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Nanoplastics impair in vitro swine granulosa cell functions

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ABSTRACT

Soil, water, and air pollution by plastic represents an issue of great concern since the particles produced by degradation of plastic materials can be ingested by animals and humans, with still uncertain health consequences. As a contribution on this crucial subject, the present work reports an investigation on the in vitro effects of different concentrations of polystyrene nanoplastics (5, 25, and 75 μg/mL) on swine granulosa cells, a model of endocrine reproductive cells. In particular, cell growth (BrDU incorporation and ATP production), steroidogenesis (17-β estradiol and progesterone secretion) and redox status (superoxide and nitric oxide production, enzymatic and non-enzymatic scavenging activity) were studied. Nanoplastics, at the highest concentration, stimulated cell proliferation ($P < 0.05$), while cell viability resulted unaffected. Steroidogenesis was disrupted ($P < 0.05$). Both enzymatic and non-enzymatic scavenging activity were increased after exposure at the highest nanoplastic dose ($P < 0.05$, $P < 0.001$). Nitric oxide secretion was increased by 25 and 75 μg/mL ($P < 0.05$) while superoxide generation was stimulated ($P < 0.001$) only by the highest concentration tested. Taken together, main features of cultured swine granulosa cells resulted affected by exposure to nanoplastics. These results raise concerns since environment nanoplastic contamination can represents a serious threat to animal and human health.

1. Introduction

Plastic pollution has become one of the most critical environmental issues given that the production of disposable plastic objects is overwhelming our ability to manage their safe recycling. This problem is most evident in countries of Asia and Africa, where waste collection systems are often inefficient or non-existent. Moreover, also the developed countries with low recycling rates are having difficulties in correctly managing plastic waste and a global agreement negotiated by the United Nations has been drawn up [1,2]. Plastics derived from fossil fuels are over a century old. The production and development of thousands of new plastic products expanded so much after the Second World War that nowadays life without plastic would be unrecognizable: not only medicine has been revolutionized by plastic life-saving devices, transports by much lighter cars and jets but almost every aspect of modern life. The comforts offered by plastic, however, have led to a throw-away culture that reveals the dark side of this material: today, disposable plastics make up 40% of all those produced every year [3]. Despite a use of a few minutes or hours for many of these items, for example plastic bags or food wraps, their persistence in the environment is hundreds of years. The increase in production has been paralleled by a rise in plastic waste which is accumulating in the oceans because of its extreme resistance and very slow degradation rates. The first plastic debris was observed in the oceans in 1960, and in the early 1970s the phenomenon was first identified as an environmental problem thus creating a growing social concern.

Depending on the diameter of plastic fragments, particles can be divided into microplastics (MPs) and nanoplas-

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tics (NPs), with MPs being less than 5 mm in diameter and NPs being 1 to 100 or 1,000 nm in diameter [4]. This definition has also been used by the European Food Security Authority [5]. MPs and NPs can be classified into primary and secondary: the first are produced on a micrometric scale and mostly used in cosmetics, toothpastes, detergents, medical devices and as fibers in clothing; the second derive from the fragmentation of macroplastics after physical (wind, waves, abrasion, temperature, UV), chemical (chemical agents) and biological (microorganisms) degradation. Interactions between the plastic waste and environmental components can degrade large pieces of plastics to smaller plastic debris. Currently, MPs are ubiquitous in all ecosystems, both terrestrial and marine. High concentrations of plastic debris have been mainly detected in subtropical oceans, rural lakes and Arctic regions after transport by sea currents [6]. These results indicate that MNPs pollution is widespread, and their biological hazard for humans and other living organisms cannot be ignored. An interesting model useful to study MNPs is represented by corals, were MPs negatively impact on coral energetics, growth and health, with adverse consequences in feeding behavior, photosynthetic performance, energy expenditure, skeletal calcification and reproductive performance [7–9]. It has been demonstrated that MPs impair immune functions of several marine animals as fishes, mussels, crabs, polychaeta, echinodermata, and corals [10]. Previous studies have reported that MNPs exposure affects reproduction in oysters [11], liver function in zebrafish, [12], and results in tissue bioaccumulation and potential organ toxicities in mice [13]. Moreover, immunotoxicity and negative intestinal effects have been demonstrated both in fishes and mammals [14]. Hou and Zhu raised concerns about critical effects of NPs on female reproduction [15]. Rolsky and Varun Lelkar [16] recently detected NPs in human tissue.

A new critical issue could derive from the widespread use of surgical masks useful to reduce the risks of SARS-Cov 2 infection [17]. Although the underlying mechanisms of MPs and NPs toxicity are still poorly understood, oxidative stress has been considered an important player of their negative effects [18,19].

In our previous studies, we demonstrated that ovarian follicles and granulosa cells represent valuable models for the study of oxidative stress [20–22]. Therefore, we have undertaken the present work to verify the effect of NPs on the main functional parameters of granulosa cells, including redox status markers. The pig was chosen as a valuable animal model for experimental medicine which is widely used in translational studies due to the strong similarities with the human in both physiological and genomic patterns [23].

2. Materials and methods

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Collection of ovaries

Swine ovaries were collected at a local slaughterhouse from 40 Large White cross-bred gilts for each of the 6 experiments (240 animals in total), (parity = 0, 8–9 mo aged, weighing about 180 kg), immediately after death. Since the stages of the estrous cycle were unknown, its evaluation was based on ovarian morphology as previously described [24,25]. Ovaries were placed into cold PBS (4°C) supplemented with penicillin (500 IU/mL), streptomycin (500 μg/mL) and amphotericin B (3.75 μg/mL), maintained in a freezer bag and transported to the laboratory within 1 h [26].

2.1.1. Granulosa cell collection

Swine ovarian follicles were classified as healthy or atretic on the basis of morphological criteria, and those with hemorrhagic, opaque or "milky" follicular fluid were excluded [27]. Accordingly to our previous studies [28–31], and on the basis of the classification of Foxcroft and Hunter [32], granulosa cells were aseptically harvested by aspiration of healthy follicles at a later stage (>5 mm diameter) with a 26-gauge needle and released in medium containing heparin (50 IU/mL). During granulosa cell collection, a gentle scraping of the follicle wall was performed with the needle in order to collect also mural cells. Cells were then centrifuged for pelleting and treated with 0.9% prewarmed ammonium chloride at 37°C for 1 min to remove red blood cells. Cell number and viability were estimated using a hemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension.

2.1.2. Granulosa cell culture and evaluation of effects induced by nanoplastics

Collected cells, as above detailed, were seeded in culture medium represented by DMEM/Ham's F12 supplemented with sodium bicarbonate (2.2 mg/mL), bovine serum albumin (BSA 0.1% w/v), penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), selenium (5 ng/mL) and transferrin (5 μg/mL), indicated hereafter as CM. Once seeded, cells were incubated for 48 h at 37°C under humidified atmosphere (5% CO_2) in the presence or absence of polystirene nanoparticles (Sigma Aldrich, catalogue N. 43302). The particle size was 100 nm. The particles are in aqueous suspension (10% WT). The examined concentrations were 5, 25, and 75 μg/mL accordingly to Poma et al [33]. At present, although the documented presence in the environment and in food products, no policy has been in place, to monitor and regulates MPS in commercial foods meant for human consumption and little is known about a real exposure [34]. However, it should be noted that total consumption and inhalation of MPs for human is approximately in an average of 700–1,050 μg of MPs per week. The ingested MPs enter the gut or lung, and smaller plastics accumulate in tissues likely in the examined range of concentrations accordingly to Choi et al [35].

2.1.2.1. Granulosa cell growth.

2.1.2.1.1. Granulosa cell proliferation. The ELISA BrdU (Roche Diagnostic, Indianapolis, In, USA) is an immunological colorimetric assay used for the quantitative analysis of cell proliferation since BrdU can be incorporated into the newly synthesized DNA of replicating cells. Granulosa cells

were plated into 96-well plates (Sarstedt, Nümbrecht, Germany) (10^4 cells/200 µL of CM), they were treated with the substance of interest and incubated overnight at 37°C in a humidified atmosphere [36]. Thereafter, they were incubated with 20 µL of BrdU for 24 h. Then cells were fixed and DNA was denatured before adding the anti-BrdU antibody, conjugated with the enzyme peroxidase. During the two h of incubation, we added 100 µL of tetra-methylbenzidine substrate (TMB) which develops the blue color after being oxidized by the enzyme in a quantity proportional to the amount of newly synthesized DNA. Finally, the reaction was stopped by the addition of 25 µL of sulfuric acid (1M) which makes the sample to turn yellow. Absorbance values were measured at 450nm using Victor Reader spectrophotometer (Perkin Elmer, Groningen, the Netherlands). To quantify viable cell number, the absorbance of each sample was related to a standard curve prepared by culturing, in quintuplicate, granulosa cells at different plating densities (from 10^3 to 10^5 viable/200 µL) for 48 h. The curve was repeated in 4 different experiments. The relationship between cell number and absorbance was linear ($r = 0.92$). Cell number/well was estimated from the resulting linear regression equation and was used to correct experimental data. The assay detection limit was 10^3 cell/well and the variation coefficient was less than 5%.

2.1.2.1.2. Granulosa cell metabolic activity. 2×10^5 viable cells were seeded in 96-well plates (Perkin Elmer, Buckinghamshire, UK) in 200 µL CM and treated with NPs for 48 h as above indicated. Cell metabolic activity was assessed using a bioluminescent assay (ATP-lites; Packard Bioscience, Groningen, Netherlands) which measures intracellular ATP levels. ATP, being present in all metabolically active cells, is a viability marker whose concentration declines very rapidly when the cells undergo either necrosis or apoptosis [37]. The ATP lite-M assay system is based on the detection of light produced by the luciferase catalyzed reaction of ATP with D-luciferin. The emitted light is proportional to the ATP concentration. Briefly, 50 µL of mammalian cell lysis solution were added to 100 µL of cell suspension. Then 50 µL of substrate solution were added to the wells and the luminescence was measured by Victor reader.

2.1.2.2. Granulosa cell steroid production. 10^4 viable cells/well were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) in 200 µL CM supplemented with 28 ng/mL androstenedione [38] and treated with NPs for 48 h as above indicated. After a 48 h incubation, culture media were collected, frozen and stored at –20°C until progesterone (P4) and estradiol 17β (E2) determination. For the direct immunoenzymatic determination of steroid levels, Estradiol ELISA and Progesterone ELISA kits (Dia.Metra s.r.l, Spello, PG, Italy) were used. The kits are based on competitive colorimetric immunoassay methods. As for Estradiol ELISA kit, sample media were incubated at 37°C for 2 h and after three washings, 100 µL of TMB substrate were added; its reaction with H_2O_2 is catalyzed by HPR enzyme present in the bound fraction. The reaction product, incubated for 30 min in the dark, develops a blue color that turns to yellow after the stop solution has been added.

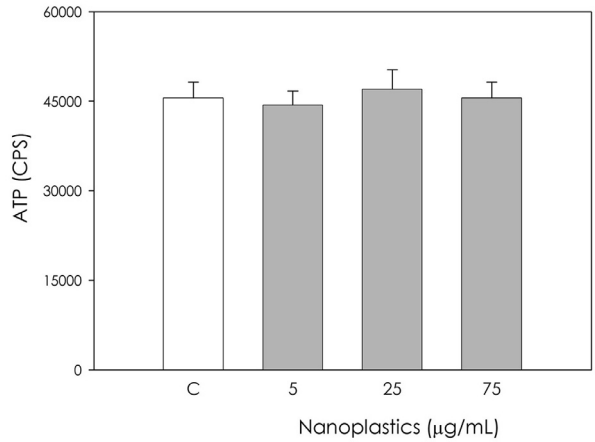

Fig. 1. Effect of the treatment with nanoplastics (5, 25, and 75 µg/mL) for 48 h on swine granulosa cell metabolic activity using ATP content assay test. Data, expressed as counts per second (CPS), represent the mean ± SEM of 6 replicates/treatment repeated in 6 different experiments.

The concentration of the hormone is calculated on the basis of a 5-point calibration curve from 0 to 2,000 pg/mL. The data are processed by the spectrophotometer; the absorbance is read at 450 nm against a reference wavelength of 620–630 nm. The sensitivity of the assay is 8.6 pg/mL; the intra-assay CV is <9%. The same principle has been used for the direct immunoenzymatic determination of progesterone; the concentration of progesterone in the sample is calculated on the basis of a 4-point calibration curve from 0 to 40.0 ng/mL. The ELISA progesterone kit requires an hour incubation at 37°C; after removing the unbound antibody, 100 µL of substrate TMB are added and the plate is left to incubate for 15 min at 37°C away from light. When the reaction is stopped, the absorbance is read at 450 nm against a reference wavelength of 620–630 nm using the Victor Reader [39]. The sensitivity of the assay is 0.05 ng/mL; the intra-assay CV is <4%.

2.1.2.3. Granulosa cell redox status.

2.1.2.3.1. Granulosa cell superoxide (O_2^-) production. O_2^- production was evaluated by WST-1 (4 - [3 - (4 - iodophenyl) – 2 - (4 - nitrophenyl) - 2H – 5 - tetrazolium] - 1,3-benzene disulfonate) test (Roche, Mannheim, Germany). The assay is based on the cleavage of the water-soluble tetrazolium salt WST-1 to a yellow-orange, water soluble formazan. Evidence exists that tetrazolium salts can be used as a reliable measure of intracellular O_2^- production [40–42]. 10^4 viable cells/200 µL of CM were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany), treated with NPs as above described and incubated for 48 h. Twenty microliter WST-1 were added to cells during the last 4 h of incubation and absorbance was then determined using the Victor reader at a wavelength of 450 nm against 620 nm.

2.1.2.3.2. Granulosa cell nitric oxide (NO) production. 10^5 viable cells/200 µL CM were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated with NPs for 48 h as above described. NO was assessed by measuring nitrite levels in culture media by the microplate method

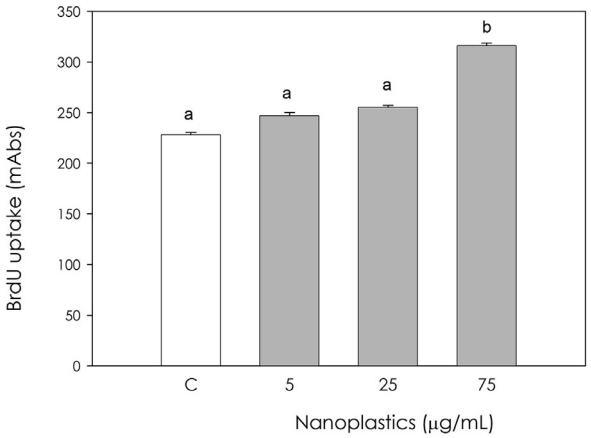

Fig. 2. Effect of the treatment with nanoplastics (5, 25, and 75 µg/mL) for 48 h on swine granulosa cell proliferation using 5-bromo-2′-deoxyuridine (BrdU) incorporation assay test. Data, expressed as milliAbs units, represent the mean ± SEM of 6 replicates/treatment repeated in 6 different experiments. Different letters on the bars indicate a significant difference ($P < 0.05$) among treatments as calculated by ANOVA and Scheffè' F test.

based on the formation of chromophore after reaction with Griess reagent, which was prepared fresh daily by mixing equal volumes of stock A (1% sulfanilamide, 5% phosphoric acid) and stock B (0,1% N-[naphthyl] ethylenediaminedihydrochloride) [43].

2.1.2.3.3. Granulosa cell superoxide dismutase (SOD) activity. SOD activity was determined by a SOD Assay Kit (Dojindo Molecular Technologies, Japan). 2×10^5 cells/200 µL CM were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated for 48 h with NPs as above detailed. After centrifugation for 10 min. at 400 × g, the surnatants were discarded and cells were lysed adding cold Triton 1% in TRIS HCl (100 µL/10^5 cells) and incubating on ice for 30 min. Cell lysates were tested without dilution and a standard curve of SOD ranging from 0.156 to 20 U/mL was prepared. The colorimetric assay was performed measuring formazan produced by the reaction between a tetrazolium salt and a superoxide anion (O_2^-), produced by the reaction of an exogenous xantine oxidase. The remaining O_2^- is an indirect hint of the endogenous SOD activity. The absorbance was determined with Victor Reader reading at 450 nm against 620 nm [44].

2.1.2.3.4. Granulosa cell nonenzymatic scavenging activity. The Ferric Reducing Activity of Plasma (FRAP) assay is a colorimetric method based on the ability of the antioxidant molecules to reduce ferric-tripiridyltriazine (Fe^{3+}TPTZ) to a ferrous form (Fe^{2+}TPTZ). Fe^{2+} is measured spectrophotometrically via determination of its colored complex with 2,4,6-Tris(2-pyridyl)-s-triazine (Fe^{2+} TPTZ). TPTZ reagent was prepared before use, mixing 25 mL of acetate buffer, 2.5 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) 10 mM in HCl 40 mM and $FeCl_3-6H2O$ solution. Briefly, 2×10^5 viable cells/200 µL CM were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated with NPs for 48 h as above described. At the end, the plates were centrifuged for 10 min at 400 × g, the supernatants were discarded and cells were lysed by adding cold Triton 0.5% (v/v) + PMSF in PBS (200 µL/well), and incubating on ice for 30 min. The test was performed on 40 µL of cell lysates added to Fe^{3+} TPTZ reagent and then incubated at 37°C for 30 min. The absorbance of Fe^{2+} TPTZ was determined by Victor Reader at 595 nm. The ferric reducing ability of cell lysates was calculated by plotting a standard curve of absorbance against $FeSO_4-7H_2O$ standard solutions [36].

2.3. Statistical analysis

The experiments were repeated at least 6 times (6 replicates/treatment). In each experiment, the ovaries were collected from 40 gilts (240 ovaries in total). Experimental data are presented as mean ± SEM; statistical differences between treatments were calculated with ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's F test; P values <0.05 were considered to be statistically significant.

3. Results

3.1. Granulosa cell growth

NPs did not affect granulosa cell viability evaluated as ATP production (Fig. 1). On the contrary, NPs significantly increased ($P < 0.05$) cell proliferation, evaluated by BrdU incorporation, but only at the highest concentration (Fig. 2). No effects were detected in the presence of the other tested concentrations.

3.2. Granulosa cell steroidogesis

Granulosa cell steroidogenesis was affected by nanoplastics. In particular, E2 secretion was significantly stimulated by the treatment with the examined concentrations ($P < 0.001$) without differences among the different doses (Fig. 3A).

In contrast, P4 secretion was inhibited ($P < 0.05$) at the two highest tested concentrations, while the lowest one was ineffective (Fig. 3B)

3.3. Granulosa cell redox status

The generation of O_2^- was significantly increased ($P < 0.001$) by NPs at the highest concentration tested while the lower concentrations were ineffective (Fig. 4).

NO output was significantly stimulated by 25 and 75 µg/mL of NPs without difference among dosages ($P < 0.05$) while the lowest concentration tested exerted a lower stimulatory effect on NO production (Fig. 5).

Superoxide dismutase activity and the scavenging activity, represented by non-enzymatic antioxidant power, were both significantly stimulated by NPs ($P < 0.001$; $P < 0.05$) (Fig. 6) ($P < 0.05$) at the highest concentration tested; cell treatment with the other dosages resulted ineffective.

4. Discussion

The term plastics includes a wide range of synthetic or semi-synthetic materials that are widely used in industry, agriculture, packing, as well as in daily life. Therefore,

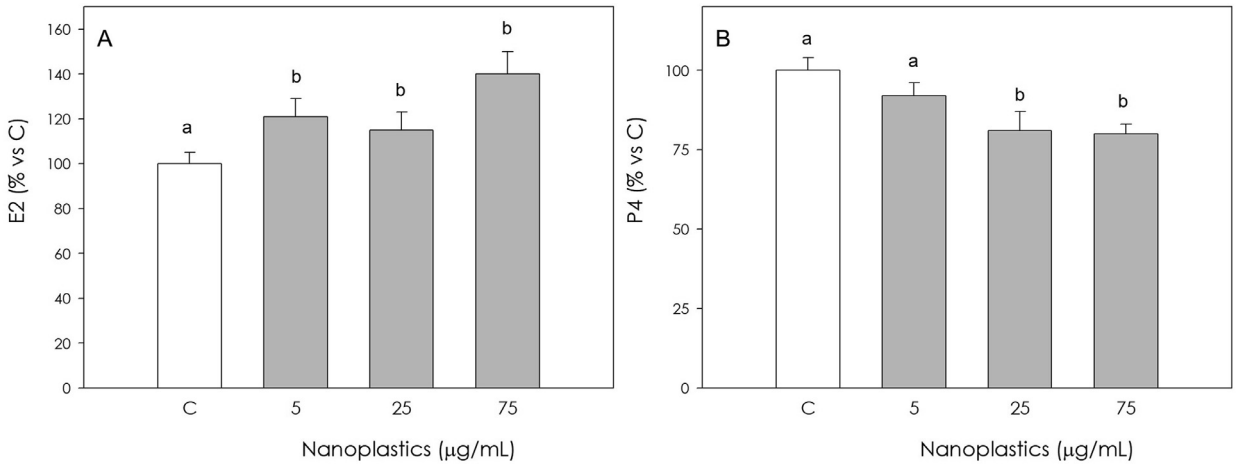

Fig. 3. Effect of the treatment with nanoplastics (5, 25, and 75 µg/mL) for 48 h on swine granulosa cell estradiol 17β (E2) production (A) or progesterone production (B) using ELISA assay. Data, expressed as % versus control (C), represent the mean ± SEM of 6 replicates/treatment repeated in 6 different experiments. Different letters on the bars indicate a significant difference ($P < 0.05$) among treatments as calculated by ANOVA and Scheffè' F test.

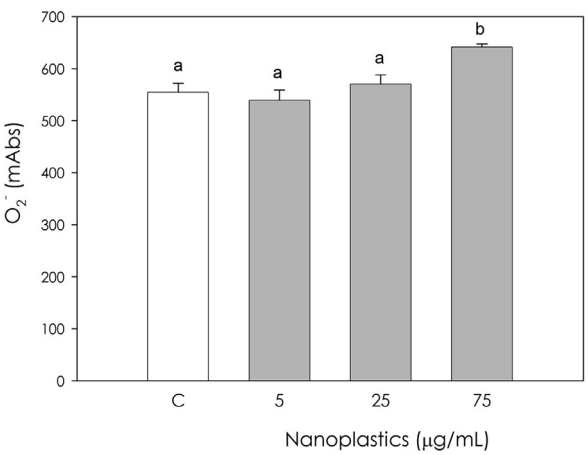

Fig. 4. Effect of the treatment with nanoplastics (5, 25, and 75 µg/mL) for 48 h on swine granulosa cell superoxide anion (O_2^-) generation using colorimetric assay. Data, expressed as milliAbs units, represent the mean ± SEM of 6 replicates/treatment repeated in 6 different experiments. Different letters on the bars indicate a significant difference ($P < 0.001$) among treatments as calculated by ANOVA and Scheffè' F test.

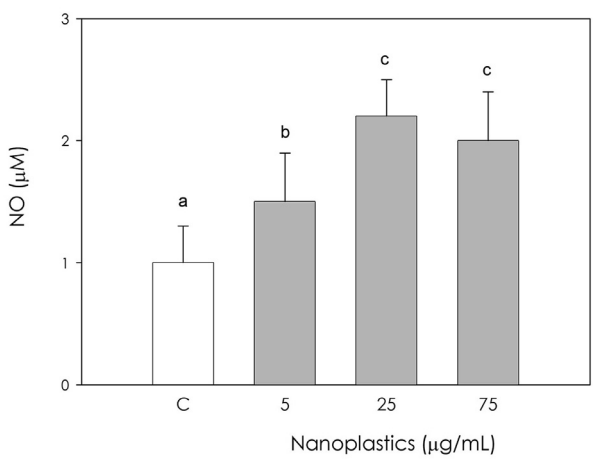

Fig. 5. Effect of the treatment with nanoplastics (5, 25, and 75 µg/mL) for 48 h on swine granulosa cell nitric oxide (NO) production using Griess Assay. Data, expressed as µM, represent the mean ± SEM of 6 replicates/treatment repeated in 6 different experiments. Different letters on the bars indicate a significant difference ($P < 0.05$) among treatments as calculated by ANOVA and Scheffè' F test.

the global production of plastics is still increasing with a current annual production exceeding 380 million tons [45]. A significant amount of plastic waste is discharged to the environment directly, or as a result of improper reuse or recycling, with an estimate of 26 billion tons by 2050 [46], thus resulting in its ubiquitous distribution in the water, soil, and atmosphere [47]. Once released to the environment, plastic debris are degraded to smaller particles known as micro- or nanoplastics [48]. Particular concern has been raised about nanoplastics (NPs) since they could be readily ingested thus entering in blood and cells [49]. Moreover, the abundant surface area of NPs makes them an excellent carrier for pollutants, such as organic contaminants and heavy metals and may serve as a novel habitat for colonization for microbial pathogens (ie, bacteria and viruses) [50]. Unfortunately, knowledge gaps still exist as regards to the determination and quantification of NPs [51] even if bioaccumulation and biomagnification occur [52]. The major concerns about NPs pollution have been raised by studies on marine organisms (revised by Ferreira et al) [53]. The effects of NPs have been also tested in vivo in mammalian models (mainly mouse) and several in vitro studies have been published using cell lines (revised by Yong et al) [54]. Present research was undertaken to investigate the effects of NPs on a primary culture of ovarian cells, a validated model of endocrine and reproductive cells [28] exploring the main functional parameters. Ovaries were collected from swine, an animal species frequently used in biomedical and translational research [55,56]. We studied the effect of NPs on granulosa cell growth, an essential process which guarantees a proper follicular development. Our results point out that

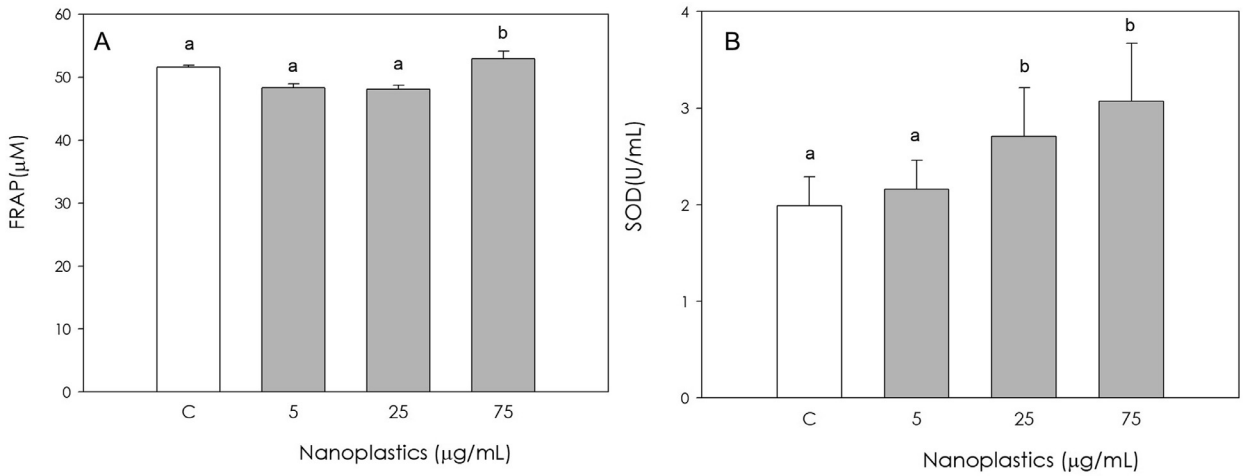

Fig. 6. Effect of the treatment with nanoplastics (5, 25, and 75 μg/mL) for 48 h on swine granulosa cell nonenzymatic scavenging activity by swine granulosa cells using the FRAP assay (A) and superoxide dismutase activity using SOD assay (B). Data, expressed as μM for FRAP and U/mL for SOD, represent the mean ± SEM of 6 replicates/treatment repeated in 6 different experiments. Different letters on the bars indicate a significant difference ($P < 0.001$; $P < 0.05$) among treatments as calculated by ANOVA and Scheffè' F test.

the examined NPs concentrations did not affect granulosa cell metabolic activity, evaluated as ATP production, but the highest concentration tested stimulated cell proliferation, in agreement with Poma et al [33] who documented NPs biological action in human fibroblast Hs27 cell line. As for the absence of effect in the presence of low concentrations, it should be noted that, in general, MPs can enter an organism either by inhalation or by ingestion. Absorption across the gastrointestinal tract is relatively low, especially for MPs, which appear to have little toxicity. However, NPs are more readily absorbed and may accumulate in the brain, liver and other tissues in aquatic species and other animals [57]. In vitro models of human uptake of nanoparticles have shown that NPs can interact with proteins, lipids and carbohydrates thus forming a 'shell' which could guarantee an easier transport across membranes by clathrin- and dynamin-dependent endocytosis or by micropinocytosis [58].

One of the main features of granulosa cell is represented by steroidogenesis, a finely tuned process that guide the follicle during its development [59]. Growing evidences indicate that endocrine disrupting chemicals interfere with ovarian steroidogenesis [60,61]. Several studies demonstrated a disruption of granulosa cell steroidogenesis induced by molecules used in plastic industry (bisphenol A, bisphenol S, phthalates) [43,62–64]. In particular, bisphenols have been found to critically impair steroidogenesis in human [62], ovine [65], swine [43,64], and murine granulosa cells [66]. Several concerns have been raised against phthalates, which have been demonstrated to affect granulosa cell steroidogenesis in human [67,68], rat [69] and mouse [70]. However, to our knowledge, at present the effect of NPs on granulosa cell steroidogenesis has not been investigated. Instead, a reproductive risk due to endocrine disturbance by NPs has been recently demonstrated in male rats by Amereh et al [71]. The authors suggested that the testicular effects documented in the study may be caused by oxidative stress. In general, enzymatic and nonenzymatic defense systems enables cells to live in an oxidative environment, performing necessary biochemical processes and even using ROS as signaling molecules. Given their high reactivity and short half-life, measurement of reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels follicular microenvironment has led to conflicting results about their role in fertility. Nevertheless, increasing research in this field has confirmed that modulation of ROS levels through ROS scavenging systems may regulate follicular development and/or survival. In addition, ROS and RNS might be involved in the initiation of apoptosis in antral follicles and are a necessary signal for ovulation [72]. Our results showed an increase in both the examined radical species and the scavenger systems, thus suggesting a NPs-induced general disruption of granulosa cell redox status. Other authors demonstrated that NPs interferes with redox status [73,74]. The effects pointed out in our cellular model, could be detrimental since adequate ROS and NO levels are crucial for a correct physiological follicle development which also relies on new vessel growth [75]. Taken together, our results demonstrate a disruptive effect of polystyrene NPs on the main functional parameters of cultured swine granulosa cells. It appears also important to better explore the effects of other plastics as nanoparticulate since several reports raise concerns [76,77]. In conclusion, we advise for the potential negative effect of polystyrene NPs on reproductive function, in particular on female fertility. Additional research is necessary to study the bioaccumulation of NPs with their adsorbed contaminants, which could eventually affect health and fertility in humans and animals.

Author contributions

Basini G. conceptualization, methodology, writing—review and editing, supervision **Bussolati S., Andriani L., Berni P.** data collection**; Grolli S., Ramoni R.** writing—review and editing **Bertini S., Iemmi T., Menozzi A.,** fund-

ing acquisition, writing—review and editing; writing **Grasselli F.,** writing—original draft preparation and review and editing; All authors have read and agreed to the published version of the manuscript.

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