Original Article

Comparison between Quality of Cryopreserved Embryos Generated from Short and Long Gamete Incubation

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Abstract

Background: The purpose was to investigate the effect of the duration of gamete incubation on fertilization rate, embryo cleavage, and embryo quality before and after freezing in mice.

Materials and Methods: Ovulated oocytes collected from superovulated mice after ip injection of PMSG and hCG were divided randomly into control and experimental groups. Oocytes from the control group were inseminated for six hours and the experimental group were inseminated for one hour, respectively. The differences in fertilization rates, embryo cleavage and percent of good quality embryos in four grades (A, B, C, D) were analyzed. Finally, two cell embryos were frozen; and after thawing, the quality of embryos from the two groups were compared.

Results: There was no difference between the two groups in regards to fertilization and cleavage rates. However, the proportion of grade A embryos was significantly higher among the experimental group (41.7%) when compared to the control group (19%). Also the proportion of grade D embryos was significantly (p=0.04) lower in the experimental group (8.3%) as compared to the control group (23.8%). In addition, percentage of good quality embryos in the experimental group did not decrease after freezing (p=0.3), however the percentage of good quality embryos were significantly decreased after freezing in the control group (p=0.01).

Conclusion: Insemination of oocytes for a short period produced embryos of superior quality than insemination for a longer period in the experimental group . Also, the effect of freezing on embryos produced from short insemination was less than the long insemination period. After freezing, a higher percentage of good quality embryos survived post thawing in mice.

Keywords: *In vitro* Fertilization, Insemination, Embryo, Mouse

Introduction

In vitro fertilization (IVF) is now widely used in human infertility treatment, and for the production of large numbers of cleaving embryos in the domestic animal industry (1, 2). Capacitated spermatozoa and mature good quality oocytes are two basic requirements for successful IVF (3). In the clinical setting, IVF technique employs a prolonged (16 to 20 hour) incubation of oocytes with spermatozoa in culture media (4). Despite many technical improvements in IVF methodology, the rate of implantation is still low (4, 5). Aitken reported that the rate of reactive oxygen species (ROS) is associated with both a high concentration of spermatozoa and prolonged duration of insemination (6). It has been noticed by Aitken that the arrest of mouse embryo development may be related to cell membrane malformation after an elevation in the concentration of ROS (6). Based on these observations, investigators tried to shorten the gamete co-culture period to observe whether this would result in a better IVF outcome (7-9). Kattera and colleagues observed a significantly lower rate of polyspermy in the group of patients with short insemination compared with standard co-incubation of sperm and oocytes (7). In addition, short insemination significantly improved embryo quality and implantation rates.

Following successful IVF methodology, several embryos may develop *in vitro* and only a limited number are transferred. The extra embryos are usually cryopreserved for future use (10). Therefore, the purpose of this experimental study was to evaluate the timing of co-incubation of gametes as well as cryopreservation of developed cleaving embryos under one setting and compare the fertilization rate and quality of embryos derived from standard co-incubation of gametes (6 hours) with short co-incubation (1 hour) in mice. The quality of embryos from each group was also compared after cryopreservation.

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Materials and Methods

This study was approved by our institute's research committee.

Collection and preparation of oocytes

Female NMRI mice aged 8-10 weeks were induced to super ovulate by i.p. injection of 10 IU pregnant mare serum gonadotropin (PMSG, Sigma, USA). Forty-eight hours later, these mice were injected i.p. with 10 IU of human chorionic gonadotropin (hCG, Serono, Swiss) to trigger ovulation. Fifteen hours later, the oviducts were excised under aseptic conditions and the cumulus-oocyte complexes were collected in T6 medium. Granulosa cells of the oocytes were removed by pipetting in T6 medium containing 80 IU/ml hyaluronidase (Sigma, USA) and washing. Mature oocytes with the first polar body were collected for the experiments. They were cultured with T6 medium containing 5% human serum albumin (HSA, Sigma, USA) in an atmosphere of 5% CO_2 in air at 37°C. The oocytes were randomly allocated to two groups: control and experimental, for short and standard insemination periods, respectively.

IVF and Embryo Cultures

A total of 200 MII oocytes were prepared. Oocytes were divided randomly to either the long (control) or short (experimental) insemination groups. In the short insemination group, oocytes were exposed to spermatozoa for 1 hour; and in the standard insemination group, oocytes were exposed to spermatozoa for 6 hours. Oocytes in both groups were checked for fertilization, cleavage rate and embryo quality. The embryos were frozen, and after thawing, were evaluated for morphological quality.

For IVF, epididymal sperm suspensions were prepared from male mice aged 8-16 weeks and preincubated for 60 minutes in T6 medium containing 5% HSA to ensure sperm capacitation. A final concentration of 2×10^6 capacitated sperm/ml was collected from the periphery of the capacitation media and introduced into 50µl microdrops of T6 medium containing the oocytes. Sperm and oocytes were incubated together for 6 hours in the control group and 1 hour in the experimental group. Then, the oocytes were washed and placed into 50µL microdrops of T6 medium supplemented with 5% HAS under mineral oil for further development at 37°C in a highly humidified, 5% CO_2 incubator. After insemination, fertilization of the oocytes was confirmed by the observation of two pronuclei 6 hours after insemination. The presence of two blastomeres at 24 hours after insemination were considered as embryo development (11). All generated embryos were evaluated and graded according to Hills criteria. The cleaved embryos were frozen by ultra rapid freezing.

Ultra rapid freezing procedure

Embryos were suspended in m-hTF based supplemented with 10% HSA equilibration medium containing 1.5 mol/L DMSO for 5 minutes and transferred to freezing medium (3.5 mol/L DMSO and 0.25 mol/L sucrose) for 2.5 minutes at room temperature. The embryos were then transferred into straws and immediately plunged into liquid nitrogen (LN) for storage. For thawing, embryos were directly inserted into a thawing medium (2.0 mol/L sucrose) for 5 minutes. Thawed embryos were transferred to 1.5 mol/L, 1.0 mol/L and 0.5 mol/L sucrose solutions for 5 minutes each, then washed twice with culture medium for 5 minutes and finally transferred to T6 medium for quality evaluation (12).

Embryo evaluation

Embryo evaluation was based on the method of Hill, et al (1989) before and after freezing. Grade A embryos were the best embryos containing even-sized, symmetrical blastomeres with no obvious fragmentation. Grade B embryos had blastomeres of uneven size or the total cytoplasm mass contained < 10% fragmentation; grade C embryos had a maximum of 50% of their cytoplasm fragmented and uneven blastomeres; and grade D embryos showed > 50% cytoplasm fragmentation (13).

Statistical analysis

Differences between data for comparison of the two groups were analyzed by chi-square test. For comparison of data before and after freezing, paired student's t test was applied. P value <0.05 was defined as statistically significant.

Results

The findings generated from this study showed that the rates of fertilization and embryo development were similar between the control and experimental groups. The fertilization rates and the number of embryos did not differ significantly between the two groups.

However, the proportion of good quality (grade A) embryos was significantly higher in the experimental group (41.7%) than in the control group (19%). On the other hand, the proportion of grade D embryos was significantly lower in the experimental group (8.3%) than in the control group (23.8%) (Table1).

Table 1: Embryo evaluation based on Hills method before freezing in short and long insemination groups

Group	Embryo grade				Total
	A	B	C	D	
Short insemination	20 (41.7)	12 (25)	12 (25)	4 (8.3)	48
Long insemination	8 (19)	10 (23.8)	14 (33)	10 (23.8)	42
p value	0.02	0.89	0.38	0.04	

Values inside parentheses represent percentages.

Table 2: Comparison of embryo grading in short and long insemination groups.

Group	Embryo grade				Total
	A	B	C	D	
Short insemination	13 (31.7)	12 (29.3)	10 (24.4)	6 (16.6)	41
Long insemination	4 (10.1)	6 (16.2)	12 (32.4)	15 (40.5)	37
p value	0.02	0.17	0.43	0.01	

Values inside parentheses represent percentages.

The results also showed that the proportion of grade A embryos was significantly higher in the experimental group (31.7%) than in the control (10.1%) group after freezing-thawing. In addition, the proportion of grade D embryos was significantly lower in the experimental (16.6%) than in the control (40.5%) after freezing (Table 2).

In addition, the results showed that the proportion of grade A+B embryos in the experimental group before cryopreservation was 66.7% and after freezing was 61%.

Therefore the proportion of grade A+B in the experimental group was not decreased by freezing and thawing (p=0.3). The proportion of grade C+D embryos in the experimental group after freezing (33.3%) didn't increase significantly when compared to those before freezing (40.8%, p=0.1) (Table 3).

Table 3: Comparison of embryo grading before and after freezing in short insemination group.

Group	Embryo grade	
	A+B	C+D
Before freezing	66.7%	33.3%
After freezing	61%	40.8%
p value	0.3	0.1

But, in the control group, the proportion of grade A+B embryos was 42.8% before freezing and after freezing the proportion was reduced to 26.3% (p=0.01). Also the proportion of grade C+D embryos was 56.8% before freezing and significantly increased after freezing (72.9%) (p=0.01) (Table 4).

Table 4: Comparison of embryo grading before and after freezing in long insemination group.

Group	Embryo grade	
	A+B	C+D
Before freezing	42.8%	56.8%
After freezing	26.3%	72.9%
p value	0.01	0.01

Discussion

In conventional IVF technology, oocytes are co-incubated with spermatozoa overnight for a period of approximately 16-18 hours. In recent years, several reports emphasized that a reduced co-incubation time of gametes in clinical IVF is beneficial or at least not detrimental to fertilization and subsequent embryo development. However, Barraud-Lange et al evaluated the effect of short gamete incubation on fertilization and embryo quality and observed that the fertilization rate was statistically lower in the short insemination group (1hour) compared to the standard insemination group (64.9% & 70.15%, p=0.039), but a nonsignificant increase was observed concerning good embryo quality rate in the short insemination group when compared to the standard insemination (14). Also Lundqvist et al reported that fertilization rate and embryo quality was significantly higher in long insemination than short insemination, but no difference was observed in further development in terms of morphology and implantation rates (15). Swenson et al reported implantation rates were similar in both groups and short incubation did not seem to have any advantage over conventional co-incubation techniques in couples undergoing *in vitro* fertilization where the male factor was nor-

mal (16).
Therefore the main objective of this study was to find out whether a shortened gamete co-incubation protocol would be of benefit to the outcome of IVF in mice. In addition, the quality of *in vitro* developed embryos generated from short versus standard insemination protocols were compared following cryopreservation .The results confirmed that the rates of fertilization and 2-cell embryo development were similar between the two groups. Gianaroli et al investigated two protocols of 1 hour and 16 hour inseminations in an IVF program (5). Their results showed that the rate of polyspermy was reduced in the short when compared with the long insemination protocol. However, the rate of fertilization and embryo quality were similar between the two groups. The rates of 4-5 cell embryos were significantly higher in short than long insemination groups. Results from other studies that compared short and long insemination protocols, showed insignificant differences in fertilization rates between brief or overnight co-incubation of gametes. However, the quality of embryos was significantly improved in the 1hour exposure group (1, 7, 17). In addition, other studies stated that short vs. long insemination techniques in IVF resulted in a similar success rates between the two groups. Therefore, it seems that 1 hour co-incubation was sufficient for progression in an IVF protocol (18, 19). In animal studies, Smantri et al also reported that 5 hour rather than 20 hour co-incubation resulted in a higher quality rate of blastocysts in bulls (20). Therefore, the majority of studies believe that long co-incubation has no advantage over short co-incubation in either experimental or clinical IVF (1, 5, 7-9, 17-20).
Cryopreservation of embryos generated from IVF has several advantages in both experimental and clinical programs. However cryopreservation may damage the cell structure; which is mainly due to sudden temperature reduction, extra and intracellular ice formation, osmotic variation, cell toxicity and cell fracturing (21, 22). Therefore, these structural alternations may permanently damage embryo blastomers, which could result in cell degeneration and necrosis (23-25). It has been noted by several investigators that cryopreservation alters the quality of embryos. Therefore it becomes necessary to evaluate and compare embryo morphology pre- and post- cryopreservation. In this regard, Selick reported that cryopreservation has a clear detrimental effect on embryo quality (26). Saito et al also stated that the rates of high quality embryos will be reduced after freezing-thawing (27). In this study, the embryo quality was compared before and after freezing in both short and long insemination groups. Our results indicated that embryos generated from short insemination IVF could tolerate the cryo environment better than embryos from the long insemination protocol. Further studies are needed to find out the exact mechanism behind this phenomenon. However, it is generally accepted that higher quality embryos form following cryopreservation (28, 29). In addition Lian et al compared the cryopreservation rates of 2-cell embryos which resulted from *in vivo* with *in vitro* embryos (30). Their finding showed that *in vivo* formed embryos were of higher quality and could tolerate cold shock during the cryopreservation process better than *in vitro* embryos. This shows that the important issue for cryotechnology is a high quality embryo to tolerate the cold shock. As a result, cryopreservation of embryos generated from short insemination, which are of the better quality, will result in a better outcome.
Recently, Bungum et al compared two short protocols of 30 seconds and 90 minutes co-incubation (8). The results showed that fertilization rates were similar in both groups. Also, the rates of grade A embryos (good embryos) were similar between the two short groups of co-incubation. However, the rate of polyspermy was significantly lower in the 30 second group. According to different studies, prolonged oocyte exposure to sperm may be detrimental because of ROS production. The damaging effects of ROS are dependent on the type and concentration, as well as the duration of exposure. Studies have reported that ROS generated by leukocytes may affect normal spermatozoa. A high leukocyte concentration may cause loss of sperm motility and membrane function in oocytes. These alterations affect the division process in oocyte development. Several animal studies have related oxidative stress to cleavage arrest or more slowly developing embryos (6, 31, 32). It seems clear that any increase in the possible toxic effects on membrane integrity is mainly dependent on the time course of sperm exposure. In addition, Kattera et al showed that cumulus cells also secret E2 and P4 into culture media during co-incubation, which have detrimental effects on zygotes (7). Therefore, in order to eliminate the effects of the aforementioned chemical release from cumulus cells, only naked oocytes were inseminated in this experimental study.

Conclusion

In conclusion, embryos generated from 1 hour

co-incubation withstand freezing stress better than embryos from the long insemination protocol in mice. Further studies on clinical cases are necessary to evaluate the outcome of short insemination IVF cycles following embryo cryopreservation.

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There is no conflict of interes in this article.

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