Glucose-6-phosphate Metabolic Preferential Destinations in Bovine Oviduct Cells

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ABSTRACT

Background: The oviduct is a dynamic organ which facilitates gamete function, fertilization and embryo development. This organ is covered by an epithelium containing ciliated and non-ciliated cells. Secretions of non-ciliated cells compose the oviduct fluid, which will nourish the early embryo. During the period of ovulation, the oviduct exhibits an active role, where the lumen provides an environment suitable for fertilization and the muscle layer contracts rhythmically to move the egg toward the uterus. In this study we aimed to investigate the content of fuel metabolites and enzyme activity assays related to the glycolytic metabolism in bovine oviduct cells such as Glucose-6-phosphate, Glycogen, Pyruvate, Hexokinase, Pyruvate kinase, Phosphoenolpyruvate carboxykinase and Glucose-6-phosphate dehydrogenase.

Materials, Methods & Results: In this sense, we divided the oviduct in 3 identical portions (anterior, medium and posterior). After division, each region was massaged and the lumen was flushed with 2mL Ca+2 and Mg+2 free PBS, pH 7.2. The material obtained was centrifuged at 3.000 x g for 5 min at 4ºC. The pellet of cells were resuspended again with 2 mL of Ca+2 and Mg+2 free PBS at pH 7.2 and sonicated 6 times at 60W. All the assays were performed in a Shimadzu U1240 spectrophotometer. The data were normalized in terms of total protein content and the assay was ran in triplicate. In the anterior region a low activity of hexokinase (HK) and pyruvate kinase (PK) and an accumulation of pyruvate were detected. Besides this, based on the glucose-6-phosphate dehydrogenase (G6PDH) activity and the amount of glycogen, the glucose-6-phosphate (G6P) could be directed to the pentose phosphate pathway, which provides protection against the toxicity of reactive oxygen species (ROS) and supplies NADPH to be used in anabolic pathways. In the oviduct’s medium portion we observed an intense glycolytic metabolism based on HK and PK activities and low pyruvate levels that could be exported to compose the lumen fluid. In the oviduct’s posterior region we detected a different metabolic profile with high gluconeogenic activity due to elevated phosphoenolpyruvate carboxykinase (PEPCK) activity glucose-6-phosphate concentration and HK activity.

Discussion: Low HK and PK activities were observed in the anterior portion, which could be due to the concentration of pyruvate. Besides this, the (G6PDH) activity, assessed together with its substrate and the amount of glycogen, suggests that cells from the anterior portion are working for the energetic maintenance of whole oviduct cells. The medium portion presents a metabolism via high HK and PK activities. An increase in PEPCK activity could be considered as a source of phosphoenolpyruvate. Accumulation of glycogen, high concentration of G6-P and (G6PDH) activity are also observed. The profile of enzymes from glycolytic and gluconeogenic pathways and their substrate together with (G6PDH) activity and G6-Phosphate lead us to believe that the preferential product produced in the posterior region is pyruvate.

Keywords: oviduct, glucose-6-phosphate, energy metabolism, glucose, glycogen.
INTRODUCTION

Oviducts are part of female reproductive tract that lie between each ovary and the tip of the adjacent uterine horn [5]. Two cell types constitute the epithelium of the oviduct: ciliated and secretory cells. Ciliated cells play an important role in the transport of oocytes, sperm and embryos, while secretory cells produce and release specific secretory materials [2,3]. The combination of a selective transudate of serum [7] and of these secretions forms the oviductal fluid [18].

In mammals, the oviduct plays the most fundamental role in the reproduction process [2]. Its importance in normal reproductive processes such as gamete final maturation, which occurs in the infundibulum [12], the sperm capacitation in the isthmus region, fertilization in the ampulla [13], and early embryo development has been emphasized by the difficulty experienced in attempting to repeat these events in vitro [7,12].

The current literature provides ample information regarding energetic metabolites in cow oviduct fluid. Nevertheless, there is little or no information available on the concentrations of key metabolites in oviduct cells in cattle.

Successful reproduction on cattle requires the knowledge of reproductive processes of the cow and the better understanding of the metabolism of cow oviduct cells due to the role that this organ plays in early embryo support and nutrition. This knowledge may be useful in correcting and adapting the composition of the media used in in vitro production of embryos.

MATERIALS AND METHODS

Oviduct collection

Cow’s oviducts were collected at a local slaughterhouse immediately after death. They were transported to the laboratory in vials with ice, and then classified as to the presence or absence of corpus luteum. The oviducts associated to ovaries with corpus luteum were dissected and equally divided into three different portions: anterior, medium and posterior (Figure 1).

Cells collection

After division, each region was gently massaged to remove cells. Then, the lumen was flushed with 2 mL Ca²⁺, and Mg²⁺ free PBS, pH 7.2. These wash-offs from oviducts were centrifuged at 3.000 x g for 5 min at 4°C. The pellet of cells were resuspended with 2 mL of Ca²⁺ and Mg²⁺ free PBS at pH 7.2 and sonicated 6 times (each time of 30 s with an interval of 30 s between the times) at 60 W. After centrifugation at 10.000 x g for 30 min at 4°C, the supernatant was aliquoted in 1 mL and lyophilized and stored at -20°C until use.

Endogenous Glucose-6-P (G6P) content

Glucose-6-phosphate was enzymatically quantified in 1 mg/mL total protein of lyophilized cells resuspended with 55 mM Tris-HCl, pH 7.6 in the presence of 5 mM MgCl₂, 2mM NAD⁺, and 0.34 U/mL glucose-6-phosphate dehydrogenase (G6PDH). The β-NADH production was detected in a spectrophotometer at 340 nm using a molar extinction coefficient of 6.22 M⁻¹, as described by Moraes et al. [20]. The data were normalized in terms of total protein content and the assay was ran in triplicate.

Endogenous Glycogen content

The lyophilized cells from each region were resuspended with ultra pure water (100 µL). They were incubated with 200 mM sodium acetate buffer, pH 4.8, 1 unit α-amyloligosidase and then incubated for 4 h at 40°C. Glucose produced from glycogen degradation was determined with an enzymatic Kit for glucose dosage in a spectrophotometer at 510 nm, according to the manufacturer’s instructions. A standard curve was used with different dilutions of glycogen, subjected to the same treatment. The data were normalized by total protein content and the assay was ran in triplicate.

Endogenous Pyruvate content

Lyophilized cells from each region were resuspended with ultra pure water (100 µL). Pyruvate was indirectly measured by the addition of lactate dehydrogenase. The NADH consumption was detected in a spectrophotometer at 340 nm [20] and the assay was ran in triplicate.

Hexokinase (HK) activity assay

Hexokinase activity was determined immediately in freshly collected cells from each oviduct portion. Cells were sonicated and centrifuged as mentioned above. Supernatant aliquots were incubated with 20 mM Tris-HCl, pH 7.5 containing 6 mM MgCl₂, 1mM ATP, 0.5 mM NAD⁺, 10 mM NaF, and the reaction was started with 2 mM glucose. The glucose-6-phosphate formed was measured by adding equal volumes of 20 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 1 unit/mL of glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides and 0.3 mM β-NAD⁺. The
production of β-NADH was detected in a Shimadzu U1240 spectrophotometer at 340 nm using a molar extinction coefficient of 6.22 M⁻¹ as described by Galina & Da Silva [6] and the assay was ran in triplicate.

Pyruvate kinase (PK) activity

Samples were prepared as described for HK activity analysis. PK activity was measured in 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 mM ADP, 0.4 mM NADH, 1 unit/mL lactate dehydrogenase and the reaction was started with 1 mM phosphoenolpyruvate (PEP). The β-NADH consumption was detected in a spectrophotometer at 340 nm using a molar extinction coefficient of 6.22 M⁻¹ as described by Worthington [27] and the assay was ran in triplicate.

Phosphoenolpyruvate carboxykinase (PEPCK) activity assay

Samples were prepared as described in the HK activity section. The supernatant was incubated with 100 mM Hepes buffer pH 7.0 containing 10 mM phosphoenolpyruvate (PEP), 0.2 mM NADH and 24 units of malate dehydrogenase. The reaction was started with 2.5 mM inoside diphosphate (IDP). The β-NADH consumption was monitored at 340 nm in a Shimadzu U1240 spectrophotometer and the physiological PEPCK activity was determined as described by Petersen et al. [22] and the assay was ran in triplicate.

Glucose-6-phosphate dehydrogenase (G6PDH) activity assay

Samples were prepared as described above for enzyme activities. The supernatant was incubated in 55 mM Tris-HCl, pH 7.8 containing 3.3 mM MgCl₂, 2 mM NAD⁺, 0.3 mM glucose-6-phosphate. The β-NADH production was detected in a spectrophotometer at 340 nm using a molar extinction coefficient of 6.22 M⁻¹ as described by Worthington [27] and the assay was ran in triplicate.

Total protein content

Total protein content was quantified according to Bradford [4] using bovine serum albumin as standard. Three samples were analyzed for each experimental point.

Statistical analysis

The bioactivities were analysed by analysis of variance (ANOVA) between groups using Tukey test (P < 0.05) for means.

RESULTS

Glucose-6-phosphate distribution in oviduct cells

Glucose-6-phosphate (G6P) quantification demonstrated that the cells from the anterior portion present approximately 356 nM. The cells from medium portion exhibited almost 2-fold this value (680 nM). On the other hand, endogenous G6P in the cells from the posterior portion is 3x and approximately 6x less than anterior and posterior portion, consecutively (Figure 2A).

Pentose-phosphate pathway in oviduct cells

The pentose-phosphate pathway was investigated by the determination of glucose-6-phosphate dehydrogenase profile (G6PDH) activity, which is the rate limiting step in this pathway [26]. In the anterior portion we observed a higher G6PDH activity (0.03 units/mg protein) in comparison to the other portions (0.025 units/mg protein - medium and 0.02 units/mg protein - posterior portion) (Figure 2B).

Figure 2. G6-Phosphate destinations - A) G6-Phosphate concentration at each oviduct portion. B) GG6-P dehydrogenase (G6PDH) activity at each oviduct portion. C) Glycogen content at different oviducts regions. The bioactivity was analyzed by analysis of variance (ANOVA) between groups using Tukey test (P < 0.05) for means.

Glycogen content

The medium portion exhibited higher glycogen level (79 µg/mg total protein). In anterior portion we observed a concentration 20% lower than that exhibited by the medium portion. In turn, in the posterior portion we observed a concentration about twice as low as that of the medium segment (30 µg/mg total protein).
Glycolysis in oviduct cells

The study of the glycolytic pathway in cow oviduct was evaluated from measurements of the activity at the initial (Hexokinase - HK) and final (Pyruvate kinase - PK) glycolysis steps. HK (Figure 3A) and PK activities (Figure 3B) in the anterior portion was about 0.02 units/mg protein and 0.14 units/mg protein, and correlated with the rise in pyruvate levels (44.4 nM/µg protein)(Figure 3C). We observed HK and PK activities 4 times higher in the oviduct medium portion (0.24 units/mg protein and 1.38 units/mg protein, respectively) (Figures 3 A & B). In the same segment we observed a smaller pyruvate concentration (10 nM/µg protein) (Figure 3C). In the posterior portion, HK activity was similar to that observed in the anterior segment (0.02 units/mg protein) and PK activity was 1.02 units/mg protein, representing a decrease in 26% as compared to the level in the medium portion (Figures 3 A & B). Pyruvate content in this piece was around 55% higher than that measured in the medium section (15.5 nM/µg protein) (Figure 3C).

Figure 3. Glycolysis pathway - A) HK activity on oviduct regions; B) PK activity at different portions. C) Pyruvate content at anterior, medium and posterior portions. The bioactivity was analyzed by analysis of variance (ANOVA) between groups using Tukey test ($P < 0.05$) for means.

Gluconeogenesis in oviduct cells

The evaluation of gluconeogenesis in the oviduct was carried out using the activity of the key enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Figure 4A). We could observe a low PEPCK activity in the anterior region, when compared with the other portions (1.5 x higher in medium and 2.5 x higher in posterior segments, respectively) (Figure 4A).

Figure 4. Gluconeogenesis - A) PEPCK activity on oviduct cells. The PEPCK activity was analyzed by analysis of variance (ANOVA) between groups using Tukey test ($P < 0.05$) for means.

DISCUSSION

In mammals, the fertilization and early embryo development occur inside the maternal organ named oviduct [11]. It has been recognized as a reproductive organ responsible for creating a microenvironment that facilitates ovum transport and maturation, sperm capacitation and fertilization [23-25]. It has been suggested that this organ is responsible for the supply of all the nutrients necessary for the early embryo development [10,12].

In this article we investigated the metabolism of glucose-6-phosphate in the bovine oviduct cells from three equally divided segments. By dividing the oviduct in three parts (Figure 1) we were able to observe that the cells from each segment of bovine oviduct exhibit distinct profiles of G6P content consumption (Figure 2A). One previous study, using co-culture systems, suggested regional differences associated with the physiological support provided by the epithelial cells from oviduct [1].

Glycolysis is the metabolic pathway that converts glucose into pyruvate. The first step in glycolysis is glucose phosphorylation by a family of enzymes called hexokinases, in order to form glucose-6-phosphate (G6P). Pyruvate kinase catalyzes glycolysis’ final reaction, which generates pyruvate and ATP. The glycolytic pathway is regulated both by enzyme activity and metabolites/substrate availability.

We established as anterior portion the oviduct region that includes the anatomical segment named infundibulum (Figure 1). The free end of infundibulum is called fimbria, and is able to capture the oocyte during ovulation. Pickup of oocytes is achieved by a massage process, done through muscular contractions, on the surface of the ovary [8]. Once captured from the surface of the ovary, the
transport of the oocyte in the oviduct is achieved by ciliary beating of the oviductal epithelial cells and by contraction of the oviductal smooth muscle [13]. The massaging and gamete transport events require energy from the cells along the oviduct. We suggest that the accumulation of pyruvate in this portion (Figure 3C) could support the energy demands from oviduct cells on the moment of ovulation, when the tissue spends intense energy capturing the oocyte and transporting it until the site of fertilization. The availability of the appropriate substrate is in turn controlling the oocyte and transporting it until the site of fertilization. The availability of the appropriate substrate in turn controlling PK activity, as its activity is low in this segment. Furthermore, the level of G6P (Figure 2A) suggests that this metabolite could be generated from another source, besides HK, since its activity is low. Classically glycogen degradation could be useful as an alternative source of G6P. Our results also indicate that G6P generated in the oviduct’s anterior portion by glycogen degradation could be preferably mobilized to supply the pentose phosphate pathway. It is supported by the level of G6P (Figure 2A) and the high G6PDH activity (Figure 2B) on this segment. The pentose phosphate pathway is a process that serves to generate NADPH and the synthesis of pentose (5-carbon backbone) sugars [9]. NADPH is used for anabolic pathways, such as lipid synthesis and also provides metabolites involved in protection against the toxicity of ROS (reactive oxygen species) [14,20]. Previous studies demonstrated the presence of antioxidant enzymes in oviduct fluid as a way to protect the developing embryo [16,19,21].

The medium portion presented an intense glycolytic metabolism via high HK (Figure 3A) and PK (Figure 3B) activities. This observation was corroborated by the detection of low concentrations of pyruvate in this segment (Figure 3C). As we mentioned before, the oviduct was equally divided for the measurements. This portion of oviduct was divided so as to include the region that is described as the presumptuous fertilization site [10]. The early bovine embryo floats during the first 2 weeks after fertilization and depends on the nutrients provided by oviduct and uterine fluids for its development, growth, and ultimately, its survival [10]. From the zygote to the 8- to 16-cell stage, pyruvate and lactate are the preferred energy sources [17]. Pyruvate was shown to play an important role in oocyte maturation, as described by Krisher & Bavister [15]. Pyruvate, glutamine, and glycine metabolism in the tricarboxylic acid cycle increases during oocyte maturation, suggesting that oxidative metabolism is the mechanism responsible for energy production during bovine oocyte maturation [15]. Based on previous physiological observations and pyruvate concentration, we suggest that oviduct cells secrete this metabolite into the lumen, to constitute oviduct fluid [10,15].

The oviduct’s posterior portion presented high PEPCK activity levels (Figure 4A), which could be a result of a decrease in glucose-6-phosphate concentration and of lowered HK activity. The main function described for this oviduct’s segment is the maintenance of embryo survival and the posterior transfer to uterus. Based on this fact we hypothesize that this segment exhibits an intense metabolic energetic demand.

In summary, we observed low HK and PK activities in the anterior oviduct region (Figure 5A), and this could be due to the accumulation of pyruvate in the anterior segment. Besides this, based on G6PDH activity and on the amount of glycogen, G6P is being directed to the pentose phosphate pathway, which could provide protection against the toxicity of ROS and supply NADPH to be used on anabolic pathways [20]. In the oviduct’s medium portion (Figure 5B) we observed an intense glycolytic metabolism based on HK and PK

Figure 5. Schematic representation of glucose metabolism control in cow’s oviduct regions.
activities and low pyruvate levels that could be exported to compose the lumen fluid. In the oviduct’s posterior region (Figure 5C) we detected a different metabolic profile with high gluconeogenic activity due to elevated PEPCK activity, low glucose-6-phosphate concentration and HK activity. These observations are schematically organized in Figure 5 and show how our hypothesis applies to different oviduct portions.

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