Low Oxygen Inhibits but Complex High-Glucose Medium Facilitates In Vitro Maturation of Squirrel Monkey Oocyte–Granulosa Cell Complexes

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Purpose: *The objectives of these in vitro maturation studies in primate cumulus-oocyte complexes (COCs) were to evaluate the effect of a reduced-oxygen environment and to compare medium with a high-glucose concentration to medium with pyruvate but no glucose.*
Methods: *COCs were retrieved from squirrel monkeys stimulated with 1 mg of follicle-stimulating hormone (FSH) for 4–6 days. Experiment 1 examined maturation after 48 hr in 5% O_2/5% CO_2/90% N_2 compared with 5% CO_2/air. The medium was CMRL-1066 containing moderate glucose (5.5 mM) supplemented with 1 mM glutamine, 0.33 mM pyruvate, 0.075 IU/ml human FSH, 5 IU/ml human chorionic gonadotropin, 75 U penicillin G/ml, and 20% fetal bovine serum. Experiment 2 in 5% CO_2/air, compared P-1 medium (pyruvate and lactate but no glucose) to Waymouth's medium (27.5 mM glucose), both with identical supplements.*
Results: *Only 3 (8%) of 37 COCs matured in 5% O_2, while 39 (49%) of 80 matured in ambient O_2. Fourteen (22%) of 64 complexes matured in P-1 medium, compared to 47 (49%) of 96 meiosis II oocytes in Waymouth's medium ($P < 0.05$).*
Conclusions: *These are the first primate studies showing detrimental effects of reduced-oxygen culture on in vitro maturation. Additionally, maturation was enhanced with complex high-glucose medium suggesting that the predominant metabolism is aerobic glycolysis.*

KEY WORDS: glucose; maturation; oocyte; oxygen; primate.

INTRODUCTION

During fetal development, germ line nuclear activity is arrested at the GO stage of meiosis I, commonly known as the germinal vesicle stage, which is equivalent to G2 in the somatic cell cycle. Once follicular growth from the primordial stage is initiated at some point in the reproductive life of the individual, development proceeds dependent on local growth factors and permissive gonadotropins (1). Dynamic processes occurring within the oocyte include growth to its full size within the follicular antrum and increases in intracytoplasmic organelle concentration such that the oocyte has the highest concentration of mitochondria of any cell in the body (2). The development of maturational competency to progress beyond the GO stage occurs with antral formation and oocyte growth in murines (3) but requires further follicular growth in ungulates and primates (4–6). Synthesis and storage of RNA, protein, and lipids for future vital development is occurring and contributes to its competency for maturation (7,8). A component of this maturational competency is the buildup of cell cycle regulatory proteins including an inactive maturation promoting factor. The additional acquisition of gonadotropin receptors on the accompanying granulosa cells enables the stimulatory effect of the luteinizing hormone (LH) surge to induce a burst of ATP production, which is transferred into the awaiting oocyte (9). This rise in ATP activates a maturation promoting factor within the oocyte by phosphorylation and dephosphorylation of selective components (10). Breakdown of the germinal vesicle membrane then occurs, and nuclear maturation progresses through meiosis I yet arrests at meiosis II with the expulsion of the first polar body. Additional cytoplasmic changes have occurred during this time including RNA translation, repositioning cortical granules beneath the cell membrane and movement of the mitochondria (11). It is this span of oocyte development from the germinal vesicle stage to the arrested state of meiosis II with one polar body visible that is generally

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considered for in vitro maturation and is evaluated in the present experiments.

There is an absence of active glycolytic enzymes within the immature oocyte necessitating dependence on substrates suitable for the Krebs cycle such as pyruvate (12). A major source of energy comes from surrounding granulosa cells, which readily metabolize glucose via the glycolytic pathway to produce ATP for transport to the oocyte along with other nutrients (13). Gap junctions between the oocyte and microvilli from surrounding granulosa cells provide the cytoplasmic communication important for supporting this growth (14). Arrested and aberrant development frequently occurs during this final stage of maturation in oocytes without adjacent granulosa cells (6, 15). Previous studies have investigated in vitro maturation of primate oocytes with varying degrees of success (6,15–17), however, none have comparatively examined the culture conditions relating to the metabolism involved in in vitro maturation. Considering the research and clinical potential of ovum banking that may likely require in vitro maturation, investigation of media favoring different metabolic pathways appeared a worthwhile goal. It is known that different species and strains require different culture conditions for in vitro maturation and development (18,19). In particular, improved development of oocytes and embryos has been reported after culture in low-oxygen environments (20,21). Additionally, studies have shown species-specific optimal balances of glucose and pyruvate in culture media (22). This report describes experiments that first examined primate oocyte maturation under reduced- or ambient-oxygen conditions. Additionally, maturation was compared in a complex medium with a high glucose concentration to support glycolysis versus a simple medium without glucose that contained only lactate and pyruvate for metabolism in the Krebs cycle.

MATERIALS AND METHODS

Two groups of experiments were done in this study. The first group examined the effect of oxygen tension in the culture environment on oocyte maturation. The second group of experiments was done at ambient oxygen tension with contrasting medium intended to emphasize glycolysis (high glucose) versus the tricarboxylic acid cycle (no glucose).

Media and Culture Conditions

The medium prepared for oocyte retrieval was modified human tubal fluid (HEPES buffered) (Irvine Scientific, Irvine, CA) with 5% fetal bovine serum and 8 U heparin/ml. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. For the first experimental group, culture for oocyte maturation was done in CMRL-1066 medium containing 5.5 m*M* glucose (Life Technologies/GIBCO, Gaithersberg, MD) supplemented with 1 m*M* glutamine, 0.33 pyruvate, 0.075 IU/ml human follicle-stimulating hormone (FSH), 5 IU/ml human chorionic gonadotropin (hCG), 75 U penicillin-G/ml and 20% fetal bovine serum. Before use, media were equilibrated overnight in their designated oxygenation conditions. A humidified incubator adjusted to 38.5°C and 5% CO_2 via an infrared sensor (FormaScientific, Marietta, OH) was adapted with a PROOX sensor and controller (Reming Bioinstruments, Redfield, NY) that injected nitrogen gas to reduce the oxygen to 5%. Fyrite CO_2 and O_2 gas analyzers (Bacharach, Pittsburgh, PA) were used to confirm the controller settings.

For the second group of experiments, either P-1 medium (Irvine Scientific) (no glucose) with 1 m*M* glutamate or Waymouth's medium (Life Technologies/GIBCO) (27.5 m*M* glucose) supplemented with 0.5 m*M* pyruvate, 1 m*M* glutamine, 10 m*M* lactate, and 50 μg/ml gentamycin sulfate (6) was used. Media were adjusted to pH 7.3–7.5 and 285–295 mOsm with Milli-Q water (Millipore, Bedford, MA) then sterilized by filtering through a 0.22-μm mesh. Both of these maturation media also contained 5 μg of purified FSH/ml, 5 IU hCG/ml, and 20% fetal bovine serum. Media again were equilibrated overnight in the incubator before use. All of the second group of experiments were done in a humidified incubator at 38.5°C with a 5% CO_2/95% air (20% O_2) environment.

Ovarian Stimulation and Oocyte Retrievals

Pubertal squirrel monkeys (*Saimiri boliviensis*) (24–34 months of age) housed indoors in group pens during the breeding season were used in the present studies. Animals (n = 33) were stimulated in experimental groups of three or four at a time with 1 mg purified FSH (Ausa International, Inc., Tyler TX) twice daily for 4–6 days, then given 500 IU hCG at night. Ten to twelve hours later on the following morning, oocyte retrievals were performed using ketamine anesthesia (35 mg/kg; AVECO, Fort Dodge, IA) (16). Laproscopically observed folllicles were aspirated

(−90 mm Hg) using a 23-gauge needle connected via warmed tubing to a vaccum trap tube positioned in a 38°C heating block. Tube contents were immediately searched for oocytes under a dissecting microscope in a humidified, 38°C chamber. For the first experiment, additional material was available from ovaries obtained from six animals involved in other studies. These animals had also been stimulated with 1 mg purified FSH for 4 days prior to sacrifice under ketamine and pentobarbital anesthesia. Ovaries were quickly minced in HEPES-buffered medium within the humidified, heated chamber. From both the laparoscopic aspirations and the minced ovaries, immature oocytes were quickly rinsed and placed in groups of three to five oocytes per 100-μl drops of medium under washed and equilibrated oil. Immature oocytes were identified by tight granulosa cell coverings or visible germinal vesicals within the cytoplasm. Oocytes from the different sources were evenly distributed between the appropriate incubation atmospheres of either 5% CO_2/95% air or 5% CO_2/5% O_2/90% N_2. For the second group of experiments, which compared maturation in simple versus complex high-glucose medium, only oocytes obtained laproscopically were utilized. Oocytes identified as having germinal vesicle breakdown, having a polar body visible at retrieval, or being degenerate/attretic were not included in these studies.

Oocyte Assessment and Statistics

Development was observed after 24 hr of culture when not obscured using an inverted microscope equipped with a Hoffman contrast-modulation optical system and the culture dishes were quickly returned to the incubator. After 46 hr of culture, oocytes were again observed and obstructing granulosa cells were removed with a pulled glass pipette when restricting the vision. Germinal vesicle breakdown and polar body expulsion were recorded as markers of the maturational progress. These markers of maturation were compared between the culture conditions at the observation points using a chi-square analysis. When $P < 0.05$, the difference was considered significant.

RESULTS

Effect of Atmosphere Oxygenation on Oocyte Maturation

One hundred seventeen immature oocytes were cultured in the first group of experiments, which examined the effect of reduced oxygen on maturation. Granulosa cells were attached to 29 (78%) of 37 oocytes cultured in 5% CO_2/5% O_2/90% N_2, while 56 (70%) of 80 oocytes had attached granulosa cells in the group cultured in 5% CO_2/air. No maturation was observed in any oocyte in 5% CO_2/5% O_2/90% N_2 after 24 hr and in only 3 (8%) of 37 maturing after 48 hr (Fig. 1). When other immature oocytes were cultured in 5% CO_2 in air for 24 hr, 3 (16%) of 19 observed had germinal vesicle breakdown. After a further 22 hr of culture, 39 (49%) of 80 oocytes matured with polar bodies visible, which was significantly more than observed in the reduced-oxygen environment ($P < 0.01$). Maturation rates of oocytes obtained from ovarian tissure were comparable to those obtained laparoscopically and were, thus, combined for analysis (5% CO_2/air, 23/49 and 16/31, respectively; 5% CO_2/5% O_2/90% air, 1/20 and 2/17, respectively; data not shown).

Effects of Complex High-Glucose Medium on Oocyte Maturation

The second group of experiments examined the effect of glucose in an atmosphere of 5% CO_2 in air. One hundred sixty immature oocytes were incubated in either P-1 medium containing no glucose or in Waymouth's medium with 27.5 m*M* glucose. Granulosa cells were attached to 50 (78%) of 64 oocytes cultured in P-1 medium and 67 (70%) of 96 oocytes cultured in Waymouth's medium. Four (12%) of 34 of the observable oocytes in the P-1 medium had undergone germinal vesicle breakdown after 24 hr of culture (Fig.

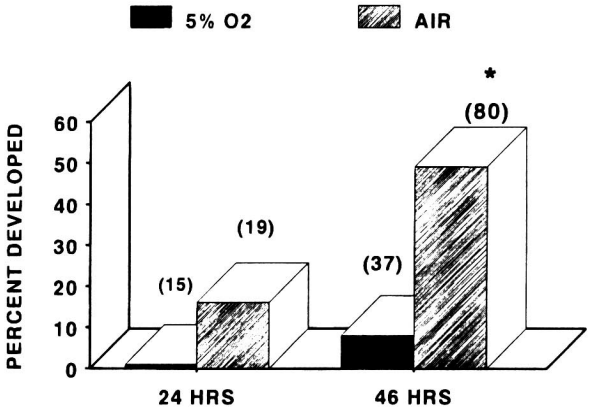

Fig. 1. Comparison of 5% CO_2/5% O_2/90% N_2 to 5% CO_2/air effects on maturation in vitro of cultured squirrel monkey oocytes. The bars display the percentage with germinal vesicle breakdown at 24 hr and percentage with a polar body visible at 48 hr. Numbers in parentheses are oocytes evalutated at that time. *$P < 0.05$.

2). With further culture for another day, 14 (22%) of 64 of the oocytes had completed maturation exhibiting polar bodies. In comparison, after 24 hr of culture in Waymouth's medium, 14 (27%) of 52 of the oocytes had germinal vesicle breakdown. Additionally, the granulosa cells were plating to a greater extent then seen in the P-1 medium (data not shown). When examined after 46 hr of culture, a significantly higher percentage of the oocytes had matured in the high-glucose medium: 47 (49%) of 96 ($P < 0.01$).

DISCUSSION

The present results showed a detrimental effect of reduced-oxygen culture conditions during in vitro maturation of squirrel monkey cumulus–oocyte complexes while cultured in CMRL medium (5 m*M* glucose). Superficially, these results would misleadingly support the hypothesis that oxidative phosphorylation is the primary metabolic pathway during this developmental stage. However, the second group of experiments, in which one condition specifically limited metabolism to the Krebs cycle and oxidative phosphorylation had inferior results, suggested that this was not the case and that a complex medium with a high glucose content supportive of glycolysis was preferred. These contrasting results can be resolved when the possibility of aerobic glycolysis is considered in which glycolysis occurs but at a very high rate such as found in rapidly dividing cells (23).

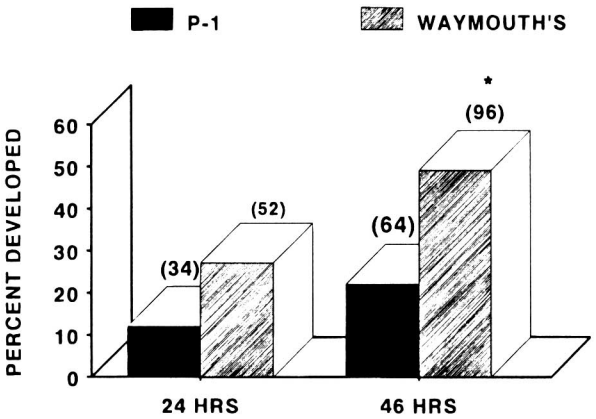

Fig. 2. Comparison of culture in P-1 medium to culture in Waymouth's medium on the rates of in vitro maturation of cultured squirrel monkey oocytes. The bars display the percentage with germinal vesicle breakdown at 24 hr and percentage with a polar body visible at 48 hr. Numbers in parentheses are oocytes evaluated at that time. *$P < 0.05$.

Estimations of the oxygen tension within the developing follicle suggest that most of the oxygen is consumed in the outer 5% of the radius, which corresponds to the outer layer of cells, resulting in only minimal oxygen levels at the oocyte (24). Although with antral formation, the follicular fluid oxygen content approximates serum tensions (25,26), the increasing layers of cumulus granulosa cells still diminish the oxygen level at the oocyte. Oxygen tension in the oviduct is low, indicating that later embryos also develop under low-oxygen concentrations (27). However, studies have reported that the glycolytic pathway in the oocyte and early embryo does not possess a full complement of active enzymes, and only the oxidative pathway is functional (12,28). This limitation has been shown with the arrest of cumulus cell-free hamster oocytes under anaerobic conditions (29). The inhibition of low oxygen tension is overcome by metabolic coupling via gap junctions with the surrounding granulosa cells, which actively utilize glycolysis (14).

The present results showed improved in vitro maturation of primate oocytes using ambient oxygen tension. This has been observed previously during the later stages of maturation of murine whole follicles or cumulus-enclosed oocytes (20, 30). These data suggest that the growing mass of follicular and cumulus cells which are contributing to the oocyte maturation requires considerable oxygen availability in vitro.

Weymouth's medium, which contains a substantial concentration of glucose, 27.5 m*M*, when supplemented with 0.23 m*M* pyruvate, has previously been shown to support adequately in vitro maturation of cumulus–oocyte complexes in marmoset monkeys, humans, pigs, and mice (6,31–33). The present results with squirrel monkey oocytes also showed improved nuclear maturation with Waymouth's medium compared to medium that only supported the Krebs cycle. When results from culturing in 5% CO_2 in the first study (CMRL medium; 5 m*M* glucose) were compared with results with Waymouth's medium (27.5 m*M* glucose), there was no difference when evaluated by the presence of one polar body. However, the cumulus morphology was not as consistently expanded with culture in CMRL compared to culture in Waymouth's medium (data not shown).

Reports of glucose concentrations in human and mouse follicular fluid are similar and range from 3.11 to 3.29 m*M* (23,34). Evaluations of oviductal fluid glucose concentrations through the human menstrual cycle, however, noted a marked decrease, from 3.11 to 0.5 m*M* at the time of ovulation, with an inverse molar relationship to rising lactate levels, emphasizing the high rate of glycoly-

sis at midcycle (35). These in vivo glucose concentrations are similar to those in the CMRL medium but much lower than found in the Waymouth's medium. This complex high-glucose medium, which promotes better maturation and morphology, may be compensating for an unknown in vitro culture artifact.

In vitro maturation is enhanced by gonadotropin supplementation (36,37) and this effect is dependent on the glucose in the medium (18,38). This also points to a primary role for glycolysis and stimulation of ATP production in the granulosa cells to provide metabolic support for the maturing oocyte.

Among the alternative possible explanations for the enhanced maturation observed when using the complex Waymouth's medium is the antioxidant potential of the glutathione in this medium. Potential oxygen toxicity from free radicals and peroxidation at ambient levels is a concern during early embryo culture. Metabolism in early embryos is primarily oxidative, and they have been shown to benefit from culture in a reduced-oxygen environment (21,39). However, metabolism in the cumulus-oocyte complex is predominantly glycolytic and the glutathione in Waymouth's medium may function to reduce potential peroxidation from ambient oxygen levels. Waymouth's medium also contains the precursors for glutathione, which may be important considering the limited permeability of glutathione into the cell (40). In support of this theory are the reports correlating the concentration of glutathione within the oocyte to successful production of pronuclei and embryonic development after maturation under conditions favoring glutathione production (41,42).

Similarly, enhanced glycolysis during in vitro maturation of murine cumulus-enclosed oocytes has resulted in high rates of lactate production and compared this state to aerobic glycolysis (38). This phenomenon is exhibited by a number of solid, fast-growing tumors and proliferating cells (43,44) and may relate to the cell's incapacity to generate ATP at a sufficiently high rate for rapid growth by oxidative phosphorylation (23). It should be noted that even with this high rate of glycolysis occurring, estradiol production is found with stimulation in vitro (38). Since some of the steroidogenic enzymes are dependent on oxidative mechanisms, it is likely that the outer granulosa cells, being exposed to a higher oxygen tension, are still able to maintain sufficient oxidative activity for steroidogenesis.

In conclusion, these are the first primate studies showing detrimental effects of a reduced-oxygen environment on oocyte in vitro maturation. Additionally, maturation was enhanced with a complex-high glucose medium compared to a simple medium containing pyruvate but not glucose. These results suggest that the high energy required for squirrel monkey oocyte in vitro maturation is supplied primarily by aerobic glycolysis.

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