Introduction

In vitro fertilization (IVF) has become an established procedure to treat infertility. However, failure of pregnancy necessitates repeating the whole procedure of IVF. Cryopreservation of the extra oocytes and embryos at -196°C would allow repeating the IVF procedure with the advantage of avoiding the inconvenient induction of ovulation and the invasive procedure of oocyte retrieval. Cryopreservation is a critical process that is affected by many factors; such as the freezing rate and temperature [1, 2], cryopreservation procedure [3-10], type and concentration of the cryoprotectant [2,11-14], and the use of pre-exposure to ethylene glycol or raffinose solution [15, 16].

However, factors like the thawing temperature and the developmental stage of the cryopreserved embryos did not attract much attention. Further, controversy exists in the few reports detected in the literature dealing with the effect of thawing temperature on the developing embryos [1, 17, 18].

The present study aimed at investigating the effect of the ultra rapid cryopreservation (vitrification) technique on the fertilized ova, 4-cell embryos and morula. It aimed also at comparing the effect of slow and fast thawing on the cryopreserved ova and embryos.

Material and Methods

Forty adult female outbred MF1 mice, (20-40 g) at age 5-6 weeks were used in this research. The animals were obtained from the Animal House, King Fahd Medical Research Center, King Abdulaziz University, Jeddah. They were maintained on a standard diet [commercial cubes containing (w/w) approx. 18% protein, 3% fat, 77% carbohydrate and 2% of an inorganic-salt mixture with a vitamin supplement (Grain Silos and Flour Mills Organization, Jeddah, Saudi Arabia)] and water *ad libitum*. The mice were kept in a controlled environment (constant temperature 24°C, and a light cycle of 14 h on/ 10 h off). Superovulation was induced by subcutaneous injection of 5 IU of pregnant mare serum gonadotropin (PMS) and, 48 hours later, with intraperitoneal injection of 5 IU of human chorionic gonadotropin (hCG). Each female was placed with a proven male in a breeding room and then examined for sperm plugs next morning (day 1). Fertilized ova were collected 24 hours after hCG injection and exposed to hyaluronidase enzyme for few minutes to free them from the cumulus cells. The ova were examined for signs of fertilization; extrusion of second polar body, formation of pronuclei or division of the zygote. The experimental work was done on 623 fertilized ova, whereas non-fertilized ova were discarded. The fertilized ova were incubated in potassium simplex optimized medium (KSOM, from Specialty Media, USA) in 15 mm culture dish (Nunc). The incubation was carried out in 20 µl droplets of the culture medium covered with paraffin oil, and kept in the CO2 incubator at 37°C and 5% CO2. The fertilized ova were divided into three groups of 200, 289, and 134, respectively. Out 200 fertilized ova in the first group, 60 were vitrified at the fertilized ova stage. Out of 289 and 134 fertilized ova in the second and third groups, 140 and 60 were vitrified at the 4-cell or the morula stage, respectively. The remaining fertilized ova were incubated without vitrification and used as control for each group.

The ultra-rapid cryopreservation (vitrification) procedure used is the method described by Kasai et al [19]. It consisted of washing the embryos in Dulbeco's phosphate buffered saline (D-PBS) and equilibrating them in the vitrification solution for 2 minutes. The vitrification solution (EFS40) consisted of 40% ethylene glycol in solution of 30% Ficoll, 0.5 M sucrose and BSA dissolved in D-PBS. The embryos were then transferred to 13mm EFS40 column in the straw. The straw was allowed to cool slowly in liquid nitrogen vapor for at least 3 min before immersing in liquid nitrogen (-196°C) for storage. The vitrified embryos were thawed by the slow

or fast warming rates. For slow thaw, the straws were kept standing on air at room temperature for 15 sec, and then immersed into a 20°C water bath. For fast thaw, the straws were agitated in a water bath at 37°C. When the sucrose solution began to melt, the straws were removed from the water bath and slowly perfused with 1ml sucrose solution, the embryos recovered and transferred to drops of hyperosmolar sucrose in culture dish. Glucose was then gradually diluted and replaced by culture medium.

The embryos were cultured for 1 hour and examined with the inverted phase contrast microscope to assess the survival rate (embryos which appear morphologically intact). The embryos were then cultured for 24 hours more and examined with the inverted phase contrast microscope to assess the in vitro development rate. The significance of difference between the cryopreserved/ thawed and control embryos was evaluated by students' t-test.

Results

The percentage of success of superovulation (% mice which gave ova) was 85%. The number of ova collected/ mice were 33 ± 19 (mean ± SD). The ova collected 24 hours after hCG injection (Day 1), following exposure to hyaluronidase enzyme for few minutes to free them from the cumulus cells, showed signs of fertilization, in the form of extrusion of second polar body or formation of pronuclei (**Figure 1**). Most of the vitrified slow thawed fertilized ova appeared morphologically normal with intact zona pellucida (70% survival rate). Some of the ova, however, appeared with ill-defined borders and widened perivitelline space. The ova showed a good capability to recover and cleave in vitro, where 57% (**Table 1**) could divide into 2-cell embryos when incubated for 24-hours (**Day 2; Figure 2**). Fast thawed fertilized ova showed results which were approximately similar to those of the slow-thawed ova (80% survival rate). They showed also a good capability to recover and cleave in vitro, where 60%

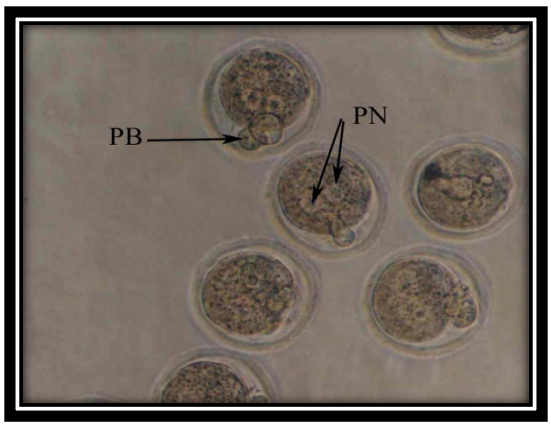

Figure 1: Fertilize *ova (control group) collected 24 hours after hCG injection (Day-1), following exposure to hyaluronidase enzyme for few minutes to free them from the cumulus cells. Most of the ova show signs of fertilization, in the form of extrusion of second polar body (PB) or formation of pronuclei (PN). Inverted phase contrast microscope x100.*

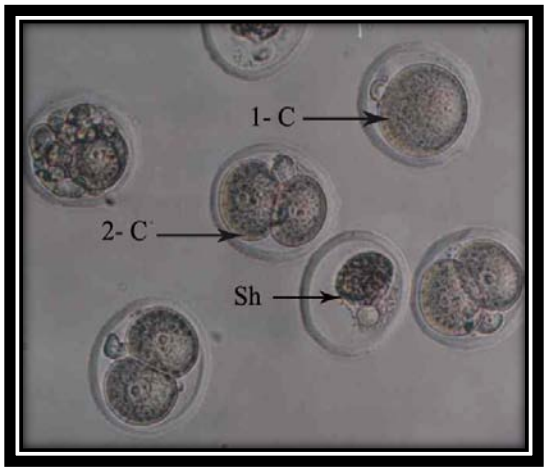

Figure 2: *Vitrified fertilized ova 24 hours after slow-thawing (Day-2). Most of the ova show good capability to recover and cleave in vitro into 2-cell embryos (2-C). One ovum shows no cleavage (1-C). The other ova are shrunken (Sh) inside their zona pellucida. Inverted phase contrast microscope x100.*

(**Table 1**) could divide into 2-cell embryos When incubated for 24-hours (**Day 2; Figure 3**).

The incubated control embryos at day-3 (4-cell embryos) showed a spherical thick zona pellucida containing four blastomeres (**Figure 4**).

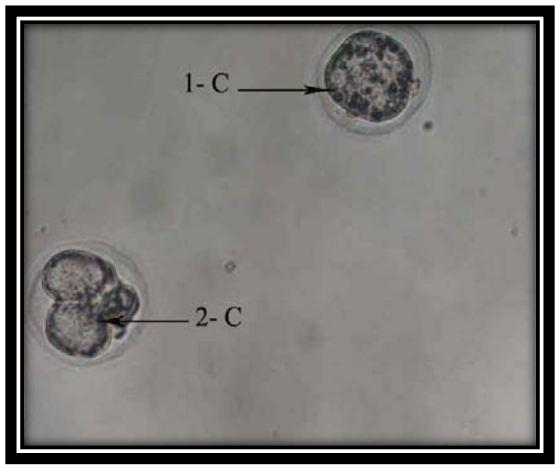

Figure 3: *Vitrified fertilized ova 24 hours after fast-thawing (Day-2). One ovum shows good capability to recover and cleave into 2-cell embryo (2-C). Another ovum does not show any cleavage (1-C). Inverted phase contrast microscope x100.*

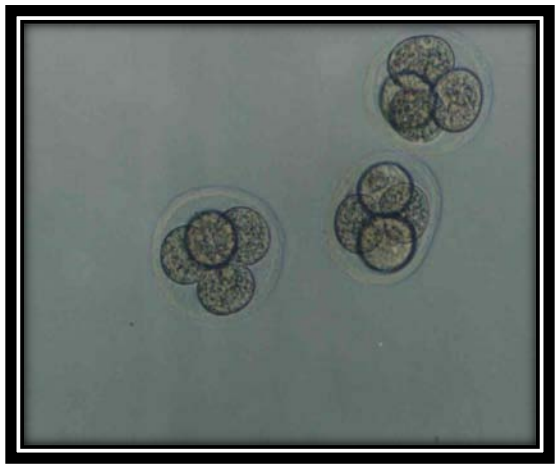

Figure 4: *Embryos at day-3(control group) after incubation in KSOM medium. The embryos show a spherical thick zona pellucida containing four blastomeres (4-cell embryos). Inverted phase contrast microscope x100.*

Most of the vitrified slow thawed 4-cell embryos (Day 3) appeared in good condition immediately after thawing with intact zona pellucida and well-defined borders of the blastomeres (86% survival rate). When they were cultured for 1-day (Day 4), 74% of the embryos were found to have recovered and further developed (**Table 2**), whereas some embryos were blocked at the 4-cell stage or showed shrunken blastomeres with ill-defined borders and widened perivitelline space (**Figure 5**).

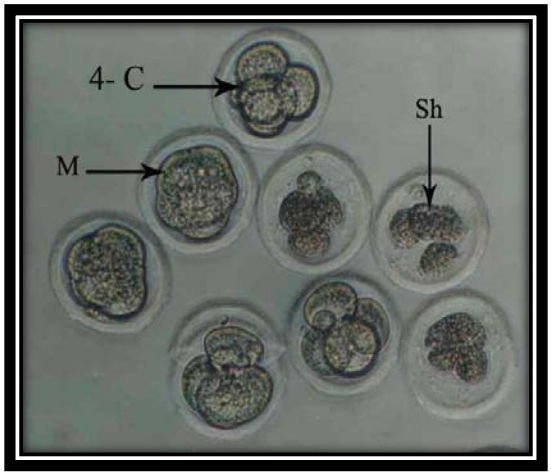

Figure 5: *Vitrified 4-cell embryos 24 hours after slow-thawing (Day-4). Some embryos appear in good condition and have developed into morula (M). Two embryos appear blocked at the 4-cell stage (4-C) with well-defined borders of the blastomeres, whereas others show shrunken blastomeres (Sh) with ill-defined borders and widened perivitelline space. Inverted phase contrast microscope x100.*

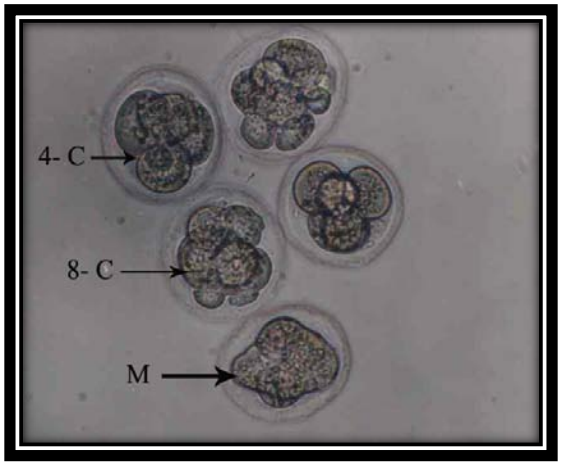

Figure 6: *Vitrified 4-cell embryos 24 hours after fast-thawing (Day-4). Most of the embryos have either developed to the stage of 8-cells (8-C) or morula (M). One embryo appears blocked at the 4-cell stage (4-C). Inverted phase contrast microscope x100.*

Similarly, most of the vitrified 4-cell embryos, immediately after fast thawing, appeared in good condition with well-defined borders of the blastomeres (94% survival rate). When examined 1-day after culture (Day 4), 80% of the embryos were found to have recovered and further developed into 8-cell embryos or morula (**Table 2**), whereas few embryos did not develop (**Figure 6**).

Most of the embryos at day-4 after incubation (control group) were in the stage of compacted morula which appeared inside the zona pellucida (**Figure 7**)

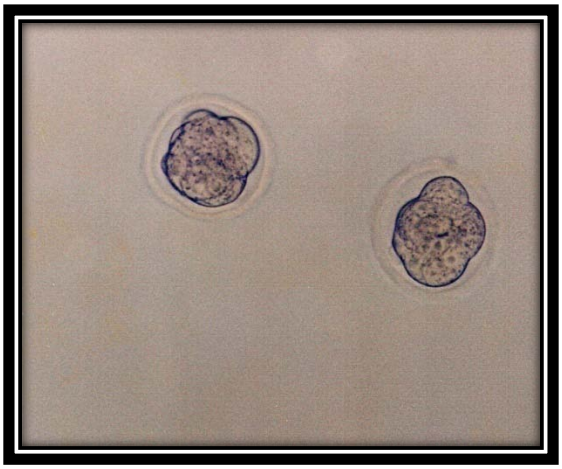

Figure: 7 *Embryos at day-4 (control group) after incubation in KSOM medium. The embryos are in the stage of compacted morula inside the thick zona pellucida. Inverted phase contrast microscope x100.*

Fifty seven percent of the vitrified slow-thawed morula appeared morphologically intact with intact zona pellucida and well defined borders of the embryos (**Figure 8**). Some embryos appeared with ill-defined borders and disintegrated or shrunken cytoplasm. When the embryos were incubated for 24 hours (Day 5), 50% were capable to survive and further develop in vitro (**Table 3**). Seventy seven percent of the vitrified fast-thawed morula appeared morphologically intact with well defined borders (**Figure 9**) and 63% were capable to recover and develop in vitro when incubated for 24h in KSOM medium (Day 5; **Table 3**).

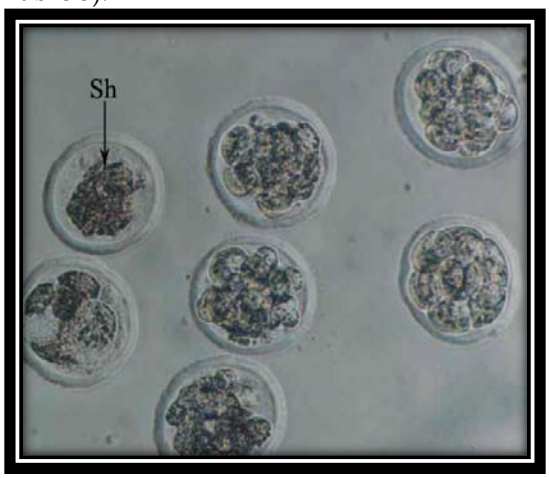

Figure 8: *Vitrified morula (Day-4) 1-hour after slow-thawing. Most of the embryos appear morphologically intact with intact zona pellucida and well defined borders of the embryos or blastomeres. Some embryos appear with ill-defined borders and disintegrated or shrunken cytoplasm (Sh). Inverted phase contrast microscope x100.*

Figure 9: *Vitrified morula (Day-4) 1-hour after fast-thawing. Most of the embryos appear morphologically intact with well defined borders. Inverted phase contrast microscope x100.*

Table 1: Survival rate and in vitro development rate of the vitrified fertilized ova following slow and fast thawing.

Feature	Control	Vitrification	
		Slow-thaw	Fast-thaw
Number of ova	140	30	30
Number of intact post-thaw (Day-1)	---	21	24
Survival rate (Day-1)	100%	70%	80%
P		0.0176*	0.0371*
Number of ova developed in vitro (Day-2)	103	17	18
In vitro development rate (Day-2)	74%	57%	60%
P		0.0029*	0.0101*

* =Significant at p<0.05

Table 2: Survival rate and in vitro development rate of the vitrified 4-cell embryos following slow and fast thawing.

Feature	Control	Vitrification	
		Slow-thaw	Fast-thaw
Number of embryos	149	70	70
Number of intact post-thaw (Day-3)	---	60	66
Survival rate (Day-3)	100%	86%	94%
P		0.0002*	0.0150*
Number of embryos developed in vitro (Day-4)	120	52	56
In vitro development rate (Day-4)	80.6%	74.3%	80%
P		0.0002*	0.0003*

* =Significant at p<0.05

Table 3: Survival rate and in vitro development rate of the vitrified morula following slow and fast thawing.

Feature	Control	Vitrification	
		Slow-thaw	Fast-thaw
Number of cryopreserved morula	74	30	30
Number of intact post-thaw (Day-4)	---	17	23
Survival rate (Day-4)	100%	57%	77%
P		0.0345*	0.0099*
Number of embryos developed in vitro (Day-5)	60	15	19
In vitro development rate (Day-5)	81%	50%	63.3%
P		0.0189*	0.0041*

* =Significant at p<0.05

Discussion

The present work investigated the effect of vitrification on the fertilized ova, the 4-cell embryos and the morula. The results of the present work could be compared with the results reported in the literature for each corresponding embryonic stage. Regarding vitrification of the fertilized ova, our results showed survival rate of 70% and 80% and in vitro development rate of 57% and 60% following slow and fast thaw, respectively. These results are more or less comparable to the results reported in the literature for the fertilized ova [6,7,20,21]. They reported that vitrification of mouse pronuclear fertilized ova resulted in a rate of survival with normal morphology that ranged from 62% to 91%, depending on the freezing rate, exposure time and type of the cryoprotectant. The average rate of development was reported to range from 33% to 66% [7,18,21,22].

Extensive work has been reported exploring the effect of vitrification on the multicellular embryos. Kono et al[23], Lee et al[3], Zhu et al[24] and Mukaida et al[11] reported a range of 29% to 92% survival rates with morphologically normal mouse embryos following vitrification. Lee et al[3] reported in vitro development rate of the vitrified mouse embryos between 17.4% and 80%, depending on the mouse strain. Also Yang et al [25] reported in vitro developmental rate of 86.6% in the vitrified-thawed mouse morula. The results of the present work are comparable to the best results reported in the literature for this embryonic stage. Our results on the 4-cell embryos showed survival rate of 86% and 94% and in vitro development rate of 74% and 80% following slow and fast thaw, respectively. Vitrification of the morula resulted in survival rate of 57% and 77% and in vitro development rate of 50% and 63% following slow and fast thaw, respectively.

The present work is peculiar in comparing the effect of vitrification-thawing on three developmental stages of early mouse embryos. Our results showed that the best survival rate and in vitro development was obtained with vitrification of the 4-cell embryos. Three comparable studies, although on different developmental stages, were found. Tao et al [26] found that vitrified-thawed compacted morula had significantly higher developmental potential than pronucleate fertilized ova and the 2-cell stage embryos ($P < 0.01$). El-Danasouri and Selman [27] found higher survival rate in vitrified eight-cell human embryos compared with embryos at seven-cell and six-cell stages (79.2%, 39.7%, and 21.1%, respectively). Zhou et al.[18] reported a developmental rate of the vitrified one-cell (52.5 to 66.7%), two-cell (63.3 to 68.9%), four-cell (81.7 to 86.4%), eight-cell embryos (90.0 to 93.3%), morula (96.7 to 100%), and the early blastocyst (98.3 to 100%). They concluded that the protocol for cryopreservation of the morula was suitable for the four-cell embryos to early blastocyst stages[18].

Considerable controversy was found in the few reports dealing with the effect of thawing temperature on the developing embryos. Dattena et al[17] found no significant difference in the development at 72 h of culture when vitrified mouse embryos were thawed at 20°C or 37°C for 6 sec or 48°C for 2 sec. At 48 h of culture the embryos thawed at 20°C had a reduced resumption rate (69.5%) while the embryos thawed at 37°C and 48°C for 2 sec had a higher resumption rate (80.0% and 82.5%). Kasai et al[1] found that moderate warming of vitrified mouse blastocysts by holding them in air for 5-30 sec gave better results than rapid warming by plunging them immediately into 25°C water. The same results were obtained even when the straws were recooled and rewarmed up to 10 times, to exaggerate the effect of cooling and warming. The results of the present work showed that better survival rates were obtained following fast thawing (80%, 94%, 77%) than that following slow thawing (70%, 86%, 57%) of the fertilized ova, 4-cell embryos and morula, respectively. Similarly, better in vitro development rates were obtained following fast thawing (60%, 80%, 63%) than that following slow thawing (57%, 74%, 50%) of the fertilized ova, 4-cell embryos and morula, respectively. These results gave

a strong proof that fast thawing is preferred to that of slow thawing in nearly all stages of early embryos.

It is concluded that the stage of the 4-cell embryos is the most feasible stage for cryopreservation of early mouse embryos using vitrification and fast thawing technique. These results may have an impact on the human IVF protocol and the idea of developing a bank of embryos or embryonic cells, where they could be vitrified for future use.

Acknowledgement

This work was supported by Grant Number 003/420 from King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

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