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The effect of vitrification device and embryonic stage on survival and developmental competence of gabali rabbit embryos

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The current study aimed to evaluate the effect of vitrification device (straw or cryotop) and embryonic stage (pronuclear or morula stages) on survivability, normality and embryo development rate into blastocysts/hatched blastocysts. A total of 24 mature rabbit does of Gabali breed (5-6 months of age, 3-3.5 kg live body weight) were used in this study as embryo donors. Rabbit does were super ovulated by PMSG and hCG. Embryos at pronuclear stage including 4-cell and 8-cell embryos were recovered by flushing oviducts 30–34 h and 40-46 h post-mating, while, embryos at late morula stage were recovered by flushing oviducts 72 h post-mating. Embryos were vitrified by straw or cryotop. Survival and normality rates were determined. The vitrified embryos were *in vitro* cultured for 5 days to record blastocyst and hatching blastocyst formation rates. Results showed insignificant differences in survival and normality rates of vitrified embryos as affected by embryonic stage, although there were a tendency of higher rates yielded from embryos at morula than at pronuclear stage (87.7 and 89.2% vs. 85.9 and 85.2%, respectively). Survival and normality rates were higher ($P<0.05$) using cryotop than straw, being 92.1 and 93.1% using cryotop versus 81.7 and 81.0% using straw, respectively. Expansion and hatching rates were higher ($P<0.05$) for vitrified embryos at morula than at pronuclear stage (80.0 and 71.5% vs. 66.0 and 54.0%, respectively) and using cryotop than straw (81.6 and 75.3 vs. 63.1 and 48.2%, respectively). It can be concluded that the feasibility of using the cryotop method to vitrify rabbit embryos rather than straw method at various developmental stages, particularly at morula stage.

Key words: Cryotop, gabali rabbit, vitrification, blastocyst, straw.

Cryopreservation for mammalian oocytes and embryos has been used for several purposes such as to preserve the genes of elite livestock, increase the efficiency of animal breeding, preserve valuable genetically modified animals and endangered species, and use germ cells effectively in assisted reproductive technology in animals and humans (Yasuyuki et al. 2011). In certain cases optimization of cryopreservation protocols for more sensitive embryos is relevant (Chrenek et al. 2014). Vitrification is routinely being used in the artificial reproductive technologies and has become increasingly used than slow freezing method (Park et al. 2009) with no or little damage to provide the chance to maintain scientifically important stocks, strains, and lines. Also, vitrification methods have been made possible to store embryos for extended periods and allowed the import and export of embryos and gametes (Habibi et al. 2013).

In rabbits, vitrification technique was used for embryo cryopreservation (Kobayashi et al. 1990) with improved general efficiency of embryo survival (Chrenek et al. 2014). Several factors were reported on the relative efficiency of cryopreservation, such as

embryo culture medium, type and concentration of cryoprotectant and cryopreservation container (Ryu et al. 2012). Using high concentration of cryoprotectants (CAPs) potentially increases toxicity to the vitrified embryos. Also, increasing the volume of vitrification solution interferes with the survival of vitrified-warmed embryos due to decreasing the cooling rate (kuwayama and kato, 2000) and to obtain a higher vitrification rate, a small volume of vitrification solution is required (Junya et al. 2010).

Several devices have been developed to produce a small volume of vitrification solution, such as electron microscope grid (Kim and Hong, 2011), open pulled straw (El-Gayar et al*.* 2010), cryoloop (Raju et al. 2009) and cryotop (Lin et al. 2010). Such devices have improved the vitrification and led to the successful production of offspring from vitrified-warmed embryos.

A cryotop is an alternative device that consists of a thin strip of plastic film (0.4 mm wide ,20 mm long, 0.1 mm thin) attach to a hard plastic holder (Kuwayama and Leibo, 2008; Kuwayama et al. 2007). In the cryotop protocol, embryos are loaded with the use of a glass capillary under the control of stereomicroscope in small volume less than 0.1 µl of vitrification medium (Kuwayama and Kato, 2000; Kuwayama et al. 2005a). Since almost all of the medium is removed before cooling, the embryo are covered with only a very thin solution layer, and then capped cryotop is plunged directly into liquid nitrogen (Kuwayama and Kato .2000).

According to the changes in cryological properties, e.g. membrane permeability and surface area to volume ratio during embryonic development, vitrification device should be optimized for embryos at various developmental stages (Lin et al. 2011). The efficiency of rabbit embryo vitrification evaluated *in vitro* (Papis et al. 1993) and/or *in vivo* (Kasai et al. 1992) depends partly on resistant of embryos at morula stage obtained from outbreed strains of rabbits. Cryopreservation of rabbit embryos was reported at morula (Naik et al. 2005), blastocyst (Lopez-Bejar and Lopez-Gatius*,* 2002) and zona-free expanded or hatching blastocyst (Cervera and Garcia-Ximenez, 2003) stages, but higher rates of development were achieved when blastocyst (but not morula) stage embryos were used.

In rabbit, the vitrification of rabbit embryos at pronuclear stage with cryotop yields a higher rate of post-warming survival than either the gel-loading tip or cryoloop (Hunter, 1995). Higher survival rate (88%) and development (52%) was obtained post-warming embryos vitrified at morula to blastocyst stages using a modified open pulled straw method (Lopez-Bejar and Lopez-Gatius*,* 2002). While, a 91% survival rate *in vitro* and 29% term development (kits/total embryos transferred) after transfer of vitrified embryos at morula stage (Naik et al. 2005).

There were limited reports of vitrification on rabbit embryos at earlier developmental stages (pronuclear to cleavage) and results were generally unsuccessful (Smorag et al*.* 1989 and Hochi et al*.* 2001), except that one study with 94% *in vitro* survival and 36% birth rates was reported on pronuclear stage embryos vitrified by cryoloop (Hochi et al*.* 2004).

Therefore, the current study was carried out to evaluate the effect of vitrification device (straw or cryotop) and embryonic stage (pronuclear or morula embryos) on survivability, normality and embryo development rate into blastocysts/hatched blastocysts.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center (ILMTC), belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

Embryos collection:

A total of 24 mature rabbit does of Gabali breed (5-6 months of age, 3-3.5 kg live body weight) were used in this study as embryo donors. Rabbit does were superovulated using injection of 20 IU PMSG/kg live weight (Foligon, Intervet International B.V., Boxmeer), followed 48 h later by 40 IU hCG/kg live weight (Pregnyl, Organon, Nile Co., Egypt), immediately after natural mating with fertile bucks belonging to the same breed.

Total of 416 rabbit embryos were used 105 and 100 at pronuclear stage as well as 108 and 103 at morula stage for straw and cryotop, respectively.

The embryos at pronuclear stage including 4-cell and 8-cell embryos were recovered by flushing oviducts 30–34 h and 40-46 h post-mating (Lin et al. 2010) and pooled as one group, while, embryos at late morula stage

were recovered by flushing oviducts 72 h post-mating (Morgan and Kane, 1993). Embryos were recovered in Petri dish containing phosphate buffer saline (PBS) supplemented with 0.2% (w/v) of bovine serum albumin (BSA) and antibiotics (10,000 IU penicillin G potassium + 10 mg streptomycin sulfate/ml) (Sigma Chemical Co., St. Louis, Mo, USA).
The recovered embryos were counted and evaluated morphologically under stereoscopic microscope after washed twice in PBS supplemented with 10% FCS and antibiotics. All recovered embryos at pronuclear or morula stages were cryopreserved by vitrification method (cryotop or straw).

Embryo vitrification:
The basal medium (BM) used for vitrification was PBS supplemented with 20 % fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). Vitrification was carried out in two steps: in the 1st step, embryos were pipetted into vitrification solution 1 (VS1) containing BM + 12.5% (v: v) ethylene glycol (EG) + 12.5% (v: v) dimethyl-sulfoxide (DMSO) + 0.5 M sucrose) in disposable sterile Petri dish for 2 min. In the 2nd step, the embryos were pipette into VS2 (BM + 20% (v: v) EG+20% (v: v) DMSO + 0.5 M sucrose) for 30 s. Then, three embryos were loaded into cryotop with <0.1 µl VS2, and immediately submerged in liquid nitrogen (Kuwayama et al. 2007; Habibi et al. 2013). Also, eight embryos were suspended in VS2, loaded in 0.25 ml plastic straws (IMV, L'Aigle, France), sealed and plunged directly into liquid nitrogen for at least 15 days.

Vitrified embryo warming:
Vitrified embryos were thawed by plunging the straw or cryotop into one ml of thawing solution (PBS + 4% BSA + 0.33 M sucrose) for 1 min, followed by serial dilutions in PBS + 0.175 M sucrose solution for 2 min to remove the intracellular cryoprotectants, then embryos were washed three times in PBS solution for 5 min per time to remove cryoprotectants at 25°C.

Evaluation of post-warming embryos:
Survival rate was calculated based on number of post-warming recovered embrys, while normal and abnormal number of vitrified embryos (normality rate) was morphologically evaluated based on abnormalities in mucin coat, intact zona pellucida, blastomeres, and refractive cytoplasm (Azadbakht and Rezazadeh , 2008).

Embryo culture:
The survived viable embryos were *in vitro* cultured in 100 µl drops of tissue culture medium-199 (TCM 199, Sigma) supplemented with 4 mg/ml BSA and 50 µg /ml of Gentamicin sulphate under mineral oil at 38°C, 95% humidity and 5% CO_2 in air to develop into the blastocyst stage. Embryos were assessed daily to record blastocyst and hatching blastocyst formation rates for 5 days. Then rate of different blastocyst stages were calculated.

Statistical analysis
Data obtained from this study were statistically analyzed using a software package (SAS, 2004). Mean differences in survival and normality rates, and development of vitrified embryos at different stages, were performed using factorial design. The significant differences among means were tested using Duncan's Multiple Range Test (1955).

RESULTS AND DISCUSSION
Post-warming survival and normality rates:
Data presented in Table 1 showed insignificant differences in survival and normality rates of vitrified embryos as affected by embryonic stage, although there were a tendency of higher rates yielded from embryos at morula than at pronuclear stage (87.7 and 89.2% vs. 85.9 and 85.2%, respectively). However, significant effect of cryodevice was obtained on survival and normality rates, being significantly ($P<0.05$) higher using cryotop than straw. The survival and normality rates were 92.1 and 93.1% using cryotop versus 81.7 and 81.0% using straw, respectively.

The effect of interaction between embryonic stage and cryodevice on survival and normality rates was insignificant, reflecting the highest survival and normality rates of vitrified embryos at morula stage using cryotop device (93.2 and 94.9%, respectively, Figs. 1 and 2).
The present results are higher than those reported by Kang et al. (1999), who reported a survival rate of rabbit embryos at pronuclear

Table 1. Post-warming survival and normality rates of vitrified rabbit embryos as affected by embryonic stage, vitrification cryodevice and their interaction.

Item	Embryoinc stage (E)		Cryodevice (C)		Interaction (ExC)
	Pronuclear	Morula	Straw	Cryotop	P-Value
Total embryos, n	205	211	213	203	-
Post-warming embryos, n	176	185	174	187	-
Survival rate, %	85.9± 0.74	87.7± 0.74	81.7± 0.74[b]	92.1± 0.74[a]	0.8065
Normal embryos, n	150	165	141	174	-
Abnormal embryos,	26	20	33	13	-
Normality rate, %	85.2± 0.74	89.2± 0.74	81.0± 0.74[b]	93.1± 0.74[a]	0.6170

a and b: Means denoted within the same row with different superscripts for each effect are significantly different at P<0.05.

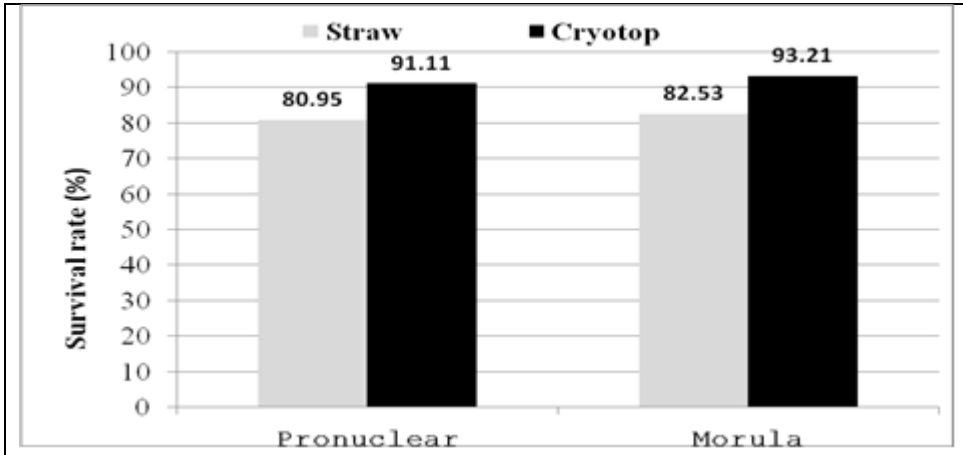

Fig. 1. Survival rate of rabbit embryos at pronuclear and morula stages vitrified by straw and cryotop at different stages.

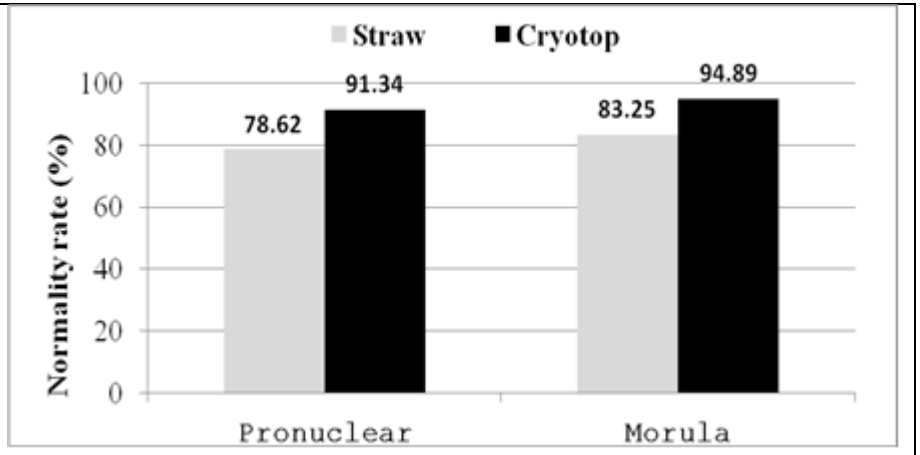

Fig. 2. Normality rate of rabbit embryos at pronuclear and morula stages vitrified by straw and cryotop at different stages.

Table 2. Developmental competence of rabbit embryos vitrified at different stages and cryodevice after thawing.

Item	Embryoinc stage (E)		Cryodevice (C)		Interaction (ExC)
	Pronuclear	Morula	Straw	Cryotop	P-Value
Cultured embryos, n	150	165	141	174	-
Expanded blastocysts, n	99	132	89	142	-
Expansion rate, %	66.0± 0.79 [b]	80.0± 0.79 [a]	63.1± 0.79 [b]	81.6± 0.79 [a]	0.0914
Hatched blastocysts, n	81	118	68	131	-
Hatching rate, %	54.0±0.83 [b]	71.5±0.83 [a]	48.2±0.83 [b]	75.3±0.83 [a]	0.0694

a and b: Means denoted within the same row with different superscripts for each effect are significantly different at P<0.05.

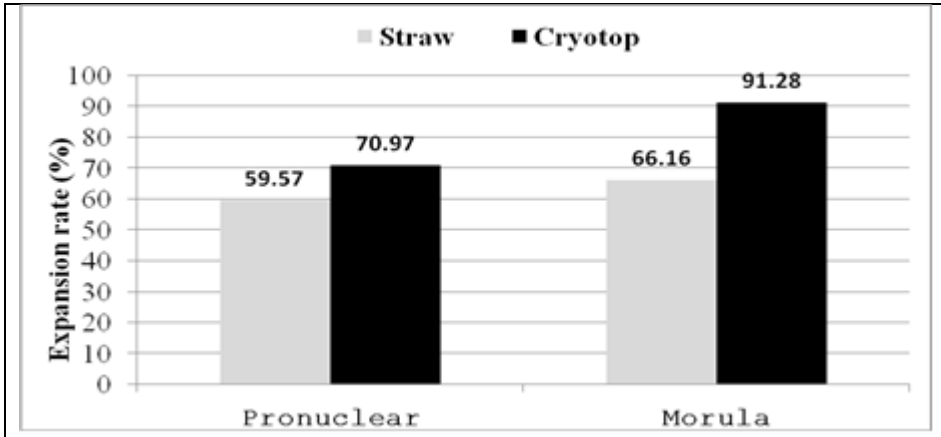

Fig. 3. Expansion rate of rabbit embryos vitrified by straw and cryotop at pronuclear and morula stages and *in vitro* cultured for 5 days

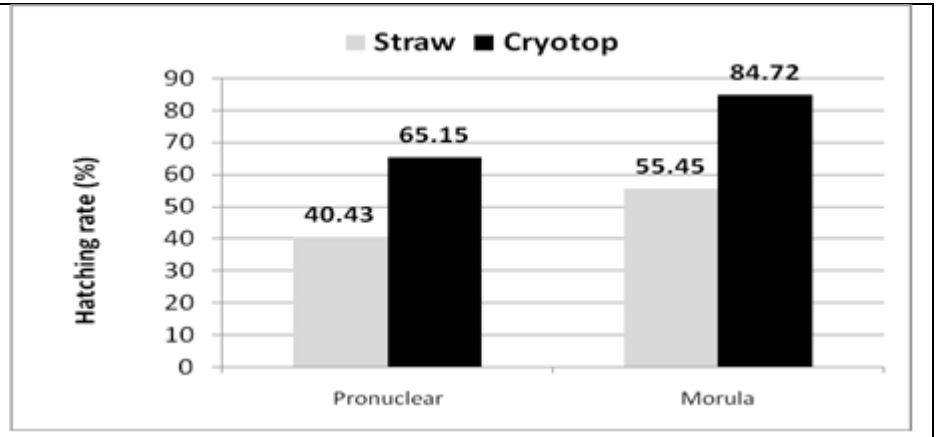

Fig. 4. Hatching rate of rabbit embryos vitrified by straw and cryotop at pronuclear and morula stages and *in vitro* cultured for 5 days

preserved by vitrification and slow freezing, being 63.3 and 57.7%, respectively. Also, koprdová et al. (2009) found that viability of rabbit embryos after short-term storage in liquid nitrogen by straw was 46.16% and after long-term storage it was 44.1%. Survival of rabbit embryos after devitrification, described in the study of Makarevich et al*.* (2008) was 58.8%.

In nearly similarity with the results of the current study, Desai et al*.* (2007) recorded post-vitrification survival rate of 85% following vitrification of human embryos at 6- to 8-cell stages on day 3 of culture. Also, Shin et al*.* (2011) found that survival rates of vitrified mouse embryos by straw was (88.4%). On the other hand, higher survival rate of mouse embryos than that obtained in the current study was recorded by Zhang et al*.* (2009), who found that the post-vitrification survival rates of vitrified mouse embryos at 2-cell, 4-cell and 8-cell stages were 96, 97 and 97%, respectively. Also, Ryu et al. (2012) found that survival rates of vitrified mouse embryos by cryotop was 97%. Ling et al*.* (2009) showed that the post-vitrification survival rates for mouse embryos at the morula and blastocyst stage were 95.4 and 96.5%, respectively. Yasuyuki et al*.* (2011) reported that the viability of the canine embryos at 4-cell to 16-cell, morula, and blastocyst stages was 90, 50 and 40%, respectively.

In accordance with the present results, Hochi et al*.* (2004) found higher post-warming survival of rabbit embryos vitrified by cryotop than gel-loading tip and cryoloop. In this respect, Hochi et al. (2004) suggested that cryotop is a better alternative cryodevice than open pulled straw. Kuwayama et al*.* (2005b) reported that use of a cryotop container improves the survival rate of vitrified-warmed embryos and has advantages over conventional vitrification procedures.

The advantages of vitrification of embryos by cryotop was attributed to using very small volumes of embryo suspension (less than 1 µl). Minimizing the volume of the solution with which embryos are vitrified might result in higher viability of the embryos post-vitrification (Yasuyuki et al. 2011). Also, the vitrification by cryotop prevents chilling injury to sensitive cells (Kuwayama et al. 2005c). Generally, problems with viability of embryos after vitrification can be caused by permeable cryoprotectants presented in vitrification solution (Shaw and Jones, 2003).

Embryo culture *in vitro*:

Results shown in Table 2 revealed significant differences in expansion and hatching rates of vitrified embryos as affected by embryonic stage, cryodevice and their interaction. Expansion and hatching rates were significantly ($P<0.05$) higher for vitrified embryos at morula than at pronuclear stage (80.0 and 71.5% vs. 66.0 and 54.0%, respectively) and using cryotop than straw (81.6 and 75.3 vs. 63.1 and 48.2%, respectively).

The effect of interaction between embryonic stage and cryodevice on expansion and hatching rates was insignificant, reflecting the highest expansion and hatching rates of vitrified embryos at morula stage using cryotop device (91.3 and 84.7%, respectively, Figs. 3 and 4).

The present trend of increasing expansion and hatching rates of embryos at morula than at pronuclear stages was reported by Lin et al*.* (2011), who found that the *in vitro* development of rabbit embryos at 8-cell and morula stages post-vitrification and warming to expanding blastocyst was 70.1 *vs.* 91.7% and hatching blastocyst was 63.7 *vs.* 86.2%, respectively. Also, Papis et al*.* (2005) reported that 59.1 % of rabbit embryos reached the advanced blastocyst stage after de vitrification. Shin et al*.* (2011) found that 88.4% of mouse embryos frozen by vitrification survived, of which 97.4% developed into blastocysts. However, hatching rates of blastocysts following vitrification-warming on cryotop was 94.7% (Ryu et al. 2012).

Such findings in the 8-cell and the morula embryos may have been due to differences between *in vitro* and *in vivo* environments in supporting embryonic development. Obviously, damage to one blastomere is much more important in pronuclear (2-cell embryos) than in 8-cell, morula or blastocyst embryos. Perhaps microfilament depolymerization agents, such as propanediol, may improve survival and development of 1-celled rabbit embryos by preventing fracture of the cytoskeleton (Vincent et al. 1990).

In mouse, Ling et al. (2009) showed that murine blastocyst formation rate of 90.3% for vitrified morulae. However, Zhang et al*.* (2009) found that the blastocyst formation rate

of the 2-cell embryos was significantly lower than of vitrified 4- and 8-cell embryos, suggesting the higher sensitivity of younger embryos. The developmental rates of vitrified 2-cell embryos to the blastocyst and hatched blastocyst stages were 69.4 and 52.6%, respectively, versus corresponding values of 93.3 and 60% for vitrified-warmed 4-cell embryos, 91.1 and 78.4% for 8-cell embryos, respectively. Cryotop vitrification for mammalian embryos is greatly influenced by the developmental stages of embryos, type of species and/or even the animal strain (Moore and Bonilla, 2006; Morato et al. 2008). Also, Kuleshova et al*.* (2001) were able to culture mouse embryos at pronuclear stage (two-cell) to the blastocyst stage at a rate of 100% when embryos were exposed up to 15 minutes in an EG based vitrification media, which contained Ficoll or Dextran.

In accordance with the present results, Cuello et al*.* (2008) found that vitrified 8-cell stage embryos had the highest blastocyst formation and hatching rates, which implied that this stage is the most appropriate form vitrification. Similar reports on the vitrification of human cleavage-stage embryos were reported by Balaban et al. (2008) and Valojerdi et al*.* (2009).

Generally, embryos at morula stage appear to be the most optimal stage for embryo vitrification by cryotop. Therefore, the current paper could be conclud that the feasibility of using the cryotop method to vitrify rabbit embryos rather than straw method at various developmental stages, particulary at morula stage.

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