Microdensitometric assay of enzymatic activities in parthenogenetically activated and in vitro fertilized bovine oocytes

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Summary

To examine the paternal genome’s role in reprogramming metabolic activity in one-cell embryos, we investigated metabolic aspects of bovine oocytes after in vitro maturation and in vitro fertilization and after in vitro parthenogenetic activation with a Ca2+ ionophore and 6-dimethylaminopurine. We assayed succinate dehydrogenase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities by microspectrophotometry in immature oocytes and oocytes after maturation, in vitro fertilization and parthenogenetic activation. Succinate dehydrogenase activity significantly increased after in vitro maturation, significantly decreased after Ca2+ ionophore activation and further decreased after 6-dimethylaminopurine treatment. Lactate dehydrogenase activity showed a significant decrease in bovine oocytes after in vitro maturation, remained unchanged in Ca2+ ionophore-treated oocytes and rose significantly after 6-dimethylaminopurine treatment. This activity was dramatically reduced after in vitro fertilization, reaching absorbance levels that were not different from those in mature and Ca2+ ionophore-treated oocytes. Glucose-6-phosphate dehydrogenase activity was significantly lower in matured oocytes compared to immature oocytes, was significantly higher after artificial activation with Ca2+ ionophore and remained constant after 6-dimethylaminopurine treatment or after in vitro fertilization. We suggest that metabolic changes involved in parthenogenetic activation are similar to those occurring after fertilization.

Key words: bovine – oocyte metabolism – parthenogenetic activation – microspectrophotometric determination

Introduction

Fertilization is a complex interaction between unusually specialized cells. The union of sperm and egg involves a series of signalling mechanisms that leads to a species-specific gamete interaction, resulting in the activation of an egg by a single sperm. The biochemical pathways of fertilization are complex because sperm behaviour is modified by the egg component, and egg activation is affected by sperm attachment and fusion.

Equally intriguing are the biological aspects of parthenogenesis. The production of young without mating and therefore without fertilization holds fascination for a wider audience. This is a normal process in several non-mammalian phyla, but rudimental parthenogenesis...
can be induced by experimental intervention in mammals and artificially activated oocytes can develop parthenogenetically at least up to the blastocyst stage (Siraussa et al., 1985).

The activity of enzymes may change drastically during the preimplantation stages of the mammalian embryo. It is generally recognized that the unfertilized egg is metabolically active, but the large increase in activity at the time of fertilization is unique.

Understanding of the energy metabolism of oocytes and preimplantation embryos is important, especially in relation to in vitro fertilization and implantation. Although there is a substantial amount of information on morphological changes in early embryos, little is known on their biochemical characteristics, especially of embryos parthenogenetically developed in vitro (Ayabe et al., 1994).

Apart from paternal contributions to the mammalian zygote after sperm-oocyte union at fertilization, as has been documented in detail by Sutovsky and Schatten (2000), it appeared interesting to investigate the paternal genome’s role in controlling metabolic behaviour in the preimplantation embryo. We examined this role in bovine oocytes after in vitro maturation and fertilization and after in vitro parthenogenetic activation. This latter experimental condition was achieved by treating oocytes with a Ca²⁺ ionophore and subsequent exposure to an inhibitor of maturation promoting factor (MPF), 6-dimethylaminopurine (6-DMAP). It is known that a rapid transient rise in the concentration of free intracellular calcium is the universal trigger of oocyte activation. It is also of interest to note that 6-DMAP completely blocks germinal vesicle breakdown (GVBD; Lonergan et al., 1997) without completion of the meiotic program, resulting in diploid parthenotes (Susko-Parrish et al., 1994). Since fertilization triggers glucose utilization via an active glycolytic pathway (Saito et al., 1994), we focused our attention on the activity of succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G6PDH). To examine these enzymatic activities, we employed microdensitometrically evaluable histoenzymological reactions. This quantitative cytochemical approach has several advantages over biochemical methods. Cell disruption is avoided and thus cell morphology is kept well preserved. Final products of quantitative cytochemical reactions due to enzyme activity are formed entirely within the cell cytoplasm, providing information on the intracellular localization of the activity of these enzymes, and their microdensitometric analysis using cytochemical staining methods in single cells provides information on cytochemically detectable enzymatic behaviour within a potentially heterogeneous cell population.

Material and methods

Oocyte collection, selection and in vitro maturation

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 2 h in Dulbecco’s phosphate balanced saline (PBS), maintained at 32–34 °C. All subsequent procedures were conducted at 36 °C. Cumulus oocyte complex (COC) activation and selection procedures were performed in TCM-199, supplemented with 1 mg/ml polystyrene alcohol (PVA), 25 mM Hepes, and 10 µg/ml heparin. All chemicals were obtained from Sigma Aldrich, Milano, Italy unless stated otherwise. The COCs were retrieved from the ovaries by aspiration of 2–5 mm follicles with an 18-gauge needle on a 10-ml syringe containing a small amount of collection medium. The final proportion of follicular fluid in the collection medium was kept as close as possible to 3:1. COCs with 3 or more complete layers of cumulus cells and a finely granulated homogeneous ooplasm were selected under a stereomicroscope. These COCs were then washed twice in the same medium used for collection and twice in the maturation medium, as specified below. The whole procedure was completed in approx 30 min. Oocytes were divided into 2 groups. The first (control) group was used to study immature oocytes immediately after collection. Cumulus was removed by hyaluronidase treatment (1 mg/ml) and vortexing in a minimum amount of medium for 3 min. These oocytes, completely freed of adherent granulosa cells were suspended in the same medium and immediately processed for cytochemical reactions. The second group of oocytes was subjected to in vitro maturation and afterwards divided into 3 subpopulations: the first subgroup was treated for in vitro activation with ionomycin alone, the second subgroup with ionomycin and subsequently with 6-DMAP, and the third subgroup was treated for in vitro fertilization. The maturation medium was TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO₃, 10% (v/v) FCS plus 0.1 iu/ml hMG (Pergovet, Serono, Rome, Italy). Groups of 20–30 oocytes were cultured in 500 µl of maturation medium for 24 h, in 4-well dishes (Nunc, Roskilde, Denmark) at 38.5 °C under 5% CO₂ in humidified air. Oocytes collected at the end of the incubation in maturation medium were decumulated by a single vortexing step for 3 min, and then kept in suspension, and processed for cytochemical reactions.

In vitro activation

To achieve developmental competence, bovine oocytes were activated using ionomycin and were then treated with an in vitro inhibitor of oocyte meiotic resumption
(6-DMAP) according to the method described by Susko-Parrish et al. (1994). After microdensitometric quantitation of enzymatic activities, the block of the second meiotic division was assessed as follows: fixed oocytes were incubated with RNAase (100 iu/ml) for 30 min at room temp and then processed for chromatin staining with propidium iodide (Dietch et al., 1982).

Oocyte in vitro fertilization

A straw containing cryopreserved bull spermatozoa (CIZ, S. Miniato, Italy) was thawed in water at 32 °C, and cells were layered on top of a 45–90% Percoll gradient with modified Tyrode’s medium (TALP), calcium free, and centrifuged for 30 min at 600 × g. The sperm pellet was washed once in the same medium, spermatozoa were counted and diluted to a final concentration of 1 × 10⁶ cells/ml fertilization medium. The fertilization medium consisted of TALP supplemented with 0.6% (w/v) fatty acid-free BSA, 10 µg/ml heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine (Parrish et al., 1988). After the maturation period, COCs were washed 3 times in TALP medium supplemented with 20 mM Hepes (pH 7.4) and cells were layered on top of a 45–90% Percoll gradient. A straw containing cryopreserved bull spermatozoa was counted and diluted to a final concentration of 1 × 10⁶ cells/ml fertilization medium. The sperm pellet was washed once in the same medium, spermatozoa were counted and diluted to a final concentration of 1 × 10⁶ cells/ml fertilization medium. The fertilization medium consisted of TALP supplemented with 0.6% (w/v) fatty acid-free BSA, 10 µg/ml heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine (Parrish et al., 1988). After the maturation period, COCs were washed 3 times in TALP medium supplemented with 20 mM Hepes (pH 7.4) and centrifuged for 30 min at 600 × g. The sperm pellet was washed once in the same medium, spermatozoa were counted and diluted to a final concentration of 1 × 10⁶ cells/ml fertilization medium.

Cytochemical reactions

Preovulatory immature oocytes, in vitro matured oocytes, oocytes activated in vitro by the calcium ionophore, oocytes maintained in meiotic arrest by 6-DMAP treatment and in vitro fertilized oocytes were processed for cytochemical detection of activity of SDH (E.C.1.3.99.1) according to Van Noorden et al. (1983 a), LDH (E.C. 1.1.1.27) and G6PDH (1.1.1.49) according to De Schepper et al. (1985). Three cytochemical control media were used: one in the absence of the specific substrates succinate, lactate (Serva, Heidelberg, Germany) or glucose-6-phosphate; a second in the absence of the coenzymes NAD and NADP; and a third in the absence of both substrates and coenzymes to determine nonspecific final reaction product formation. Tetranitro BT (TNBT) was the final electron acceptor in all 3 cytochemical reactions.

Determination of enzyme activities

One hundred measurements were performed for each oocyte population (immature, in vitro matured, treated with Ca²⁺ ionophore alone, treated with Ca²⁺ ionophore and 6-DMAP) with the exception of in vitro fertilized oocytes. In that case, we evaluated 30 oocytes. The mean absorbance of the formazan precipitated in individual oocytes (Fig. 1) was analyzed with a Universal Microspectrophotometer System (Zeiss, Milano, Italy) using a measuring diaphragm of 0.16 mm and step sizes of Δx = 1.5 µm, and Δy = 2.5 µm. Readings were taken with a 10× objective (N.A. = 0.25) and a 10× eyepiece at a wavelength of 534 nm which yields the maximum absorbance of precipitated TNBT formazan (Van Noorden and Tas, 1980; 1981; Van Noorden et al., 1983 a, b).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) with the Scheffe test for multiple comparisons. Unless otherwise specified, differences of p ≤0.01 were regarded as statistically significant.

Results

Oocytes had a well-preserved morphology and the coloured end product (formazan) produced by enzyme activities precipitated entirely within the cytoplasm of the cells. In addition, precipitated formazan was restricted to the interior of the cells and formazan was never found in the incubation media, indicating that leakage of enzyme molecules or reduced intermediates or formazan from the cell did not occur. Formazan production could not be detected microdensitometrically in immature, matured, activated and fertilized oocytes incubated in the cytochemical control media, indicating that nonspecific formazan production did not occur.

SDH activity was significantly increased (p ≤0.05) in bovine oocytes after in vitro maturation, significantly decreased after Ca²⁺ ionophore activation, with a further decrease after 6-DMAP treatment. The absorbance values for SDH activity of in vitro fertilized oocytes were similar to the values of those incubated in medium containing 6-DMAP (Fig. 1). The 3-D absorbance profile of formazan precipitated due to SDH activity in an individual immature oocyte (Fig. 2A) was markedly higher than in an in vitro fertilized oocyte (Fig. 2B). These 3-D profiles were obtained on the basis of individual cytophotometric data provided by the microdensitometer.

LDH activity showed a significant drop in bovine oocytes after in vitro maturation, remained unchanged in Ca²⁺ ionophore treated oocytes and rose significantly after 6-DMAP treatment. LDH activity was...
strongly reduced after in vitro fertilization, and was not statistically different from matured and Ca\textsuperscript{2+} ionophore-treated oocytes (Fig. 1).

The microdensitometric data showed that G6PDH activity significantly decreased after maturation and increased significantly after artificial activation with the Ca\textsuperscript{2+} ionophore, although the activity remained significantly lower than in immature oocytes. G6PDH activity remained steady after 6-DMAP treatment. In fertilized oocytes, G6PDH activity was not different from that of diploid activated 6-DMAP-treated oocytes (Fig. 1).

**Discussion**

In vitro fertilization is employed frequently to study the physiology and morphology during fertilization. In vitro activation and fertilization have become routine procedures to study reproduction. Activation of metabolite transport and protein synthesis occurs somewhat later, and is followed by fusion of the sperm and egg pronuclei, initiation of DNA synthesis, and, ultimately, cell division. Many of these events can be parthenogenetically induced by various agents. Inhibition of second polar body extrusion and the presence of only one pronucleus were observed in the majority of oocytes incubated with 6-DMAP after ionomycin treatment (Loi et al., 1998). There is evidence that Ca\textsuperscript{2+} plays a role in regulating MPF activity and exit from meiosis is controlled by Ca\textsuperscript{2+} signals in eggs of mammalian species as well including bovine oocytes (Collas et al., 1993). In bovine oocytes, GVBD is blocked when oocytes are exposed to 6-DMAP (a protein kinase inhibitor; Fulka et al., 1991; Lonergan et al., 1997; Saeki et al., 1997). Ionomycin treatment followed by 6-DMAP treatment causes transition from meiosis to mitosis without com-

![Fig. 2. Absorbance profiles in 3-D of bovine oocytes processed for the cytochemical detection of SDH activity. A, Immature oocyte with a mean absorbance of 0.58 and a diameter of 138.8 \( \mu \text{m} \). B, In vitro fertilized oocyte with a mean absorbance of 0.33 and a diameter of 150 \( \mu \text{m} \).](image-url)
pletion of meiosis. Furthermore, it should be emphasized that 6-DMAP may also be important in inhibiting phosphorylation which is necessary for the spindle apparatus or microfilaments, thus preventing expulsion of the second polar body (Susko-Parrish et al., 1994).

Our cytochemical data of the activity of enzymes involved in energy metabolism in bovine oocytes undergoing in vitro maturation followed by ionomycin-6-DMA treatment, or fertilization in vitro may be interpreted as follows. The significant increase in SDH activity in the late stages of incubation in maturation medium until complete meiotic oocyte maturation indicates an increase in the absolute number of mitochondria per oocyte in mouse oocytes as described by Saito et al. (1994). It is difficult to establish the significance of the steep decline in SDH activity in oocytes after calcium ionophore and 6-DMAP treatment and in in vitro fertilized oocytes. This finding is not in agreement with those found in mouse embryos (Ayabe et al., 1994). We may assume that there are biological differences between the metabolism of energy-rich substrates in early stages of bovine preimplantation embryos and the metabolism in embryos of other species. However, we found a decrease in the activity of the enzymes as well as in vitro fertilized oocytes. It may be proposed that this enzymatic activity is affected by paternal genomic imprinting during early development, but further investigations are needed.

The high LDH activity in immature oocytes is probably related to ATP production by the formation of lactate from pyruvate as energy source, as was previously suggested for bovine oocytes during their growth phase (Ferrandi et al., 1993) and was found in adult and prepuberal bovine oocytes as well (Rieger and Loskutoff, 1994; Gandolfi et al., 1998). The significant decrease in LDH activity during maturation of bovine oocytes may be a result from disappearance of the enzyme protein itself as has been observed in the mouse after the second embryonic day (Spielman et al., 1974 a, b; Ferrandi et al., 1993), indicating earlier aerobic utilization of glucose as energy source in bovine oocytes than in mouse oocytes. Obviously, the requirements for nutrients in optimized bovine egg cultures may not be the same as for mouse eggs. The low LDH activity in Ca2+ ionophore-activated oocytes and in fertilized ova needs further confirmation but supports the possibility that LDH synthesis stops at the end of the preimplantation period as has been observed during mouse oogenesis and preimplantation development (Mangia et al., 1976). We are not able to explain whether the high LDH activity after 6-DMAP treatment reflects either posttranslational activation of the enzyme or a net increase in the number of enzyme molecules per oocyte.

The higher activity of G6PDH, the key enzyme of the pentose phosphate pathway, in preovulatory bovine oocytes may be related to biosynthetic processes such as lipid synthesis, or production of riboses and deoxyriboses for RNA and DNA synthesis, as we have previously reported (Ferrandi et al., 1993). The significant decline in G6PDH activity after oocyte maturation to levels not significantly different from those after treatment with Ca2+ ionophore and 6-DMAP and those of fertilized oocytes is in agreement with the findings in mouse oocytes as described by Mangia et al. (1976). The significant increase in G6PDH activity after chemical parthenogenetic activation and after fertilization may be related to the fact that the pentose phosphate shunt is the main source of NADPH which is needed to keep intracellular glutathione reduced (GSH). GSH plays an important role in the control of oxidative stress during fertilization and it has been suggested that activated oxygen species play a physiological role in interactions of sperm cells and the zona pellucida (Aitken et al., 1989). Therefore, generation of superoxide complexes by sperm cells and oocytes appears to be a physiological phenomenon (Miesel et al., 1993). GSH has another important role too because it is necessary for transformation of the disulphide-stabilized sperm nucleus into the male pronucleus after fertilization (Calvin et al., 1986). Furthermore, there is growing evidence that substantial levels of GSH are necessary for later events in embryogenesis as was reported by Calvin et al. (1986). In addition, maintenance of levels of GSH may be particularly important during development and rapid growth to enable responses of the embryo to a variety of exogenous insults (Hales and Brown, 1991).

Although caution is needed in the interpretation of the present results, our cytochemical profiles of SDH, LDH and G6PDH activity in bovine oocytes after chemical parthenogenetic activation suggest that some of the morphofunctional changes involved in diploid parthenogenetic activation may be similar to those occurring after fertilization of eggs.

References

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