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EFFECT OF SUPEROXIDE DISMUTASE(SOD) ON PRONUCLEUS FORMATION OF PORCINE OOCYTES FERTILIZED IN VITRO

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ABSTRACT

This study was undertaken to evaluate the effects of superoxide dismutase (SOD) on pronucleus formation in porcine oocytes fertilized in vitro by frozen-thawed spermatozoa. No differences were found in penetration rates when SOD was added to maturation or fertilization medium at any level tested in first and second experiments. Pronucleus formation rates were higher ($P<0.05$) when SOD at 10 and 100 units was added to the maturation medium (46 and 53%, respectively) compared with the controls (26%). On the other hand, when the fertilization medium was supplemented with SOD at different concentrations (1, 10 and 100 units/ml), pronucleus formation rates (55, 52 and 50%) were significantly higher ($P<0.05$) than in the control group. In third experiment, the oocytes were cultured in medium with (1 unit/ml) or without SOD for 8, 16, 24 and 32 h after insemination. The penetration rates had a tendency to increase as time of sperm-oocyte culture was prolonged. No significant differences, however, were observed in penetration rates between groups with and without SOD. On the other hand, the pronucleus formation rates were higher in medium with than without SOD at 8 (7 vs 0%), 16 (14 vs 3%), 24 (48 vs 16% ; $P<0.01$) and 32 h (49 vs 22% ; $P<0.05$). These findings demonstrate the advantage of culture with SOD on pronucleus formation in porcine oocytes penetrated by spermatozoa. However, SOD does not affect penetration rates and polyspermy.

Key words : porcine, superoxide dismutase, pronucleus formation, in vitro fertilization

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INTRODUCTION

Several investigators have demonstrated the ability of porcine oocytes matured and fertilized in vitro to develop normally (4,11,14), and the birth of piglets from embryos produced in vitro has been reported (5,23). Despite these achievements, the in vitro development of in vitro matured and fertilized porcine oocytes to the blastocyst stage is poor. The failure of most in vitro matured and fertilized porcine oocytes to develop normally is attributable to abnormalities of fertilization, including polyspermy and asynchronous pronuclear formation (4). Asynchronous pronuclear formation is characterized by a delay in male pronuclear development following the normal formation of the female pronucleus after fertilization. Meanwhile, it has been reported that the co-culture of oocytes with follicular cells (10) and the culture of oocytes in porcine follicular fluid (25) improve the ability of porcine oocytes to form male pronucleus following sperm penetration in vitro. In addition, the effect of culture media used for nuclear maturation of porcine oocytes in vitro has been reported by several investigators (10,12,24). However, polyspermy and male pronuclear formation still prominent problems for in vitro fertilization of porcine oocytes (19). It is not known if the low incidence of pronucleus formation is related to conditions present at the time of in vitro culture or to the in-vitro characteristics of the male and female gametes before co-incubation.

The gaseous environment is another important component of a culture system, and commonly, 5% CO_2 with 95% humidified air is used (8). However, the O_2 concentration of the oviduct is about one-third that of the atmosphere (2). Embryos cultured in high oxygen tension may produce more free radicals which are detrimental to embryo development. Reducing oxygen tension to 5 to 8% increases blastocyst development (7). Free radicals can be degraded by enzymes such as SOD, catalase and taurine. The presence of SOD and taurine, which serve as radical scavengers in culture medium, have been found to have beneficial effects on embryonic development in vitro for bovine (8), and SOD was found to be beneficial during in vitro maturation and fertilization of porcine oocytes (17). Although correlations have been reported between the effectiveness of SOD and sperm penetration, the importance of their action for pronucleus formation has not been elucidated.

The objectives of the study was to determine whether or not the addition of SOD to maturation and fertilization medium could increase synchronous pronuclear formation in porcine oocytes.

MATERIALS AND METHODS

Oocyte Maturation

Porcine ovaries were collected from a local slaughterhouse and kept in saline (NaCl, 0.9% w/v ; penicillin (100,000 IU/L) ; streptomycin (100 mg/L) ; and amphotericin B (250 μg/L; Sigma Chemical, St-Louis, MO, USA) at 32 to 37℃. Cumulus-oocytes complexes (COC) were aspirated from 1- to 5-mm follicles with a 10-ml syringe with an 18-g needle. Oocytes were washed 3 times in Hepes-buffered Tyrode's medium(TLH) and once in maturation medium. Oocytes with a compact and complete cumulus were introduced into droplets of maturation medium (10 oocytes/50-μℓ droplet), covered with mineral oil and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 42 to 44 h. The maturation medium consisted of TCM-199 with Earle's salt (Gibco, Lab., NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes (Sigma), 10% fetal calf serum (FCS), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamycin (Sigma), 1 μg/ml FSH (Sigma), 5 μg/ml LH (Sigma), 1 μg/ml estradiol 17β (Sigma) and 10% (v/v) porcine follicular fluid (PFF).

Sperm Preparation

Pooled ejaculate from boar were frozen, and the straws were later thawed by immersion in a 37℃ waterbath for 30 sec. Thawed spermatozoa were diluted with 2 ml of BTS (Beltsville Thawing Solution) and equilibrated in air-tight tubes at 37℃ in a water bath for 10 min. After equilibration, the 2 ml of semen were placed over 2 layers of Percoll (65 and 70%) and centrifuged at 2000×g for 15 min at 20℃. The spermatozoa in the 65% Percoll layer were carefully collected, washed in preincubation medium by suspension and centrifugation twice of 250 ×g for 10 min and resuspended in preincubation medium.

In Vitro Fertilization

After the final wash, the concentration of motile spermatozoa was adjusted to 25×10^6/ml. The fertilization medium was TCM-199 supplemented with 3 mM glucose, 3 mM Ca-lactate, 0.2 mM Na-pyruvate and 10% FCS. The final concentration of spermatozoa was adjusted to 1×10^6 cells/ml motile sperm cells during fertilization.

Experimental Design

In the first experiment, follicular oocytes were cultured with different concentrations (0, 1, 10, 100 and 1,000 units/ml) of SOD for maturation. Suspensions (50 $\mu\ell$) of spermatozoa were added to droplets of 50 $\mu\ell$ fertilization medium without SOD, containing 5 oocytes each.

In the second experiment, to evaluate the effects of SOD on sperm penetration and pronucleus formation in vitro, suspensions (50 $\mu\ell$) of spermatozoa were inseminated in 50 $\mu\ell$ fertilization medium with different concentrations (0, 1, 10, 100 and 1,000 units/ml) of SOD. Oocytes were cultured in vitro in maturation medium without SOD.

Finally, in the third experiment, the effect of SOD on sperm penetration and pronucleus formation rates at various times after insemination was examined. The sperm-oocytes were cultured in fertilization medium with (1 unit/ml) or without SOD for 8, 16, 24 or 32 h after insemination. Oocytes that were matured in medium without SOD were used, and the SOD concentration (1 unit/ml) producing the highest rate of pronucleus formation in second experiment was supplemented in fertilization medium.

Evaluation of Oocyte Fertilization

At 20 to 22 h in the first and second experiments and at various times (8, 16, 24 and 32 h in the third experiment) after insemination, the oocytes were mounted, fixed (acetic acid : ethanol 1:3) for 2 to 3 days and stained with 1% aceto-orcein in 40% acetic acid water solution. Penetration and pronucleus formation were examined with a light microscope at x 200 and 500 magnification. Oocytes were considered to be penetrated when spermatozoa with a swollen head or pronuclei were found in the vitellus together with a female pronucleus. Chi-square analysis with the Yates correction was used to test the significance of individual comparisons for the rates of penetration and pronucleus formation.

RESULTS

In the first experiment, when oocytes were matured with different concentrations of SOD and then inseminated, the penetration rates were 56, 61, 62, 59 and 54% for 0, 1, 10, 100 and 1,000 units/ml of SOD, respectively (Figure 1). The proportions of oocytes with pronucleus formation were significantly higher ($P<0.05$) in oocytes matured with 10 (46%) and 100 (53%) units/ml than with 0 (26%) and 1,000 (27%) units/ml SOD. However, polyspermy rates (24~40%) were not significantly different in oocytes matured with different concentrations of SOD.

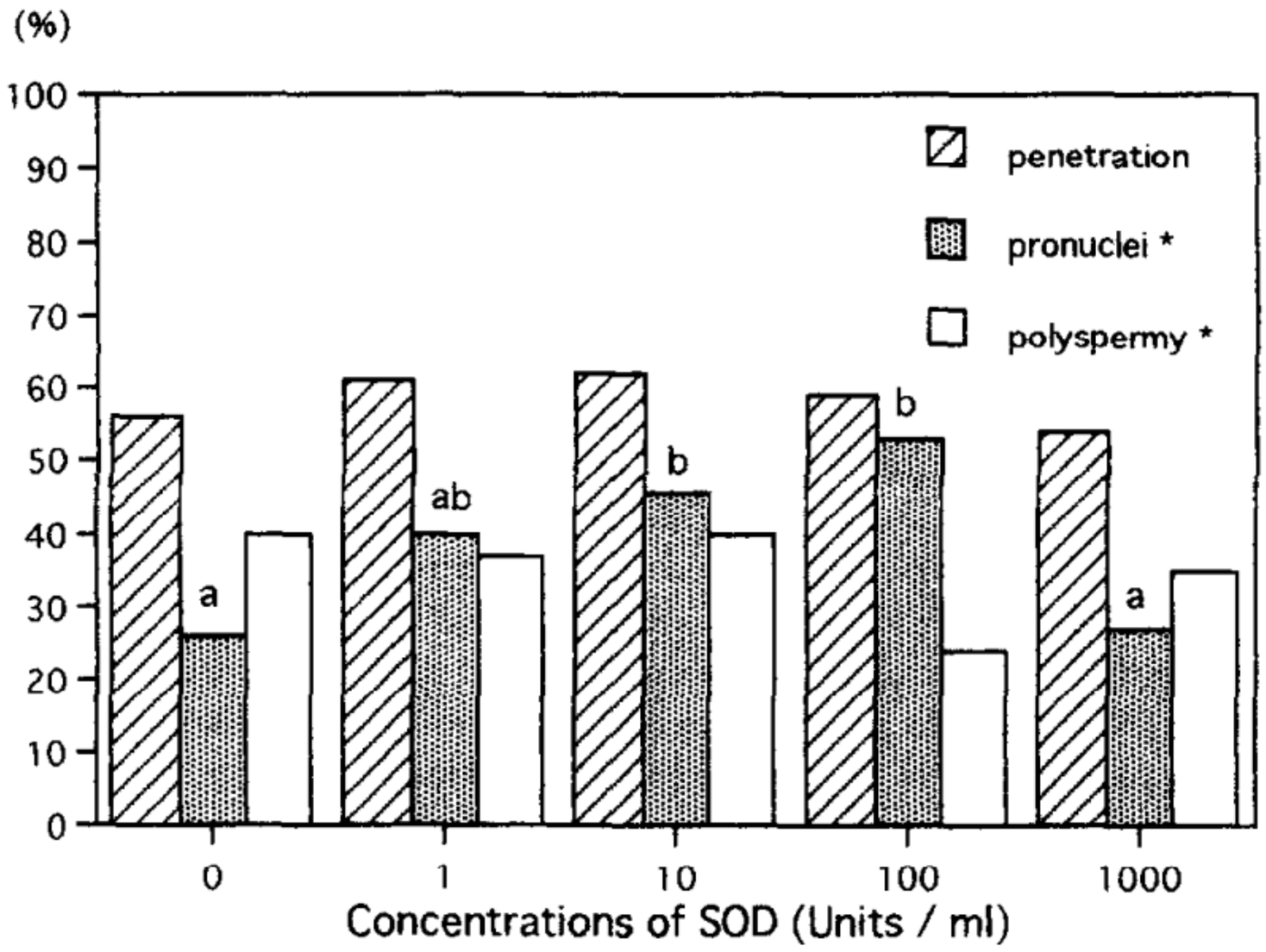

Figure 1. The effect of different concentrations (0, 1, 10, 100 and 1000 units / ml) of superoxide dismutase during maturation on pronucleus formation in porcine oocytes fertilized in vitro without superoxide dismutase. *Percentage of total number of oocytes penetrated. Different letters(a, b) indicate a significant difference ($P<0.05$). (n = 469 oocytes , 3 replicates).

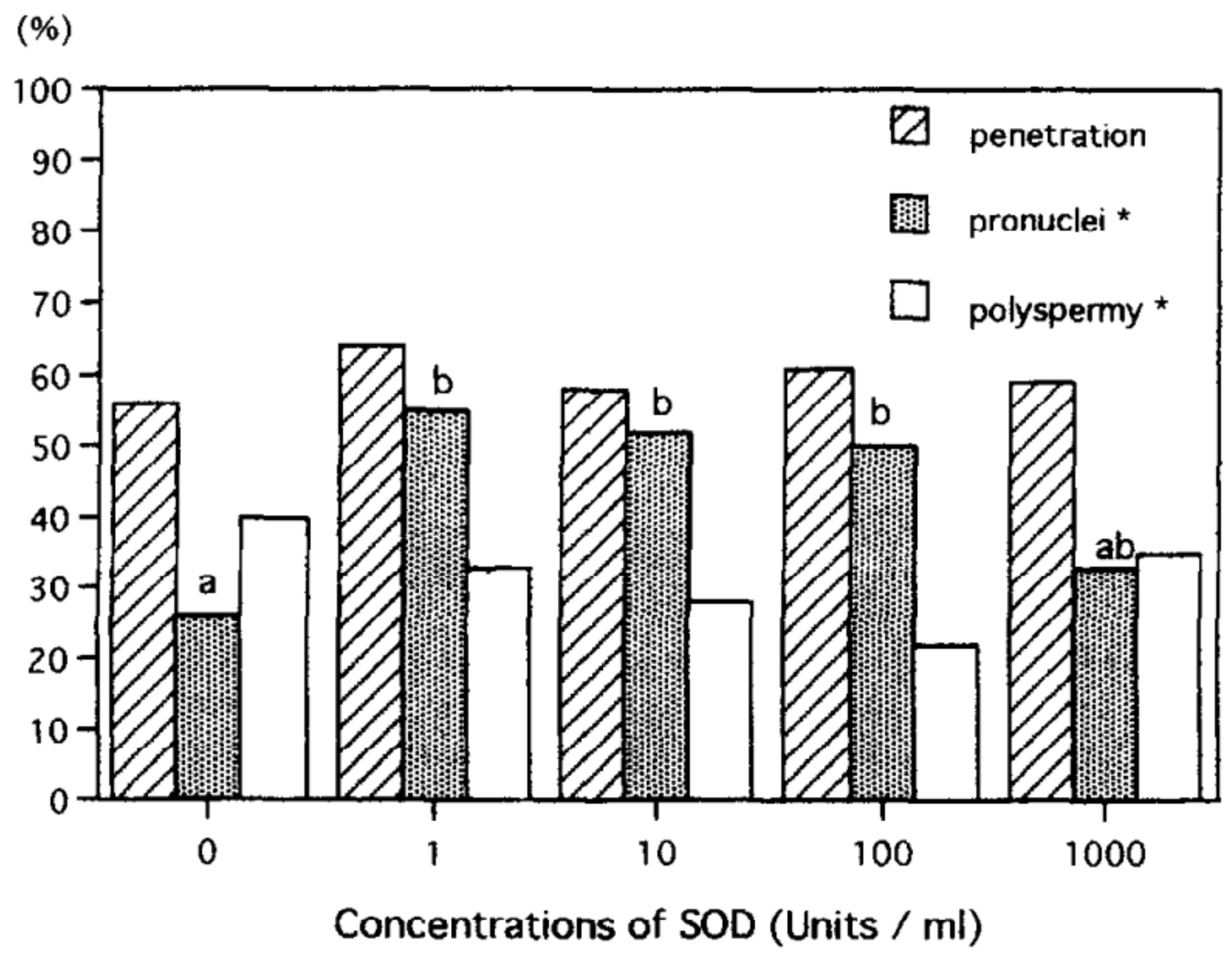

Figure 2. The effect of different concentrations(0, 1, 10, 100 and 1000 units/ml) of superoxide dismutase during the fertilization on pronucleus formation in porcine oocyte penetrated in vitro. Oocytes used were matured in medium without superoxide dismutase for fertilization in vitro. *Percentage of total number of oocytes penetrated. Different letters(a, b) indicate a significant difference($P<0.05$). (n=450 oocytes, 3 replicates).

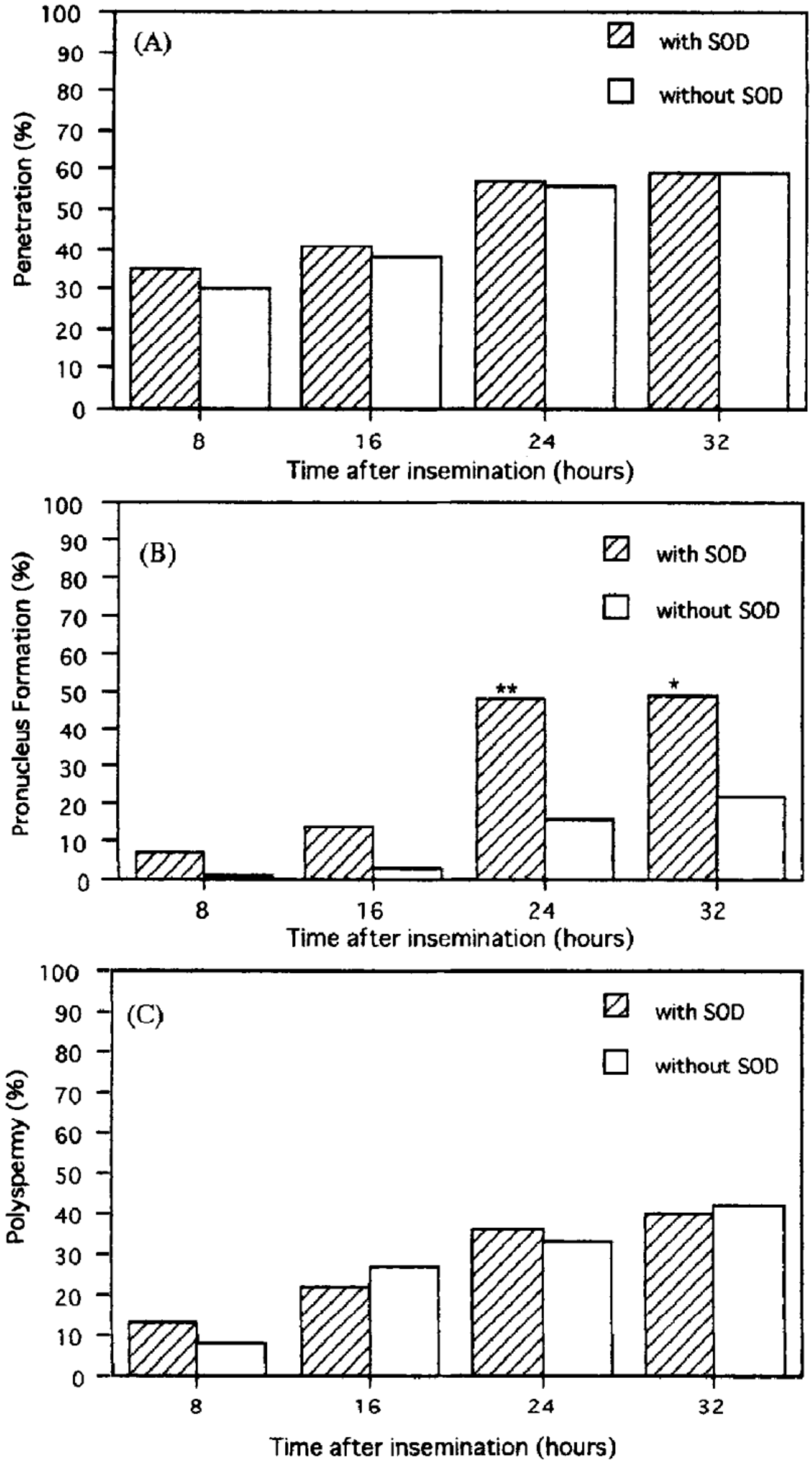

Figure 3. The effect of superoxide dismutase (1 unit / ml) on (A) penetration , (B) pronucleus formation and (C) polyspermy at various times after insemination in porcine oocytes matured without superoxide dismutase. The proportions of pronucleus formation (B) and polyspermy (C) are the percentage of the total number of oocytes penetrated. *($P<0.05$). **($P<0.01$), difference between with and without superoxide dismutase. (n=686 oocytes , 3 replicates).

In the second experiment, when SOD (1, 10 and 100 units/ml) was used to supplement the fertilization medium, concentrations and pronucleus formation rates (55, 52 and 50%) were significantly higher ($P<0.05$) than in medium without SOD (Figure 2). There were no significant differences in the penetration rates (56 to 64%) of oocytes fertilized in medium with various concentrations of SOD, and the proportions of polyspermy were also not significantly different regardless of the concentration of SOD added to the fertilization medium.

In the third experiment (Figure 3), the oocytes were cultured in medium with (1 unit/ml) or without SOD for 8, 16, 24 and 32 h after insemination. The penetration rates had a tendency to increase as time of sperm-oocyte culture was prolonged. No significant differences, however, were observed in penetration rates with and without SOD supplementation. On the other hand, pronucleus formation rates were higher in medium with that than without SOD at 24 (48 vs 16%; $P<0.01$) and 32 (49 vs 22%; $P<0.05$) h after insemination. The proportions of polyspermy gradually increased with time after insemination, but no effects of SOD was observed.

DISCUSSION

In mammals, all cells are exposed to the risk of injury by active oxygen, which is formed when molecular oxygen is utilized as an electron acceptor during oxidoreductive reactions in the cells. Superoxide anion is a major species of active oxygen and is formed by enzymes such as monomine oxidase, xanthine oxidase, and aldehyde oxidase and by auto-oxidation reactions such as those of ubiquinone, catechols, ferredoxins and hemoproteins (15,18). Superoxide anions react with proteins or lipids located in the vicinity of the O^{2-} generated. Inactivation of enzymes and lipid peroxidation in the membrane would be expected without adequate defense mechanisms against active oxygen. Superoxide dismutase catalyzes the dismutation of superoxide free radical anion as follow : $2H^{+} + 2O_2 \rightarrow H_2O_2 + O_2$. The enzyme has also been found to be ubiquitous among oxygen-metabolizing organisms(9) and is the major radical scavenger in the defense against oxygen toxicity. Thus the biological effects of SOD on porcine oocytes appears to be due to catalytic activity of this enzyme, scavenging the superoxide free radicals generated within the culture medium.

The results of this study show that supplementation of media with SOD for in vitro maturation and fertilization of porcine oocytes affects the ability of oocytes to form the male and female pronucleus after sperm penetration. Addition of SOD to both maturation and fertilization medium was effective in promoting this ability. The limited capacity of male pronucleus formation

in porcine oocytes matured and penetrated in vitro has been described by several investigators (10,21,22,24,26). In the present study, we show that it is possible to maintain the rate of pronucleus formation after insemination in oocytes matured with 10 and 100 units/ml of SOD. It has been previously shown that the pronucleus formation capacity of porcine oocytes can be maintained by the composition of the maturation medium (25) and by physical interaction with oviductal cells (1). Umaoka et al. (20) demonstrated the presence of SOD in the oviduct fluid or oviduct epithelium by biochemical (15) or immunohistochemical (8) analysis. Taking these results into consideration, it is suggested that pronucleus formation is protected from the oxidative stress under SOD-rich conditions in the oviduct fluid. The effectiveness of SOD on pronucleus formation for mouse pronuclear embryos is suggested by Noda et al. (15). They have demonstrated that SOD attenuates the 2-cell block in mouse embryos cultured in vitro. This enzyme, when added to culture medium, allows mouse embryos to undergo cleavage past the 2-cell block (3).

In the present study, pronucleus formation was possible when oocytes were cultured at and after 8 h of insemination in fertilization medium with SOD. The present results indicate that addition of SOD during in vitro fertilization (Figure 1) is helpful for pronucleus formation at any time after insemination. In the bovine, a high proportion (about 50%) of pronucleus formation occur 7 h after insemination (16). This rapid formation of pronuclei, however, does not occur in porcine oocytes. It is difficult to explain at present exactly why pronucleus formation was delayed in medium with SOD by 16 h after insemination.

Spermatozoa cultured with or without SOD in this study showed similar degrees of penetrability. Moreover, there were no differences between penetration rates in oocytes cultured with and without SOD at various times after insemination. A high incidence of polyspermy in porcine oocytes matured in vitro has been reported by many investigators (6,13,26). In the present study, the proportions of oocytes penetrated with more than 1 sperm cell were high in medium with SOD (22 to 40%) and without (40%). Since a high proportion of the oocytes cultured with spermatozoa for 8, 16, 24 and 32 h were polyspermic in the medium with (13, 22, 36 and 40%) versus without SOD (8, 27, 33 and 42%), it appears that SOD does not have a role in decreasing polyspermy of porcine spermatozoa under the experimental conditions of the study. However, there are no reports to date showing the penetrability of porcine oocytes cultured in medium with SOD.

In conclusion, these findings demonstrate the advantage of culture with SOD on pronucleus formation in porcine oocytes penetrated by spermatozoa. However, SOD does not affect penetration rates and polyspermy.

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