieved by aspirating 2–8 mm follicles from abattoir-derived ovaries using a 21 G butterfly needle connected to a 50 mL Falcon tube via a silicon plug. The plug was linked to a vacuum pump (KNF Laboport, Merck, Italy) set at −50 mmHg. After searching under a stereoscope, compact COCs with at least 4-5 layers of cumulus cells and a homogeneous, finely granulated cytoplasm were selected and washed twice in HSOF (Hepes 20 mM Synthetic Oviductal Fluid). The selected COCs were then randomly assigned to different experimental groups for PS-NPs exposure during IVM. In experiment 1 (Exp1), COCs were matured in presence of 100 nm PS-NPs (catalogue N. 43302) at concentrations of 0 (PS0), 5 (PS5), 50 (PS50), 100 (PS100), and 200 (PS200) μg/mL and subsequently analysed for nuclear maturation, cytoplasmic maturation, and intracellular levels of reactive oxygen species (ROS) and glutathione (GSH). In experiment 2 (Exp. 2), COCs were exposed to fluorescent 30 nm PS-NPs (catalogue N. L5155) at concentration of 0 (fPS0), 5 (fPS5), 35 (fPS35), and 70 (fPS70) μg/mL of and analysed for nuclear maturation, cytoplasmic maturation, and particle internalization. The experimental design is illustrated in Fig. 1.

The basal maturation medium consisted of TCM 199 supplemented with 10% foetal bovine serum (FBS), 10 ng/mL epidermal growth factor (EGF), 100 ng/mL insulin-like growth factor-1 (IGF-1), 1.2 mM L-cysteine, 1 mM sodium pyruvate, 75 μg/mL kanamycin, and 0.1 IU/mL porcine FSH-LH (Pluset, Calier, Como, Italy). Groups of 25–30 COCs were matured in 1 mL of maturation medium for 22 h at 38.5°C in humidified air at 5% CO_2. Within each replicate, oocyte culture for all experimental groups was conducted simultaneously.

2.2. Evaluation of nuclear maturation and incorporation of PS-NPs

After IVM, in both Exp. 1 (5 replicates, ovaries collected in June [range 17.7–29.2°C, average 23.4°C, THI 70.6] and July [range 20.8–33.8°C, average 27.4°C, THI 75.9]) and Exp. 2 (4 replicates, ovaries collected in June and July), expanded COCs were gently pipetted to mechanically remove cumulus cells. Denuded oocytes were stained with bisbenzimide (Hoechst 33342) at 10 μg/mL in PBS (Phosphate Buffered Saline) for 15 min at room temperature in the dark. Oocytes were then washed in PBS, mounted on microscope glass slides, covered with a coverslip, and examined under an epifluorescence microscope (Eclipse E, Nikon, Italy), equipped with a UV-2A (330–380 nm) excitation filter, to assess the meiotic stage. Oocyte displaying an extruded polar body and a metaphase plate (metaphase II stage, MII) were classified as mature. Oocytes with nuclear configurations ranging from the germinal vesicle stage to metaphase I were considered immature, while those with deteriorated or non-visible nuclear material were classified as degenerate.

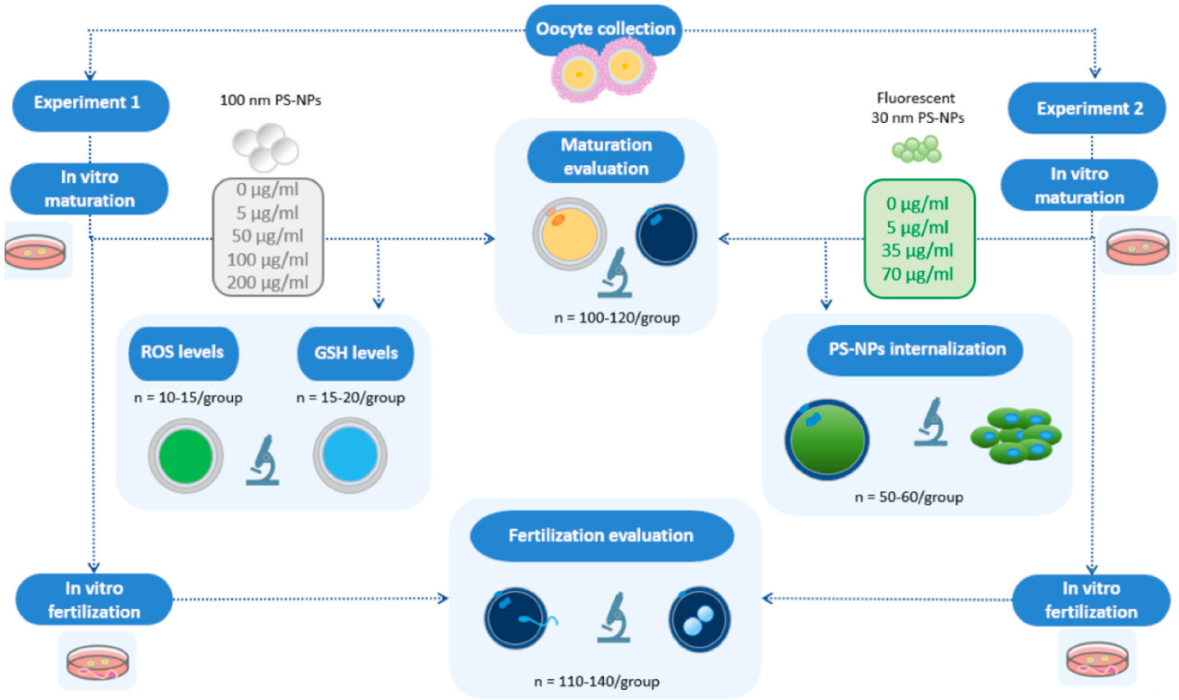

Fig. 1. Experimental Design. Schematic representation of two experiments on bovine oocyte in vitro maturation (IVM) in presence of polystyrene nanoplastics (PS-NPs). Experiment 1 tested 100 nm PS-PNs at concentrations of 0, 5, 50, 100, 200 μg/mL, while Experiment 2 used fluorescent 30 nm PS-NPs at 0, 5, 35, 70 μg/mL. In both experiments, nuclear configuration after IVM was analysed to assess maturation rates, along with the evaluation of cytoplasmic maturation through fertilization parameters following in vitro fertilization. Additional analyses in Experiment 1 included the measurement of intracellular levels of reactive oxygen species (ROS) and glutathione (GSH). In experiment 2, the internalization of fluorescent PS-NPs was examined.

For oocytes matured in presence of fluorescent 30 nm PS-NPs (Exp. 2), nanoparticle incorporation into oocytes and cumulus cells was confirmed by detecting fluorescence signals under an epifluorescence microscope equipped with a FITC (465–495 nm) excitation filter. Digital images were captured (Digital Sight camera, DS-U3, Nikon Europe BV, The Netherlands) using NIS-Elements D3.2 Laboratory Imaging software (Nikon Europe BV, The Netherlands). Image settings remained consistent across all samples for each analysis. Images of fluorescent oocytes and cumulus cells were analysed with a widely used open-source software (FIJI ImageJ). The area of interest was selected, as well as the background. Then, area, mean, minimum and maximum, integrated density, raw integrated density and length were calculated. The corrected total cell fluorescence (CTCF) was calculated using the formula: CTCF (pixel) = Integrated Density – (Area of selected cell x Mean fluorescence of background readings) [15]. Final values are expressed as arbitrary units.

2.3. Evaluation of cytoplasmic maturation

Cytoplasmic maturation was assessed by evaluating the ability of the oocyte to sustain sperm decondensation and male pronucleus formation, and insemination parameters after in vitro fertilization (IVF) (Exp.1, 5 replicates, ovaries collected in May [range 13.1–23.8°C, average 18.3°C, THI 63.8]; Exp.2, 5 replicates, ovaries collected in March [range 7.0–15.6°C, average 11.0°C, THI 52.8] and April [range 10.9–21.0°C, average 15.9°C, THI 61.9]).

After maturation, COCs were washed twice in HSOF and transferred into 0.5 mL of fertilization medium, consisting of SOF medium supplemented with 6 mg/mL bovine serum albumin (BSA), 1 μg/mL heparin, 20 μl/mL penicillamine-hypotaurine-epinephrine (PHE). Bovine motile spermatozoa were separated using a Percoll discontinuous density gradient (45–90%) and diluted to a final concentration of 1×10^6/mL (Exp.1) or 1.5×10^6/mL (Exp2). Oocytes and spermatozoa were co-incubated overnight (18–19 h) at 38.5°C, in modified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. After removing cumulus cells by gentle pipetting, denuded oocytes were washed twice in HSOF, stained with bisbenzimide as previously described, mounted, and examined under an epifluorescent microscope (UV-2A excitation filter). The following parameters were evaluated: i) penetration rate (number of fertilized oocytes/total inseminated), ii) monospermy rate (number of oocytes with a single sperm head or male pronucleus/total fertilized), iii) total fertilization efficiency (number of monospermic oocytes/total inseminated), iv) two-pronuclei rate (number of oocytes with two pronuclei/total inseminated).

2.4. Detection of ROS and GSH levels

At the end of IVM with non-fluorescent 100 nm PS-NPs (Exp.1, 3 replicates), expanded COCs were denuded and incubated for 30 min in the dark at room temperature in phosphate PBS supplemented with 0.1% polyvinyl alcohol (PVA) and 10 μM 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, Italy) for intracellular ROS detection or 10 μM 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen, Italy) for GSH level evaluation. Oocytes were then washed in PBS+PVA, mounted on glass slides with a vaseline anticompression layer, covered with coverslips, and examined under a Nikon Eclipse E400 epifluorescence microscope equipped with FITC (465–495 nm) and UV-2A (330–380 nm) excitation filters. Images were captured, and fluorescence intensity was analysed as previously described.

2.5. Statistical analysis

Data are presented as percentages or mean ± standard deviation. The Shapiro-Wilk test was used to assess normality. IVM and IVF data were analysed using a binomial generalized linear model (GLM) with logit link and Wald pairwise tests, while fluorescence intensity in both experiments was evaluated using a GLM with a gamma distribution and a log link, followed by Wald pairwise tests. All analyses were performed using IBM SPSS Statistics 29 (IBM Corporation, Milan, Italy). Statistical significance was set at $P < 0.05$.

3. Results

For Exp. 1, with 100 nm PS-NPs, 523 oocytes were used for evaluation of nuclear maturation, 683 oocytes underwent IVF for evaluation of cytoplasmic maturation, and 159 oocytes were stained for determination of GSH and ROS intracytoplasmic content. For experiment 2, with fluorescent 30 nm PS-NPs, a total of 450 oocytes were used for evaluation of maturation efficiency, of which 179 were evaluated for PS-NPs incorporation, and 461 oocytes underwent IVF for evaluation of cytoplasmic maturation.

3.1. Experiment 1

Detailed results of the evaluation of nuclear maturation of oocytes exposed to various concentrations of 100 nm PS-NPs are summarized in Table 1. The maturation rate (oocytes reaching the MII stage) was higher ($P < 0.05$) for PS0 group than PS100 and PS200 groups. Conversely, these groups exhibited the highest rates of immature oocytes ($P < 0.05$). Moreover, the degeneration rate was significantly increased in PS200 group ($P < 0.05$), while an intermediate value was observed in PS100 group ($P > 0.05$).

Results from the evaluation of cytoplasmic maturation based on IVF parameters are presented in Table 2. The addition of NPs showed a tendence to reduce ($P = 0.098$) the penetration rate, with PS0 group exhibiting the highest percentage, while no significant differences were observed between groups in the monospermy rate, total fertilization efficiency, and degeneration rate. Notably, the number of oocytes with two pronuclei was higher ($P < 0.05$) in the absence of PS-NPs.

ROS levels (Fig. 2A) were significantly increased by PS-NPs ($P < 0.05$), with higher levels in PS100 group compared to the PS0 and the PS5 groups ($P < 0.05$), and a tendency toward significance compared to the PS50 group ($P = 0.069$). A similar trend was observed in the PS200 group, which showed higher ROS levels compared to the PS0 and the PS5 ($P < 0.05$) groups, but not compared to the PS50 group.

GSH levels (Fig. 2B) were significantly decreased by PS-NPs ($P < 0.05$), with higher levels in PS0 group compared to the PS5, PS50, and PS200 ($P < 0.05$) groups, but not compared to the PS100 group.

3.2. Experiment 2

Results of the nuclear maturation evaluation are presented in Table 3. The maturation rate was significantly affected by the presence of fluorescent 30 nm PS-NPs ($P < 0.05$). The fPS70 group exhibited a significantly lower proportion of MII oocytes compared to the other groups groups ($P < 0.05$), with the highest rates of immature and degenerate oocytes ($P < 0.05$)

The internalization of fluorescent 30 nm PS-NPs was directly related to the exposure concentration ($P < 0.001$) in both oocytes (Fig. 3A) and

Table 1
Evaluation of nuclear maturation of bovine oocytes cultured in the presence of 100 nm PS-NPs.

Group	Total oocytes	Mature (%)	Immature (%)	Degenerate (%)
PS0	107	67 (62.6)^{a}	30 (28.0)^{a}	10 (9.3)^{a}
PS5	109	62 (56.9)^{a}	38 (34.9)^{ab}	9 (8.3)^{a}
PS50	106	63 (59.4)^{a}	36 (34.0)^{ab}	7 (6.6)^{a}
PS100	101	32 (31.7)^{b}	55 (54.5)^{c}	14 (13.9)^{a,b}
PS200	100	35 (35.0)^{b}	45 (45.0)^{bc}	20 (20.0)^{b}

a vs b vs c $P < 0.05$. Experimental groups: PS0 (control), PS5 (5 μg/mL), PS50 (50 μg/mL), PS100 (100 μg/mL), and PS200 (200 μg/mL) 100 nm PS-NPs.

Table 2
Evaluation of cytoplasmic maturation of bovine oocytes cultured in the presence of 100 nm PS-NPs.

Group	Total oocytes	Penetration (%)	Monospermy (%)	Total efficiency (%)	2 Pronuclei (%)	Degenerate (%)
PS0	129	113 (87.6)	102 (90.3)	102 (79.1)	60 (46.5)^{a}	1 (0.8)
PS5	142	109 (76.8)	105 (96.3)	105 (73.9)	47 (33.1)^{bc}	6 (4.2)
PS50	143	110 (76.9)	97 (88.2)	97 (67.8)	45 (31.5)^{bc}	10 (7.0)
PS100	130	104 (80.0)	94 (90.4)	94 (72.3)	46 (35.4)^{b}	7 (5.4)
PS200	139	104 (74.8)	99 (95.2)	99 (71.2)	34 (24.5)^{c}	6 (4.3)

a vs b vs c $P < 0.05$. Experimental groups: PS0 (control), PS5 (5 μg/mL), PS50 (50 μg/mL), PS100 (100 μg/mL), and PS200 (200 μg/mL) 100 nm PS-NPs.

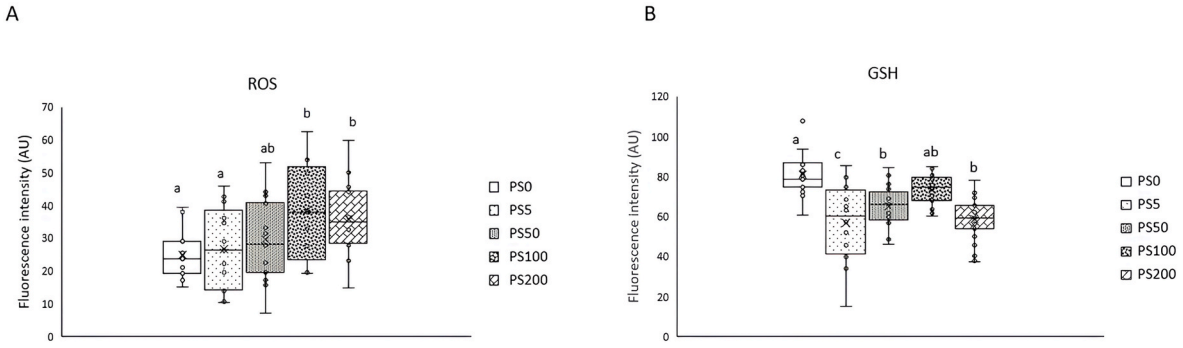

Fig. 2. Oocyte oxidative metabolism. Box plot showing the relative fluorescence intensity levels (arbitrary units) regarding intracellular levels of (A) ROS, and (B) GSH, in bovine mature oocytes derived from PS0 (ROS n = 11 and GSH n = 21), PS5 (ROS n = 13 and GSH n = 15), PS50 (ROS n = 15 and GSH n = 21), PS100 (ROS n = 10 and GSH n = 18), and PS200 (ROS n = 14 and GSH n = 21) groups. Different letters show statistical differences ($P < 0.05$). Experimental groups: PS0 (control), PS5 (5 μg/mL), PS50 (50 μg/mL), PS100 (100 μg/mL), and PS200 (200 μg/mL) 100 nm PS-NPs.

Table 3
Evaluation of nuclear maturation of bovine oocytes cultured in the presence of fluorescent 30 nm PS-NPs.

Group	Total oocytes	Mature (%)	Immature (%)	Degenerate (%)
fPS0	117	72 (61.5)^{a}	33 (28.0)^{b}	12 (10.3)^{ab}
fPS5	118	85 (72.0)^{a}	25 (34.9)^{b}	8 (6.8)^{a}
fPS35	110	73 (66.4)^{a}	34 (34.0)^{a}	3 (2.7)^{ab}
fPS70	105	46 (43.8)^{b}	41 (45.0)^{bc}	18 (17.1)^{b}

a vs b vs c $P < 0.05$. Experimental groups: fPS0 (control), fPS5 (5 μg/mL), fPS35 (35 μg/mL), and fPS70 (70 μg/mL) fluorescent 70 nm PS-NPs.

cumulus cells (Fig. 3B).

Results regarding the evaluation of cytoplasmic maturation through IVF are summarized in Table 4. A significant reduction in penetration rate was observed only in the fPS70 group ($P < 0.05$), while the fPS35 group exhibited the lowest monospermy rate ($P < 0.05$). The fPS0 and fPS5 groups showed comparable fertilization efficiency, whereas the fPS35 and fPS70 groups displayed lower rates ($P < 0.05$). The number of oocytes with two pronuclei was similar across all groups. Degeneration rate was negatively affected by the presence of PS-NPs ($P < 0.05$), as indicated by the significantly increased value observed in fPS70 group ($P < 0.05$).

4. Discussion

The purpose of the present study was to evaluate whether polystyrene nanoparticles could affect the in vitro maturation of bovine oocytes, focusing on nuclear and cytoplasmic maturation and the degree of internalization into the COC. The findings indicate that exposure to 100 and 30 nm PS-NPs during IVM impairs nuclear and cytoplasmic maturation, with the most pronounced negative effects observed at higher concentrations of 100 nm PS-NPs (100 and 200 μg/mL) and 30 nm PS-NPs (70 μg/mL). These results highlight the dose-dependent toxicity of these nanoparticles. Additionally, PS-NPs were internalized by oocytes in a concentration-related manner.

MPs have been detected in human and bovine follicular fluid, with mean concentrations of 122.3 and 38.6 particles/mL, respectively [16]. MP polymers accounted for 0.8–23.5 % of the total number of identified particles, with 7 polymers most abundant in human follicular fluid (polyvinyl chloride - PVC, polyethylene - PE, polystyrene - PS, polypropylene - PP, polyurethane - PU, rubber - RUB, and acrylonitrile butadiene styrene - ABS), and 10 polymers (PVC, PE, cellulose acetate - CE, PS, PP, silicon - SL, nylon - NL, polyester - PES, ABS, and polyamide - PA) most abundant in bovine follicular fluid [16]. Nonetheless, limitations in detection methods, such as the 0.3 μm threshold of Raman spectroscopy, likely led to an underestimation of MPs [16], entirely missing nanoparticles. The lack of reliable methods for isolating and characterizing MNPs from biological tissues and fluid has resulted in limited knowledge regarding their bioaccumulation in livestock and humans. Consequently, the impact of plastic pollution on ecosystems, health, and fertility warrants further investigation.

The observed reduction in nuclear maturation, indicated by fewer oocytes reaching the MII stage and increased degeneration rates, align with previous studies demonstrating PS-MNPs interference in murine oocytes. In mice, orally administered PS-MPs, while not affecting the GVBD rate, reduced the proportion of oocytes capable of extruding the first polar body, a crucial step for completing maturation, and impaired oocyte survival [17]. Similarly, while exposure of mouse oocytes to 50 nm PS-NPs during IVM (0, 10, 50, 100 μg/ml) did not affect meiotic resumption, it inhibited meiotic maturation at the dosage of 100 μg/ml, by compromising key aspects such as spindle assembly and chromosome alignment [14]. These disruptions may explain the decreased developmental competence observed in this study. Furthermore, the increased degeneration rates suggest that high concentrations of PS-NPs exacerbate cellular stress, potentially activating apoptotic pathways, as reported for other mammalian cells [3]. This align with report of increased apoptosis in murine oocytes exposed to PE-MPs, leading to a higher risk of cell death [18].

A single study in cattle oocytes demonstrated that PS-MPs (0.3 μm at 0.01 μg/mL and 1.1 μm at 0.1 μg/ml) compromised nuclear maturation and zona pellucida structure without affecting degeneration rates [16]. In the present study, no damage to the zona pellucida was observed. Differences in biological material sources poses limitations in the

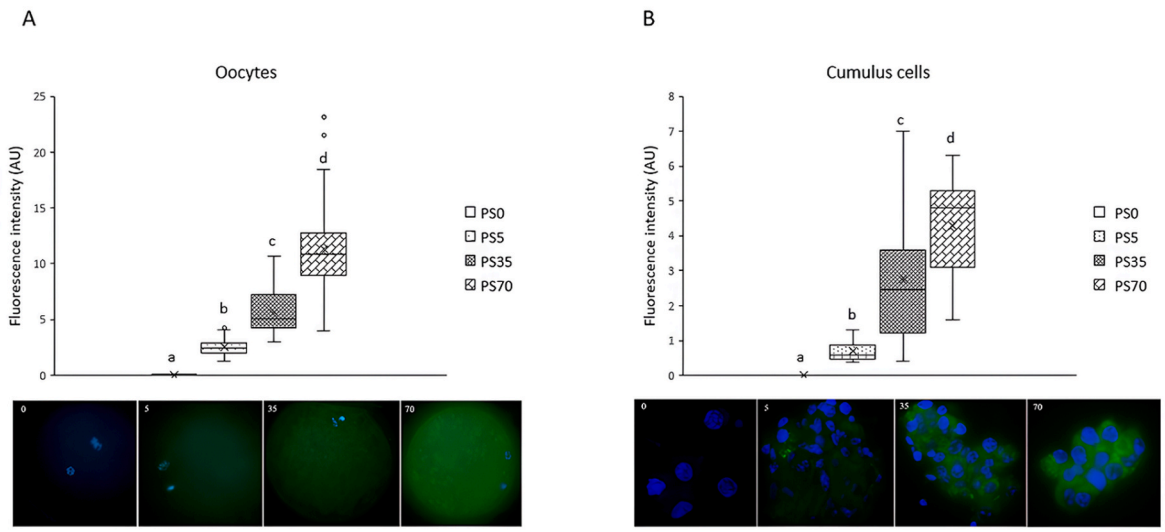

Fig. 3. PS-NPs Internalization. Box plot showing the relative fluorescence intensity (arbitrary units) of intracellular fluorescent 30 nm PS-NPs in (A) oocytes and (B) cumulus cells from in bovine mature COCs in fPS0 (n = 12), fPS5 (n = 64), fPS35 (n = 56), and fPS70 (n = 47) groups. Different letters indicate statistically significant differences ($P < 0.05$). Experimental groups: fPS0 (control), fPS5 (5 μg/mL), fPS35 (35 μg/mL), and fPS70 (70 μg/mL) fluorescent 30 nm PS-NPs. Representative images illustrate PS-NPs internalization in (A) oocytes and (B) cumulus cells at different concentrations, as observed under an epifluorescence microscope to assess fluorescence intensity.

Table 4
Evaluation of cytoplasmic maturation of bovine oocytes cultured in the presence of fluorescent 30 nm PS-NPs.

Group	Total oocytes	Penetration (%)	Monospermy (%)	Total efficiency (%)	2 Pronuclei (%)	Degenerate (%)
fPS0	110	102 (92.7)[a]	99 (97.1)[a]	99 (90.0)[a]	63 (57.3)	1 (0.9)[a]
fPS5	119	106 (89.1)[a]	104 (98.1)[a]	104 (87.4)[a]	63 (52.9)	5 (4.2)[a]
fPS35	119	104 (87.4)[a]	93 (89.4)[b]	93 (78.2)[b]	73 (61.3)	6 (5.0)[a]
fPS70	113	83 (73.5)[b]	80 (96.4)[a]	80 (70.8)[b]	62 (54.9)	30 (26.5)[b]

a vs b $P < 0.05$. Experimental groups: fPS0 (control), fPS5 (5 μg/mL), fPS30 (35 μg/mL), and fPS70 (70 μg/mL) fluorescent 30 nm PS-NPs.

comparison between laboratories, and oocyte quality may influence susceptibility to PS-MNPs toxicity. Indeed, high degeneration rates were observed in that study, even in the control group, and maturation rates included metaphase I oocytes. Nonetheless, very low concentrations of PS-MPs were used. Interestingly, the proteomic analysis of the oocytes revealed alterations in the abundance of proteins regulating critical functional pathways, such as apoptosis, mitochondrial damage, mitochondrial apoptosis, microtubule disorganization, organelle disorganization, spindle disorganization, and nuclear maturation [16]. However, only 13 out of 2060 proteins were differentially expressed, indicating that cumulus cells play an important role in controlling the effects of PS-MPs on the oocyte [16].

PS-NPs also impaired oocyte quality by disrupting granulosa cell function in mice, leading to reduced fertilization rates [19]. Moreover, PS-NPs disrupted maturation in mouse oocytes by incorporating into the endoplasmic reticulum and disturbing intracellular translation mechanisms during meiotic maturation, which may adversely affect subsequent fertilization processes [14]. Exposure to PS-NPs affected cytoplasmic integrity, confirming that PS-NPs not only affect oocyte morphology but may alter fundamental biological functions, increasing the risk of abnormalities in embryonic development and potential reproductive issues [14]. Indeed, PE-MPs induced a reduction in mitochondrial functionality, along with downregulation of actin and the expression of the Juno protein on the oocyte membrane, critical for fertilization [18]. In this study, cytoplasmic maturation was compromised, with PS-NPs exposure reducing the fertilization efficiency during IVF. Although total fertilization efficiency showed less pronounced changes with larger PS-NPs, two pronuclei formation was impaired at higher PS-NPs concentrations, indicating toxic effects on oocyte cytoplasmic processes. On the other hand, smaller PS-NPs affected penetration only at the highest concentration, with a reduction in monospermy for the intermediate concentration, without significant effects on two pronuclei formation. Considering that IVF parameters for smaller particles were assessed 4 h later than for larger PS-NPs (due to practical timing of lab activities), it is possible that PS-NPs exposure affects the kinetics of fertilization, delaying rather than fully impairing the formation of two pronuclei. Moreover, the slightly different effects observed between small and large PS-NPs may reflect size-dependent interactions with intracellular components, where smaller particles, due to greater surface area and potentially higher cellular uptake, might interfere more subtly but persistently with oocyte activation and cytoskeletal remodeling.

Oxidative stress plays a central role in nanoplastic-induced cytotoxicity [3]. In porcine oocytes, PS-NPs induced ferroptosis via increased oxidative stress and altered lipid metabolism, resulting in failed maturation [20]. In the present study, elevated ROS levels in PS-NPs-treated oocytes confirmed oxidative stress as a primary mechanism of toxicity. Although antioxidant defences were present, reduced GSH levels indicated their insufficiency in counteracting the heightened ROS production. Previous studies have reported that exposure to MNPs induces oxidative stress by increasing ROS levels and decreasing GSH, without necessarily triggering significant apoptosis [17]. While mitochondrial appeared largely preserved, alterations in mitochondrial membrane potential and a reductions in endoplasmic reticulum calcium levels were observed, further highlighting the detrimental impact on calcium reserves and oocyte viability [17]. Oral intake of polyethylene MPs similarly increased ROS levels in oocytes and embryos, leading to oxidative stress, mitochondrial dysfunction, and apoptosis [18]. A concomitant decrease in GSH levels suggested impairment of antioxidant defence mechanisms by PE-MPs [18]. Furthermore, oocytes co-cultured with

cumulus cells pre-exposed to PS-NPs exhibited excessive ROS accumulation and reduced mitochondrial membrane potential [19]. These findings emphasize the need to further investigate molecular pathways linking oxidative damage to meiotic and cytoplasmic dysfunction, particularly the roles of mitochondrial impairment and depletion of intracellular antioxidant reserves in amplifying cellular stress responses.

We found that PS-NPs internalization in cumulus cells and mature oocytes was proportional to the exposure concentration. The interaction between cumulus cells and oocytes is critical for oocyte development and maturation. Cumulus cells support oocyte development by transferring essential nutrients, signalling molecules, and regulatory factors through gap junctions and paracrine mechanisms [21]. Although limited, current evidence suggests cumulus cells may facilitate PS-NPs transport into oocytes. In a study assessing polymeric nanoparticle internalization in bovine COCs [21], both intact and partially denuded COCs (with only the corona radiata) were examined. Nanoparticles of varying size penetrated the oocyte, with a higher uptake observed in COCs with intact cumulus layers [22]. Transfer appeared to occur via both transcellular (through cumulus cells and transzonal projections) and paracellular (through zona pellucida) routes [22]. In mice, PS-NPs have been shown to enter primary cumulus cells and cause cellular dysfunction [19], which in turn adversely affected oocyte quality, as indicated by reduced maturation rates in co-cultured oocytes [19]. Although 25 nm PS-NPs readily entered murine cumulus cells, they did not penetrate the oocyte through the zona pellucida within 12 h at 100 μg/mL exposure [19], suggesting that the observed decline in oocyte quality resulted primarily from compromised granulosa cell function [19]. Conversely, when mouse denuded oocytes with intact zona pellucida were directly exposed to fluorescent PS-MNPs for 24 h, PS-NPs, but not PS-MPs, penetrated the zona pellucida and localized within the cytoplasm in a concentration-dependent manner [14]. Furthermore, PS-NPs remained trapped within the cytoplasm for extended periods [14]. Particle size also influenced PS uptake in bovine oocytes during IVM (23 h at 10 μg/mL) [23]. While 200 nm PS-MPs were internalized by only a few cumulus cells, 50 nm PS-NPs were taken up by both cumulus cells and oocytes, although nuclear maturation was not significantly affected [23]. These findings collectively highlight the importance of concentration and particle size in evaluating MNPs reproductive toxicity.

The bovine oocyte model used in this study offers unique advantages for reproductive toxicology. In addition to aligning with the 3Rs principles (replacement, reduction, refinement), it closely mirrors human reproductive physiology in terms of oocyte size, developmental timelines, and mono-ovulatory behaviour [12]. Its relevance to livestock systems also emphasizes the agricultural implications of nanoplastic exposure. A decline in oocyte quality, as observed here, could compromise cattle reproductive performance, a key component for sustainable livestock production [12].

Our findings raise concerns regarding the bioavailability and potential tissue accumulation of nanoplastics, which may impact fertility and overall health across species [2,4]. These results highlight the need for further research into long-term effects, including chronic exposure scenarios and environmentally relevant concentrations. Additional investigations into other nanoplastic types, varying in size and composition, are also warranted.

A final consideration regarding the suboptimal maturation rates observed in this study concerns the season of sampling. For nuclear maturation evaluation, oocytes were collected during June and July, a period characterized by increasing ambient temperatures and THI values above 70, which are known to induce heat stress in cattle. Heat stress has been shown to negatively affect estrous cyclicity, oocyte quality, and pre-implantation embryo development, ultimately compromising cattle reproductive performance [24]. On the other hand, IVF experiments were performed during the cooler months of March, April, and May, and the slightly improved fertilization rates observed in Exp. 2 may be attributed to the higher sperm concentration used during IVF.

5. Conclusion

In conclusion, our findings demonstrate that PS-NPs impair bovine oocyte nuclear and cytoplasmic maturation, with adverse effects intensifying at higher concentrations. This study provides valuable insights into the reproductive toxicity of nanoplastics, emphasizing their potential threat to both livestock and human reproductive health.

CRediT authorship contribution statement

Merlo Barbara: Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Alessandro Marino Volsa:** Investigation. **Laura Tovar:** Investigation, Data curation. **Margherita Gaiani:** Investigation, Data curation. **Penelope Maria Gugole:** Investigation. **Emilia Attolini:** Data curation. **Iacono Eleonora:** Writing – review & editing, Validation.

Funding

Tovar L's PhD fellowship was supported by the Italian Ministry of University and Research (MUR) under the National Recovery and Resilience Plan (NRRP, ex D.M. 118/23).

Declaration of competing interest

I have nothing to declare.

Acknowledgments

The authors would like to thank Marcella Spinaci and Diego Bucci for providing 100 nm PS-NPs and access to the sonicator, and 'La Regione Emilia-Romagna - Settore Prevenzione Collettiva e Sanità Pubblica' for funding Volsa AM's fellowship.

References

[1] Webb HK, Arnott J, Crawford RJ, Ivanova EP. Plastic Degradation and Its Environmental Implications with Special Reference to Poly(ethylene terephthalate). Polymers 2013;5:1–18. https://doi.org/10.3390/polym5010001.
[2] Lai H, Liu X, Qu M. Nanoplastics and Human Health: Hazard Identification and Biointerface. Nanomaterials (Basel) 2022;12:1298. https://doi.org/10.3390/nano12081298.
[3] Banerjee A, Shelver WL. Micro- and nanoplastic induced cellular toxicity in mammals: A review. Sci Total Environ 2021;755:142518. https://doi.org/10.1016/j.scitotenv.2020.142518.
[4] Shi C, Liu Z, Yu B, Zhang Y, Yang H, Han Y, et al. Emergence of nanoplastics in the aquatic environment and possible impacts on aquatic organisms. Sci Total Environ 2024;906:167404. https://doi.org/10.1016/j.scitotenv.2023.167404.
[5] Kik K, Bukowska B, Sicińska P. Polystyrene nanoparticles: Sources, occurrence in the environment, distribution in tissues, accumulation and toxicity to various organisms. Environ Pollut 2020;262:114297. https://doi.org/10.1016/j.envpol.2020.114297.
[6] Liang J, Ji F, Wang H, Zhu T, Rubinstein J, Worthington R, et al. Unraveling the threat: Microplastics and nano-plastics' impact on reproductive viability across ecosystems. Sci Total Environ 2024;913:169525. https://doi.org/10.1016/j.scitotenv.2023.169525.
[7] Krisher RL. In vivo and in vitro environmental effects on mammalian oocyte quality. Annu Rev Anim Biosci 2013;1:393–417. https://doi.org/10.1146/annurev-animal-031412-103647.
[8] Asimaki K, Vazakidou P, van Tol HTA, Oei CHY, Modder EA, van Duursen MBM, et al. Bovine In Vitro Oocyte Maturation and Embryo Production Used as a Model for Testing Endocrine Disrupting Chemicals Eliciting Female Reproductive Toxicity With Diethylstilbestrol as a Showcase Compound. Front Toxicol 2022;4.
[9] Lazzari G, Tessaro I, Crotti G, Galli C, Hoffmann S, Bremer S, et al. Development of an in vitro test battery for assessing chemical effects on bovine germ cells under the ReProTect umbrella. Toxicol Appl Pharmacol 2008;233:360–70. https://doi.org/10.1016/j.taap.2008.08.019.
[10] Luciano AM, Franciosi F, Lodde V, Corbani D, Lazzari G, Crotti G, et al. Transferability and inter-laboratory variability assessment of the in vitro bovine oocyte maturation (IVM) test within ReProTect. Reproduct Toxicol 2010;30:81–8. https://doi.org/10.1016/j.reprotox.2010.01.015.

[11] Beker van Woudenberg A, Gröllers-Mulderij M, Snel C, Jeurissen N, Stierum R, Wolterbeek A. The bovine oocyte in vitro maturation model: A potential tool for reproductive toxicology screening. Reproduct Toxicol 2012;34:251–60. https://doi.org/10.1016/j.reprotox.2012.05.098.
[12] Santos RR, Schoevers EJ, Roelen BA. Usefulness of bovine and porcine IVM/IVF models for reproductive toxicology. Reproduct Biol Endocrinol 2014;12:117. https://doi.org/10.1186/1477-7827-12-117.
[13] He Y, Yin R. The reproductive and transgenerational toxicity of microplastics and nanoplastics: A threat to mammalian fertility in both sexes. J Appl Toxicol 2024;44:66–85. https://doi.org/10.1002/jat.4510.
[14] Park S, Jeon H-J, Choi DY, Oh JS. Polystyrene nanoparticles incorporate into the endoplasmic reticulum and disturb translation during meiotic maturation in mouse oocytes. Toxicol in Vitro 2022;82:105380. https://doi.org/10.1016/j.tiv.2022.105380.
[15] El-Sharkawey. Calculate the corrected total cell fluorescence (CTCF). ResearchGate; 2016. https://doi.org/10.13140/RG.2.1.1307.8008. [Accessed 20 January 2025].
[16] Grechi N, Franko R, Rajaraman R, Stöckl JB, Trapphoff T, Dieterle S, et al. Microplastics are present in women's and cows' follicular fluid and polystyrene microplastics compromise bovine oocyte function in vitro. eLife 2023;12. https://doi.org/10.7554/eLife.86791.1.
[17] Liu Z, Zhuan Q, Zhang L, Meng L, Fu X, Hou Y. Polystyrene microplastics induced female reproductive toxicity in mice. Jf Hazardous Mater 2022;424:127629. https://doi.org/10.1016/j.jhazmat.2021.127629.
[18] Zhang Y, Wang X, Zhao Y, Zhao J, Yu T, Yao Y, et al. Reproductive toxicity of microplastics in female mice and their offspring from induction of oxidative stress. EnvironPollut 2023;327:121482. https://doi.org/10.1016/j.envpol.2023.121482.
[19] Xue Y, Cheng X, Ma Z-Q, Wang H-P, Zhou C, Li J, et al. Polystyrene nanoplastics induce apoptosis, autophagy, and steroidogenesis disruption in granulosa cells to reduce oocyte quality and fertility by inhibiting the PI3K/AKT pathway in female mice. J Nanobiotechnol 2024;22:460. https://doi.org/10.1186/s12951-024-02735-7.
[20] He Y, Yu T, Li H, Sun Q, Chen M, Lin Y, et al. Polystyrene nanoplastic exposure actives ferroptosis by oxidative stress-induced lipid peroxidation in porcine oocytes during maturation. J Animal Sci Biotechnol 2024;15:117. https://doi.org/10.1186/s40104-024-01077-6.
[21] Lakshmanan M, Saini M, Nune M. Exploring the innovative application of cerium oxide nanoparticles for addressing oxidative stress in ovarian tissue regeneration. J Ovarian Res 2024;17:241. https://doi.org/10.1186/s13048-024-01566-2.
[22] Gonçalves DR, Leroy JLMR, Van Hees S, Xhonneux I, Bols PEJ, Kiekens F, et al. Cellular uptake of polymeric nanoparticles by bovine cumulus-oocyte complexes and their effect on *in vitro* developmental competence. Europ J Pharmaceut Biopharmaceut 2021;158:143–55. https://doi.org/10.1016/j.ejpb.2020.11.011.
[23] Jiayi Yang MJJB. First evidence of nanoplastic uptake by the maturing oocyte. First Evidence of Nanoplastic Uptake by the Maturing Oocyte 2022;19. 0–0.
[24] Gómez-Guzmán JA, Parra-Bracamonte GM, Velazquez MA. Impact of Heat Stress on Oocyte Developmental Competence and Pre-Implantation Embryo Viability in Cattle. Animals 2024;14:2280. https://doi.org/10.3390/ani14152280.