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Examination of the safety of intracytoplasmic injection procedures by using bovine zygotes

Mutsuro Motoishi, Kazufumi Goto[1], Keiko Tomita, Shoji Ookutsu and Yoshihiko Nakanishi

Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, Kagoshima 890, Japan

[1]To whom correspondence should be addressed

We have evaluated the safety of intracytoplasmic sperm injection (ICSI) procedures by using bovine zygotes. Bovine zygotes were injected with a small amount (2–3 pl) of either medium alone or medium containing polyvinylpyrrolidone (PVP) (sham-ICSI, without spermatozoon) using the same procedure as ICSI, and the subsequent in-vitro embryonic development and embryo quality (number of cells/blastocyst) were examined. Control zygotes which had not been injected were similarly evaluated after in-vitro development. The sham-ICSI of either medium alone or medium containing PVP into bovine zygotes had no harmful effects on the rate of normal fertilization and on the rate of development to hatched blastocyst stage compared with those of controls ($P > 0.05$). In addition, no harmful effects were observed in the number of cells per blastocyst (embryo quality). The results suggest, for the first time, that the ICSI procedures currently used for animal and human ICSI are neither detrimental to embryonic development nor detrimental to embryo quality.

Key words: bovine oocyte/intracytoplasmic sperm injection/polyvinylpyrrolidone/zygote

Introduction

Intracytoplasmic sperm injection (ICSI) is a promising technique for treating patients with severe male subfertility (Van Steirteghem *et al.*, 1993a, b) and for the conservation of endangered species (Goto, 1993). Normal live offspring were born following ICSI in rabbit (Hosoi *et al.*, 1988), bovine (Goto *et al.*, 1990), human (Palermo *et al.*, 1992), sawfly (Hatakeyama *et al.*, 1994) and mouse (Ahmadi *et al.*, 1995) but the safety of ICSI procedures has not been fully established. Since the injection of a small amount of medium (1–3 pl) containing polyvinylpyrrolidone (PVP) into the oocyte, together with the spermatozoon, is unavoidable, concerns have been expressed regarding the safety of using PVP for this purpose (Fischel *et al.*, 1993). Recently, Bras *et al.* (1994) reported that some commercially-produced PVP solutions are toxic, causing failed embryo development after ICSI in the mouse zygotes. However, the mouse oocyte is significantly smaller (80 μm) than either human or bovine oocytes (120 μm). The needle for mouse sperm injection has to be larger to accommodate the large mouse spermatozoon. This poses a technical problem which has resulted in damage to 22% of injected mouse oocytes (Ahmadi *et al.*, 1995). In addition, because of the large size of the mouse spermatozoon, more medium is inevitably injected into the oocyte during mouse ICSI. Furthermore, the plasma membrane of mouse oocyte is fragile compared with that of human and bovine oocytes (Kimura and Yanagimachi, 1995; K.Goto, unpublished observations). All of these factors could possibly play a role in reducing the number of viable embryos obtained following ICSI in mouse (Goto *et al.*, 1991; Ahmadi *et al.*, 1995).

We, therefore, conducted an experiment to examine not only the safety of using PVP but also the safety of the ICSI procedure itself (breaking of cytoplasmic membrane, aspiration of ooplasm and injection of medium into ooplasm) on bovine zygotes which are both similar to human oocytes in size and have a similar tolerance to the ICSI procedure (<5% of bovine oocytes are damaged by ICSI). For this purpose, a small amount (2–3 pl) of either medium alone or medium containing PVP was injected into bovine zygotes by using the same procedure as ICSI, and the subsequent in-vitro embryonic development and embryo quality (number of cells/blastocyst) were examined.

Materials and methods

Preparation of zygotes

Bovine oocytes surrounded by cumulus cells were matured and inseminated *in vitro* (Goto *et al.*, 1988) in an incubator (5% CO_2, 95% air). After 6 h insemination, the presumptive zygotes (defined as the whole population of oocytes used for insemination) were treated with 0.1% hyaluronidase to remove cumulus cells.

Intracytoplasmic injection of medium into zygotes (sham-ICSI)

Sham-ICSI (ICSI without spermatozoon) was performed according to the method described by Goto *et al.* (1990) with slight modifications. Briefly, the zygote was held in place on the holding pipette (inner diameter 20 μm, outer diameter 100 μm) with the polar body in either the 6 or 12 o'clock position, and the injection pipette (inner diameter 6–7 μm, outer diameter 8–9 μm) approached the zygote from the 3 o'clock position. The pipette was then introduced deeply into the cytoplasm, which was then aspirated up to a visible break of the cytoplasmic membrane. A small amount (about 2–3 pl) of either medium [12.5 mM HEPES tissue culture medium (TCM) 199 with Earle's salts; Gibco, Grand Island, NY, USA] supplemented with 5% calf serum, or medium containing 6.6% PVP (K90; Wako, Osaka, Japan) was then injected into the ooplasm. Control zygotes were not subjected to the injection procedure.

Culture of sham-ICSI and control zygotes

The sham-ICSI and control zygotes were washed with culture medium (12.5 mM HEPES TCM 199 + 1% calf serum + 1 mM sodium

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Table I. Fertilization rate of bovine presumptive[a] zygotes injected with medium alone or medium containing polyvinylpyrrolidone (PVP) (experiment 1). Figures in parentheses alone are percentages

Treatment	No.[b]	No. zygotes fertilized	
		Total (≥2PN)	Normal (2PN + one sperm tail)
Control	96	90 (93.8)	64 (66.7)
Medium	93	85 (91.4)	66 (71.0)
Medium + PVP	93	84 (90.3)	64 (68.8)

[a]Defined as the whole population of oocytes used for insemination.
[b]Sum of four replicates.
PN = pronucleus.
Control = no injection.
Medium = 12.5 mM HEPES tissue culture medium 199 + 5% fetal calf serum.
Medium + PVP = medium + 6.6% PVP.
Differences within columns were not significant ($P > 0.05$).

Table II. Development of bovine presumptive zygotes injected with medium alone or medium containing polyvinylpyrrolidone (PVP) (experiment 2). Figures in parentheses are percentages

Treatment	No.[a]	No. developed to			
		Cleaved (Day 3)[b]	Morula (Day 4–6)	Blastocyst (Day 6–8)	Hatching (Day 8–12)
Control	92	64 (69.6)	24 (26.1)	14 (15.2)	5 (5.4)
Medium	89	61 (68.5)	24 (27.0)	16 (18.0)	10 (11.2)
Medium + PVP	90	65 (72.2)	24 (26.7)	15 (16.7)	7 (7.8)

[a]Sum of four replicates.
[b]Day 0 = day of insemination.
Control = no injection.
Medium = 12.5 mM HEPES tissue culture medium 199 + 5% calf serum.
Medium + PVP = medium + 6.6% PVP.
Differences within columns were not significant ($P > 0.05$).

pyruvate) and transferred into 0.5 ml of culture medium in a 4-well multidish (Nunc, Roskilde, Denmark) covered with mineral oil (Squibb, Princeton, NY, USA). The dish with the zygotes (20–25 per well) was placed in an incubator (39°C, 5% O_2, 5% CO_2 and 90% N_2) either for 12 h (experiment 1), for 12 days (experiment 2) or for 8 days (experiment 3). In experiment 1, the zygotes were fixed in methanol:acetic acid (3:1, v/v) and stained with 1% aceto-orcein for microscopic assessment of fertilization (Goto *et al.*, 1988). The zygotes were considered to be fertilized when two or more pronuclei and sperm tail(s) were seen in the ooplasm. The proportion of normal fertilization (one male pronucleus + one sperm tail + one female pronucleus) was also recorded. In experiment 2, the proportions of zygotes that cleaved and developed to morula, blastocyst and hatching blastocyst stages were examined 3, 4–6, 6–8 and 8–12 days after insemination respectively. In experiment 3, zygotes that developed to blastocysts were fixed and stained 8 days after insemination and the number of cells was determined according to the method described by Tokumaru *et al.* (1989).

Statistical analysis

Each experiment was replicated 4 or 5 times (different occasions) each with an appropriate control group. The proportion of embryos that developed to each stage was compared by a χ^2 test incorporating Yates' continuity correction. Differences in the mean number of cells per blastocyst between groups were assessed by Student's t-test.

Table III. Development of bovine presumptive zygotes injected with medium alone or medium containing polyvinylpyrrolidone (PVP) (experiment 3). Figures in parentheses are percentages

Treatment	No.[a]	No. developed to:		
		Cleaved (Day 3)[b]	Morula (Day 4–6)	Blastocyst (Day 8)
Control	111	96 (86.5)	36 (32.4)	22 (19.8)
Medium	110	89 (80.9)	32 (29.1)	25 (22.7)
Medium + PVP	114	97 (85.1)	29 (25.4)	20 (17.5)

[a]Sum of five replicates.
[b]Day 0 = day of insemination.
Control = no injection.
Medium = 12.5 mM HEPES tissue culture medium 199 + 5% calf serum.
Medium + PVP = medium + 6.6% PVP.
Differences within columns were not significant ($P > 0.05$).

Table IV. Cell number of blastocysts obtained in experiment 3

Treatment[a]	No. blastocysts examined[b]	Cell number[c]/blastocyst
Control	22	115.3 ± 6.5
Medium	22	100.5 ± 6.4
Medium + PVP	20	109.8 ± 5.7

[a]See Table III for treatment explanation.
[b]Sum of five replicates.
[c]Mean ± SEM.
Differences within columns were not significant ($P > 0.05$).

Results

The injection of a small amount (2–3 pl) of either medium or medium containing PVP into zygotes had no detrimental effect on the rates of total and normal fertilization compared with those of non-injected control zygotes (Table I). No detrimental effects were observed in the proportions of zygotes that cleaved and developed to morula, blastocyst and hatching blastocyst stages compared with those of control (Tables II and III). There were no significant ($P > 0.05$) differences in the number of cells per blastocyst between control and injected groups (Table IV).

Discussion

PVP solution has been commonly used as a sperm diluent for ICSI, and apparently normal offspring have been born following ICSI in the rabbit (Hosoi *et al.*, 1988), bovine (Goto *et al.*, 1990), human (Palermo *et al.*, 1992) and mouse (Ahmadi *et al.*, 1995). However, the possibility exists that the techniques themselves are detrimental to embryonic development and embryo quality. Using mouse zygotes, Bras *et al.* (1994) suggested that some commercially-prepared PVP solutions may be detrimental to zygote development. The present results suggest that intracytoplasmic injection of either medium or medium containing PVP to bovine zygotes had no such detrimental effects on either the subsequent development of zygotes *in vitro* or on embryo quality. In a separate experiment, we have recently transferred blastocysts (obtained by the same method described in this paper) developed from PVP-injected zygotes into two recipient cows (two embryos/recipient), one

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of which became pregnant (currently at 75 days of pregnancy). These results suggest that PVP can be used safely for ICSI if its non-toxicity is first carefully confirmed. Further evidence for the safety of PVP in the ICSI procedure has been demonstrated by karyotyping bovine embryos produced by ICSI (Goto, 1993) and by determination of the incidence of major malformations in neonates (Bonduelle *et al.*, 1994); neither indicated any increase in abnormality compared with the incidence in the normal population. Furthermore, the normality of ICSI offspring in the post-pubertal period has been demonstrated by the birth of normal calves from ICSI-derived females (Goto and Yanagita, 1995). Ray *et al.* (1995) examined the potential mutagenic effect of PVP on cultured human somatic cells, at the concentration used for ICSI. They suggested that PVP did not cause DNA lesions resulting from sister chromatid exchanges, and provided reassuring evidence concerning its safety in human ICSI. However, Dozortsev *et al.* (1994, 1995) reported that PVP at a high concentration stabilized the sperm membrane, thus retarding the rate of sperm decondensation, and hence, increasing the time span of the fertilization event following ICSI. Thus, the use of an unnecessarily high concentration of PVP should be avoided. The effect of PVP on the sperm decondensation process could not be evaluated in this study, because the bovine zygotes used had already completed the decondensation process.

The present results show that the components of the ICSI procedure themselves, such as the breaking of cytoplasmic membrane, the aspiration of ooplasm and the injection of medium containing PVP into the ooplasm of zygotes, do not cause any detrimental effect on either fertilization rate or subsequent in-vitro embryo development and quality. These findings are encouraging since the ICSI procedures described here are already widely-used in human infertility programmes all over the world.

In summary, our results suggest that the ICSI procedures currently used in animals and humans have no detrimental effects on embryonic development or embryo quality.

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