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Platelets are involved in *in vitro* swine granulosa cell luteinization and angiogenesis

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ABSTRACT

During corpus luteum formation, impressive biological events take place to guarantee the transition from original follicular to luteal cells and to support required massive angiogenesis. It has been demonstrated that these phenomena resemble those essential for wound healing. After ovulation, blood vessels release their content in the antral cavity and coagulation takes place. Involvement of platelets in corpus luteum growth has been hypothesized both in human and in rat. On this basis, using platelet lysate (PL), a blood derivative with a higher platelet concentration, we aimed to assess a potential involvement of platelets in swine granulosa cell luteinization and on new blood vessel growth. Our results demonstrate, for the first time in the swine, that platelets could be directly involved in granulosa cell physiological luteinization, since the treatment with PL shifted steroid production from estradiol 17β to progesterone. Moreover, PL stimulated angiogenesis. Nitric oxide could be involved in these effects. These results are important to clarify complex intrafollicular molecular machinery. A better understanding of these mechanisms can be useful to develop more focused therapeutic strategies to contrast sow infertility. In addition, since the pig represents a model for translational studies, collected data could be of interest for human medicine because reproductive pathologies such as Polycystic Ovary Syndrome (PCOS) and endometriosis are often accompanied by platelet dysfunctions.

1. Introduction

Blood platelets, which are produced by differentiation and maturation of megakaryocytes in the bone marrow, gather at blood vessel injuries and form he initial platelet plug by adhesion, activation and aggregation. Apart from their fundamental role in primary hemostasis, increasing evidence suggests that platelets are also involved in the regulation of tissue repair and remodeling (Sorrentino et al., 2015).

The biological events that take place in the ovary are unique since the transition of a preovulatory follicle into a corpus luteum happens by means of a complex process involving mechanisms similar to those involved in wound healing.

Just before follicular rupture, granulosa cells shift their main hormonal product from estrogen to progesterone. After ovulation, these cells, being in contact with migrating endothelial cells and producing extracellular matrix, undergo hypertrophy to differentiate into luteal cells. At the same time, dramatic centripetal angiogenesis starts after destruction of follicular basement membrane.

Granulosa cells have been demonstrated to be actively involved in regulating neoangiogenesis owing to the release of several pro- and anti-angiogenic factors (Grasselli et al., 2003; Bianco et al., 2005; Basini et al., 2007, 2008, 2011, 2014a). After penetrating the

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granulosa layer, several blood vessels open into the antral cavity and plasma fluid and platelets flow into the antrum, where coagulation takes place. It has been hypothesized that platelets could play a role in corpus luteum development both in the human and the rat (Furukawa et al., 2007; Sleer and Taylor, 2007).

Present research was undertaken to evaluate if platelet lysate (PL), a blood-derived product in which platelets are concentrated in plasma above the baseline of that in the whole blood, can play a role in luteinization of swine granulosa cell collected from follicles > 5 mm. Moreover, since corpus luteum formation is strictly associated with angiogenesis, we also evaluated potential effects of PL on new vessel growth by means of a validated fibrin gel angiogenesis bioassay (Basini et al., 2014b,c). A better knowledge of these physiological events, which are relevant in the reproductive processes of the female, might help in the design of therapeutic strategies focused on treating corpus luteum dysfunctions in different species.

2. Materials and methods

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

2.1. Porcine platelet rich plasma (PRP) and platelet lysate (PL) preparation

For the preparation of porcine PRP, 50 ml samples of venous blood of three animals were collected at the local abattoir from the jugular vein immediately after slaughter. To avoid coagulation, a citrate dextrose solution (ACD, 3.8% w/v) was used at a 1:10 ratio with blood. Whole blood was first centrifuged at 200*g* for 15 min at room temperature (RT) and the plasma fraction was collected. Red blood cells and buffy coat rich in white blood cells were discarded. The plasma fraction was then centrifuged at 900*g* for 20 min at RT obtaining a Platelet Poor Plasma (PPP) as a supernatant and a platelet pellet. After removing the PPP, the pellet was re-suspended in a minimum volume of PPP (1 ml) and platelets were counted with a cell counter (CELL-DYN 3500R hematology analyzer Abbott, Roma). Platelets were then diluted at a final concentration of 1.0×10^9 cell/mL to generate PRP. Aliquots of 0.5 ml of PRP were than prepared and stored at −20 °C until used for cell culture experiments. Platelet lysate (PL) was prepared by thawing PRP at 37 °C and centrifuging for 20 min at 10,000 *g* to remove cell debris. Finally, platelet lysate was filtered by a 0.22 μm sterile syringe filter.

2.2. Effect of platelet lysate (PL) on granulosa cell function

2.2.1. Granulosa cell collection and culture

Swine ovaries were collected at a local abattoir from 20 Large White cross-bred gilts. The stages of their estrous cycle were unknown. 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. Follicles were classified as healthy or atretic on the basis of morphological criteria and those with hemorragic, opaque or "milky" follicular fluid were excluded (Basini et al., 2014a). Healthy follicles were furtherly classified on the basis of their size according to Basini et al. (2008, 2014a). Granulosa cells were aseptically harvested by aspiration of follicles > 5 mm with a 26-gauge needle and released in medium containing heparin (50 IU/ml). 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 haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension.

According to the different experimental procedures described below, cells were seeded in DMEM/Ham's F12 (CM) supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1%), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml) and immediately after an equal volume of PL was added. Final concentration of PL was 50%, corresponding to 0.5×10^9 platelet/ml of culture medium. In control cultures PL was substituted by an equal volume of Platelet Poor Plasma (PPP) prepared as described above.

2.2.2. Granulosa cell viability

2×10^5 cells were seeded in 96-well plates in 100 μl CM and treated with PL or PPP for 48 h as indicated above. Cell viability was assessed using a bioluminescent assay (ATP-lite; Packard Bioscience, Groningen, Netherlands) which measures intracellular ATP levels as an indicator of cell numbers. ATP is a cell viability marker because it is present in all metabolically active cells with a very rapid decrease of concentration when the cells undergo necrosis or apoptosis. The ATP lite-M assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin (Basini et al., 2012). The luminescence was measured in a Victor luminometer (Perkin Elmer, Boston MA, USA). The results were recorded in counts per second (CPS). The assay detection limit was 10^3 cells/well and the variation coefficient was less than 5%.

2.2.3. Granulosa cell proliferation

10^4 cells/well were seeded in 96-well plates in 100 μl CM and treated with PL or PPP for 48 h as indicated above. Cell proliferation was evaluated by 5-bromo-2′-deoxyuridine (BrdU) incorporation assay test (Roche, Mannheim, Germany). After 44 h of incubation in the presence (PL) or absence (PPP) of treatment, 20 μl BrdU were added to each well, then culture media immediately removed and a DNA denaturing solution was added in order to improve the accessibility of the incorporated BrdU for antibody detection as detailed in our paper (Basini et al., 2017).

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