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Original Research Article

Copper nanoparticles supported on charcoal and betacellulin – Two novel stimulators of ovarian granulosa cell functions and their functional interrelationships

Alexander V. Sirotkin [a,*], Barbora Loncová [a], Zuzana Fabová [a], Michaela Bartušová [a], Iris Martín-García [b], Abdel Halim Harrath [c], Francisco Alonso [b,**]

[a] Department of Zoology and Anthropology, Constantine the Philosopher University, Nitra, 94974, Slovakia
[b] Instituto de Síntesis Orgánica (ISO) and Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Alicante, Apdo. 99, 03080, Alicante, Spain
[c] Department of Zoology, College of Science, King Saud University, Riyadh, 11451, Saudi Arabia

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ABSTRACT

The present experiments are aimed to examine the effect of copper nanoparticles supported on charcoal (CuNPs/C), growth factor betacellulin (BTC) and their interrelationships in the control of ovarian cell functions. Porcine ovarian granulosa cells were cultured in the presence of CuNPs/C (0, 1, 10 or 100 ng/ml), BTC (100 ng/ml) and the combination of both, CuNPs/C + BTC. Markers of cell proliferation (BrDU incorporation), of the *S*-phase (PCNA) and G-phase (cyclin B1) of the cell cycle, markers of extrinsic (nuclear DNA fragmentation) and cytoplasmic/mitochondrial apoptosis (bax and caspase 3), and the release of progesterone and estradiol were assessed by BrDU test, TUNEL, quantitative immunocytochemistry and ELISA.

Both CuNPs/C and BTC, when added alone, increased the expression of all the markers of cell proliferation, reduced the expression of all apoptosis markers and stimulated progesterone and estradiol release. Moreover, BTC was able to promote the CuNPs/C action on the accumulation of PCNA, cyclin B1, bax and estradiol output.

These observations demonstrate the stimulatory action of both CuNPs/C and BTC on ovarian cell functions, as well as the ability of BTC to promote the action of CuNPs/C on ovarian cell functions.

1. Introduction

The application of copper nanoparticles (CuNPs) in techniques, animal production and medicine is growing now [1–3]. Among other effects of CuNPs, their ability to prevent oxidative stress and promote the release of growth factors and steroid hormones, cell proliferation, as well as to suppress apoptosis, could be useful for wound healing [4], stimulation of bone and cartilage formation [5], animal growth [1] and ovarian cell functions [6–8]. On the other hand, the toxic effect of some CuNPs on non-ovarian [2,9,10] and ovarian [6,8,11–13] cells, associated with changes in hormone biosynthesis and regulators of the cell cycle and apoptosis have been reported. The character of the effect of CuNPs on ovarian cells depends on their morphology and support [8]. For example, CuNPs supported on charcoal (CuNPs/C) were able to promote ovarian cell functions – to increase viability, proliferation and steroid hormones release and to reduce apoptosis by cultured porcine ovarian granulosa cells. Other five tested CuNPs inhibited these functions or affected not all the measured ovarian cell parameters [8].

The application of CuNPs as biostimulators of female reproductive processes require further studies on the character and mechanisms governing the effect of CuNPs promoters of ovarian cell functions. Furthermore, understanding the interrelationships between these CuNPs with other biostimulators, which can additionally promote female reproductive processes, could be promising from both biological and practical viewpoints.

The potential enhancers of the stimulatory effect of CuNPs on the ovary could be growth factors. Although CuNPs can increase the release of growth factors from cultured porcine granulosa cells [6], the mutual interrelationships between growth factors and CuNPs in the up-regulation of ovarian cell functions have not been studied yet. One of

* Corresponding author.Department of Zoology and Anthropology, Constantine the Philosopher University, Nitra, 949 74, Slovakia.
** Corresponding author.Instituto de Síntesis Orgánica (ISO) and Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Alicante, 03080, Alicante, Spain.
E-mail addresses: asirotkin@ukf.sk (A.V. Sirotkin), falonso@ua.es (F. Alonso).

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the growth factors promoting basic functions could be betacellulin (BTC), which belongs to the family of epidermal growth factors. There is evidence, that BTC can be a mediator of luteinizing hormone (LH) action on the ovary [14], a promoter of maturation of the oocyte-cumulus complex [14–16] ovulation [17], of prostaglandin production [15,18] and proliferation in cultured healthy [15,19] and cancer [20] cells; conversely, BTC suppressed some functions of cultured feline ovarian cells [21]. To the best of our knowledge, the action of BTC on porcine ovarian cells, as well as its possible interrelationships with CuNPs in the control of functions of these cells have not been reported yet.

The aim of the present study was: (1) to verify the effect of CuNPs/C, which stimulatory action on porcine ovarian cell functions has been previously demonstrated [8]; (2) to detect the action of BTC on these cells; and (3) to examine the possible functional interrelationships between CuNPs/C and BTC in the control of cultured porcine ovarian granulosa cell functions. For this purpose, we analyzed markers of proliferation, apoptosis and secretory activity of porcine ovarian granulosa cells, which were cultured in the presence of CuNPs/C and BTC, either separately and in combination (CuNPs/C + BTC). The following markers and regulators of the cell functions have been analyzed: BrDU incorporation (cell proliferation [22], PCNA (*S*-phase of mitosis) [23], cyclin B1 (M and G-phase of mitosis) [22,24], DNA fragmentation (extrinsic apoptosis, nuclear DNA fragmentation) [25], bax, caspase 3 (cytoplasmic/mitochondrial apoptosis) [26], and progesterone and estradiol (ovarian cell proliferation, apoptosis, ovarian folliculo- and luteogenesis [27–29]).

2. Material and methods

2.1. Preparation of copper nanoparticles supported on charcoal (CuNPs/C)

Copper(II) chloride (97 %, Sigma-Aldrich, St. Louis, USA), lithium powder (MEDALCHEMY S.L.), 4,4′-di-*tert*-butylbiphenyl (DTBB, Sigma-Aldrich) and activated charcoal (Norit CA1, Sigma-Aldrich) were commercially available. Tetrahydrofuran (THF) was dried in a Sharlab PS-400-3MD solvent purification system using an alumina column. In a typical procedure [30]: anhydrous copper (II) chloride (134 mg, 1 mmol) was added to a suspension of lithium (14 mg, 2 mmol) and DTBB (27 mg, 0.1 mmol) in dry THF (2 ml) at room temperature under an argon atmosphere. The reaction mixture, which was initially dark blue, rapidly changed to black, indicating that a suspension of copper nanoparticles was formed. This suspension was diluted with THF (18 ml), followed by the addition of activated charcoal (1.28 g). The resulting mixture was stirred for 1 h at room temperature, filtered, and the solid was successively washed with water (20 ml) and THF (20 ml), and dried under vacuum.

2.2. Isolation and culture of granulosa cells

Ovaries were obtained from Landrace prepubertal gilts (6–8 months of age) at the local slaughterhouse of Chovmat F.U. in Rastislavice (Nové Zámky, Slovakia). Within the 6 h of the slaughter, the ovaries were transported to the laboratory in a thermos with sterile physiological solution (0.9 % NaCl). The granulosa cells were aspirated with a syringe from the porcine ovarian follicles (2.5–6 mm diameter) without visible signs of atresia. The cells were isolated by centrifugation (10 min at 1500 rpm), washed in sterile DMEM/F12 1:1 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F12, BioWhittakerTM, Verviers, Belgium), and resuspended in the same medium with 10 % of fetal bovine serum (South America Origin, Bio-West, Inc., Logan, UT, USA) and 1 % of an antibiotic and antimycotic solution (Sigma–Aldrich, St. Louis, MO, USA). The initial concentrations of the cells before setting up the culture ranged from 10^5 to 10^6 cells per ml. The cell suspension was dispensed in: (a) 24-well plates (Nunc, A/S, Roskilde, Denmark, 1 ml suspension/well) for enzyme-linked immunosorbent assay (ELISA) test; (b) 96-well plates for BrdU (bromodeoxyuridine), TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) (Brand GmbH, Wertheim, Germany) and AlamarBlue (Thermo Fisher Scientific, 100 μl/well) assays; or (c) 16-well chamber slides (Nunc, Inc., International, Naperville, IL, USA; 200 μl/well) for immunocytochemistry. All cells were precultured in a medium at 37 °C under 5 % CO_2 in humidified air until a confluent monolayer (80 %) was formed; usually, 2 days of preculture was needed. After 48 h of preculture, the medium was replaced with a fresh medium of the same composition. In addition, in a fresh medium, the experimental groups received BTC (0, 100 ng/ml; Sigma-Aldrich) with or without CuNPs/C (0, 1, 10, 100 ng/ml). These doses were comparable with those used in the previous experiments with BTC [14–16,18–20] and CuNPs [6–8,11–13]. All substances were dissolved in a culture medium immediately before the experiment. The control groups were represented by cells with no exogenous treatment. After 2 days of culture, the cells were processed for the BrdU and TUNEL assay quantitative immunocytochemistry, and the medium was processed for the enzyme immunoassay (ELISA). The cell concentration was determined by counting on an automated cell counter (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. BrdU assay

Cell proliferation, based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis, was determined by using the colorimetric cell proliferation ELISA (Roche Diagnostics GmbH, Roche Applied Science, Germany), according to the manufacturer's instructions. The reaction products were quantified by measuring the absorbance at 450 nm using an ELISA reader (Thermo Fisher Scientific Multiskan FC, Vantaa, Finland).

2.4. TUNEL assay

DNA fragmentation induced in the cell culture was measured by using the TUNEL assay (HT TiterTACS™ Apoptosis Detection Kit; Trevigen, Gaithersburg, MD, USA), following the manufacturer's instructions. The absorbance was measured at 450 nm by using an ELISA reader (Thermo Fisher Scientific) after adding 0.2 N HCl. As a negative control, cells were labeled without the transferase terminal deoxynucleotidyl transferase (TdT), and positive controls were generated by using TACS-Nuclease for 1 h at 37 °C before hydrogen peroxide treatment.

2.5. Immunocytochemical analysis of the presence of proliferation and apoptosis markers

The presence of markers of proliferation (PCNA and cyclin B1) and apoptosis (bax, caspase 3) were detected via immunocytochemistry, as previously described [7,29], by using primary mouse monoclonal antibodies against either PCNA, cyclin B1, bax, or caspase 3 (1:500 dilution in PBS; Santa Cruz Biotechnology, Inc., CA, USA), and secondary swine antibody against mouse IgG (1:1000 dilution; Santa Cruz Biotechnology, Inc.) labeled with horseradish peroxidase (Servac, Prague, Czech Republic). The cells labeled with horseradish peroxidase were stained with 3.3′-diaminobenzidine (DAB) substrate (Roche Diagnostics GmbH). The cells treated without the primary antibody were used as negative controls. The number of stained cells was determined based on the brown coloration of DAB peroxidase by using a light microscope. The ratio of stained cells to the total number of cells was determined.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of progesterone and 17β-estradiol were analyzed in 25- or 100-μl aliquots of the incubation medium using ELISA, according to the manufacturer's instructions (LDN Immunoassays and Services, Nodhorn, Germany).

The cross-reactivity of antiserum against progesterone with other steroids were as follows: ≤1.1 % to 11-desoxycorticosterone, ≤0.35 % to pregnenolone, ≤0.30 % to 17α-hydroxyprogesterone, ≤0.20 % to corticosterone, <0.10 % to estriol, 17β-estradiol, testosterone, cortisone and 11-desoxycortisol, <0.02 % to DHEA-S and cortisol. The sensitivity of the assay was 0.045 ng/ml. The intra- and inter-assay coefficients of variations were <5.4 and <5.59 respectively. All the assays of hormone concentration in the incubation medium were performed in duplicates.

The antiserum against estradiol used in estradiol ELISA cross-reacted ≤9.5 % to fulvestrant, ≤4.2 % to estrone, ≤3.8 % to E2-3-glucuronide, ≤3.6 % to E2-3-sulphate, ≤0.4 % to estriol, and <0.1 % to androstenedione, 17-hydroxyprogesterone, corticosterone, pregnenolone, E2-17-glucuronide, progesterone and testosterone. The sensitivity of this ELISA was 6.2 pg/ml, with the intra- and inter-assay coefficients of variation being <6.4 and <4.5, respectively.

All assays of hormone concentration in the incubation medium were performed in duplicates. The accuracy of these ELISAs was tested for culture medium samples through dilution experiments.

2.7. Statistical analysis

The data from this study are reported as the means of the values, which were obtained in three separate experiments performed on separate days with different groups of granulosa cells, with each experiment including, at least, six ovaries. Each experimental group was represented by four culture wells containing ovarian granulosa cells. For the imunocytochemical analyses, the proportion of cells containing antigene was calculated for, at least, 1000 cells per well. The optical density of the signals was calculated by BrdU and TUNEL analyses to a percentage respect to the control, according to the instructions of the kits manufacturers. For the ELISA, the blank control values of the media-containing wells were subtracted from the corresponding values, excluding any nonspecific background (less than 10 % of the total values). The rates of hormone secretion were calculated per 10^6 viable cells/day. Significant differences between the groups were determined by using the Shapiro–Wilk normality and Student's t tests, as well as with the one-way ANOVA followed by Tukey's tests, with SigmaPlot 11.0 (Systat Software, GmbH, Erkrath, Germany). Differences were considered statistically significant at P levels less than 0.05 ($P < 0.05$).

3. Results

CuNPs/C was prepared by the chemical reduction of copper(II) chloride with metal lithium powder, in the presence of a catalytic amount of DTBB as an electron carrier [30], followed by the addition of activated charcoal (see the Experimental Section) [30]. The copper nanoparticles were composed of Cu_2O and CuO in a *ca.* 1:1 ratio, with a size of *ca.* 6 nm [7]; supporting information).

3.1. CuNPs effect on ovarian cell functions

The addition of CuNPs/C significantly increased the incorporation of BrdU (Fig. 1A), accumulation of PCNA (Fig. 1B) and cyclin B1 (Fig. 1C) in all the doses added. On the other hand, CuNPs/C reduced the DNA fragmentation (Fig. 1D) and the accumulation of bax (Fig. 1E) and caspase 3 (Fig. 1F). CuNPs/C promoted the release of progesterone at the doses of 1 and 10 ng/ml (Fig. 1G) and that of estradiol at all the doses added (Fig. 1H).

3.2. BTC effect on ovarian cell functions

The presence of BTC significantly increased the incorporation of BrdU (Fig. 1A), accumulation of PCNA (Fig. 1B) and cyclin B1 (Fig. 1C), but reduced DNA fragmentation (Fig. 1D) and accumulation of bax (Fig. 1E) and caspase 3 (Fig. 1F). Moreover, BTC was found to significantly promote the progesterone (Fig. 1G) and estradiol (Fig. 1H) output.

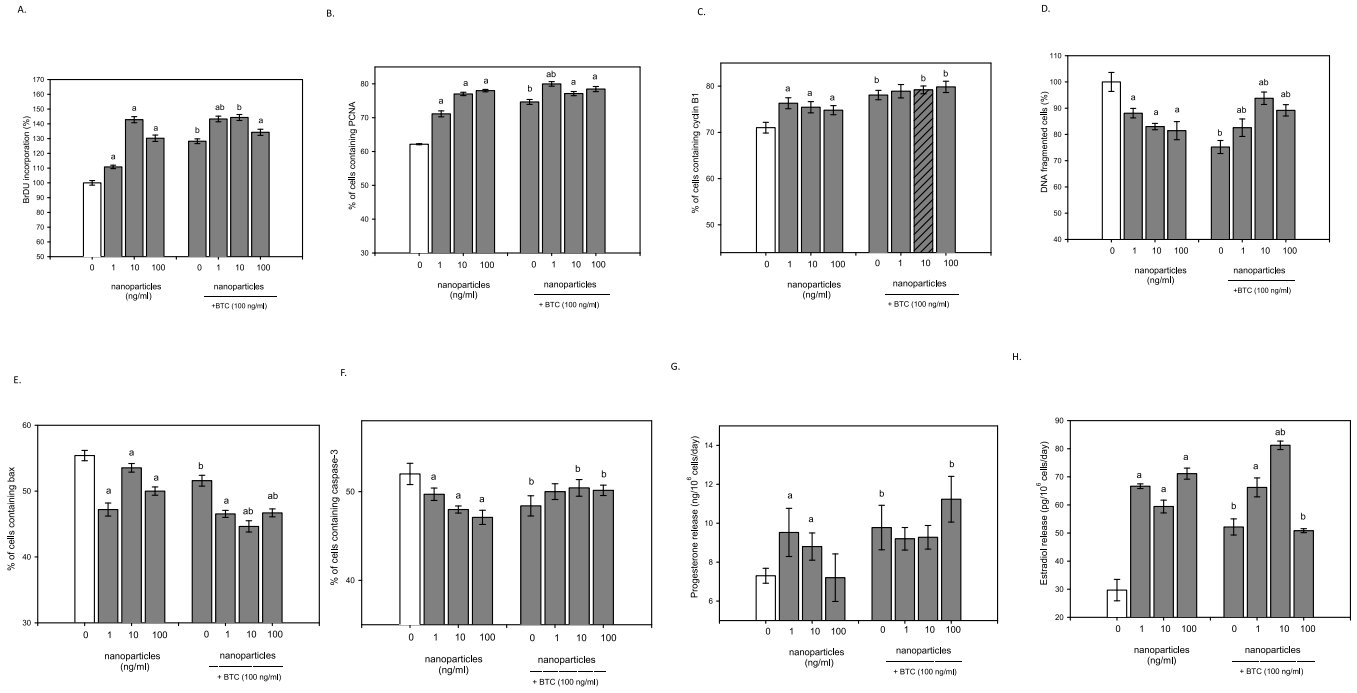

Fig. 1. Effects of copper nanoparticles supported on charcoal (CuNPs/C), betacellulin (BTC) and their combinations on BrdU incorporation (A), accumulation of PCNA (B), cyclin B1 (C), DNA fragmentation (D), accumulation of bax (E), of caspase 3 (F) and the release of progesterone (G) and estradiol (H) by cultured porcine ovarian granulosa cells. The results show (a) the effects of nanoparticles - the significant $P < 0.05$) differences between the cells cultured with and without nanoparticles and (b) the effect of betacellulin - the significant ($P < 0.05$) difference between the corresponding groups of cells cultured with and without betacellulin. The results are expressed as the mean ± SEM.

3.3. BTC promotes CuNPs effect on ovarian cell functions

CuNPs/C (at a dose of 1 ng/ml) significantly increased BrdU (Fig. 1A) and the accumulation of PCNA (Fig. 1B) in presence of BTC, when compared with what observed for CuNPs/C alone. BTC promoted the stimulatory action of CuNPs/C (at 10 and 100 ng/ml) on cyclin B1 (Fig. 1C), but CuNPs/C failed to reduce the DNA fragmentation in the presence of BTC (Fig. 1D). The inhibitory action of CuNPs/C (at 10 or 100 ng/ml) on the accumulation of bax (Fig. 1E), but not of caspase 3 (Fig. 1F) was significantly more pronounced when CuNPs/C was administrated together with BTC. The presence of BTC did not substantially modify the stimulatory effect of CuNPs/C on progesterone release (Fig. 1F), but increased the stimulatory effect of CuNPs/C (at a dose of 10 ng/ml) on the estradiol output (Fig. 1G).

4. Discussion

4.1. CuNPs effect on ovarian cell functions

The character of the CuNPs/C action on cultured porcine ovarian granulosa cells confirms the CuNPs/C effects observed on these cells in the previous experiments [8]. The ability of CuNPs/C to increase the incorporation of BrdU (marker of cell proliferation), accumulation of PCNA (marker and promoter of *S*-phase of mitosis) and cyclin B1 (marker of G-phase of mitosis) observed in our experiments suggests that CuNPs/C is a potent promoter of ovarian cell proliferation at the two phases of the cell cycle. On the other hand, the ability of CuNPs/C to reduce DNA fragmentation, bax and caspase 3 indicate its suppressive action on both extrinsic (nuclear DNA fragmentation) and intrinsic (cytoplasmic/mitochondrial) apoptosis. Finally, the increased progesterone and estradiol outputs after the addition of CuNPs/C suggest that CuNPs/C can be a promoter of ovarian steroidogenesis. The increased release of progesterone (marker of luteinisation of ovarian follicle) [27] and estradiol (marker of healthy, growing, not atretic follicle) [27] indicates that CuNPs/C could be a promoter of both ovarian folliculogenesis and luteogenesis. Estradiol and progesterone are known promoters of ovarian cell proliferation, and inhibitors of their apoptosis and the resulted follicular atresia [27]. Therefore, the fact that the CuNPs/C promotion of ovarian cell proliferation and suppression of their apoptosis, as observed in the present experiments, could be mediated by its ability to stimulate the release of the steroid hormones, cannot be ruled out.

The stimulatory action of CuNPs/C on porcine ovarian cell proliferation and steroidogenesis, and its inhibitory influence of their apoptosis suggest a potential applicability of these CuNPs as stimulator of reproduction and fecundity in this farm animal species. However, this hypothesis requires validation by some in vivo studies.

4.2. BTC effect on ovarian cell functions

In the present experiments, the addition of BTC was found to increase the incorporation of BrdU, accumulation of PCNA and cyclin B1, the release of both progesterone and estradiol, but reduced DNA fragmentation and accumulation of bax. The stimulatory action of BTC on BrdU, PCNA and cyclin B1 denotes that BTC can stimulate the proliferation of porcine ovarian cells via up-regulation of promoters of the *S*-phase and G-phase of mitosis. This effect is in line with the previous reports dealing with the ability of BTC to promote the proliferation of murine [15] and chicken [19] ovarian granulosa cells, but not with the anti-proliferative action of BTC on feline granulosa cells [21], reported previously. This is the first demonstration of the involvement of BTC in the up-regulation of the proliferation of porcine ovarian cells. Moreover, this is the first evidence that BTC can suppress both extrinsic and intrinsic apoptosis of porcine ovarian cells. This observation corresponds to the previous observation of the ability of BTC to reduce intrinsic apoptosis in feline ovarian cells [21]. On the other hand, the present observations oppose those of [21] on the suppressive action of BTC on cultured feline ovarian cells. The stimulatory action of BTC on progesterone and estradiol – promoters of ovarian cell proliferation and suppressors of their apoptosis – indicates that steroid hormones could mediate the BTC action on these processes. Taken together, the data available now denote the direct and species-specific influence of BTC on ovarian cells. Moreover, the stimulatory action of BTC on porcine granulosa cell proliferation and steroidogenesis, and its suppressive effect on both intrinsic and extrinsic apoptosis, suggest that BTC could be a direct stimulator of ovarian cell functions in this species. If the stimulatory action of BTC on ovarian functions could be confirmed by further in vitro and in vivo experiments, BTC could be considered as a novel regulator and biostimulator of female reproductive processes, at least, in several species. This fact would lend weight to the potential application of BTC for the promotion of fecundity in some farm animal species.

4.3. BTC promotes CuNPs effect on ovarian cell functions

The similarity in the effects of BTC and CuNPs/C points to comparable mechanisms of action of these molecules on ovarian cells. Moreover, in the present experiments, BTC was able to promote the action of CuNPs/C on the majority of the analyzed parameters: BrdU, PCNA, cyclin B1, bax and estradiol release, but not on accumulation of caspase 3. The similar and additive action of BTC and CuNPs/C, as well as the ability of BTC to promote the main effects of CuNPs/C, indicate a potential applicability of the combination of these synergists for the stimulation of porcine ovarian cell functions and fecundity in this farm animal species. This suggestion should be verified by further in vivo studies on the effects of BTC, CuNPs/C and their combinations on porcine reproductive processes.

The possible targets of both BTC and CuNPs/C in female reproductive system are summarized in Fig. 2. Taken together, the present preliminary data indicate that both BTC and CuNPs/C, independently, and their combination could be biostimulators of porcine ovarian cell functions, which applicability in porcine breeding is worth of further examination.

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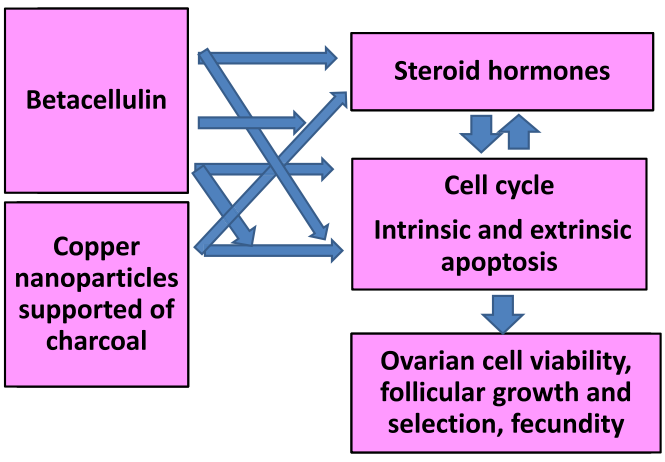

Fig. 2. The possible targets of betacellulin and copper nanoparticles supported on charcoal in female reproductive system. Details are in the main text.

Data availability

The primary data could be provided on request.

Ethics statement

Not applicable. This research did not include any human subjects and animal experiments.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Alexander V. Sirotkin: Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Barbora Loncová:** Validation, Investigation. **Zuzana Fabová:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Michaela Bartušová:** Investigation. **Iris Martín-García:** Resources, Investigation. **Abdel Halim Harrath:** Writing – review & editing, Project administration, Funding acquisition. **Francisco Alonso:** Writing – review & editing, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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