Development of mammalian embryos exposed to mixed-size nanoparticles

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Summary

Inhaled or ingested ultrafine nanoparticles and their effects on early pregnancy remain polemic. The objectives of the study were: (a) to determine the embryotoxic effects of nanoparticles at the 2-cell stage and (b) to localize the internalized nanoparticles in the blastocyst. Thawed mouse 2-cell embryos (no. = 128) were exposed to either mixed-size polystyrene-based nanoparticles (11 million/ml) or control G1.3 medium and assessed after 72 hours. Additionally, blastocysts (no. = 146) were exposed to nanoparticles and analyzed. The results showed that the nanoparticles did not inhibit 2-cell embryo development to the blastocyst stage (89.4 vs 96.8%; treated vs control). There were no differences in hatching (34.8 vs 43.5%), implantation (13.6 vs 24.2%) and degeneration (10.6 vs 3.2%). Delayed exposure to nanoparticles showed similar percent hatching (40.7 vs 47.3%) and implantation (17.6 vs 20.0%). Although nanoparticles were internalized, embryo development was not inhibited suggesting a lack of embryotoxicity. During hatching, the larger nanoparticles adhered to the extruding blastocyst, preferentially on trophoblasts, but interference was insignificant. Exposure to polystyrene-based nanoparticles at the concentration tested are not associated with embryonic loss.

Key words: First trimester; Blastocyst; Preimplantation embryo; Nanoparticles; Nanotoxicity.

Introduction

Nanoparticles are ultrafine particles (UF or UFP, PM 0.1, particulate matter or particle mass < 100 nm diameter) synthesized from materials such as cadmium selenide [1, 2], gold [3], silver [4], perylene [5], polystyrene [6], carbon [7-8], iron oxide [9], silica [10], titanium dioxide [11], or organics such as latex [12], polylactic acid [13], polyglycolic acid [14] and polyalkylcyanoacrylate [15]. Nanoparticles are increasingly being used in new technological tools such as nano-tagged antibodies to identify molecules or organisms, identification of sequences, nanoshells to deliver drugs for cancer or gene therapy [16], tissue implants and cellular imaging [17, 18].

Nanoparticles are also found in sunblocks, talc, polish, toners and released airborne through automotive and power plant emissions as pollutants composed of either carbon, sulfates, nitrates [19, 20], aluminum, silicon, or titanium [21]. Recent studies have associated pollutant nanoparticles with reduced fetal growth [22] and genetic abnormalities in infants [20] suggesting mutagenic toxicity. However, in vitro studies of nanoparticles injected into non-mammalian Xenopus embryos have demonstrated a lack of toxicity [2]. More information is needed to understand the effects of nanoparticles on mammalian embryogenesis. The null hypothesis here was that nanoparticles did not have an effect on early embryo development. The study design involved using polystyrene-based fluorescent nanoparticles of various sizes to emulate exposure to mouse embryos. The objectives were: (a) to assess the development of 2-cell embryos exposed to nanoparticles and (b) to localize the nanoparticles after interaction with blastocysts in vitro.

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Materials and Methods

Preparation of mouse embryos

Cryopreserved two-cell stage mouse embryos were obtained from a commercial source (Embryotech Laboratories, Wilmington, MA) and thawed according to the supplier's instructions. Briefly, each straw containing the frozen embryos was warmed at room temperature (23°C) for 2 min followed by rapid warming at 37°C for 2 min. The embryos were expelled out of the straws and washed through two changes of prewarmed G1.3 culture medium (Vitrolife Fertility Systems, Göteborg, Sweden) supplemented with 10% synthetic serum substitute (SSS, Irvine Scientific CO., Santa Ana, CA). The 2-celled embryos thawed from different straws were pooled and equally divided into Falcon 3037 culture dishes (Becton Dickinson, Franklin Lakes, NJ), each with 1 ml of supplemented G1.2 medium containing either 0 (control) or 11.0 million/ml centrifuge-washed fluorescent nanoparticles (constellation, Molecular Probes Inc., Eugene, OR) and incubated (5% CO_2 in air mixture at 37°C for 24 hours). The nanoparticles were supplied as a kit containing mixed-size ultrafine polystyrene particles from 40 nm to over 120 nm microsphere-size with different fluorescent colors corresponding to the particle size. The smallest particles fluoresced green or red and the size verified against 40 nm carboxylated polyacrylonitrile nanobeads (Fluka Chemie AG GmbH, Buch, Switzerland).

After incubation, a preliminary assessment of each group of embryos was made and the cell-stages noted. After four days of incubation, the groups of embryos were scored according to stage of development. Embryonic stages were visually confirmed using phase contrast microscopy and recorded. The experiment was conducted in triplicates to control for culture conditions. A portion of the control embryos at the early blastocyst stage was divided into separate culture dishes with supplemented G1.3 medium containing either 0 or 11.0 million/ml nanoparticles (delayed exposure group) and incubated for an additional 48 hours at 37°C in a 5 % CO_2 in air mixture. At the

end of the incubation period, the developmental stages of the blastocysts (early, expanded, hatching, implanted on the culture dish or degenerated) were assessed and recorded. Degenerated blastocysts were characterized by dark shrunken cytoplasm retracted from the zona pellucida, presence of vacuoles, blebbing and granulation. Each group of blastocysts was pipetted onto a clean glass slide with vaseline droplet posts. The vaseline droplet posts were placed where the four corners of the cover slip were located to reduce excessive pressure on the blastocysts similar to the procedure used in the sperm penetration assay to examine oocytes. The location, color and size of the nanoparticles in each blastocyst were determined using UV-epifluorescence microscopy techniques.

Statistical analysis

The results are presented as percentages of embryo stage development and significant difference analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA) and chi-square statistics. A difference with $p < 0.05$ was considered significant.

Results

There was no difference between control and nanoparticles-exposed embryos in terms of development to the blastocyst stage (Table 1). Although numerically fewer exposed-exposed embryos hatched when compared with the control, it was not significant. The percentages of 2-cell embryos developing to the blastocyst stage were 89.4 and 96.8% for the exposed-exposed and control groups, respectively. Interestingly, nanoparticles-exposed 2-cell embryos that reached the hatching stage had similar percent implantation when compared with the control embryos (Table 1).

Table 1. — *The effect of multi-sized nanoparticles on the in vitro development of 2-cell and blastocyst-stage mouse embryos.*

Group	No.	Early or expanded (%)	No. at hatching (%)	Hatched and implanted (%)	No. of degenerated (%)
Exposure at 2-cells:					
Control	62	18 (29.0)	27 (43.5)	15 (24.2)	2 (3.2)
Nanoparticles	66	27 (40.9)	23 (34.8)	9 (13.6)	7 (10.6)
Exposure at blastocyst:					
Control	55	16 (29.1)	26 (47.3)	11 (20.0)	2 (3.6)
Nanoparticles	91	33 (36.3)	37 (40.7)	16 (17.6)	5 (5.5)

When exposure to the nanoparticles was delayed until the blastocyst stage, there were no significant differences in hatching, implantation or degeneration. Large nanoparticles (> 100 nm) were not associated with the blastocysts while smaller nanoparticles in hatched blastocysts were localized at either the trophoblast cells alone (16/29; 55.2%) or at both the trophoblast and inner cell mass cells (13/29; 44.8%). There were no hatched blastocysts with nanoparticles localized solely at the inner cell mass cells.

Discussion

Mouse embryos at the 2-cell and blastocyst stages exposed to mixed-size nanoparticles did not differ in developmental capacity when compared with control embryos. The results are consistent with the study on amphibian Xenopus embryos [2] that did not demonstrate toxicity. However, abnormalities occurred at high concentrations of over five billion nanoparticles per cell. At low concentrations, they were found in all cell types such as the somites, neurons and axons, neural crest, ectoderm and endoderm. The nanoparticles were initially located in the cytoplasm of early stage embryonic cells but translocated to the nuclei at the mid-blastula stage [2]. The present study design utilized mixed-size nanoparticles similar to the natural situation. Interestingly, embryos exposed at the 2-cell stage demonstrated a trend towards less hatching albeit the sample size precluded significance. Delaying nanoparticle exposure until the blastocyst stage did not affect hatching and implanting on the dish suggesting that molecular processes involved with these events were completed before the blastocyst stage. Problems with embryonic hatching have been observed in women over 35 years of age whereby assisted hatching becomes necessary following in vitro fertilization procedures. The association between older female patients and the effects of accumulated exposure to the type and size of the nanoparticles on fertility remain unknown.

Fluorescent microscopy showed that smaller nanoparticles were internalized by endocytosis or pinocytosis. Internalized nanoparticles could be distinguished from external adhering nanoparticles by their reduced quantum scattering of Rayleigh or dimmer fluorescence, same plane of focus with cell organelles and the lack of Brownian movement [4]. The results suggest that the internalized nanoparticles did not affect cellular processes or expression of factors needed for development. It has been reported that internalized nanoparticles caused an increase in reactive oxygen species (ROS), elevated intracellular calcium, activation of transcription factors [7] and apoptosis [11]. Conversely, nanoparticles were shown to stabilize mRNA and to stimulate protein release [23]. However, the present data could not corroborate the reported cellular activities.

Another interesting observation was the preferential localization of the nanoparticles to the trophoblast cells. Few nanoparticles were located in the inner cell mass. A possible explanation might be the larger cell dimension of the trophoblast accommodating the internalized nanoparticles. This suggests that the tightly packed inner cell mass layer of cells either prevented uptake of nanoparticles or they actively expelled the internalized nanoparticles during compaction phase.

The presence of nanoparticles or ultrafine particles in the environment is a topic of interest and has sparked a new branch of science termed nanotoxicology by Donaldson and colleagues [24, 25]. Recent epidemiological studies have suggested an association between genetic abnormalities in infants and exposure to pollutants in utero [19, 22, 26]. Moreover, it has been reported that more than ten million ultrafine particles are ingested per person every day and internalized by intestinal lymphoid aggregates [21]. Other conditions associated with expo-

sure to nanoparticles include inflammatory bowel disease, ulcerative colitis, Crohn's disease [21], exacerbations of ischemic and/or arrhythmic cardiac diseases [27], pulmonary inflammation [28, 29] and accumulation in the central nervous system after translocating along axons of the olfactory nerves [8].

Synthesized nanoparticles demonstrate little toxicity and have been used to tag antibodies, identify of gene sequences, deliver drugs for cancer or gene therapy and used in cellular imaging [16-18, 30-33]. It should be noted that nanoparticles containing polystyrene were used in this study and that nanoparticles derived from other materials might behave differently. Nevertheless, more studies are needed to catalog the effects of different types, concentrations and sizes of nanoparticles during embryo development.

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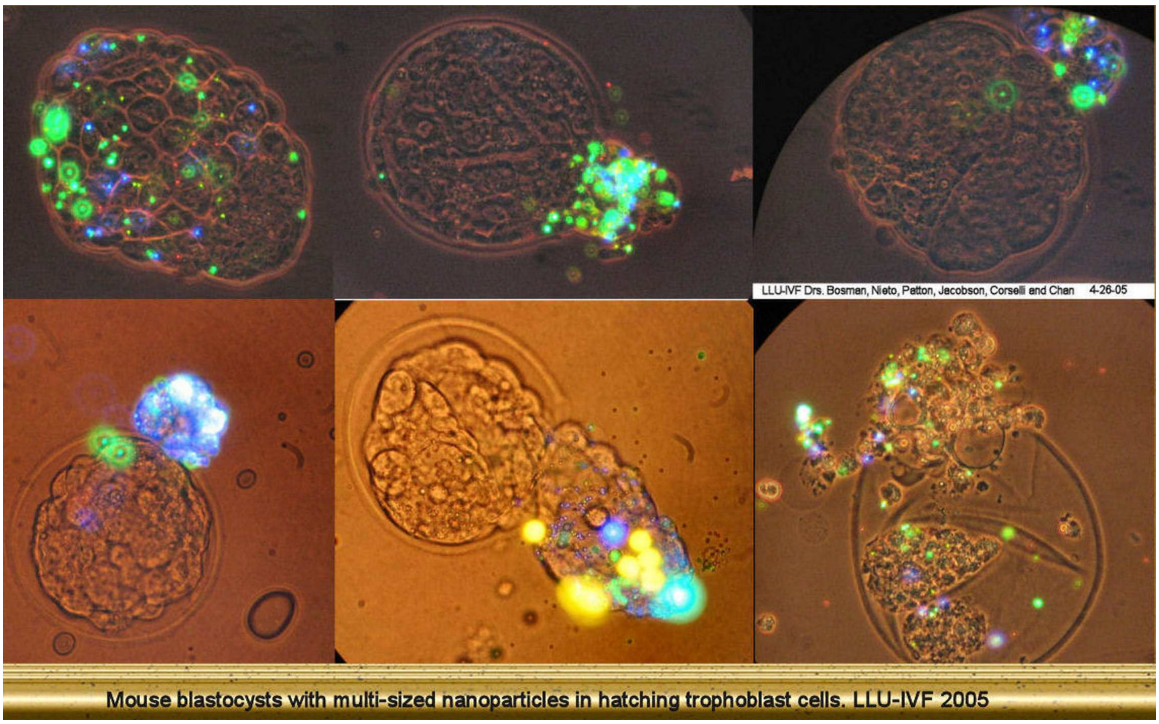

Mouse blastocysts with multi-sized nanoparticles in hatching trophoblast cells. LLU-IVF 2005