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REGULATION OF SPERM CAPACITATION

by

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ABSTRACT

REGULATION OF SPERM CAPACITATION

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Capacitation is an essential process before fertilization. This project was designed to study the control mechanisms of sperm capacitation. The specific aims were to examine (1) the relationship between oxygen consumption and the progress of capacitation in boar spermatozoa, and (2) the role of glucose and the requirement for reactive oxygen species for mouse sperm fertilizing ability.

The first study demonstrated a surge of "capacitation respiration" in boar spermatozoa during capacitation. Oxygen consumption decreased after capacitation was achieved. In addition, a modified chlortetracycline (CTC) fluorescence assay for boar spermatozoa was adopted. The progress of capacitation and the acrosome reaction was verified by an *in vitro* fertilization assay and transmission electron microscopy (TEM).

In the second study, the role of glucose in fertilization was examined. Glucose, in addition to being a substrate for glycolysis, facilitates sperm capacitation and the acrosome reaction by generating H_2O_2 through the pentose phosphate pathway and the activity of NADPH oxidase. A moderate amount of H_2O_2 was shown to be essential for the fertilizing ability of mouse spermatozoa. Both insufficient and excessive amounts of H_2O_2 decreased sperm capacitation, the acrosome reaction, and fertilization.

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I also thank my parents and my husband Andrew Huang for their consideration, encouragement, and support.

PREFACE

This dissertation is divided into two chapters. Chapter I describes a study of the relationship between oxygen consumption and the progress of capacitation in boar spermatozoa. Chapter II presents the research on the role of glucose and the requirement for reactive oxygen species for mouse sperm fertilizing ability. Each chapter includes an abstract, a literature review and hypothesis, a section on materials and methods, the results, discussion, and references.

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CHAPTER I

THE TIME COURSE RELATIONSHIP BETWEEN OXYGEN CONSUMPTION, CAPACITATION, AND FERTILIZING ABILITY OF BOAR SPERMATOZOA

ABSTRACT

This study examined the relationship between oxygen consumption, fertilizing ability, and the progress of capacitation and the acrosome reaction of boar spermatozoa. Ejaculated boar spermatozoa were incubated in either MM199, a capacitation-supporting medium, or Modena, a boar semen extender that does not support capacitation.

The chlortetracycline (CTC) fluorescence assay for mouse spermatozoa was adopted to examine the progress of capacitation and the acrosome reaction in boar spermatozoa. Four CTC patterns associated with the progress of capacitation and the acrosome reaction were identified. In Modena, P(I) and P(II), representing fresh spermatozoa, were the predominant patterns observed throughout incubation. In MM199, pattern P(III), representing capacitated spermatozoa, was predominant after 4 h of incubation. The percentages of P(IV), acrosome reacted spermatozoa, were low in both media throughout incubation. The population of P(IV) increased when ionophore A23187 was added to spermatozoa incubated in MM199.

At the beginning of incubation, spermatozoa utilized more oxygen in MM199 than in Modena ($p < 0.05$). After 4 h, oxygen consumption of spermatozoa in MM199 began to decrease when the sperm fertilizing ability reached maximum levels. At 8 h, the same amount of oxygen consumption

was observed in MM199 and in Modena.

This study demonstrated that the surge of "capacitation respiration", associated with the acquisition of fertilizing ability, preceded the increase in beat/cross frequency and was independent of sperm motility.

LITERATURE REVIEW AND HYPOTHESIS

Changes in oxygen consumption of sperm fertilization have been associated with the progress of capacitation. Increases in oxygen consumption have been reported in rabbit and cock spermatozoa after incubation in the female reproductive tract, which is known to support sperm capacitation, or after *in-vitro* incubation in the presence of uterine or oviduct fluid [1-11]. However, decreases in oxygen consumption have been reported in capacitating mouse spermatozoa [12].

The inconsistency in the rate of oxygen consumption observed in many previous studies could be due to the sampling time in relation to the progress of capacitation. In fact, capacitation itself occurs at different rates depending on the species and incubation conditions [13, 14]. In addition, within a single sperm collection, individual spermatozoa capacitate at different rates. For example, in a given capacitation-supporting medium, a small percentage of mouse spermatozoa may acquire fertilizing ability in as little as 15 min [15] while the majority requires a period of 1 to 2 h to complete capacitation [16-20].

In this study, a computer-assisted automatic oxygen sensor, SEN16 (designed by Dr. Henry Wang, Chemical Engineering Department, University of Michigan, and Process Biotechnology, Inc.), was used to continuously

record oxygen consumption in the sperm culture. This sensing system recorded real time of oxygen consumption throughout the entire progress of sperm capacitation. The chlortetracycline fluorescence (CTC) assay, which has been developed to examine membrane changes as a measurement of sperm capacitation and the acrosome reaction in mouse [20, 21], human [22], equine [23], and monkey spermatozoa [24], was adopted and verified by transmission electron microscopy (TEM) to evaluate boar spermatozoa. The changes in oxygen consumption were also related to the development of sperm fertilizing ability assessed by the *in vitro* fertilization assay.

MATERIALS AND METHODS

Media and chemicals

Boar sperm capacitation medium, modified medium 199 (MM199), was made as described by Cheng *et al.* [25]. It contained 88% (v/v) medium 199 (with Hanks' salts, L-glutamine, and 25 mM HEPES buffer; Gibico, Gaitherburg, MD), 12% (v/v) heat-inactivated fetal calf serum (FCS), 2.3% (w/v) bovine serum albumin (BSA), 3.05 mM D-glucose, 2.91 mM calcium lactate, 0.91 mM sodium pyruvate, and 1% (v/v) antibiotics solution (250 units/ml penicillin; 0.25 mg/ml streptomycin; 0.5 mg/ml neomycin). The pH of MM199 was adjusted to 7.8 for boar sperm capacitation. The egg-maturation medium contained medium 199 supplemented with 10% (v/v) heat-inactivated FCS, 5 μg/ml NIH-LH-S22, 2.5 μg/ml NIH-FSH-S13, 20 ng/ml NIH-prolactin-S9, and 1 μg/ml estradiol-17β [25]. The pH of the egg-maturation medium was adjusted to 7.8. The fertilization medium was modified from MM199 by adding 2.01 mM caffeine and its pH was adjusted to 7.4 [26]. The modified BMOC-2 medium, which supports the development of fertilized eggs to the 2-cell or 4-cell stage, consisted of 94.9 mM NaCl, 4.78 mM KCl, 1.29 mM $CaCl_2 \cdot 2H_2O$, 1.18 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7H_2O$, 25.07 mM $NaHCO_3$, 41.47 mM sodium lactate, 0.26 mM sodium pyruvate, 5.55 mM D-glucose, 0.4% (w/v) BSA, and 1% (v/v) antibiotics [25]. The pH

of modified BMOC-2 medium was adjusted to 7.4. Modena, a commercial product (provided by Swine Genetics International, LTD., Cambridge, Iowa), contained citric acid, sodium bicarbonate, EDTA, glucose, Tris, L-cysteine, BSA, lincomycin, and spectinomycin. Modena, as an extender for artificial insemination and for semen storage, was used in this study as a control medium to support sperm motility and viability, but not capacitation. All chemicals used in this study, except where indicated, were purchased from Sigma Chemical Company, St. Louis, MO.

Sperm collection and treatments

Boar semen was obtained from mature boars by the gloved-hand technique at the Swine Teaching and Research Center of Michigan State University (MSU). Freshly collected semen was filtered through three layers of cheesecloth to remove gelatinous materials and was transported to the laboratory in a 35-36°C water bath. Sperm cells were washed by adding an 5 ml of the test medium (MM199 or Modena) into 5 ml semen, followed by centrifugation at 1200 x g for 5 min. The supernatant was discarded each time. The sperm pellet was resuspended in another 5 ml test medium for a second washing and then resuspended in 5 ml test medium. A volume of 0.5 ml washed spermatozoa was then diluted six times and 20 μl of the diluted sperm samples were placed on a 20 μm-depth cell-counting chamber for sperm motion

assay and videomicroscopy using a CellSoft™ sperm analyzer (Cryo Resources, Montgomery, NY). The parameters of the sperm motion assay included concentration, motility, velocity (total distance travelled divided by the total time the cell was tracked), linearity (the ratio of the straight-line distance to the actual track distance), amplitude of the lateral head (ALH) displacement (deviation of the sperm head from the mean trajectory), and beat/cross frequency (the number of beats per second). One beat is defined as each time the sperm cell crosses the computer-calculated curval mean.

The device for testing oxygen consumption was composed of six oxygen sensors connected to a computer with SEN16 software (designed by Dr. Henry Wang, Chemical Engineering Department, University of Michigan, and Process Biotechnology, Inc.). To measure sperm oxygen consumption, the 6-transwells culture dish (Costar, Combridge, MA) was used. The oxygen consumption of spermatozoa was recorded at 5 min intervals for 8 h with 4 ml sperm suspension per transwell at a concentration of 14 x 10^6 cells/ml in a 37°C incubator. The amount of oxygen dissolved in the medium was detected by the sensor. Sperm oxygen consumption was expressed as the percent of oxygen remaining in the medium. The amount of dissolved oxygen at 0 time, before spermatozoa were added to the transwell, was considered 100%.

Maturation of swine eggs in vitro

Fresh ovaries were obtained from the Meat Laboratory at Michigan State University. Eggs were collected from ovarian follicles (2-4 mm in diameter), sliced by a sharp surgery knife, and released into phosphate buffer solution (PBS). The method and medium for egg-maturation were based on those described by Staigmiller and Moor [27]. Ten to twenty five egg-cumulus complexes were washed free of follicular fluid with PBS and transferred into a 35 mm-diameter petri dish containing 2 ml of the egg maturation medium. Egg-cumulus complexes were incubated at 5% CO_2 in air, in a 39°C incubator for 48 h before eggs were separated from cumulus cells by gently flushing with a micropipet. The matured eggs were then rinsed with the fertilization medium before being introduced into sperm suspension for *in vitro* fertilization.

In vitro fertilization

Spermatozoa were collected and preincubated in the capacitation-supporting medium (pH 7.8) or Modena as described before. To assess the progress of capacitation, spermatozoa were sampled at 2, 4, 6, and 8 h for the *in vitro* fertilization assay. Each fertilization petri dish contained 2 ml fertilization medium (pH 7.4), 5 x 10^4 cells/ml preincubated spermatozoa, and 20-30 *in-vitro* matured eggs. After 6 h incubation at 39°C, eggs were washed with a micropipet to eliminate excess spermatozoa and transferred to 2 ml modified BMOC-2 for an additional 32-34 h of incubation at 39°C to allow 2-

cell or 4-cell development [26].

Examination of fertilization rate

The whole-mount technique and the two-staining system (DAPI fluorescence and Lacmoid-stain) were used to evaluate the fertilization status of eggs [25, 26]. Eggs were first stained with 0.001% DAPI (4', 6-diamidino-2-phenylindole) in modified BMOC-2 medium for 30-60 min at 37°C and then washed in the same medium with a micropipet. Five to ten eggs were wet mounted on each clean microscope slide by gently placing them beneath a No. 1 cover slip (22 x 22 mm) supported at each corner by four spots of petroleum jelly (Vaseline) and sealed on two sides with a 1:1 mixture of paraffin wax and petroleum jelly. Each whole-mount was first examined for the presence of DAPI stained pronuclei with a Nikon Optiphot microscope equipped with a 100 W mercury bulb, 365/10 nm excitation filter, 400 nm dichroic mirror, and 400 nm barrier filter. To examined the presence of swollen sperm heads and remanent tails, each whole-mount was immersed for 48 h in a Coplin jar containing a 1:3 mixture of glacial acetic acid and absolute ethyl alcohol. After fixation, the slides were stained with 1% lacmoid in 45% glacial acetic acid, which enhances the appearance of the eggs and pronuclei as viewed by phase contrast microscopy.

Criteria for evaluating fertilization ensured that the results were not

distorted by parthenogenic activation or degenerative fragmentation of eggs. Eggs in the single cell stage were not considered fertilized unless both female and male pronuclei with the remnant of sperm tail were visible. Eggs which had cleaved to 2 or 4 cells were considered fertilized only if nuclei were found in each cells. The presence of a swollen sperm head with remnant tail and female nucleus or pronucleus in an egg was taken as a sign of fertilization.

The chlortetracycline (CTC) fluorescence assay

A chlortetracycline (CTC) assay, modified from the procedures described by Ward and Storey [20], was used to identify the progress of capacitation and acrosome reaction of boar spermatozoa. After washing and resuspension in the test medium, MM199 or Modena, spermatozoa were incubated at a concentration of 1 x 10^8 cell/ml for 7 h at 37°C and sampled hourly for the CTC assay.

For CTC staining, 20 μl of 500 μM CTC in a chilled buffer of 20 mM Tris, 130 mM NaCl, and 5 mM cysteine were added to a warm (37°C) microscope slide followed by 20 μl of sperm suspension and mixed thoroughly. After 10 sec, sperm cells were fixed with 10 μl of 12.5% glutaraldehyde in Tris buffer (pH 7.8) and mixed again. A coverslip was then applied for observation.

Spermatozoa were illuminated with a 100 W mercury bulb and examined

with a Nikon Optiphot microscope equipped with a 380-425 nm excitation filter, a 520 nm barrier filter, and a 510 nm dichroic mirror. A minimum of 100 sperm cells were analyzed for each sample.

Transmission electron microscopy (TEM)

The percentage of acrosome reacted spermatozoa identified via the CTC assay matched the percentage of acrosome reacted spermatozoa observed via TEM after 1 and 5 h of incubation in MM199. Samples were also examined after 7 h incubation with ionophore A23187 introduced to the incubation medium at 5 h. Sperm samples were centrifuged at 600 x g for 10 min and the pellet was resuspended in 4% glutaraldehyde [28, 29]. The final sperm concentration was adjusted to 2 x 10^8 cell/ml. After fixation, spermatozoa were washed three times with PBS (pH 7) at 15 min intervals. Post-fixation was achieved by gentle agitation with 1% OsO_4 for 1.5 h followed by three washings with H_2O at 15 min intervals. Spermatozoa were then dehydrated by graduated ethanol (25, 50, 75, 100%) and embedded in 65°C 100% resin. After 36 h, sperm blocks were sectioned (90 nm), mounted on copper grids and stained with saturated aqueous uranyl acetate and calcined lead citrate. Specimens were examined on JEOL 100 CX II TEM/Scan (Nicon, Garden, NY), at 100 KV accelerating voltage and 25,000 x magnification, for evidence of the acrosome reaction. Sperm heads with an attached midpiece were

selected for evaluation. A minimum of 100 sperm cells were observed for each sample.

Statistics

Angular transformation was performed for the mean of the sperm penetration rate, the dissolved oxygen consumed (%), and the sperm population showing different CTC fluorescence patterns. The transformed data were analyzed by one-way analysis of variance (ANOVA) using SigmaStat 50. Sperm linearity, motility, and beat/cross frequency were analyzed by the same statistical model. The statistics for ALH displacement (μm) and velocity (μm/second) were performed by ANOVA without transformation. Multiple comparisons between groups were determined by Least Significant Differences (LSD) for the comparisons of multiple means. Pearson's Correlation Analysis was used to evaluate the correlation between the CTC fluorescence pattern and TEM results.

RESULTS

Oxygen consumption of spermatozoa remained constant in Modena during the 8 h incubation, while that in MM199 was significantly higher during the first 4 h and decreased to the same level as that in Modena by the 8th h (Fig. 1-1). Sperm velocity, ALH displacement, and linearity were the same in MM199 as in Modena at all sampling times. The beat/cross frequency, although the same at 0 and 2 h, was significantly higher in MM199 at 4 and 6 h than that in Modena ($p < 0.05$) (Fig. 1-3). Whiplash-like tail beating and head-to-head aggregation were observed after 4 h in MM199, while spermatozoa in Modena swam freely without any signs of hyperactivation or aggregation throughout the observation (Fig. 1-1). Sperm motility was 10% lower in MM199 than in Modena at 7 h. At 12 h, however, only 8% of the spermatozoa remained motile in MM199, compared with 63% in Modena (Fig. 1-2).

Spermatozoa acquired little fertilizing ability after incubation for up to 8 h in Modena. The fertilizing ability of spermatozoa in MM199, on the other hand, increased significantly and reached maximum levels during the first 4 h of incubation before it decreased again at 8 h (Table 1).

Four CTC fluorescent patterns, P(I) to P(IV), were identified (Fig. 1-4). P(I) and P(II) were the predominant patterns observed in fresh or pre-

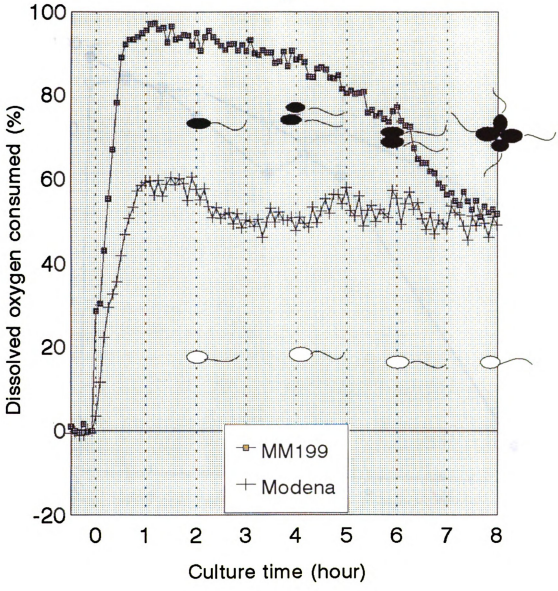

Figure 1-1. The surge of oxygen consumption and the aggregation* of boar spermatozoa during the progress of capacitation at a concentration of 14 x 10^6 cells/ml.

* Sperm symbols represent the progress of aggregation from 1 spermatozoa swimming alone to an aggregation of 2 swimming, and to a stationary aggregation of more than 2 spermatozoa.

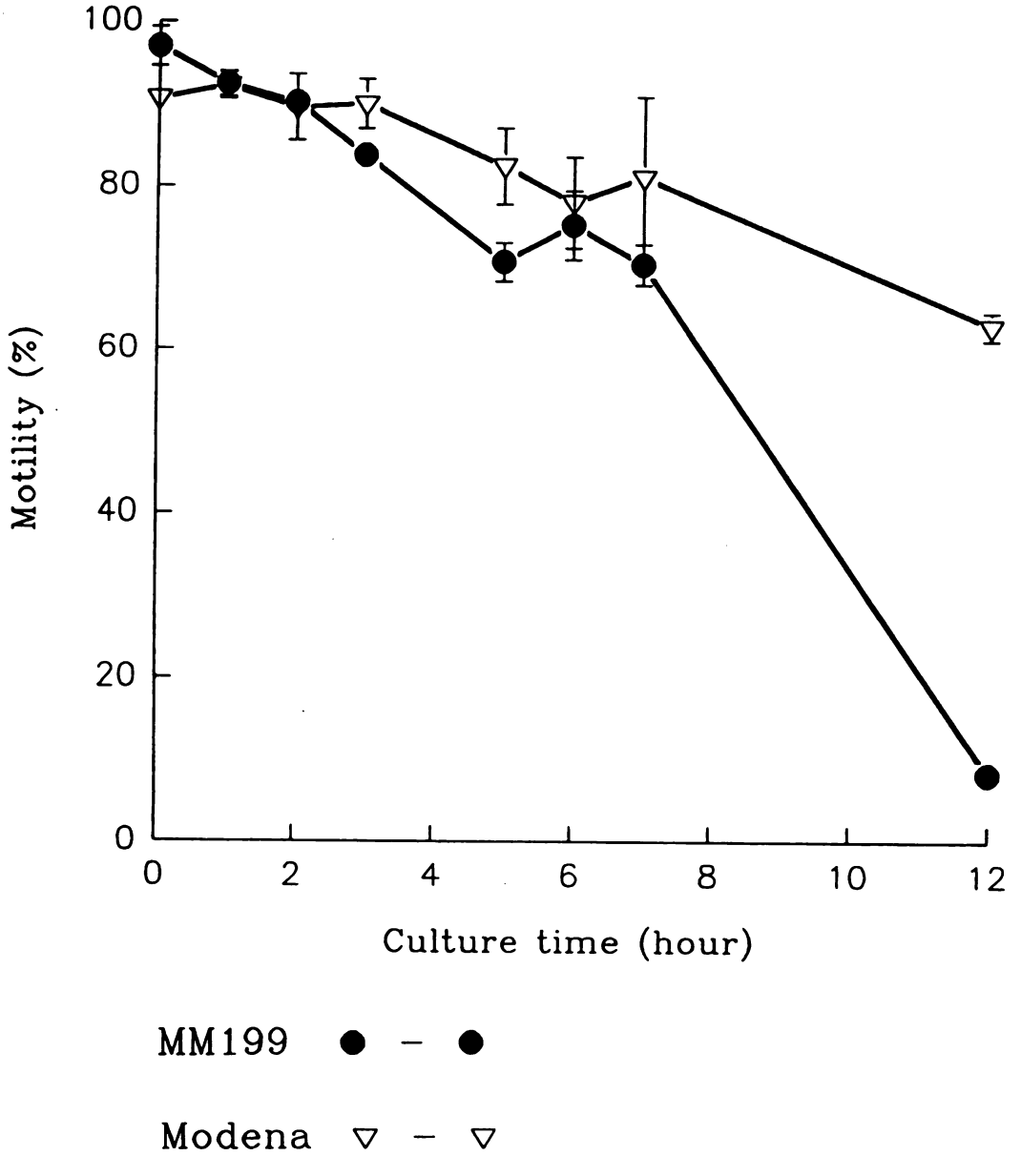

Figure 1-2. Motility of boar spermatozoa incubated in MM199 (●) and in Modena (▽) at 37°C for 12 h at a concentration of 14 x 10^6 cells/ml. Each point represents mean ± S.E.M. of 3 experiments. A minimum of 100 spermatozoa were assessed in each sample.

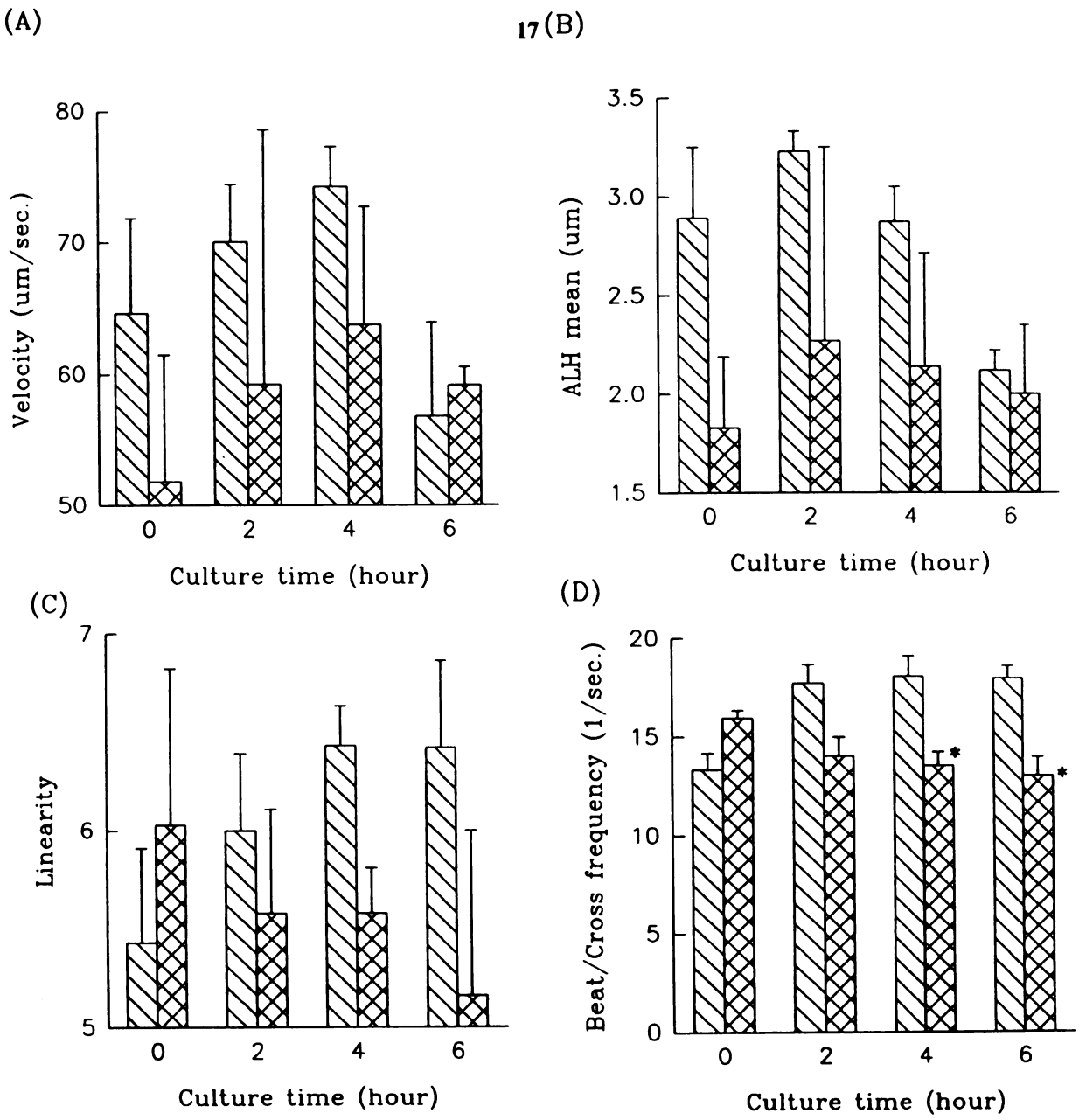

Figure 1-3. Boar sperm motion parameters of (A) velocity (μm/sec.), (B) amplitude of the lateral head displacement (μm), (C) linearity, and (D) beat/cross frequency (Hz, 1/sec.) in MM199 and Modena for 6 h of incubation. Each bar represents mean $\pm$ S.E.M. of 3 experiments. A minimum of 100 spermatozoa were assessed in each sample.

* P< .05 for MM199 vs. Modena.

Table 1-1. Effect of preincubation in MM-199 and Modena on the fertilizing ability of boar spermatozoa.

Preincubation time[a] (hour)	Fertilization rate[b] (Mean ± S.E.M.)[c]	
	MM-199[d]	Modena
2	48/151 (32.3 ± 2.7)[e]	10/148 (7.2 ± 1.0)[h]
4	29/40 (73.8 ± 2.5)[f]	2/40 (5.4 ± 1.0)[h]
6	120/155 (77.3 ± 1.5)[f]	17/150 (11.2 ± 3.5)[h]
8	26/40 (65.1 ± 1.6)[g]	1/40 (3.3 ± 0.6)[h]

[a] Boar spermatozoa were preincubated for 2, 4, 6, or 8 hours in MM-199 or Modena before used for inseminating in vitro matured eggs.

[b] Total number of eggs penetrated by spermatozoa / Total number of eggs evaluated.

[c] Values are means ± S.E.M. of the percentages of eggs fertilized in three experiments.

[d] Modified Medium 199

[efg] Means with different superscripts differ ($p < 0.05$).

[h] Means with the same superscript are not significantly different ($p > 0.05$).

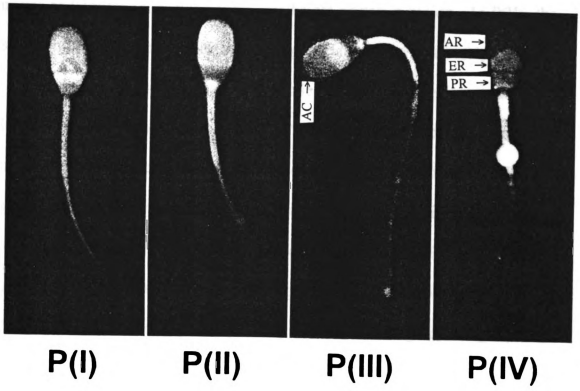

Figure 1-4. Boar sperm chlortetracycline (CTC) fluorescence patterns. P(I) and P(II) represent fresh spermatozoa, P(III) represents capacitated spermatozoa, and P(IV) represents acrosome reacted spermatozoa.

AR: anterior region; PR: posterior region; ER: equatorial region; AC: acrosome cap

capacitated samples. P(I) was characterized by a bright anterior region and a bright acrosome cap, with a relatively faint posterior region and a dark half circle at the equatorial region. P(II) was characterized by equally bright anterior and equatorial regions and a less bright posterior region. In P(II), the distinction between the anterior and the equatorial regions was barely visible. In P(III), the anterior region was faint, while the equatorial region remained relatively bright. Unlike other patterns, P(III) appeared to include a serious of transition patterns differentiated by the size of the faint spot in the anterior region. Some P(III) sperm had a small faint area in the anterior region adjacent to the equatorial region, while others had a relatively large faint area. The presence of the acrosome cap was frequently noticeable in those exhibiting P(III). P(IV) was characterized by a very faint anterior region, a slightly bright equatorial region and a faint posterior region. The acrosome cap appeared to be missing in the sperm exhibiting P(IV), and the sperm head appeared to be smaller than those exhibiting P(I), P(II), and P(III).

The changes in the distribution of the sperm population in the four fluorescence patterns are shown in Figure 1-5. In MM199 at all sampling times, less than 10% of the spermatozoa exhibited P(I)(Fig. 1-5A). The predominant population changes occurred in those exhibiting P(II) and P(III): populations P(II) declined throughout the incubation time, while P(III)

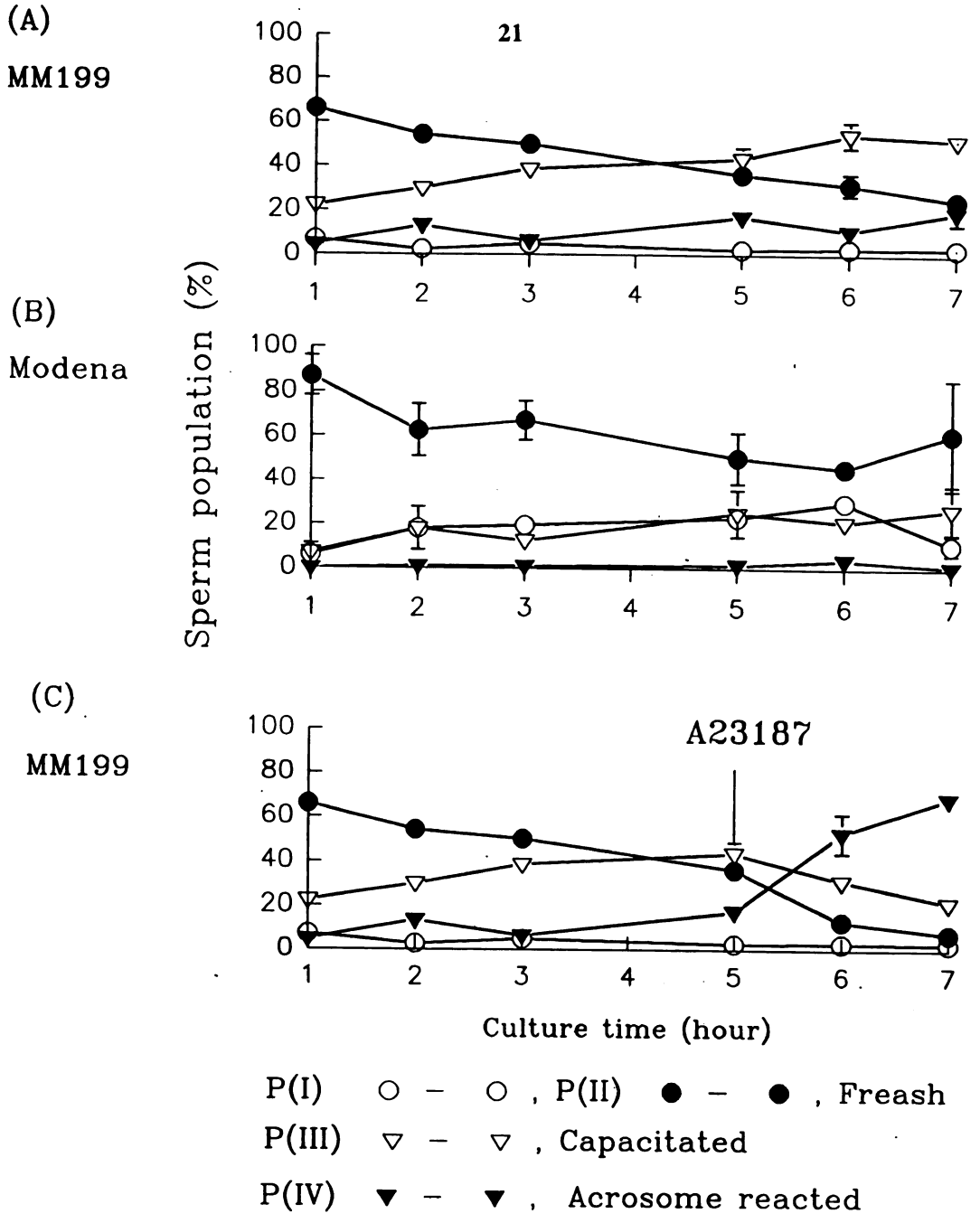

Figure 1-5. Fluorescence patterns of boar spermatozoa when incubated in (A) MM199, (B) Modena, and (C) MM199 with ionophore A23187 added at 5 h of incubation. P(I):○, P(II):●, P(III):▽, and P(IV):▼. Each point represents mean ± S.E.M. of 3 experiments. A minimum of 100 spermatozoa were observed in each sample.

increased. Only a small increase in population P(IV) was observed.

In Modena, the majority of spermatozoa exhibited P(II) in all samples (Fig. 1-5B). Although the percentage of sperm exhibiting P(II) decreased 22% between 3 h and 6 h, it continued to be more than 50% of the total population at 7 h. The population exhibiting P(I) and P(III) remained relatively low. The population of spermatozoa exhibiting P(IV) did not change during the observation and remained at less than 5%.

In a separate treatment, ionophore A23187 was added to MM199 sperm culture at 5 h and immediately washed twice with MM199 for further incubation. At 6 h, the P(II) population decreased from 36% to 13%, and P(III) from 44% to 31%, while the P(IV) population increased from 17% to 53% (Fig. 1-5C). Two hours later, 69% of the sperm population exhibited P(IV).

The percentage of spermatozoa exhibiting intact and acrosome reacted membranes was further examined by TEM to verify CTC fluorescence patterns (Fig. 1-6).

A match was observed between the percentage of P(IV) and the percentage of acrosome reacted spermatozoa assessed via TEM. Using TEM, no acrosome reacted spermatozoa were observed at 1 h. At 5 h, 13% of spermatozoa in MM199 were acrosome reacted. After exposure to ionophore A23187, 64% of the population were acrosome reacted. A high correlation

Figure 1-6. Transmission electron microscopic photographs of (A) fresh spermatozoa with intact and compact acrosome cap, (B) acrosome reacted spermatozoa with swollen and loose acrosome cap, and (C) acrosome reacted spermatozoa without acrosome cap.

was observed between the P(IV) and acrosome reacted spermatozoa ($r^2=0.96$) (Fig. 1-7).

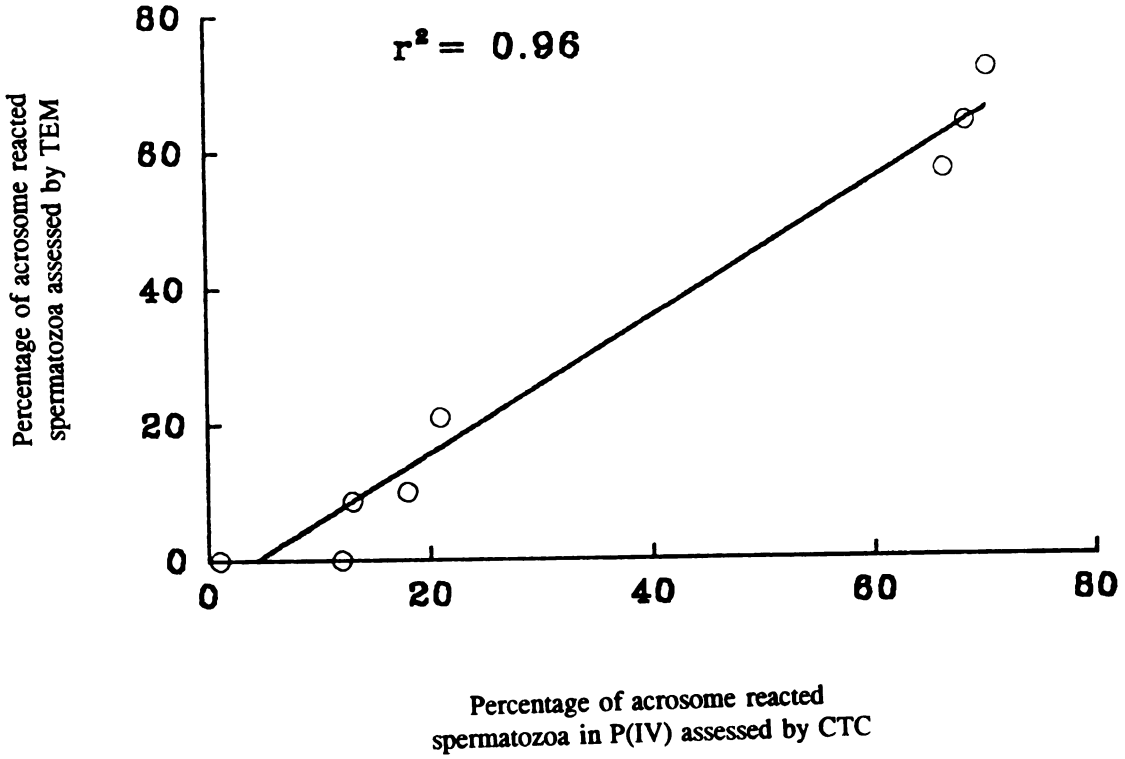

Figure 1-7. Correlation between the percentages of spermatozoa showing chlortetracycline (CTC) fluorescence acrosome reacted spermatozoa and the percentages of acrosome reacted spermatozoa assessed by transmission electron microscopy (TEM).

DISCUSSION

Results from this study demonstrated that capacitating spermatozoa consumed significantly more oxygen than non-capacitating spermatozoa ($p < 0.05$). The low oxygen consumption of spermatozoa in Modena, a semen extender for storage, was associated with prolonged motility, the lack of signs of capacitation and acrosome reaction, and the lack of fertilizing ability. In MM199, sperm fertilizing ability doubled between 2 and 4 h. The surge of "capacitation respiration", occurred during the first 4 h of incubation, decreased after spermatozoa acquired the maximum fertilizing ability. At 2 h, spermatozoa were swimming alone. At 4 h, head-to-head aggregation and vigorous whiplash-like beating of the tails were first observed.

By 12 h, most spermatozoa lost their motility in MM199. This was expected because capacitated spermatozoa have a limited life-span and are more fragile and short lived than non-capacitated spermatozoa [30]. Sperm motility during the first 8 h of incubation was the same in the capacitation-supporting medium and the non-cpapcitation-supporting medium. The surge in oxygen consumption observed during "capacitation respiration" was, therefore, independent of sperm motility.

Hydrogen peroxide has been shown to facilitate human and hamster sperm capacitation [31, 32]. It can be generated by either the two-electron

reduction of oxygen (O_2) (Reaction 1) or by the superoxide anion ($O_2^{\cdot -}$) dismutation reaction (Reaction 2 and 3):

$$O_2 + 2\,e + 2\,H^+ \rightarrow H_2O_2 \quad (1)$$

$$O_2 + e \rightarrow O_2^{\cdot -} \quad (2)$$

$$2\,O_2^{\cdot -} + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Part of the oxygen consumed during "capacitation respiration" in this study may contribute to H_2O_2 generation. This would account for the higher oxygen consumption in the capacitation-supporting medium than in the non-capacitation-supporting medium. During capacitation, the membranes of spermatozoa undergo several biochemical changes, including a decrease in the net negative charge within the plasma membrane [33], an increase in membrane permeability for calcium ions [34], the acceleration of membrane lipid peroxidation [35], and the loss of specific membrane components such as decapacitation factors from the seminal plasma [36-38]. Hydrogen peroxide may facilitate membrane lipid peroxidation, thus destablizing the plasma and acrosomal membranes. In addition, lipid peroxidation may increase the adherence ability of the sperm membrane and induce head-to-head aggregation.

The changes of spermatozoa during capacitation are morphologically invisible. The CTC assay adopted for this study has been used previously in

several other species [20-24]. This is the first report that describes the fluorescent patterns for boar sperm capacitation and the acrosome reaction. In Modena, patterns P(I) and P(II), representing pre-capacitated spermatozoa, were the predominant patterns observed throughout incubation. In MM199, P(III) became the predominant pattern after 4 h of incubation while the percentage of P(II) decreased. The emergence of P(III) at 4 h corresponded with maximum fertilizing ability.

The percentage of spermatozoa exhibiting P(IV) were low in both test media because neither were designed to support the acrosome reaction. The population of P(IV) increased after exposure to ionophore A23187, a compound known to induce the acrosome reaction [39, 40]. This was verified by TEM which identified the morphological changes of acrosome reacted spermatozoa.

In summary, a surge of "capacitation respiration" was observed in sperm culture before spermatozoa acquired maximum fertilizing ability. The high rate of oxygen consumption is independent of sperm motility. In addition, a modified CTC assay was developed to assess capacitation and acrosome reaction in boar spermatozoa.

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CHAPTER II

THE RELATIONSHIP BETWEEN GLUCOSE AND THE REQUIREMENT FOR REACTIVE OXYGEN SPECIES IN MOUSE SPERM FERTILIZING ABILITY

ABSTRACT

Glucose is essential for capacitation and fertilization of mouse gametes *in vitro*. Although glucose-6-phosphate (G-6-P) also supported maximum fertilization, no other simple carbohydrate tested, including fructose, fructose-6-phosphate (F-6-P), pyruvate, and lactate, did so. However, in the presence of xanthine, xanthine oxidase, and catalase (XXOC) or H_2O_2, both fructose and F-6-P supported maximum fertilization.

Effects of XXOC, H_2O_2, and carbohydrates on sperm capacitation and the acrosome reaction were examined with the chlortetracycline (CTC) fluorescence assay. Glucose, but not fructose, supported the maximum rate of sperm capacitation and the acrosome reaction. However, fructose in the presence of XXOC or H_2O_2 also supported maximum progress capacitation and acrosome reaction. Both insufficient and excessive amounts of H_2O_2 decreased sperm capacitation and acrosome reaction.

To examine the pathway through which glucose generates H_2O_2 in sperm cells, 6-aminonicotinamide, a G-6-P dehydrogenase inhibitor, and apocynin, a NADPH oxidase inhibitor, were added to sperm suspensions in glucose-containing medium. Sperm capacitation, the acrosome reaction, and fertilization were inhibited by both inhibitors. These inhibitory effects were nullified by XXOC. Results from this study supported the hypothesis that

glucose, in addition to being a substrate for glycolysis, facilitates sperm capacitation and the acrosome reaction by generating H_2O_2 through the pentose phosphate pathway and the activity of NADPH oxidase.

LITERATURE REVIEW AND HYPOTHESIS

Glucose has been shown to be essential for *in vitro* capacitation and fertilization of mouse gametes [1-3]. No other carbohydrate tested, including fructose, lactate, and pyruvate, could substitute for glucose in supporting fertilization. Glucose has been implicated in the initiation of the acrosome reaction and the whiplash motility of spermatozoa associated with fertilizing ability [2]. Culture media that are frequently used to support maximum *in vitro* fertilization, such as modified Krebs-Ringer bicarbonate solution [4], M-16 medium [5] and modified Tyrode's medium [6], contain three carbohydrates: pyruvate, lactate and glucose. Glucose alone is known to support maximum capacitation and fertilization [1], while pyruvate or lactate alone does not. Furthermore, despite the ability of mouse spermatozoa to metabolize fructose, little fertilizing ability was supported by fructose alone [1, 2].

Recently, other researchers have studied the role of various reactive oxygen species in sperm fertilizing ability. Superoxide anion, generated by exogenous xanthine and xanthine oxidase, has been demonstrated to trigger human sperm hyperactivation and capacitation [7-10]. Hydrogen peroxide has also been shown to promote human [11, 12] and hamster sperm capacitation [13] *in vitro*. In this study, glucose as a substrate for reactive oxygen species production was examined, based on the hypothesis that the critical role of

glucose in supporting sperm fertilizing ability is as a substrate for NADPH production in the pentose phosphate pathway. NADPH oxidase is responsible for the production of superoxide anion ($O_2^{\cdot -}$), which is then converted to H_2O_2 [14, 15].

MATERIALS AND METHODS

Materials

The test media containing different carbohydrates were modified based on M-16 medium [5]. Original M-16 contains 5.56 mM D-glucose, 22 mM sodium lactate, 0.33 mM sodium pyruvate, 40 mg/ml BSA, 94.66 mM NaCl, 1.19 mM K_2HPO_4, 4.78 mM KCl, 1.71 mM $CaCl_2 \cdot 2\ H_2O$, 1.19 mM $MgSO_4 \cdot 7\ H_2O$, 25 mM $NaHCO_3$, 100 IU/ml potassium salt of penicillin G, and 100 IU/ml streptomycin sulphate. The media were adjusted to pH 7.4. All chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

In vitro fertilization

Epididymal spermatozoa were collected from mature B_6D_2-F_1 mice and incubated with test medium at 37°C, 5% CO_2 in air, and 100% humidity before being used for insemination.

Oocytes in the cumulus mass were collected from superovulated B_6D_2-F_1 females 21-42 days of age. Superovulation was achieved by intraperitoneal injections of 10 IU pregnant mare serum gonadotropin (PMSG) followed 48-50 h later by 10 IU hCG. Oocytes in cumulus mass were collected into test media from the ampulla 12 to 13 h after hCG injections. Spermatozoa, after 1.5 h preincubation in the test medium, were added to newly collected oocytes and

incubated for 24 h before fertilization was examined. The final sperm concentration at insemination was 1-5 x 10^6cells/ml.

To examine fertilization, eggs were stained with 37 μM bisBenzimide Hoechst for 0.5 h before they were examined with a Nikon Optiphot microscope, equipped with a 100 W mercury bulb, 365/10 nm excitation filter, 400 nm dichroic mirror, and 400 nm barrier filter. Eggs containing 2 cells with a nucleus in each cell were recorded as fertilized. Eggs with one cell and two pronuclei were also recorded as fertilized. Eggs containing only one cell and one nucleus were considered non-fertilized. Fragmented and degenerated eggs were also considered non-fertilized.

Treatments

In the first of a series of experiments, M-16 medium was modified to contain only one of the carbohydrates at the indicated concentration: 5.56 mM glucose, 5.56 mM fructose, 5.56 mM fructose-6-phosphate (F-6-P), 5.56 mM glucose-6-phosphate (G-6-P), 5.56 mM sodium pyruvate, 22 mM sodium pyruvate, or 22 mM sodium lactate. When glucose, lactate, or pyruvate was eliminated from M-16 medium, additional NaCl was added to maintain osmolarity [2].

In the second series of experiments, 0.125 mM xanthine (X) and 0.0125 units/ml xanthine oxidase (XO) were added to the sperm suspension. After

incubation for 15 min, 34 μg/ml catalase (C) was added to selectively remove H_2O_2 [7-9]. Spermatozoa were then incubated for a total of 90 min before insemination. After 2 h, eggs were washed with fresh medium and incubated for another 22 h before examination for fertilization. At 45 min and 90 min of preincubation, sperm capacitation and the acrosome reaction were examined using the chlortetracycline (CTC) fluorescence assay [16, 17].

In the third series of experiments, concentrations of H_2O_2 ranging 0.025 mM to 5 mM were added with 5.56 mM fructose to the test media. The results were used to determine the optimal concentration of H_2O_2 in glucose-free medium for fertilization. Sperm capacitation, the acrosome reaction, as well as sperm motion parameters were examined at three selected H_2O_2 concentrations. For the sperm motion assay, a 20 μl aliquot of sperm suspension taken at 90 min of incubation was placed on a CellSoft 20 μm chamber and analyzed with a CellSoft computer-assisted digital image analysis system (CRYO Resources Inc., New York). A minimum of 100 sperm cells were analyzed to obtain percent of motile spermatozoa and curvilinear velocity.

In the fourth series of experiments, 6-aminonicotiamide (6-AN), an inhibitor of glucose-6-phosphate dehydrogenase [18, 19], was added to the sperm suspension at 2 μM with and without XXOC in 5 different treatments before and after sperm capacitation: (1) the control treatment consisted of M-16 medium, (2) 6-AN was added to the control medium at 0 min, (3) 6-AN was

added at 0 min and XXOC at 45 min, (4) 6-AN and XXOC were added at 0 min, and (5) 6-AN was added at 45 min. At 90 min, eggs were inseminated with spermatozoa *in vitro* to assess fertilizing ability. In order to see the effect of these treatments on the percentages of capacitated and acrosome reacted sperm in a sperm population, sperm samples were taken from each treatment at 45 min and 90 min for CTC assays. A parallel experimental design substituted 10 μM apocynin, an inhibitor of NADPH oxidase [20-22], for 6-AN.

Statistics

Angular transformation was performed for the discrete quantitative parameters of percent motile spermatozoa, velocity, the percentage of eggs fertilized *in vitro*, and the percentage of capacitated and acrosome reacted spermatozoa. Those transformed data passing homogeneity of variance and normality tests were analyzed with the Student-Newman-Keuls multiple pairwise comparison by one way analysis of variance (ANOVA) using SigmaStat 50 (1992).

RESULTS

In M-16 medium, 83.9 ± 6.4% of eggs were fertilized. In the presence of glucose or G-6-P alone, 88.6 ± 7.6% and 77.9 ± 5.5% of the eggs were fertilized, respectively (Table 2-1). The absence of pyruvate and lactate did not significantly change the percentage of fertilized eggs. As in previous studies [1-3], when fructose, pyruvate, or lactate alone was present in the medium, no fertilization occurred (Table 2-1).

To test the hypothesis that glucose supports sperm fertilizing ability by generating reactive oxygen species, glucose in the medium was substituted with xanthine, xanthine oxidase, and catalase (XXOC). In the absence of carbohydrates, XXOC alone did not support fertilization (Table 2-2). When XXOC was combined with fructose, F-6-P, or glucose, maximum fertilization was obtained. The combination of XXOC with pyruvate or lactate supported fertilization up to 42.1 ± 7.1% and 41.1 ± 1.0%, respectively, which is about half of the fertilization supported by fructose with XXOC, F-6-P with XXOC, glucose with XXOC, G-6-P, or glucose alone.

When catalase was eliminated from the fructose and XXOC treatment,

Table 2-1. Effect of individual carbohydrates on sperm fertilizing ability.

Source of carbohydrates	No. of eggs observed	Eggs fertilized (%)[a]
Control[b]	129	83.9 ± 6.4[c]
Glucose (5.56 mM)	111	88.6 ± 7.6[c]
G-6-P (5.56 mM)	74	77.9 ± 5.5[c]
Fructose (5.56 mM)	110	1.6 ± 1.4[d]
F-6-P (5.56 mM)	61	1.1 ± 2.1[d]
Pyruvate (5.56 mM)	87	1.2 ± 2.1[d]
Pyruvate (22 mM)	96	1.9 ± 1.7[d]
Lactate (22 mM)	68	1.9 ± 3.2[d]

[a] After 90 min of preincubation, spermatozoa were incubated with eggs for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization. Values are means ± S.E.M. of 3 experiments.

[b] Control medium, M-16, contained 5.56 mM glucose, 0.33 mM pyruvate, and 22 mM lactate.

[cd] Means with different superscripts differ ($p < 0.05$).

Table 2-2. Effects of the xanthine oxidase system and carbohydrates on fertilization of mouse gametes.

Treatment	No. of eggs observed	Eggs fertilized (%)[a]
Glucose (5.56 mM)	87	88.5 ± 3.2[d]
Glucose (5.56 mM), XXOC[b]	90	86.7 ± 2.5[d]
Fructose (5.56 mM), XXOC	83	87.1 ± 4.0[d]
F-6-P (5.56 mM), XXOC	33	79.1 ± 7.3[d]
Pyruvate (5.56 mM), XXOC	74	37.0 ± 6.0[e]
Pyruvate (22 mM), XXOC	51	42.1 ± 7.1[e]
Lactate (22 mM), XXOC	83	41.1 ± 1.0[e]
XXOC only[c]	91	0[f]

[a] After 90 min of preincubation, spermatozoa were incubated with eggs for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization. Values are means ± S.E.M. of 3 experiments.

[b] XXOC represents the xanthine oxidase system: xanthine (X), xanthine oxidase (XO), and catalase (C). Spermatozoa were incubated with X and XO for 15 min before addition of catalase. They were then incubated for 75 min before being used for fertilization.

[c] No carbohydrate was present in the medium.

[def] Means with different superscripts differ ($p < 0.05$).

only 14.5 ± 3.7% of the eggs were fertilized (Table 2-3). Fructose and catalase without XXO also did not support fertilization. Therefore, the combination of XXO with catalase, which converts H_2O_2 to H_2O, was necessary to achieve maximum fertilization, suggesting possible deleterious effects of excess H_2O_2 on spermatozoa. This observation, however, does not indicate if small amounts of H_2O_2, generated from XXOC, played a role in supporting sperm fertilizing ability in the absence of glucose.

When different concentrations of H_2O_2 (0.025 mM, 0.075 mM, 0.3 mM, 1 mM, 3 mM, and 5 mM), instead of XXOC, were added to the media containing 5.56 mM fructose, 0.3 mM of H_2O_2 was the concentration that supported maximum fertilization (Fig. 2-1). The dose-response relationship clearly suggested that any concentration less than 0.3 mM was not sufficient to support sperm fertilizing ability, while any concentration greater than 0.3 mM was toxic (Fig. 2-1). The percent of motile spermatozoa and curvilinear velocity (Fig. 2-2) were the same in 0.3 mM and lower concentrations. At higher concentrations, H_2O_2 decreased sperm motility and velocity.

The addition of 6-AN to the M-16 medium at 0 time of sperm incubation decreased sperm fertilizing ability to 34.8 ± 2.6% ($p<0.05$) (Table 2-4).

Table 2-3. Effects of xanthine and xanthine oxidase on fertilization.

Treatment	No. of eggs observed	Eggs fertilized (%)[a]
Fructose, C[b]	107	10.4 ± 2.6[e]
Fructose, XXOC[c]	112	76.1 ± 5.6[f]
Fructose, XXO[d]	104	14.5 ± 3.7[e]

[a] After 90 min of preincubation, spermatozoa were incubated with eggs for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization. Values are means ± S.E.M. of 3 experiments.

[b] C represents catalase.

[c] XXOC represents the xanthine oxidase system: xanthine (X), xanthine oxidase (XO), and catalase (C).

[d] XXO represents the combination of xanthine and xanthine oxidase.

[ef] Means with different superscripts differ ($p < 0.05$).

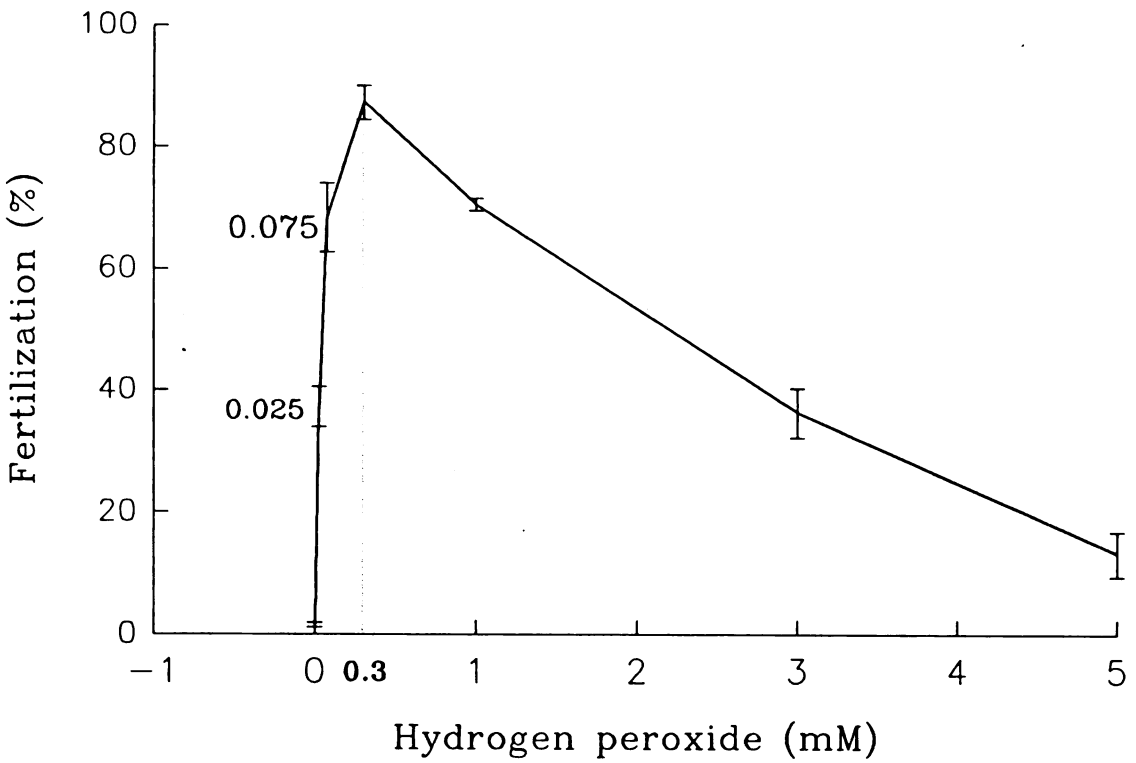

Figure 2-1. Fertilization of mouse gametes in the presence of hydrogen peroxide and fructose. During 90 min of incubation, spermatozoa were treated with H_2O_2 (0.025 mM - 5 mM) at 0 time in the fructose medium. After 90 min of preincubation, spermatozoa were incubated with eggs in the fructose medium for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization.

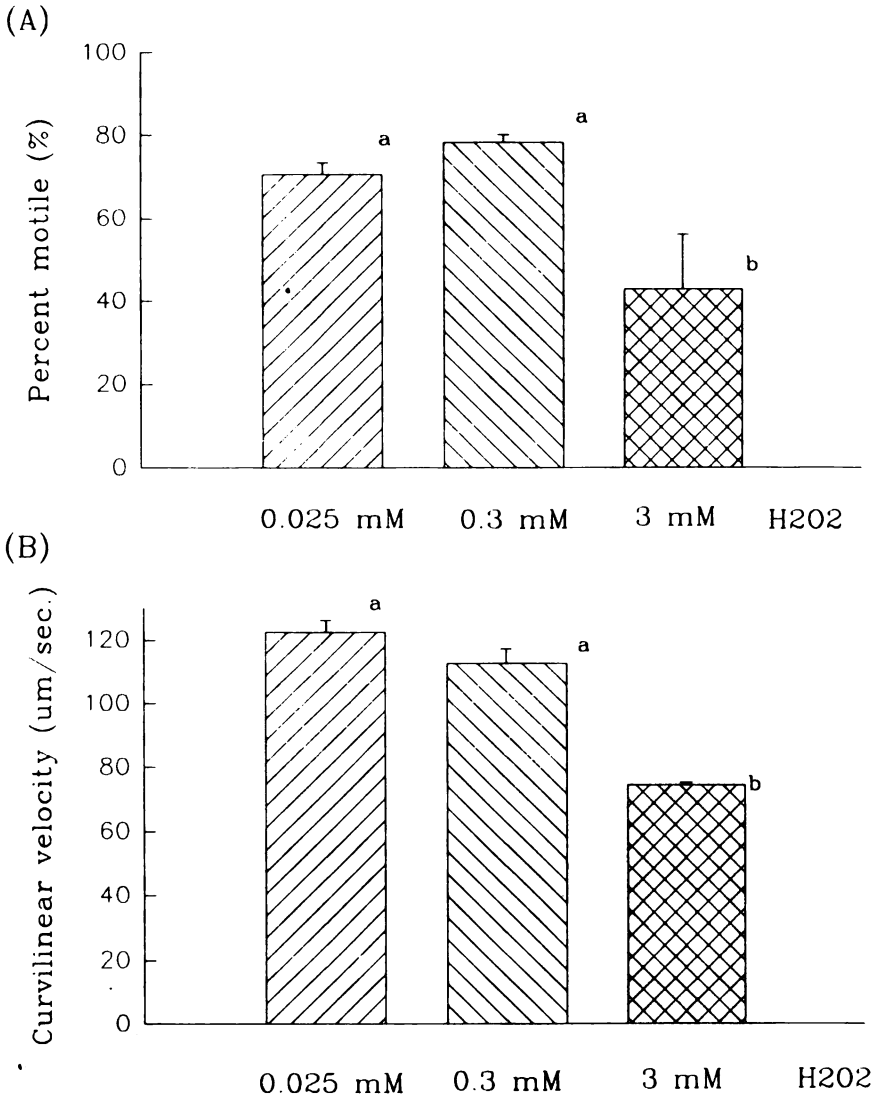

Figure 2-2. Effects of hydrogen peroxide on (A) sperm motility and (B) curvilinear velocity. During 90 min of incubation, spermatozoa were treated with H_2O_2 at 0 time in the fructose medium. For sperm motion assay, 20 μl of sperm suspension taken at 90 min of incubation was analyzed with a CellSoft computer-assisted digital image analysis system (CRYO Resources Inc., New York). A minimum of 100 sperm cells were analyzed to measure motility and curvilinear velocity.

[ab] Means with different superscripts differ ($p < 0.05$).

Table 2-4. Inhibitory effect of 6-aminonicotinamide on fertilization.

Treatments[a]		No. of eggs observed	Fertilization[b] (%)
0 min	45 min		
----	----	100	95.0 ± 2.9[e]
6-AN[c]	----	77	34.8 ± 2.6[f]
6-AN	XXOC[d]	111	65.1 ± 3.2[g]
6-AN, XXOC[d]	----	103	91.8 ± 3.7[e]
----	6-AN	84	81.5 ± 1.4[h]

[a] During 90 min of sperm incubation, spermatozoa were treated with 6-AN or XXOC at either 0 or 45 min. Both the media of sperm incubation and fertilization were M-16 medium.

[b] After 90 min of preincubation, spermatozoa were incubated with eggs for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization. Values are means ± S.E.M. of four experiments.

[c] 6-AN is the abbreviation of 6-aminonicotinamide, which is an inhibitor of glucose-6-phosphate dehydrogenase.

[d] XXOC represents the xanthine oxidase system: xanthine (X), xanthine oxidase (XO), and catalase (C).

[efgh] Means with different superscripts differ ($p < 0.05$).

When spermatozoa were exposed to 6-AN at 0 time with XXOC added at 45 min 65.1 $\pm$ 3.2% eggs were fertilized. The presence of 6-AN and XXOC at 0 time resulted in 95.0 $\pm$ 2.9% fertilization. These demonstrated that reactive oxygen species generated from XXOC overrode the 6-AN inhibition of glucose-6-phosphate dehydrogenase.

In the parallel experiment, the addition of apocynin to the M-16 medium at 0 time of sperm incubation decreased sperm fertilizing ability to 33.9 $\pm$ 3.1% ($p < 0.05$) (Table 2-5). The additional presence of XXOC at 0 time and 45 min also reversed the inhibitory effect on fertilization as observed in the 6-AN/XXOC experiments.

Table 2-5. Inhibitory effect of apocynin on fertilization.

Treatments[a]		No. of eggs observed	Fertilization[b] (%)
0 min	45 min		
----	----	116	91.0 ± 2.4[e]
Apocynin[c]	----	79	33.9 ± 3.1[f]
Apocynin	XXOC[d]	106	71.9 ± 6.3[g]
Apocynin, XXOC[d]	----	103	63.4 ± 5.2[g]
----	Apocynin	109	82.6 ± 0.9[h]

[a] During 90 min of sperm incubation, spermatozoa were treated with apocynin or XXOC at either 0 or 45 min. Both the media of sperm incubation and fertilization were M-16 medium.

[b] After 90 min of preincubation, spermatozoa were incubated with eggs for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization. Values are means ± S.E.M. of four experiments.

[c] Apocynin is an inhibitor of NADPH oxidase.

[d] XXOC represents the xanthine oxidase system: xanthine (X), xanthine oxidase (XO), and catalase (C).

[efgh]Means with different superscripts differ ($p < 0.05$).

DISCUSSION

Neither fructose, pyruvate, or lactate, alone, supported fertilization, as has been demonstrated in previous studies [1, 2]. The lack of fertilization in the absence of glucose was also associated with the lack of both sperm capacitation and the acrosome reaction. The progress of capacitation and the acrosome reaction, conducted concurrently in Dr. Chou's lab by Kathy Li, was examined by the CTC assay [15]. The percentages of capacitated and acrosome reacted spermatozoa in fructose alone were significantly lower ($p < 0.05$) than those in either glucose or fructose with XXOC. The inhibitor of G-6-P dehydrogenase, 6-AN, also decreased the rate of sperm capacitation and acrosome reaction. The addition of XXOC partially reversed the inhibitory effects of 6-AN on capacitation and acrosome reaction. The addition of 6-AN at 45 min, after significant amounts of spermatozoa had already capacitated, did not inhibit the acrosome reaction to the same extent as when it was added at 0 time. This optimal concentration of H_2O_2 (0.3 mM) for supporting fertilization also supported maximum capacitation and acrosome reaction.

Hoppe [1] examined the ability of mouse spermatozoa to use fructose as an energy source for fertilization. The rate of sperm CO_2 production in the presence of 5.55 mM fructose was about 50% of that in the presence of 5.55 mM glucose. Fructose is apparently sufficient as an energy source for sperm

fertilizing ability, because, in this study, either fructose or fructose-6-phosphate supported maximum fertilization in the presence of XXOC. Fructose with 0.3 mM H_2O_2 in the medium also supported maximum fertilization. The difference between glucose and fructose, therefore, lies not in their role as a substrate for glycolysis, but as an electron source for the generation of reactive oxygen species.

De Lamirande and Gagnon [7-9] have previously reported that XXOC triggered hyperactivation and capacitation of human spermatozoa. Both $O_2^{\cdot -}$ and H_2O_2 are known products of XXO [23]. In an additional study, superoxide dismutase (SOD), which converts $O_2^{\cdot -}$ to H_2O_2, prevented XXO-induced hyperactivation. This lack of hyperactivation in the presence of SOD could be due to either H_2O_2 toxicity or the lack of $O_2^{\cdot -}$. The current studies in our laboratory demonstrated that H_2O_2 alone could replace XXOC in supporting sperm capacitation and fertilization in the absence of glucose.

In fact, H_2O_2 was also shown to promote human and hamster sperm capacitation [11, 13]. Low concentrations of H_2O_2 (0.01 to 0.1 mM) have been proven to maintain human sperm hyperactivation and sperm-zona pellucida binding capacity. It also enhanced the acrosome reaction. In the presence of 0.4 mM Fe^{2+}/2.0 mM ascorbic acid, lipid peroxide in human spermatozoa increased 4.6-fold without significantly modifying free sulfhydryl groups and sperm motility parameters. The peroxidative condition also increased sperm

binding capacity to zona pellucida and fertilizing potential by 50% [24].

On the other hand, spermatozoa are also highly sensitive to oxidative damage because of their high polyunsaturated fatty acid content and their relatively low activity of antioxidant enzymes [25-28]. Excess amounts of endogenous reactive oxygen species, $O_2^{\cdot -}$ or H_2O_2, have been associated with low motility in human spermatozoa [25-27]. Lipid-peroxidation induced by XXO was associated with decreased motility in human spermatozoa [28]. This effect was not influenced by the addition of SOD or scavengers for either hypochlorous acid or hydroxyl radicals. The cytotoxic species was also shown to be extremely stable and could be completely eliminated by catalase, which reduces hydrogen peroxide to water and oxygen. Excess amounts of exogenous H_2O_2 has also been shown to inhibit the acrosome reaction and reduce hyperactivation [11, 12].

The presence of NADPH oxidase, as a membrane-bound superoxide-anion generating enzyme on human spermatozoa, has been proposed before [12]. Results from this study now support the hypothesis that glucose functions as a substrate for the pentose phosphate pathway to generate NADPH, which, as a substrate for NADPH oxidase, produces $O_2^{\cdot -}$ and H_2O_2. These results also demonstrate that there is no net flow from F-6-P to G-6-P, since glucose or G-6-P supported maximum fertilization, while fructose, F-6-P, or other simple carbohydrates did not. Furthermore, in order to maintain a high ATP/AMP

ratio, which is necessary for sperm motility, elevated glycolysis and diminished substrate flow toward gluconeogenesis would be expected.

The role of the pentose phosphate pathway and NADPH in fertilization, was first suggested by Fraser and Quinn [2]. However, in their experiments, the combination of exogenous NADPH and pyruvate did not support fertilization, nor did the stimulation of endogenous NADPH production via exogenous $NADP^+$ and malate. The lack of supporting data was attributed to the lack of uptake of nucleotides by the sperm membrane. In this study, 6-AN, an inhibitor of glucose-6-phosphate dehydrogenase, decreased sperm capacitation, the acrosome reaction, and fertilization. The inclusion of XXOC or H_2O_2 in the culture medium reversed the 6-AN effect. A parallel experiment was conducted with apocynin, an inhibitor of NADPH oxidase. Its similar effects and reversibility by XXOC or H_2O_2 were also observed. Results of these two experiments demonstrated the essential roles of G-6-P dehydrogenase and NADPH oxidase in sperm fertilizing ability.

This study also demonstrated the importance of glycolysis in fertilization, a hypothesis proposed earlier by Hoppe [1] and Fraser and Quinn [2]. Unlike fructose, pyruvate or lactate, even in the presence of XXOC, only supported sub-maximum fertilization.

In conclusion, glucose plays a dual role in supporting mouse sperm fertilizing ability. It functions as a substrate for glycolysis, and as a substrate

for the pentose phosphate pathway for generating H_2O_2.

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