Biol. Stud. 2015: 9(1); 57–66 • DOI: https://doi.org/10.30970/sbi.0901.419
www.http://publications.lnu.edu.ua/journals/index.php/biology

UDC 620.3:591.13:636.92

EFFECT OF SILVER NANOPARTICLES ON MATURATION OF RABBIT'S OOCYTES CO-CULTURED WITH GRANULOSA CELLS *IN VITRO*

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Silver nanoparticles are widely used in different fields of medicine despite the lack of information on their influence on animal's reproductive system, mammalian gametes and embryos. We investigated the effect of different concentrations of silver nanoparticles (0, 0.01, 0.1, 1 and 10 μg/mL) on maturation of rabbit's oocytes co-culture with granulosa cells in vitro. For this purpose, we synthesized small (11.28±0.32 nm) spherical silver nanoparticles with different composite agents: polyvinylpyrrolidone and bovine serum albumin. Our results have shown that silver nanoparticles at the concentration of 10 μg/ml inhibited granulosa cells proliferation, but did not influence the oocytes maturation to metaphase-2. The loss of granulosa cells viability was confirmed by the release of calcium and lactate dehydrogenase in the culture medium. Analysis of the data showed that silver nanoparticles in concentration of 0–10 μg/mL did not influence on progesterone and cholesterol concentration in culture medium. We have hypothesized that less toxic effect of silver nanoparticles on oocytes is caused by the presence of zona pellucida with different mechanisms of cellular uptake.

Keywords: silver nanoparticles, oocytes, granulosa cells, rabbits

INTRODUCTION

Rapid progress in nanotechnology was accompanied by a lack of information concerning impact of nanoproducts on the environment, human and animal health. Silver nanoparticles (AgNPs) are widely used in different fields of medicine as antimicrobial and antiviral agents [18, 9], in treating wounds [5], in cancer therapy [26] or in creation of biomaterials [29]. Therapeutic potentials of these nanoparticles have been explored extensively despite the lack of information on their mechanism of action at molecular and cell level [22]. Several studies have confirmed that AgNPs induce cytotoxicity and genotoxity in several cell types [28, 2]. The results of AshaRani et al. (2008) with silver nanoparticles on Zebrafish embryonic model suggest that silver nanoparticles induce a dose-dependent toxicity in embryos, which hinders normal development [4]. However, silver nanoparticles did not affect chicken embryo development [35] and their bone structure [31], but can cause inflammation in liver [30]. Li et al. (2010) showed that silver nanoparticles increased apoptosis, decreased cell numbers and decreased success implantation rates in mouse blastocyst [23].

However, interactions of nanomaterials with animal's reproductive system, mechanism of their action on mammalian gametes and embryos remain unanswered. In the present study, rabbit's primary-derived granulosa cells (GC) and oocytes were chosen as a model system for testing toxicity of silver nanoparticles. We investigated the effects of different concentrations of AgNPs on maturation of rabbit's oocytes co-culture a with granulosa cells *in vitro*.

MATERIALS AND METHODS

Silver nanoparticles were synthesized using modified procedure, as described by Solomon et al. (2007) [33]. All chemicals for AgNPs synthesis were purchased from Sigma-Aldrich. 0.001 M silver nitrate ($AgNO_3$) was added to 0.002 M sodium borohydride ($NaBH_4$) at proportion 1 : 3. Color of solution changed from colorless to yellow, indicating the formation of nanoparticles. To prevent agglomeration and to stabilize AgNPs, we used polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) as capping agents. Solutions of AgNPs with stabilizing agent were incubated at room temperature overnight for binding composites with particles surface. To remove traces of $NaBH_4$, $NaNO_3$ and unbound stabilizing agents, nanoparticles were pelleted by centrifugation at 25000 g for 50 min. The pellets were washed twice in ultrapure water and sonicated to prepare homogeneous nanoparticles suspension.

Size and morphology of AgNPs were studied via ultraviolet-visible spectroscopy (UV-Vis) and transmission electron microscopy (TEM). To determine peaks of surface plasmon absorption, AgNPs-PVP and AgNPs-BSA were scanned in the range of 350–500 nm on a spectrophotometer. TEM samples were prepared by placing a drop of solution with AgNPs directly on a carbon grid, and imaging was done at 200 kV. Size distribution of the particles was estimated using TEM images by measurement of diameters of at least 100 nanoparticles.

Cumulus-oocyte complex and granulosa cells were obtained from ovaries of rabbits slaughtered at the abattoir. The ovaries were transported to the laboratory within 1 h in saline with 50 μg/mL of gentamicin at 38 °C. The follicular fluid with granulosa cells was aspirated and centrifuged at 800 g for 5 min. The precipitated cells were carefully resuspended in TCM 199 containing antibiotics and 10 % fetal calf serum (FCS). The oocytes were screened under microscope for having compact cumulus cells and clear cytoplasm. High quality oocytes were thoroughly washed in TCM 199 medium with 10 % FCS, 0.2 mM pyruvate and 50 μg/mL of gentamicin randomly assigned to experimental groups. Granulosa cells were disaggregated by 30 IU hyaluronidase in PBS and separated by centrifugation at 900 g for 5 min. Maturation media consisted of TCM 199 supplemented with sodium bicarbonate, 10 % FCS, 5 μg/mL FSH, 50 μg/mL LH, 50 μg/mL gentamicin. 20 high quality oocytes and 1×10^6 granulosa cells/ml were co-cultured in the cultural dish.

Appropriate volume stock solution of nanoparticles (AgNPs-PVP and AgNPs-BSA) was added to the cultures to obtain the concentrations of AgNPs: 0, 0.01, 0.1, 1 and 10 μg/mL. Oocytes and granulosa cells with different concentrations of silver nanoparticles were co-cultured in 100 μL drops under mineral oil in CO_2 incubator for 24 h at 38 °C.

After 24 h, the maturation of oocytes was assessed under stereo zoom microscope (Nicon, Japan) according to nuclear maturation and evidence of polar body [11]. Number of viable granulose cells was counted after Trypan blue staining [8].

After 24 h, culture medium was sampled by centrifugation at 900 g for 5 min at 4 °C. Samples were used to determine concentration of calcium (Ca), cholesterol and lactate dehydrogenize activity (LDH) using commercially available kits according to manufacturer's instruction (Human GmbH, Germany). The concentration of progesterone was assessed by enzyme immunoassay analyze using DRG Instruments GmbH kit (Germany) according to the manufacturer's protocol. All tests were measured by immunoassay and biochemical analyzers.

All experiments were done in triplicate and the results were presented as mean ± standard deviation. All statistical analyses were performed by using the Minitab 15 English statistical software package. Differences between groups were determined by Student *t* tests.

RESULTS AND DISCUSSION

The nanoparticles with BSA and PVP showed good stability and uniform dispersion throughout the test period. Agglomeration and precipitation were not observed. In contrast, AgNPs without capping agents were agglomerated after storage (Fig. 1, *A*). UV-visible spectroscopy is one of the most widely uses techniques for particle size characterization of silver nanoparticles [27]. It was found that both absorption spectrums AgNPs-PVP and AgNPs-BSA (Fig. 1, *B*) showed surface plasmon absorption maximum at ~ 400 nm. These results indicate that both preparations are mainly composed of small spherical silver nanoparticles [2]. Transmission electron microscopy analysis confirmed that the nanoparticles obtained in this study were spherical in shape and mono-dispersed (Fig. 1, *C*). Size distribution analysis showed that sizes of composite nanoparticles lie in the range from 3 to 20 nm with an average diameter of 11.28±0.32 nm (Fig. 1, *D*).

Relationship between oocytes and granulosa cells is essential for the development and function of ovarian follicles and promotes the production of mature oocytes competent to undergo fertilization, and give rise to a healthy preimplantation embryo development [17]. Granulosa cells provide essential nutrients (L-alanine and L-histidine and products of glycolysis etc.) for growth and development of oocyte and regulate their transcriptional activity [25]. On the other hand, expression of specific genes in granulosa cells correlates with the developmental competence of the oocyte. It is known that oocyte promotes granulosa cell proliferation and differentiation through bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) [15]. *In vitro* co-cultures of primary-derived granulosa cells and oocytes provide good reproductive models to determine possible effects of toxins and pollutants, especially nanomaterials [13, 24]. In this study we investigated the effects of different concentrations AgNPs with PVP and BSA on maturation of rabbit's oocytes co-culture with granulosa cells *in vitro*.

No significant changes in proliferation activity granulosa cells were found between the control (0 μg/mL) group and groups with silver nanoparticles at concentrations of 0.01, 0.1 and 1 μg/mL after 24 h at *in vitro* cultivation (Fig. 2, *A*). However, AgNPs-PVP and AgNPs-BSA at concentration 10 μg/mL significantly ($p<0.05$) decreased the cells viability. Number of viable cells decreased to 1.02±0.05 and $1.04\pm0.05\times10^6$/ml after exposure to AgNPs-PVP and AgNPs-BSA respectively compared to the control value $1.25\pm0.03\times10^6$/ml. Cytotoxic effects of silver nanoparticles were demonstrated in normal human lung fibroblasts and human glioblastoma cells (Starch-Capped AgNPs at dose 25–200 μg/mL with size 6–20 nm) [3]; in mouse spermatogonia stem cells (5–100 μg/mL AgNPs with size of 15 nm) [6]; in rat liver cells (10–50 μg/mL AgNPs with size 100 and

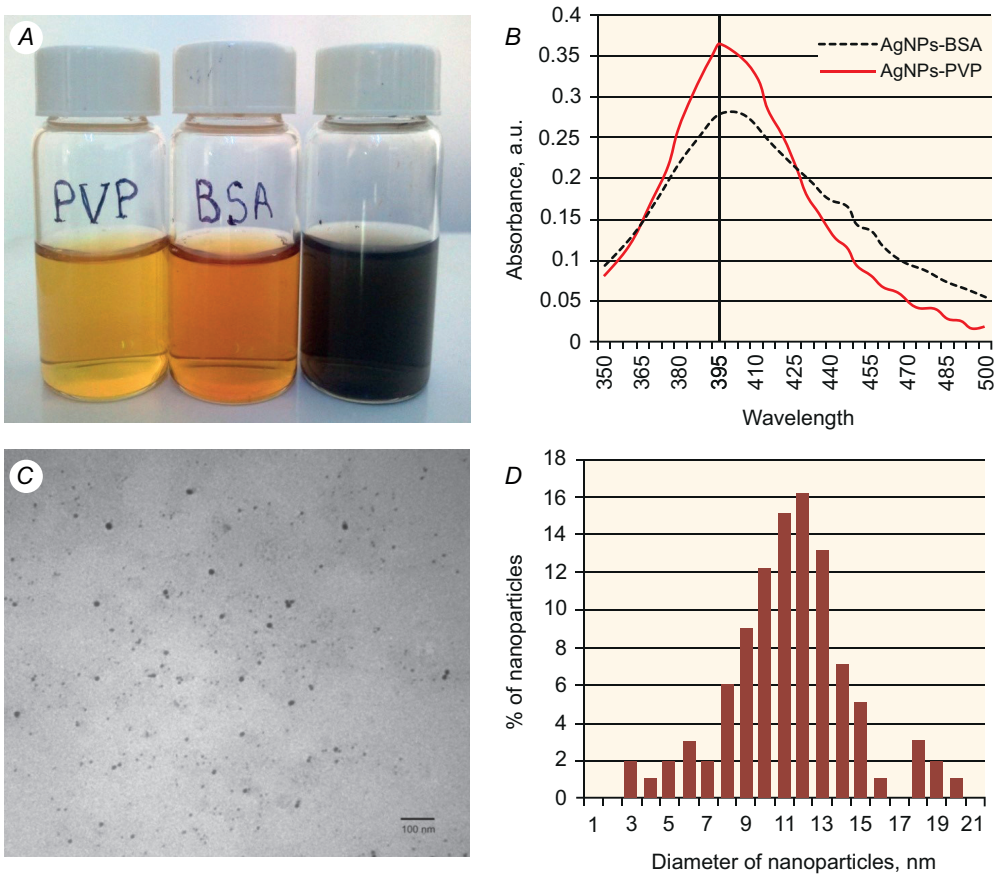

Fig. 1. Characterization of nanoparticles: *A* – from left to right: AgNPs with PVP, AgNPs with BSA and aggregation of AgNPs without capping agent; *B* – UV-vis spectrums; *C* – TEM image of AgNPs-PVP and *D* – size distribution

Рис. 1. Характеристика наночастинок: *A* – зліва направо: AgNPs з PVP, AgNPs з BSA і агрегація AgNPs без стабілізуючих речовин; *B* – UV-vis спектри; *C* – TEM зображення AgNPs-PVP і *D* – розподіл за розміром

15 nm) [14]; in mouse peritoneal macrophages (0.2–1.6 μg/mL AgNPs with FCS 68.9±30.3 nm) [28] etc. Cytotoxic mechanism of AgNPs action on mammalian cells is based on oxidative stress and inflammation caused by the generation of reactive oxygen species [2, 7]. Park et al. (2010) showed that 1.6 μg/mL AgNPs decreased intracellular glutathione level and increased NO secretion in mouse peritoneal macrophage cells [28]. Generation of reactive oxygen species causes reduced ATP content, mitochondrial damage, chromosomal aberrations, DNA damage and cell cycle abnormalities. In addition, Carlson et al (2008) observed the release of inflammatory mediators (TNF-R, MIP-2, and IL-1) into the culture media by alveolar macrophages after 24 h of exposure to silver nanoparticles with the size of 15 nm [7]. Toxic effect of AgNPs may be related to the ionization of silver from the surface of silver nanoparticles and to the direct effects of nanoparticles. The study of Kolesarova et al. (2011) on porcine ovarian cell indicated that silver ions have direct effects on cell proliferation and apoptosis through the influence on expression of growth factor IGF-I, cyclin B1 and caspase-3 [20].

Our results have demonstrated that 24 h exposure to silver nanoparticles at the concentration of 10 μg/mL resulted in a significant increase ($p<0.05$) in LDH activity of cultural medium (Fig. 2, *B*). LDH activity of cultural medium increased to 124.3±4.10 and 125.3±6.49 IU/L after exposure to AgNPs-PVP and AgNPs-BSA at the concentration of 10 μg/mL respectively compared to the control value 108.3±1.45 IU/L. This fact indicates to plasma membrane destabilization and LDH leakage into the culture medium from granulosa cells. Braydich-Stolle et al (2005) showed a slight increase in LDH leakage in spermatogonia stem cells with silver nanoparticles at the concentration of 2.5 μg/mL, indicating that they might promote cell apoptosis rather than necrosis [6]. However, the results of Carlson et al. (2008) demonstrated plasma membrane disruption in alveolar macrophages after treatment of 25 and 75 μg/mL of AgNPs [7].

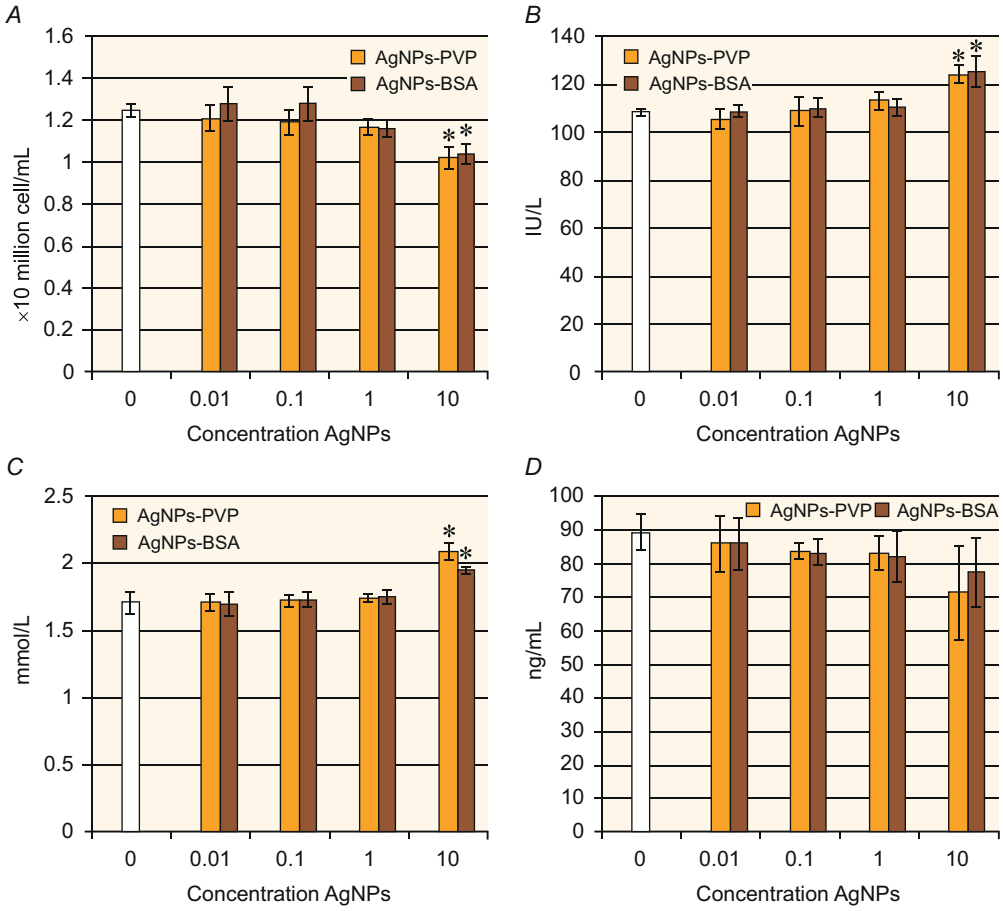

Fig. 2. Effect of AgNPs on granulosa cells proliferation and biochemical parameters of culture medium after 24 hours of maturation: *A* – number of viability cells; *B* – activity of LDH; *C* – Ca concentration; *D* – progesterone concentration (*– $p<0.05$; **– $p<0.01$)

Рис. 2. Вплив наночастинок срібла на проліферацію клітин гранульози та біохімічні показники культурального середовища після 24-х годин дозрівання: *A* – кількість життєздатних клітин; *B* – активність лактатдегідрогенази; *C* – концентрація Са; *D* – концентрація прогестерону (*– $p<0,05$; **– $p<0,01$)

Destabilization and disruption of plasma membrane in granulosa cells are confirmed by a release of calcium into the culture medium. Ca concentration increased significantly in the culture medium after 24 h of cultivation with silver nanoparticles at the concentration of 10 μg/mL (Fig. 2, *C*). Ca concentrations of cultural medium were 2.09±0.06 and 1.95±0.03 mmol/L in groups with AgNPs-PVP and AgNPs-BSA respectively compared to 1.71±0.08 mmol/L in the control group. AshaRani et al. (2009) showed that AgNPs and Ag^+ ions, which get released from the nanoparticles, may be involved in cell signalling cascades with the activation of Ca^{2+} release that further activates catabolic enzymes and damages mitochondrial membranes [2]. These data were confirmed by Haase et al (2012) on primary neurons and astrocytes cells, where they found increasing intracellular calcium levels [12].

Analysis of the data showed that silver nanoparticles in the concentration of 0–10 μg/mL did not influence steroidogenesis in granulosa cells. No significant differences in concentration of progesterone and cholesterol were found between the control group and the groups with AgNPs (Fig. 2, *D*, *A*). Toxicological study with gold nanoparticles showed a decrease in estradiol secretion by rat ovarian granulosa cells after 24 h incubation, as compared with untreated cells [34]. Kolesarova et al (2010) showed that silver ions at the highest dose (1.0 mg/mL) did not affect progesterone output in porcine ovarian granulosa cells, but at lower doses (0.09–0.5 mg/mL) they decreased the release of progesterone [19]. These results indicated that silver nanoparticles, through the output of silver ions, can be a suppressor of ovarian steroidogenesis and a potential risk factor for reproductive functions regulated by steroid hormones.

There was no significant difference in number of oocytes maturated to metaphase-2 in all groups with different concentrations of silver nanoparticles in the culture medium, as compared to the control group (Fig. 3, *B*). Although the concentrations of nanoparticles in this study was low (0.01–10 μg/ml), but lower concentrations of AgNPs 14–20 ng/ml showed toxic effects on embryos development in Zebrafish embryos [16]. Our results showed that silver nanoparticles with PVP and BSA at the concentration of 10 μg/ml inhibited granulosa cells proliferation, but did not influence oocytes maturation to metaphase-2 (Fig. 3, *C*, *D*). We have hypothesized that the less toxic effect of silver nanoparticles on oocytes was caused by the presence of zona pellucida with different mechanisms of cellular uptake. In the somatic cells the uptake of silver nanoparticles occurs mainly through endocytosis and macropinocytosis [32]. In zebrafish embryos, silver nanoparticles can passively diffuse via chorionic pore canals, creating a specific negative effect on embryonic development in a dose-dependent manner [21]. Several researchers reported the ability of AgNPs to penetrate through the blood-brain and blood-testis barrier and to get distributed in the gonads [1, 36]. The results of Ghorbanzadeh et al. (2011) demonstrated the reduction of number of primary follicles in rats, which received silver nanoparticles in the doses of 1–10 mg/kg via intraperitoneal injection [10]. Future research is needed to investigate kinetics and mechanism of AgNPs uptake through zona pellucida in mammalian oocytes and embryos.

It was found that different parameters (chemical composition of nanoparticles, size, surface modification, shape etc.) influence their biological activity and toxic potential [37]. Both AgNPs-PVP and AgNPs-BSA can affect the female reproductive system through toxic effect on granulosa cells viability and functions. Future investigations are needed to precisely estimate molecular mechanisms of action of AgNPs on mammalians gametes and embryos with the prospect of its use in medicine.

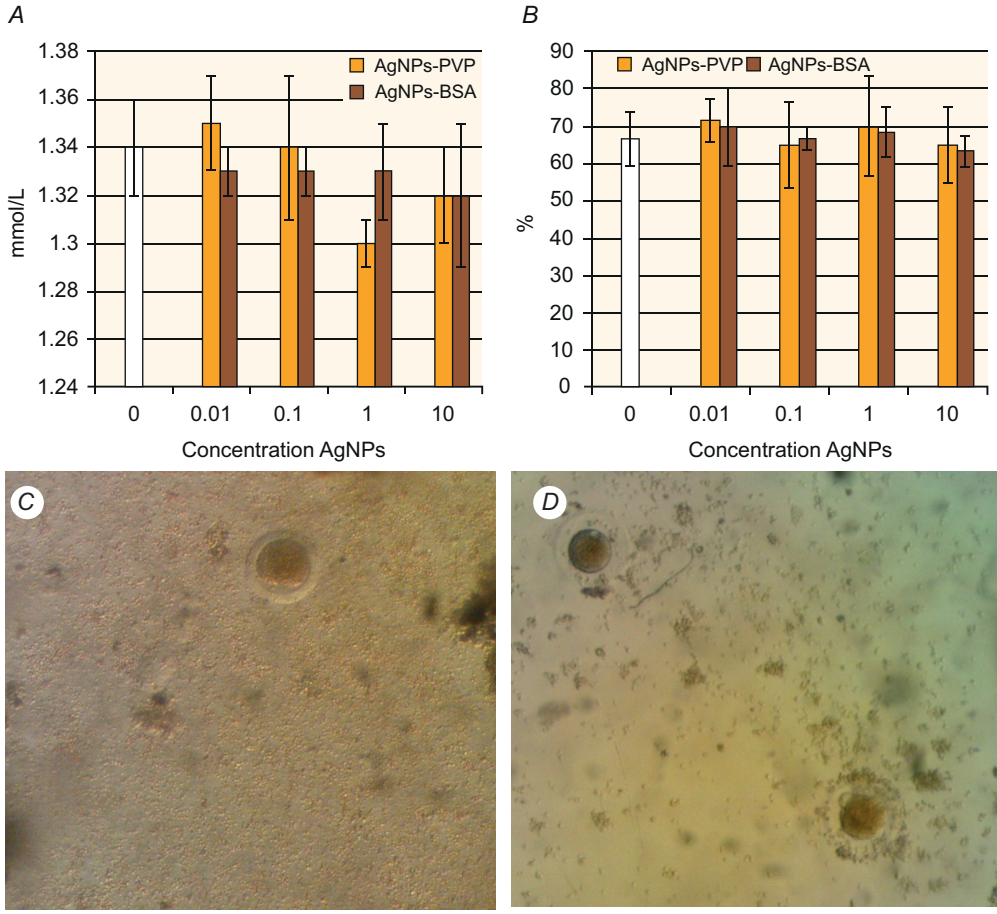

Fig. 3. Effect of AgNPs on cholesterol concentration and maturation of oocytes to metaphase-2: *A* – cholesterol concentration; *B* – percentage of maturating oocytes to metaphase-2; *C* – light microscopy image of maturating oocyte to metaphase-2 on monolayer of granulosa cells without AgNPs; *D* – oocytes and granulosa cells with 10 μg/ml AgNPs-PVP. Magnification – 7.5×10

Fig. 3. Вплив наночастинок срібла на концентрацію холестеролу та дозрівання ооцитів до метафази-2: *A* – концентрація холестеролу; *B* – відсоток ооцитів, дозрілих до метафази-2; *C* – світлова мікроскопія ооцитів, дозрілих до метафази-2 на моношарі клітин гранульози без наночастинок срібла; *D* – ооцити і клітини гранульози з 10 мкг/мл AgNPs-PVP. Збільшення – 7,5×10

CONCLUSIONS

We used small spherical nanoparticles with two different composite agents: PVP and BSA. Both AgNPs-PVP and AgNPs-BSA showed similar toxic effects on granulosa cells. Our results show that the concentration of silver nanoparticles of 10 μg/ml inhibits granulosa cells proliferation but does not influence oocytes maturation to metaphase-2.

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ВПЛИВ НАНОЧАСТИНОК СРІБЛА НА ДОЗРІВАННЯ ЯЙЦЕКЛІТИН КРОЛІВ ПІД ЧАС КОКУЛЬТИВУВАННЯ З КЛІТИНАМИ ГРАНУЛЬОЗИ *IN VITRO*

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Наночастинки срібла широко використовують у різних галузях медицини, незважаючи, що інформації про їх вплив на репродуктивну систему тварин, гамети і ембріони ссавців немає. Ми досліджували вплив різних концентрацій наночастинок срібла (0, 0,01; 0,1; 1 і 10 мкг/мл) на дозрівання ооцитів кролів при кокультивуванні з клітинами гранульози *in vitro*. Для цього ми синтезували невеликі (11,28±0,32 нм) сферичні наночастинки срібла з різними композитними речовинами: полівінілпіролідоном і бичачим сироватковим альбуміном. Наші результати показали, що наночастинки срібла в концентрації 10 мкг/мл пригнічують проліферацію клітин гранульози, але не впливають на дозрівання ооцитів до метафази-2. Наночастинки срібла

в концентрації 0–10 мкг/мл не впливають на стероїдогенез, але при концентрації 10 мкг/мл достовірно ($p<0,05$) зменшують число життєздатних клітин гранульози. Втрата життєздатності клітинами гранульози підтверджується вивільненням кальцію і лактатдегідрогенази в культуральне середовище. Аналіз даних показав, що наночастинки срібла в концентрації 0–10 мкг/мл не впливають на концентрацію прогестерону та холестеролу в культуральному середовищі. Було зроблено припущення, що менш токсичний вплив наночастинок срібла на ооцити викликаний наявністю zona pellucida із іншими механізмами клітинного поглинання.

Ключові слова: наночастинки срібла, ооцити, клітини гранульози, кролі.

ВЛИЯНИЕ НАНОЧАСТИЦ СЕРЕБРА НА СОЗРЕВАНИЕ ЯЙЦЕКЛЕТОК КРОЛИКОВ ПРИ КОКУЛЬТИВИРОВАНИИ С КЛЕТОК ГРАНУЛЕЗЫ *IN VITRO*

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Наночастицы серебра широко используются в различных областях медицины, несмотря на отсутствие информации об их влиянии на репродуктивную систему животных, гаметы и эмбрионы млекопитающих. Мы исследовали влияние различных концентраций наночастиц серебра (0, 0,01, 0,1, 1 и 10 мкг/мл) на созревание ооцитов кроликов при кокультивировании с клетками гранулёзы *in vitro*. Для этого мы синтезировали небольшие (11,28±0,32 нм) сферические наночастицы серебра с различными композитными веществами: поливинилпирролидоном и бычьим альбумином. Наши результаты показали, что наночастицы серебра в концентрации 10 мкг/мл угнетают пролиферацию клеток гранулёзы, но не влияют на созревание ооцитов в метафазе-2. Наночастицы серебра в концентрации 0–10 мкг/мл не влияют на стероидогенез, но при концентрации 10 мкг/мл достоверно ($p<0,05$) уменьшают число жизнеспособных клеток гранулёзы. Потеря жизнеспособности клеток гранулёзы подтверждается высвобождением кальция и лактатдегидрогеназы в культуральную среду. Анализ данных показал, что наночастицы серебра в концентрации 0–10 мкг/мл не влияют на концентрацию прогестерона и холестерола в культуральной среде. Мы предположили, что менее токсичное воздействие наночастиц серебра на ооциты вызвано наличием zona pellucida и иными механизмами клеточного поглощения.

Ключевые слова: наночастицы серебра, ооциты, клетки гранулёзы, кролики.

Одержано: 23.03.2015