Glucose and Its Role in Generating Reactive Oxygen Species Required for Mouse Sperm Fertilizing Ability

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ABSTRACT : Effects of xanthine (X), xanthine oxidase (XO), and catalase (C), H_2O_2, and carbohydrates on sperm capacitation, acrosome reaction, and fertilizing ability *in vitro* were examined. Glucose alone, but not fructose, supported the maximum rate of sperm capacitation and acrosome reaction. However, in the combination of X, XO, and C (XXOC) or H_2O_2, fructose alone also supported maximum capacitation, acrosome reaction, and fertilization. Either insufficient or excessive amounts of H_2O_2 decreased sperm capacitation and the acrosome reaction. In order to understand how glucose generates H_2O_2 or other reactive oxygen species in sperm cells, 6-aminonicotinamide, an inhibitor of the pentose-phosphate pathway (PPP), and apocynin, an inhibitor of NADPH oxidase, were added to sperm suspensions in glucose-containing medium. Results appeared that sperm capacitation, acrosome reaction, and fertilization were consequently inhibited by either one of these compounds. These inhibitory effects were nullified by addition of XXOC. These results support the hypothesis that glucose, in addition to being a substrate for glycolysis, facilitates sperm capacitation and the acrosome reaction by generating reactive oxygen species through G-6-P dehydrogenase and NADPH oxidase. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 6 : 748-756*)

Key Words : Sperm, Fertilization, Fructose, Glucose, Superoxide Anion, Hydrogen Peroxide, Glucose-6-Phosphate Dehydrogenase, NADPH Oxidase

INTRODUCTION

Glucose has been shown to be essential for *in vitro* capacitation and fertilization of mouse gametes (Hoppe, 1976; Fraser and Quinn, 1981; Sakkas et al., 1993; Urner and Sakkas, 1996). No other carbohydrate tested, including L-glucose, 2-deoxyglucose, 3-O-methyl glucoside, 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 (Fraser and Quinn, 1981). Glucose metabolism has also recently been shown to be essential for mouse sperm and oocyte membrane fusion (Urner and Sakkas, 1996). Culture media that are frequently used to support maximum proportion of eggs fertilized *in vitro*, such as modified Krebs-Ringer bicarbonate solution (Whittingham, 1968), M-16 medium (Whittingham, 1971) and modified Tyrode's medium (Fraser, 1983), contain three carbohydrates: pyruvate, lactate and glucose. Glucose alone is known to support maximum capacitation and fertilization (Hoppe, 1976), 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 (Hoppe, 1976; Sakkas et al., 1993).

The role of various reactive oxygen species in sperm fertilizing ability has been recently studied. Superoxide anion, generated by exogenous xanthine and xanthine oxidase, has been demonstrated to trigger human sperm hyperactivation and capacitation (de Lamirande et al., 1993; de Lamirande and Gagnon, 1993a, b). Hydrogen peroxide has also been shown to promote human (Griveau et al., 1994; Oehninger et al., 1995) and hamster sperm capacitation (Bize et al., 1991) *in vitro*. In this study, the role of glucose in reactive oxygen species production was examined, based on the hypothesis that a critical role of glucose in supporting sperm fertilizing ability is acting as a substrate for reactive oxygen species generated via glucose-6-phosphate (G-6-P) dehydrogenase and NADPH oxidase pathway (Badway and Karnovsky, 1980; Smith and Curnutte, 1991).

MATERIALS AND METHODS

Materials

The test media were modified M-16 medium (Whittingham, 1971) with the carbohydrate component replaced with specific carbohydrates. Original M-16 contains 5.56 mM D-glucose, 22 mM sodium lactate, 0.33 mM sodium pyruvate, 40 mg/ml bovine serum albumin (BSA), 94.66 mM NaCl, 1.19 mM K_2HPO_4, 4.78 mM KCl, 1.71 mM $CaCl_2 \cdot 7H_2O$, 1.19 mM $MgSO_4 \cdot 2H_2O$, 25 mM $NaHCO_3$, 100 IU/ml potassium salt of penicillin G, and 100 IU/ml streptomycin

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sulphate. The media were adjusted to pH 7.4. The test chemicals introduced were glucose, sodium lactate, sodium pyruvate, apocynin, 6-aminonicotinamide (6-AN), xanthine/xanthine oxidase (XXO), catalase (C), and H_2O_2 etc., with various combinations. 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℃, 5% CO_2 in air, and 100 % humidity before being used for insemination (Chou and Cook, 1994).

Oocytes in the cumulus mass were collected from superovulated B6D2-F1 females 21-42 days of age (Chou and Cook, 1994). Superovulation was achieved by intraperitoneal injections of 10 IU pregnant mare serum gonadotropin (PMSG) followed 48-50 h later by 10 IU human chorionic gonadotropin (hCG). Oocytes in cumulus mass were collected from the ampulla into test media 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 they were subjected to cytological examination. The final sperm concentration at insemination was $1\text{-}5\times10^6$ cells/ml.

At the end of 24 h fertilization *in vitro*, eggs were stained with 37 μM bisBenzimide Hoechst No. 33258 for 0.5 h and cytological examination was performed under 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 still in the single cell stage were not considered as being fertilized unless both male and female pronuclei with the residual of sperm tail were visible. Eggs which had cleaved into 2-cell stage were considered as being normally fertilized only if a nucleus within each blastomere were found. Fragmented and degenerated eggs were also considered as non-fertilized.

Treatments

In the first series of experiments, M-16 medium was modified to contain 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 giucose-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 as added for proper maintenance of its osmolarity (Fraser and Quinn, 1981).

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. Xanthine and xanthine oxidase were introduced as a source of exogenous reactive oxygen species (Hill and Massey, 1981). After incubation for 15 min., 34 μg/ml catalase (C) was added to selectively remove H_2O_2 (de Lamirande et al., 1993; de Lamirande and Gagnon, 1993a, b). Spermatozoa were incubated for a total of 90 min. before insemination. Two h after insemination, eggs had been washed with fresh medium and incubated for another 22 h before they were subjected to cytological examination for evaluation of their fertilization status. At 45 min. and 90 min. of preincubation, sperm capacitation and the acrosome reaction were examined using the chlortetracyline (CTC) fluorescence assay (Ward and Storey, 1984; Chou and Cook, 1995).

In the third series of experiments, six concentrations of H_2O_2, 0.025 mM, 0.075 mM, 0.3 mM, 1 mM, 3 mM, and 5 mM were added to a test media containing 5.56 mM fructose to determine the optimal concentration of H_2O_2 in the absence of glucose. Based on the results, three concentrations of H_2O_2 were then selected to test the effects of exogenous H_2O_2 on sperm capacitation, the acrosome reaction, and sperm motion parameters. A 20 μl aliquot of sperm suspension was taken at 90 min. after the initial incubation and placed on a CellSoft 20 μm chamber for evaluation of sperm mobility with a CellSoft sperm analyzer (CRYO Resources Inc., New York). A minimum of 100 sperm cells were analyzed against each sample tested.

In the fourth series of experiments, 2 μm 6-aminonicotiamide (6-AN), an inhibitor of the pentose-phosphate pathway (Rush and Alberts, 1986; Aw and Rhoads, 1994; Lange and Proft, 1970) or 10 μm apocynin, an inhibitor of NADPH oxidase (Stolk et al., 1994; Engels et al., 1992), was added to the sperm suspension in M-16 with or without the supplementation of XXOC according to 5 different treatments as follows: (1) the control treatment consisted of M-16 only, (2) 6-AN or apocynin was added at 0 time of the sperm preincubation (i.e. sperm capacitation), (3) 6-AN or apocynin was added at 0 time followed by supplementation of XXOC at 45 min., (4) 6-AN or apocynin combined with XXOC were added at 0 time, and (5) 6-AN or apocynin was added at 45 min. post the initial sperm preincubation. For understanding whether these treatments affect the percentages of spermatozoa appeared to be capacitated and acrosome reacted *in vitro*, sperm samples of each treatment were taken by 45 and 90 min. after their initial preincubation and subjected to the CTC assay, respectively. A parallel experimental design substituted 10 μm apocynin, an inhibitor of NADPH oxidase (Stolk et al., 1994; Engels et al., 1992), for 6-AN.

Statistics

Angular transformation was performed for the discrete quantitative parameters including the percentage

of motile spermatozoa, velocity, the percentage of eggs fertilized *in vitro*, and the percentage of capacitated and acrosome reacted spermatozoa etc. 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 which contains glucose, pyruvate and lactate, 83.9±6.4% of the eggs were fertilized. In the presence of glucose alone or G-6-P alone, 88.6±7.6% or 77.9±5.5% of the eggs were fertilized, respectively (table 1). The removal of pyruvate and lactate from M-16 did not significantly change the percentage of eggs fertilized. As in other studies (Hoppe, 1976; Fraser and Quinn, 1981; Sakkas et al., 1993), when fructose, pyruvate, or lactate, each alone was present in the medium, the fertilization rate was found to be less than 2.0% (table 1).

Table 1. Effect of individual carbohydrates on sperm fertilizing ability

Sources of carbohydrates	No. of eggs observed	Egg 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.
[b] Control medium, M-16, contained 5.56 mM glucose, 0.33 mM pyruvate and 22 mM lactate.
[c,d] Means with different superscripts differ ($p<0.05$).
Values are means±SEM of 3 experiments.

To test the hypothesis that glucose supports sperm fertilizing ability by generating reactive oxygen species, glucose in the medium was substituted with XXOC in combination with other carbohydrates. Without carbohydrates, XXOC did not support fertilization (table 2). When XXOC was added to fructose- or F-6-P-containing medium, 87.1±4.0% or 79.1±7.3% of fertilization rate was obtained, respectively. The addition of XXOC into the medium containing either pyruvate or lactate would also result in a significant increase of fertilization rate, up to 42.1±7.1% and 41.1±1.0%, respectively.

Table 2. Fertilization of mouse gametes in the presence of xanthine oxidase system and carbohydrates

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.
[b] XXOC represents the xanthine oxides system : xanthine (X), xanthine oxides (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.
[d,e,f] Means with different superscripts differ ($p<0.05$).
Values are means±SEM of 3 experiments.

As for the progress of sperm capacitation and acrosome reaction, in the fructose-containing medium, less than 30% of sperm cells were capacitated at 45 min. and acrosome reacted at 90 min. (figure 1). However, when XXOC was added to this medium, more than 45% of sperm cells achieved both their capacitation and acrosome reaction at the respective time courses. Similarly, high proportion of capacitated and acrosome reacted spermatozoa were also found in those mouse sperm which had been preincubated in the glucose-containing medium without XXOC supplementation.

The supplementation of XXOC seems essential for mouse spermatozoa to gain their maximum potency for fertilization *in vitro* when the fructose-containing medium was used. Xanthine/xanthine oxidase (XXO) is a source of superoxide, while catalase converts H_2O_2 to H_2O. Individually, either XXO or C supported minimal fertilization and of which only 14.5±3.7% and 10.4±2.6% of eggs were found to be fertilized, respectively (table 3).

When different concentrations of H_2O_2, ranging from 0.025 mM to 5 mM, were added to the fructose-containing medium, 0.3 mM supported the maximum amount of fertilization (figure 2). The dose-response relationship suggested that concentrations less than 0.3 mM were not sufficient to support sperm fertilizing ability, while those greater than 0.3 mM inhibited fertilization (figure 2). The optimal concentration

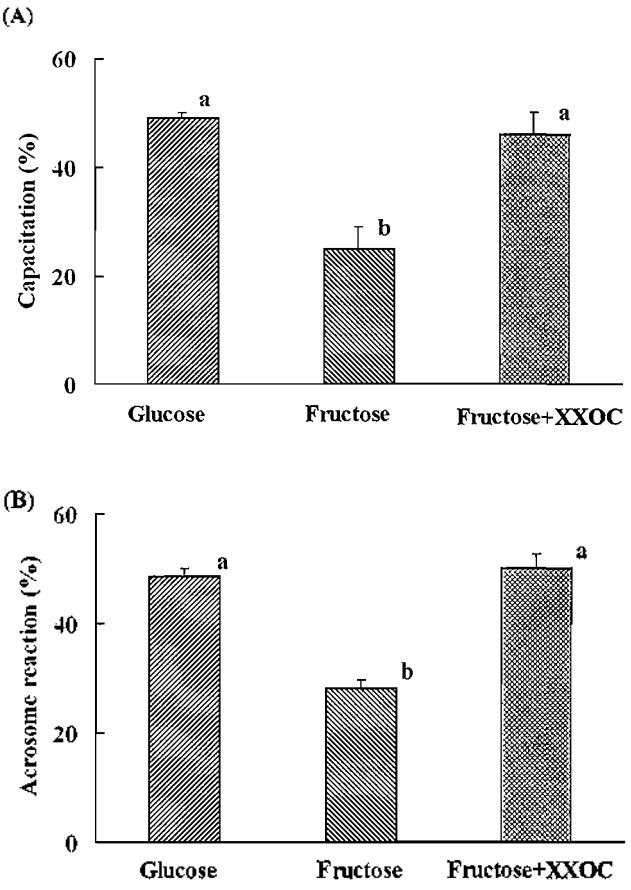

Figure 1. Percentage of (A) capacited and (B) acrosome reacted spermatozoa in the medium of glucose, fructose, or fructose with XXOC*. Capacitated spermatozoa displayed bright fluorescence over the anterior region of the head and midipece with a band which lacked fluorescence at the posterior region of the head. At least 100 spermatozoa per sample were examined at 45 min of incubation. Acrosome reacted fluorescence over the entire head. At least 100 spermatozoa per sample were examined at 90 min of incubation.

[a,b] Means with different superscripts differ ($p<0.05$).

* XXOC represents the xanthine oxidase system: xanthine (X) oxidase (XO), and catalse (C).

Table 3. Effect of catalase in xanthine oxidase system on fertilization

Treatment	No. of eggs observed	Eggs fertilized (%)[a]
Fructose, C[b]	107	10.4 ± 2.6^{e}
Fractose, 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.

[b] C represents catalase.

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

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

[e,f] Means with different superscripts differ ($p<0.05$).

Values are means±SEM of 3 experiments.

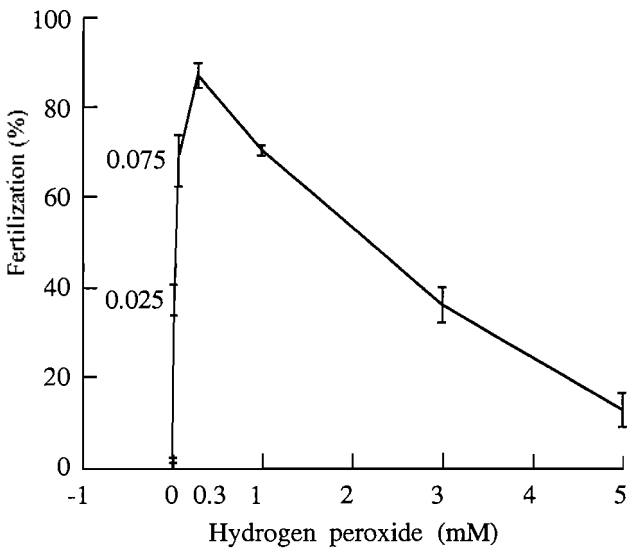

Figure 2. Fertilization of mouse gametes in the presence of hydrogen peroxide and fructose. During 90 min of incubation, spermatozoa were treated with H_2O_2 (0.025 mM-54 mM) at 0 min in the fructose medium. After 90 min of preincuabtion, spermatozoa were incubated with eggs in the fructose medium for 2 h. Eggs were then washed with fresh medium and incubated for another 22 h before examination for fertilization.

of H_2O_2 for fertilization also supported maximum proportion of spermatozoa to be capacitated and acrosome reacted, according to those data obtained from the CTC assay (figure 3). The percentage of motile spermatozoa and curvilinear velocity (figure 4), however, was the same in 0.3 mM and lower concentrations. At higher concentrations, sperm motility and velocity decreased.

The addition of 6-AN to the M-16 medium at 0 time of the sperm incubation resulted in a significant decrease of fertilizing rate ($95.0\pm2.9\%$ vs $34.8\pm2.6\%$, $p<0.05$) (table 4). Similarly, proportions of those capacitated and acrosome reacted spermatozoa were both significantly decreased by 45 and 90 min. after they had been exposed to the M-16 medium containing 6-AN (figure 5). When spermatozoa were exposed to 6-AN at 0 time and with XXOC added at 45 min., $65.1\pm3.2\%$ eggs were fertilized (table 4).

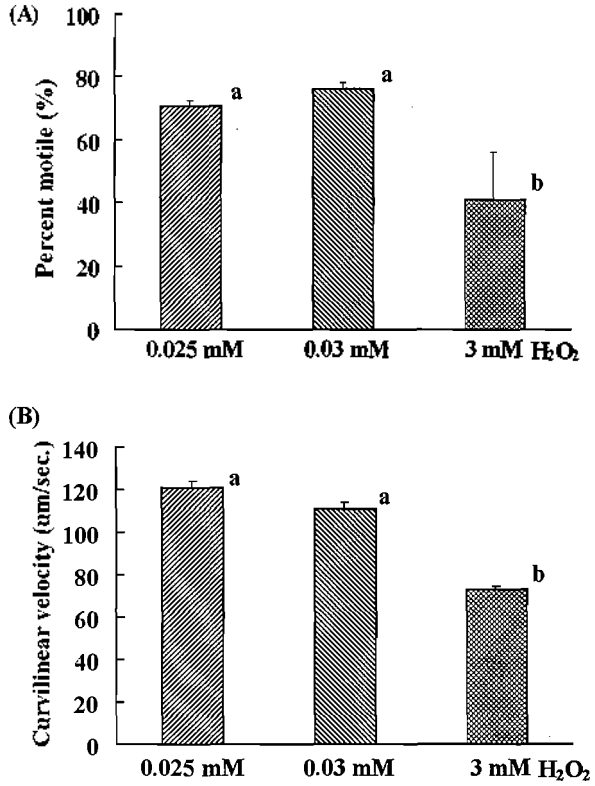

Figure 3. Effect of hydrogen peroxide on (A) capacitation and (B) acrosome reaction of mouse spermatozoa in fructose. Capacitated spermatozoa displayed bright fluorescence over the anterior region of the head. At least 100 spermatozoa per sample were examined at 45 min of incubation. Acrosome reacted spermatozoa displayed bright fluorescence on the midpiece and diminished fluorescence over the entire head. At least 100 spermatozoa per sample were examined at 90 min of incubation.
[a,b,c] Means with different superscripts differ ($p<0.05$).

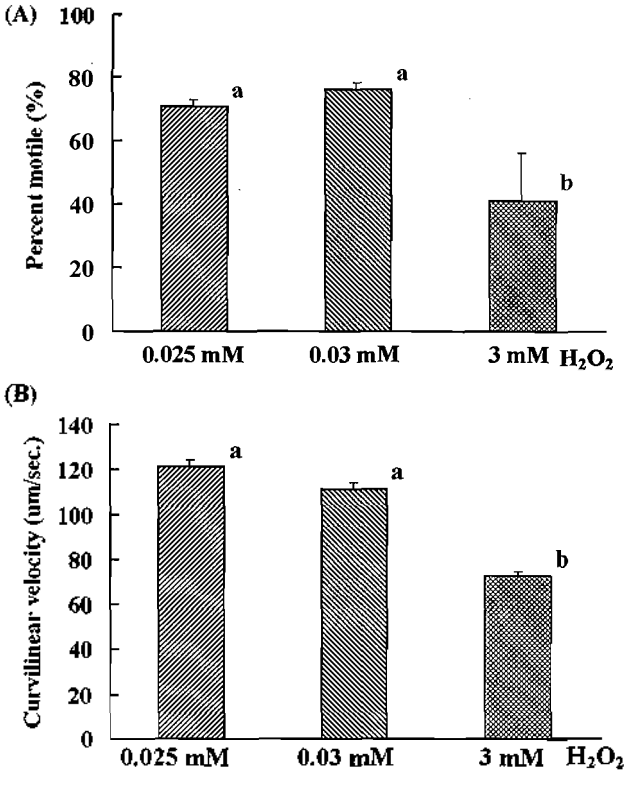

Figure 4. Effects of hydrogen peroxide on (A) sperm motily and (B) curvilinear velocity. During 90 min of incubation, spermatozoa were treated with H_2O_2 at 0 min in the fructose medium. For sperm motion assay, 20 μl of sperm suspension taken at analysis system (CRYO Resources Inc., New York). A minimum of 100 sperm cells were analyzed to measure motility and curvilinear velocity.
[a,b] Means with different superscripts differ ($p<0.05$).

The addition of XXOC at 45 min. also partially reversed the inhibitory effects of 6-AN on capacitation and acrosome reaction (figure 5). The simultaneous addition of 6-AN and XXOC at 0 time resulted in 95.0±2.9% fertilization and maximum capacitation and acrosome reaction. These data suggest that reactive oxygen species generated from XXOC overrode the inhibitory effect of 6-AN. In addition, when 6-AN was added to the sperm incubation at 45 min., after most sperm cells had attained capacitation (Chou and Cook, 1995), only minimal inhibition of fertilization and capacitation/acrosome reaction was observed (table 4, figure 5).

The addition of apocynin to the M-16 medium at different time points during sperm incubation resulted in similar results as that observed with 6-AN treatment. When added at 0 time, sperm fertilizing ability decreased to 33.9±3.1% ($p<0.05$) (table 5). The addition of XXOC at 0 time minimized the inhibitory effect of apocynin on fertilization; 63.4± 5.2% of the eggs were fertilized. When apocynin was added at 45 min., 82.6±0.9% of the eggs were fertilized. The percentages of capacitated sperm at 45 min. and acrosome-reacted sperm at 90 min. also corresponded to the fertilization rates observed in the 6-AN treatment (figure 6).

DISCUSSION

Results from this study suggest that the difference between glucose and fructose in supporting mouse sperm fertilizing ability lies in their ability to generate reactive oxygen species, which is essential for sperm capacitation. Fructose provided sufficient energy for sperm fertilizing ability, as long as reactive oxygen species were present. Observations in this study also supported the hypothesis that glucose provides the reducing power in the pentose-phosphate pathway (PPP),

Table 4. Inhibitory effect of 6-aminonicotinamide on fertilization

Treatment[a]		No. of eggs observed	Eggs fertilized (%)[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.

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

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

[e,f,g,h] Means with different superscripts differ ($p<0.05$).

Values are means ± SEM of four experiments.

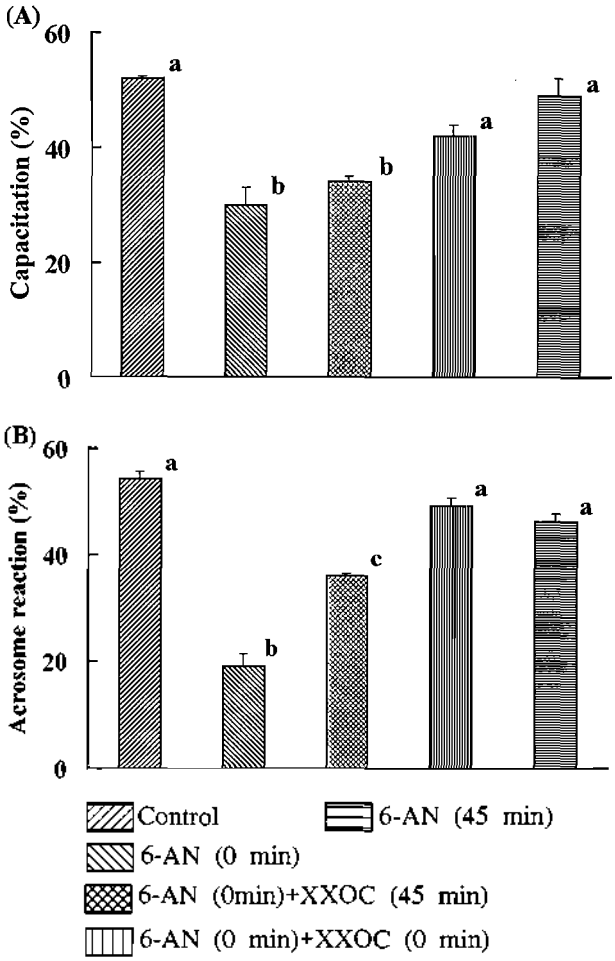

Figure 5. Inhibitory effect of 6-aminonicotinamide (6-AN) on (A) cpacitation and (B) Acrosome reaction. During 90 min of incubation, spermotozoa were treated with 6-AN or XXOC* at either 0 or 45 min in M-16 medium. Capacitated spermatozoa displayed bright fluorescence over the anterior region of the head and midpiece with a band which lacked fluorescence at the posterior region of the head. At least 100 spermotozoa per sample were examined at 45 min of incubation. Acrosome reacred spermtozoa displayed bright fluorescence on the midpiece and diminished fluorescence over the entire head. At least 100 spermatozoa per sample were examined at 90 min of incubation.

[a,b,c] Means with different superscripts differ ($p<0.05$).

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

generating reactive oxygen species via NADPH oxidase.

Urner and Sakkas (1999) proved the glucose metabolism to be essential for successful gamete fusion in the mouse. Mouse epididymal spermatozoa have a functional PPP, implying that they produce NADPH and ribose 5-phosphate. Sperm were still able to fuse with the oocyte when glucose was replaced by NADPH, suggesting that sperm need to produce NADPH via the PPP in order to obtain fertilizing ability.

Swezey and Epel (1995) have demonstrated that PPP activity is of importance shortly after sperm penetration into oocyte in the sea urchin. They have also shown that NADPH produced by the PPP activity is used for the generation of H_2O_2. Hydrogen peroxide was suggested to play a role on the modifications of membrane during fertilization.

Reactive oxygen species have been shown to promote human and hamster sperm capacitation (Bize et al., 1991; de Lamirande et al., 1993; de Lamirande and Gagnon, 1993a, b; Griveau et al., 1994; Oehninger et al., 1995). Exogenous H_2O_2 (0.01 to 0.1 mM) has also been shown to maintain human sperm hyperactivation, enhance the acrosome reaction, and facilitate sperm-zona pellucida binding capacity. In the presence of 0.4 mM Fe^{2+}/2.0 mM ascorbic acid, lipid peroxide formation in human spermatozoa increased 4.6-fold without significantly modifying free sulfhydryl groups and sperm motility parameters (Kodama et al., 1996). The peroxidative condition also increased sperm binding capacity to zona pellucida and fertilizing potential by 50%. In the present study, either XXOC or H_2O_2 maximized sperm fertilization when fructose alone was present in the medium.

On the other hand, spermatozoa are also sensitive to oxidative damage because of their high polyunsaturated

Table 5. Inhibitory effect of apocynin on fertilization

Treatment[a]		No. of eggs observed	Eggs fertilized (%)[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.

[c] Apocynin is an inhibitor of NADPH oxidase.

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

[e,f,g,h] Means with different superscripts differ ($p<0.05$).

Values are means ± SEM of four experiments.

fatty acid content and the relatively low activity of antioxidant enzymes (Aitken, 1987; Alvarez et al., 1987; Iwasaki and Gagnon, 1992; Aitken, 1993). The present study demonstrates that without catalase, excess amounts of reactive oxygen species generated from XXO diminished sperm fertilizing ability. High H_2O_2 concentration, 3 mM, also diminished sperm capacitation, acrosome reaction, and *in vitro* fertilization, although opitimal H_2O_2 concentration, 0.3 mM, supported maximum fertilization. Lipid-peroxidation has also been associated with decreased motility of human spermatozoa treated with XXO (Aitken, 1993). Excess amounts of exogenous H_2O_2 have been shown to inhibit the acrosome reaction and reduce hyperactivation in human spermatozoa as well (Griveau, 1994; Oehninger, 1995). When human sperm exposed to increasing levels of oxidative stress caused through the stimulation of endogenous oxidant generation with NADPH or direct exposure to H_2O_2 (Aitken et al., 1998). However, at low levels of oxidative stress, DNA fragmentation was significantly reduced while the rates of sperm-oocyte fusion were significantly enhanced (Aitken et al., 1998).

Based on the parameters examined in this study, G-6-P was as effective as glucose in supporting sperm capacitation, acrosome reaction, and fertilization, while F-6-P appeared to function the same as fructose in mouse spermatozoa. Neither pyruvate nor lactate supported maximum fertilization, even in the presence of XXOC, supporting the earlier hypothesis that glycolytic substrates are essential for sperm fertilizing ability (Hoppe, 1976; Fraser and Quinn, 1981). There also appeared to be no functional isomerization of

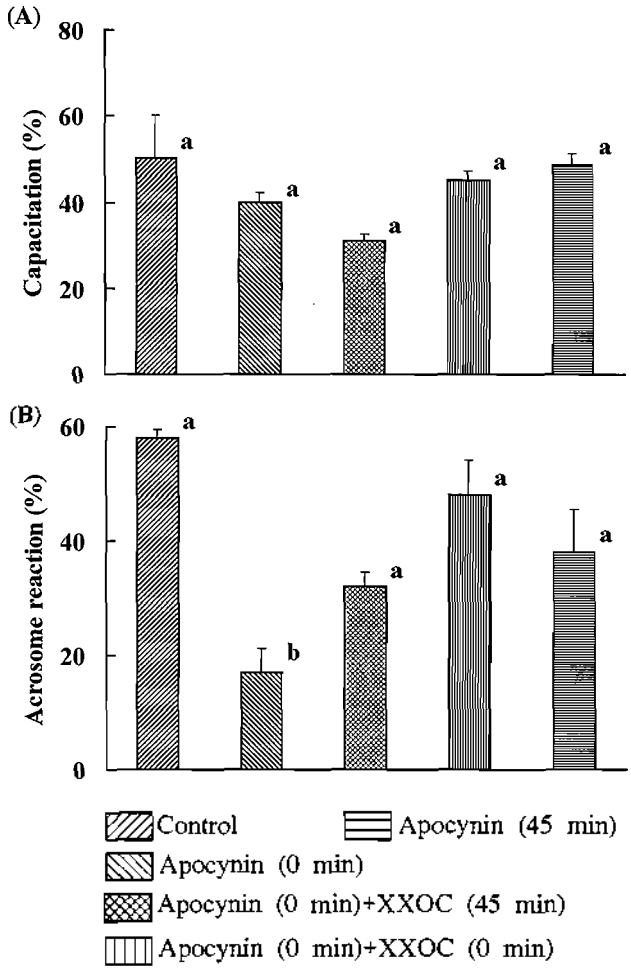

Figure 6. Inhibitory effect of apocynin on (A) cpacitation and (B) arosome reaction. During 90 min of incubation, spermotozoa were treated with apocynin or XXOC* at either 0 or 45 min in M-16 medium. Capacitated spermatozoa displayed bright fluorescence over the anterior region of the head and midpiece with a band which lacked fluorescence at the posterior region of the head. At least 100 spermotozoa per sample were examined at 45 min of incubation. Acrosome reacted supermatozoa displayed bright fluorescence on the midpiece and diminished fluorescence over the entire head. At least 100 spermatozoa per sample were examined at 90 min of incubation.

[a,b] Means with different superscripts differ ($p<0.05$).

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

F-6-P to G-6-P in the glycolysis/ gluconeogenesis pathway of mouse sperm, since glucose or G-6-P supported maximum fertilization, while fructose, F-6-P, pyruvate, and lactate did not. This direction of carbohydrate metabolism is consistent with the requirement of a high ATP/AMP ratio for sperm motility; glycolysis rather than gluconeogenesis

dominates the metabolism and energy flow.

This study also provided evidence that glucose generates reactive oxygen species via the PPP and NADPH oxidase. The presence of NADPH oxidase, as a membrane-bound superoxide-anion generating enzyme on human spermatozoa, has been proposed by de Lamirande et al. (1993). In the present study, apocynin, an inhibitor of NADPH oxidase, and 6-AN, an inhibitor of the pentose-phosphate pathway, inhibited fertilization. Exogenous reactive oxygen species generated from XXOC overcame the inhibitory effect of both compounds. Thus, glucose plays a dual role in supporting mouse sperm fertilizing ability. It functions not only as a substrate for glycolysis, but also generates reactive oxygen species required for capacitation.

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