Nat. Volatiles & Essent. Oils, 2021; 8(6): 2031-2041

NVEO
Natural Volatiles &
Essential Oils

Consequences Of Vitrification On Survivability And Subsequent Development Of Shami Goat's Early Embryos In Different Cryopreservation Protocols

Raed Kawkab Al – Muhja[1*] , Ali Abd Al-jabbar Ibrahim[1] , Omar Mardenli[2] , Ali Sami Amin Al-Tawash[1] , Hawraa Rahim Kadhim[1] , Ghassan Al-Shammas[3] , Hawraa G.T. Al-Shebani[1]

[1]Department of Animal Production, Faculty of Agriculture, University of Al-Qadisiah, Iraq

[2]Department of Animal Production, Faculty of Agriculture, University of Aleppo, Syria

[3]Department of Animal Production, Faculty of Agriculture, University of Damascus, Syria

Abstract

Due to the necessity to preserve the in vitro produced embryos of pure breeds such as Shami goat, vitrification of early embryos contributes to the preservation process significantly. In the current study, the effectiveness of vitrification on Shami goat early embryos was evaluated according to the concentration of cryoprotectant and equilibrium time. A total of 850 embryos (2-4 cell stage) were randomly distributed into 6 groups (treatments): A1, A2 and A3 groups were vitrified in a cocktail of dimethyl sulfoxide (13% v/v), ethylene glycol (13% v/v) and sucrose (2M) across three equilibrium times (A1: 8 minutes, A2: 10 minutes and A3:12 minutes). B1, B2 and B3 treatments were vitrified in a cocktail of dimethyl sulfoxide (15% v/v), ethylene glycol (15% v/v) and sucrose (3 M) across three equilibrium times (B1: 8 minutes, B2: 10 minutes and B3:12 minutes). Results showed that the frozen - thawed embryos in B2 treatment achieved the best rates of survivability (93.29%; $p < 0.01$) , cleavage (73.15%; $p < 0.01$), degeneration (6.71%, $p < 0.01$), blastocyst (53.21%, $p = 0.01$), arrest (7.33%, $p < 0.01$) and hatched blastocyst (37.93%, $p = 0.03$). No significant differences were noticed in morula, early and expanded blastocyst stages. It is concluded from the current study that vitrifying the blastomeres of Shami goats in a cocktail containing dimethyl sulfoxide (15% v/v), ethylene glycol (15% v/v) and sucrose (3 M) at an equilibration time of 10 minutes contributes to obtaining the highest survival rates after freezing and the highest possible rates of blastocysts.

Key words: cryoprotectant, early embryo, equilibration, goat, vitrification.

Introduction

The current period is characterized by a wide development in the assisted reproductive technology (ART) field, especially the freezing of gametes and early embryos of many mammalian species. The technology of embryo cryopreservation has contributed significantly to facilitating technical work in various fields (e.g., cloning, embryo splitting and embryo transfer) that aim to improve the reproductive efficiency of herds. Implicitly, freezing techniques are classified into two main parts: slow freezing and flash-freezing (vitrification). In general, the vitrification process is a low-cost and low-equipment

technique. In addition, vitrification is a fast method and requires a high concentration of cryoprotectants (Lawson et al., 2016; Do et al., 2016). Many theoretical schools have moved away from introducing cryoprotectants as a single agent because of the severe cytotoxic effect caused by these agents on gametes, embryos and cellular tissues. In the vitrification technique, many cryoprotectants have been introduced. These cryoprotectants have proven to be effective and positive in reducing as much as possible the deterioration of gametes and early embryos. Among the most important cryoprotectants that have been commonly used are dimethyl sulfoxide (DMSO), ethylene glycol (EG) and a group of sugars, the most important of which is sucrose (Villamil et al., 2012; Nateghi et al., 2017; Souza et al., 2018). The Shami goat breed is one of the breeds that has gained international fame due to its distinct and promising genetic potential. The breed is spread in several Arab countries such as Syria, Iraq, Jordan and Palestine. The demand for this breed has increased for several purposes, including genetic improvement due to its ability to reach high productivity levels (meat and milk). For the above reasons, it was necessary to establish a genetic bank for this pure breed to preserve the standard characteristics. Therefore, the current study aimed to study the feasibility of vitrification of Shami goat early embryos according to several protocols that are variant in cryopreservation concentration and equilibrium time to reach the highest possible survival rates of frozen-thawed embryos.

Material and methods

Ovaries and oocytes collection

Ovaries were collected from governmental and local slaughterhouses in the city of Aleppo during the breeding season. Ovaries were removed from the carcasses of Shami goats, whose ages ranged between 3 to 5 years and placed in a thermos containing 9% saline solution at 39 ^{0}C. The ovaries were transferred directly to the laboratory (Biotechnology Laboratory of Aleppo University) within a period not exceeding 30 minutes. Oocytes were obtained by aspiration method from follicles with diameters ≥ 3 mm. The aspirated cumulus oophorus complexes (COCs) were transferred to four – well dishes containing tissue culture medium 199 (TCM- 199) at 39 ^{0}C in preparation for further work. Oocytes with 3 or more layers of cumulus cells were selected, while oocytes with abnormal shapes and that were characterized by cytoplasmic decomposition, cytoplasmic decline, demolition of the zona pellucida and cytoplasmic granulation were excluded.

In vitro embryo production

The procedures of in vitro embryo production protocols (in vitro maturation, fertilization and culture) were carried out according to previous protocols (Abdel-Gawad et al., 2016; Mardenli et al.,2021) with some modifications. Briefly, the oocytes were transferred to four-well dishes containing the maturation medium. Each well contained 5 to 15 oocytes. The maturation medium consisted of TCM -199 supplemented with epidermal growth factor (5 µg/ml), follicle-stimulating hormone (5 µg/ml), luteinizing hormone (50 µg/ml), cysteamine (100 µM). The dishes containing the oocytes and the culture medium were transferred to the incubator. The maturation period lasted for 35 hours under regular conditions of 38.5°C with 5% CO2, 90 N2 and saturated humidity. Following maturation, the oocytes were prepared for in vitro fertilization. The culture medium specified for fertilization consisted of a solution of tyrode albumin lactate pyruvate (TALP) plus 0.2 mm sodium pyruvate, 25 mm sodium

bicarbonate, 5 mg/ml bovine serum albumin (BSA), 10 mg/ml heparin, 13 mm sodium lactate and 50 µl/ml penicillamine-hypotaurine- epinephrine (PHE). Oocytes were aspirated from the maturation solutions and washed three times with TALP solution to remove the cumulus cell. Straws of frozen-thawed semen from tested Shami bucks were used to fertilize the oocytes. Motile sperms were separated by centrifugation in Percoll gradient (45:90%) for 30 min at 700 x gravity. The spermatozoa count was adjusted to give a final concentration of 6 x 10^5 sperm/mL in the TALP medium. Next, the oocytes were transferred to four-well dishes containing the fertilization medium with sperms (5 oocytes in each well) and transferred to the incubator. The fertilization period lasted for 18 hours at the previous condition of incubation. Fertilization was monitored by investigating the male and female pronuclei (zygote) under an electron microscope with 300x magnifications. Following fertilization, presumptive zygotes were washed with TCM-199 twice, transferred to four -well dishes containing the culture medium (TCM-199) and placed in the incubator at the previous condition of incubation. Cleaved embryos at the 2-4 cell stage were investigated at day 3 of embryo culture. The resulting blastomeres were vitrified for three weeks and rewarmed. After thawing, the frozen – thawed blastomeres were returned to the incubator to complete the subsequent divisions. The resulting morula and blastocyst stage were investigated on days 7,8 and 9 of culture.

Experimental design

The vitrification process was done using a mixture of three cryoprotectants (DMSO**,** EG and sucrose) as shown in Table 1**.** A total of 850 embryos (2-4 cell stage) were randomly distributed into 6 treatments (A1, A2, A3, B1, B2 and B3), where 415 embryos were vitrified in the first vitrification solution (A), while the remaining 435 embryos were vitrified in the second vitrification solution(B). According to the time intervals of the equilibrium stage, the embryos were subjected to three intervals of 8 minutes (A1 and B1 treatments),10 minutes (A2 and B2 treatments) and 12 minutes (A3 and B3 treatments). The experiment follows the two-factor experimental design (type of vitrification solution and time interval of the equilibrium stage) for a set of parameters (survivability, cleavage, degeneration and developmental stage of embryo)

Table. 1. Composition (v/v, %) of cryopreservation solutions used in vitrification of Shami goat's early embryos.

Cryopreservation solution	Equilibrium solution					Vitrification solution			
	DMSO	EG	Sucrose	TCM-199	FCS*	DMSO	EG	Sucrose	TCM-199
A	7.5	7.5	2 M	70	15	13	13	2 M	74
B	7.5	7.5	3M	70	15	15	15	3M	70

*: FCS: fetal calf serum

Vitrification and rewarming (thawing)

Embryos were frozen and thawed as explained in previous papers (Malmir et al., 2020; Mardenli et al., 2020) with some modifications. In some detail, the embryos were subjected to the equilibrium solutions

according to the specified time intervals for each treatment. Five embryos were placed in each drop of the equilibrium solution. During the exposure period, the straws were prepared as shown in Figure 1. After exposure to the equilibrium solution, the embryos were placed with the vitrification solution in the designated place in the straw. Next, the straws were prepared with the final form taking into account that the vitrification solution exposure period was one minute. The straws were immediately immersed in liquid nitrogen and left for three weeks. The thawing process was done by keeping the straws in the air at room temperature at 30°C for 30 seconds. Next, the straws were immersed in a warm water bath at 38 °C for 50 seconds. The contents of the straws were emptied into four-well dishes. After emptying, the embryos were transferred to other four-well dishes and washed across three stages (washing solutions) containing the first washing solution (PBS medium plus 1 M propanediol+0.2 M sucrose), the second washing solution (PBS medium plus 0.5 M propanediol +0.2 M sucrose) and the third washing solution (PBS medium). Each stage of the previous washing lasted 5 minutes.

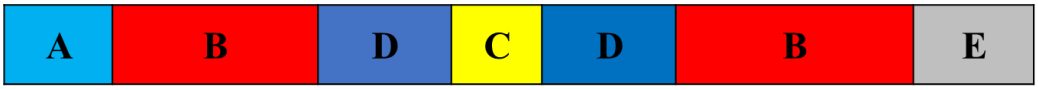

Fig. 1. Schematic diagram for traditional straws of vitrification. A: writing and numbering place, B: vitrification solution, D: air bubble, C: frozen embryos with vitrification solution, E: place of closing.

Survivability determination

The survivability of the embryos was determined based on morphological appearance according to Dhali et al. (2000). Frozen – thawed embryos were examined under a stereomicroscope (X300). Embryos whose cells were characterized by sphericity, symmetry, transparency, the integrity of the zona pellucida and the absence of signs of deterioration were selected.

Statistical analysis

The data of the experiment were formatted in an excel sheet and then analyzed using the chi-square test (crosstabs procedure) (Drebee and Razak, 2018). comparisons were made across rates of studied parameters using Fisher's test (Drebee et al, 2014). Probability values were considered significant at a level less than 5%. For the previous purpose, the SAS statistical software was used (SAS Institute 2017).

Reagents

All chemicals used were from Sigma Chemical Co (St. Louis, USA) unless mentioned otherwise.

Results

Survivability, cleavage and degeneration

The data in Table 2 indicate absolute significant differences (p=0.000) among survival rates across different treatments of frozen - thawed embryos. In general, the rates exceeded 66%. Frozen - thawed embryos of B2 treatment significantly outperformed their counterparts (93.29%%), followed by the B3 treatment (77.93%). Upon completion of embryo culture down to the blastocyst stage, it was also observed that the highest rate of cleavage was achieved by the frozen - thawed embryos of the B2

treatment (73.15%; p= 0.000). The rates of degeneration differed significantly(p=0.000) across the various treatments of embryos, where the rates ranged between 6.71% (B2 treatment) and 33.83% (A1 treatment).

Table. 2. Rates of survivability, cleavage and lysis of Shami goat early embryos vitrified in different levels of cryoprotectants

Treatment*	Equilibrium time (minute)	2-16 cell stage	Morphologically normal		Cleaved		Degenerated	
		No.	No.	%	No.	%	No.	%
A1	8	133	88	66.17^{a}	56	42.11^{a}	45	33.83^{a}
A2	10	147	101	68.71^{a}	68	46.26^{ab}	46	31.29^{a}
A3	12	135	97	71.85^{a}	57	42.22^{a}	38	28.15^{ab}
B1	8	141	106	75.18^{ab}	75	53.19^{b}	35	24.82^{ab}
B2	10	149	139	93.29^{c}	109	73.15^{c}	10	6.71^{c}
B3	12	145	113	77.93^{b}	77	53.10^{b}	32	22.07^{b}
p			0.000		0.000		0.000	

Values without a common superscript (a, b and c) within a column are different at the assigned probability. NS: not significant. *: A1, A2and A3 treatments follow up the vitrification solution A: 13% DMSO, 13% EG and 2 M sucrose. B1, B2 and B3 treatments follow up the vitrification solution B: 15% DMSO, 15% EG and 3 M sucrose.

Developmental stage and arrest

Rates of morula, blastocyst and arrest are presented in Table 3. During the arrest in the morula stage, the embryos showed a very similar developmental potential. The difference between the highest and the lowest rate was 9%. Although the frozen – thawed embryos in B2 treatment achieved the highest rates (39.44%), this superiority was not statistically significant. On the contrary, the frozen – thawed embryos in treatment B2 continued to outperform the blastocyst stage (53.21%; p=0.01), while the lowest rates were achieved by the embryos of A3 treatment (29.82%). There were significant differences (p=0.000) among the rates of the arrest of different treatments of frozen- thawed embryos. Due to the higher rates of morula and blastocysts in treatment B2, the arrest rate was the lowest (7.33%) in contrast to A2 treatment (16.32%).

Table. 3. Rates of morula, blastocyst and arrest of Shami goat early embryos vitrified in different levels of cryoprotectants

Treatment*	Equilibrium time (minute)	Cleaved	Developmental stage				Arrest (pre - morula stage)	
			Morula		Blastocyst			
		No.	No.	%	No.	%	No.	%
A1	8	56	17	30.3	17	30.36^{a}	22	12.32^{a}

				6				
A2	10	68	22	32.35	22	32.35[a]	24	16.32[b]
A3	12	57	21	36.84	17	29.82[a]	19	10.83[a]
B1	8	75	27	36.00	28	37.33[b]	20	15.00[b]
B2	10	109	43	39.44	58	53.21[c]	8	7.33[c]
B3	12	77	29	37.66	31	40.26[b]	17	13.09[ab]
p			NS		0.001		0.000	

Values without a common superscript (a, b and c) within a column are different at the assigned probability. NS: not significant. *: A1, A2and A3 treatments follow up the vitrification solution A: 13% DMSO, 13% EG and 2 M sucrose. B1, B2 and B3 treatments follow up the vitrification solution B: 15% DMSO, 15% EG and 3 M sucrose.

Developmental stages of blastocyst

Table 4 shows the rates of the basic developmental stages of the Shami goat blastocyst stage following vitrification. In the early and extended blastocyst stages, the rates were relatively similar among embryos frozen by different treatments. Despite the insignificant differences under the effect of the type of treatment and the equilibrium time, it was noted that the rates of early blastocysts were relatively high (the highest value was 58.82%; embryos of A1treatment) compared to the extended blastocyst stage (the highest value was 47.06%; embryos of A3 treatment). In the hatched blastocyst stage, embryos of the B2 treatment achieved the highest rate (37.93%; p=0.03) compared to the other treated embryos followed by the embryos of B1 treatment (28.57%).

Table. 4. Rates of blastocyst stages of Shami goat early embryos vitrified in different levels of cryoprotectants

Treatment*	Equilibrium time (minute)	Blastocyst	Blastocyst stage					
		No.	Early		Expanded		Hatched	
A1	8	17	10	58.82	4	23.53	3	17.65[b]
A2	10	22	12	54.55	8	36.36	2	9.09[a]
A3	12	17	8	47.06	8	47.06	1	5.88[a]
B1	8	28	8	28.57	12	42.86	8	28.57[c]

B2	10	58	20	34.48	16	27.59	22	37.93 d
B3	12	31	11	35.48	13	41.94	7	22.58 bc
p			NS		NS		0.03	

Values without a common superscript (a, b and c) within a column are different at the assigned probability. NS: not significant. *: A1, A2and A3 treatments follow up the vitrification solution A: 13% DMSO, 13% EG and 2 M sucrose. B1, B2 and B3 treatments follow up the vitrification solution B: 15% DMSO, 15% EG and 3 M sucrose.

Discussion

Reaching high survival rates, as well as maintaining the quality of transferable embryos, are key goals that workers in embryo cryopreservation technology seek to achieve. Perhaps the technology of vitrification, after the remarkable appearance in the world of cryopreservation of gametes and early embryos, has developed to a great extent. Regardless of the methods of vitrification, the type, concentration and condition of the cryoprotectant (single or shared) play a prominent and fundamental role in the success of vitrification (Amorim et al., 2011). In addition, the equilibration time is an insurmountable factor in order to enhance the effectiveness of vitrification and reach high survival rates of early embryos. In our current study, through the bilateral interaction between the vitrification treatment and the equilibration time, it was clearly shown that the frozen- thawed embryos in a cocktail of DMSO, EG and sucrose (B2 treatment) at an equilibration time of 10 minutes led to the highest rates of cleavage, morula and blastocysts and also produced the lowest rates of lysis (Tables 2,3 and 4) . The present results can be explained by the synergistic effect of cryoprotectants in reducing their toxicity levels in the cellular membrane. In this context, several scenarios can govern the relationship among the concentrations of cryoprotectants, their cytotoxicity, the equilibration time, reaction of embryonic cells, viability and quality of the embryos resulted in post vitrification (Sinha, 2009; Fahy, 2010; Do et al., 2016). Several theoretical approaches are sensitive to the freezing of embryos at the blastomere stage. In our study, we have frozen embryos at the blastomere stage. This stage of embryo is characterized by the large size of the cells and the high amounts of water, which leads to an increase in the sensitivity to cold impact, severe damage to the cells and the degeneration of vitality. Thus, the lower concentrations of cryoprotectants in treatments A1 and B1 treatments in our study will contribute to maintaining high water amounts inside embryonic cells during the equilibrium and the vitrification phases, which consequently led to lower rates of survival and subsequent division post thawing. On the other hand, high concentrations of cryoprotectants lead to a decrease in the amount of water in the cells at the expense of the higher concentration and consequently a higher level of cytotoxicity in the embryo cells, which leads to higher rates of degeneration, lower rates of survival and subsequent division (A3 and B3 treatments) (Son et al., 2003). However, the supportive participatory effect of cryoprotectants is one of the factors that are no less important than other factors in raising survival rates and rates of subsequent division down to the blastocyst stage. Notably, the participatory effect of the shared cryoprotectants in

the protocol implies a set of new emerging interactions, but the most important is the benefit of the positive characteristics of each type of cryoprotectant. More specifically, DMSO plays a role in facilitating EG permeability, which has a positive efficiency on spindle polymerization and protectivity (Lucena et al., 2006). EG is characterized by a low cytotoxicity effect on different stages of the embryo (Kasai et al., 1992), rapid diffusion and quick equilibration through the cellular membrane especially the zona pellucida (Emiliani et al., 2000). Sucrose ($C_{12}H_{22}O_{11}$) effectively promotes the osmotic activity (reduces osmotic shock) inside the embryonic cell during vitrification (Isachenko et al., 2003; Mouttham and Comizzoli, 2016).

In light of the results of our current study, the scenario that emerges according to the relationship among the concentration of the cryoprotectant agent, the time of penetration of the cryoprotectant molecules inside the embryo cells (permeability of the plasma membranes) and the reaction of cells and osmotic stress, in fact, is an overlapping causal relationship. Therefore, the equilibration period determines to a large extent the level of cryoprotectant concentrations in the embryonic cells (the longer the period, the higher the cryoprotectant concentration), which in turn is the main determinant of the degree of resistance and survival of the embryo in the post thawing stage (Arav et al., 2000; Liebermann and Tucker, 2002; Moussa et al., 2014; Morató and Mogas, 2014).

In general, the results of our current study are somewhat different from what is found in the literature. At a vitrification protocol consisting of DMSO (10%), EG (10%) and sucrose(1M), the embryos were subjected to two equilibration stages (1 and 5 minutes), the early and expanded blastocyst rates were 0% and 0%, respectively. Changing concentrations to DMSO (20%), EG (20%) and sucrose(1M); the embryos were subjected to the same equilibration stages, the early and expanded blastocyst rates were 23% and 0%, respectively. At another vitrification protocol consisting of DMSO (25%), EG (25%) and sucrose(1M), the early and expanded blastocyst rates reached 75% and 70%, respectively (Souza et al., 2017). In a study conducted by Varago et al. (2006), using a cocktail of EG (20%) + DMSO (20%) and sucrose (0.5 M), the rates of early and expanded blastocyst were 23.5% and 44.1%, respectively; after subjecting the embryos to equilibrium time of 40 seconds. Zhou et al. (2005) subjected mouse embryos to equilibrium intervals of 15, 25, 35, 45 and 60 seconds, the rates of embryos with normal morphology were 100% for each time interval, respectively, the rates of blastocyst were 100%, 100%, 95.1% and 91.7%, respectively, while the hatched blastocyst rates were 16.7%, 62.3%, 46.7%, 39.3%, and 18.3%, respectively; vitrification solution consisted of 10% (v/v) EG, 10% (v/v) DMSO and 171.2 g/L sucrose. The study of Takagi et al., 2004 showed that exposing embryos to equilibrium solution (10% DMSO + 10% EG + 20% fetal calf serum (FCS) for 2 min, the rates of blastocyst reached 93%; the vitrification solution consisted of 20% DMSO + 20% EG + 0.6 M sucrose. In a vitrification environment consisting of 16.5% EG, 16.5% DMSO and 0.5 M sucrose, the frozen – thawed embryos at 3 minutes of equilibrium achieved survival rates ranged between 73% and 90%, while rates of expanded blastocyst ranged between 54% and 89%, the rates of hatched blastocyst ranged between 4% and 57% (Garza et al., 2020).

Conclusion

The current study revealed that exposing the embryos for an equilibrium period of 10 minutes in a cocktail containing7.5% DMSO, 7.5% EG and 3M sucrose and vitrifying them in a cocktail containing

15% DMSO, 15% EG and 3M sucrose would raise the survival rates of the embryos after freezing, as well as the high rates of the hatching blastocyst stage.

References

Abdel-Gawad, E.M.M., Abdel-Halim, B.R., Helmy, N.A., Badr, A.F. (2016). Effect of Cryoprotective Solutions, Ethylene Glycol, Dimethyle-sulfoxide and Ficoll 70 with Different Combination Ratios on Vitrification of Bovine Oocytes and Embryos Produced in vitro. Asian Journal of Animal and Veterinary Advances, 11(10):608-6019. doi: 0.3923/ajava.2016.608.619

Amorim, C. A., Curaba, M., Van Langendonckt, A., Dolmans, M.-M., Donnez, J. (2011). Vitrification as an alternative means of cryopreserving ovarian tissue. Reproductive BioMedicine Online, 23, 160–186.

Arav, A., Zeron, Y., Ocheretny, A. A. (2000). New device and method for vitrification increases the cooling rate and allows cryopreservation of bovine oocytes. The Riogenology, 53:248

Dhali, A., Manik, R.S., Das, S.K., Singla, S.K., Palta, P.(2000). Verification of buffalo (Bubalus bubalisi) oocytes. Theriogenology, 53: 1295-1303.

Do, V.H., Walton, S., Catt, S., Taylor-Robinson, A.W. (2016). Requirements for Cryopreservation of In Vitro-Produced Bovine Embryos by a Standard Method of Vitrification. J Vet Sci Anim Husb, 4(1): 102 doi: 10.15744/2348-9790.4.102

Drebee, H.A. and Razak, N.A.A.. 2018. Measuring the Efficiency of Colleges at the University of Al-Qadisiyah-Iraq: A Data Envelopment Analysis Approach, Jurnal Ekonomi Malaysia, 52(3): 163-179.

Drebee, H.A., N.A.A Razak,. and M.Z.A. Karim. 2014. Is there an overlapping market between national car producers in Malaysia? Jurnal Ekonomi Malaysia, 48(1): 75-85.

Emiliani, S., Van den Bergh, M., Vannin, A.S., Biramane, J., Englert, Y. (2000). Comparison of ethylene glycol, 1,2-propanediol, and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos, and blastocysts. Hum Reprod, 15:905-910.

Fahy, G.M. (2010). Cryoprotectant toxicity neutralization. Cryobiology, 60(3 Suppl):S45-53. doi: 10.1016/j.cryobiol.2009.05.005.

Garza, D., Camacho, M,. Gauly, M., Holtz., W.(2020). Vitrification of mouse blastocysts by open or closed system and warming in sucrose-containing or sucrose-free diluent. CryoLetters, 41 (3): 135-139.

Isachenko, V., Alabart, J. L.; Dattena, M., Nawroth, F., Cappai, P., Isachenko, E., Cocero, M. J., Olivera, J., Roche, A., Accardo, C., Krivokharchenko, A., Folch, J.(2003). New technology for vitrification and field (microscopefree) warming and transfer of small ruminant embryos. Theriogenology, 59 (5-6): 1209-1218.

Kasai, M., Hamguchi, Y., Zhu, S.E., Miyake, T., Sakurai, T., Machida, T.(1992). High survival of rabbit morulae after vitrification in an ethylene glycol based solution by a simple method. Biol Reprod, 46:1042-1048

Lawson, A., Ahmad, H., Sambanis, A. (2011). Cytotoxicity effects of cryoprotectants as single- component and cocktail vitrification solutions. Cryobiology, 62(2): 115-122

Liebermann, J., Tucker, M.J. (2002). Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. Reproduction, 124:148-52

Lucena, E., Bernal, D.P., Lucena, C., Rojas, A., Moran, A., Lucena, A.(2006). Successful ongoing pregnancies after vitrification of oocytes. Fertil Steril, 85: 108-111

Malmir, M., Ghafarizadeh, A., Jenabi, M., Faraji, T. (2020). The Effects of Two Different Freezing Method on Survival and Development of Pronuclear Stage Human Embryos. Biomedical Journal of Scientific & Technical Research , Volume 32, 3, pp 24939-24934. DOI: 10.26717/BJSTR.2020.32.005242

Mardenli O, Mohammad, M.S., Hassooni, H.A. (2020). Efisiensi dimetil sulfoksida dan etilena glikol pada perkembangan subsequen embrio domba Awassi yang divitrifikasi. JITV, 25(2):60-67. doi: http://dx.doi.org/10.14334/jit

Mardenli, O., Al-Kerwi, M.S.M., Alolo, A.Y. (2021). The effect of follicle size and cryoprotectants on nuclear maturation and early embryonic development of vitrified - thawed Awassi sheep oocytes (Ovis aries). Veterinarski Arhiv 91 (5): 483-493, 2021. doi: 10.24099/vet.arhiv.1063

Morató, R., Mogas, T. (2014). New device for the vitrification and in-straw warming of in vitro produced bovine embryos. Cryobiology, 68(2): 288–293. doi:10.1016/j.cryobiol.2014.02.010

Moussa, M., Shu, J., Zhang, X. et al. (2014). Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. Sci. China Life Sci, 57: 903–914 https://doi.org/10.1007/s11427-014-4689-z

Mouttham, L., Comizzoli, P. (2016). Presence of sucrose in the vitrification solution and exposure for longer periods of time improve post-warming follicle integrity in cat ovarian tissues. Reproduction in Domestic Animals, 52: 224–229. doi:10.1111/rda.12847

Nateghi, R., Alizadeh, A., Jafari Ahangari, Y., Fathi, R., Akhlaghi, A. (2017). Ethylene Glycol and Dimethyl Sulfoxide Combination Reduces Cryoinjuries and Apoptotic Gene Expression in Vitrified Laying Hen Ovary. Biopreservation and Biobanking, 15(6): 519–528. doi:10.1089/bio.2017.0078

SAS Institute. 2017. SAS/STAT® 14.3 User's Guide. Cary, NC 27513 (USA): SAS Institute

Sinha, S. (2009). Role of Cryopreservation in Assisted Reproductive Technology (ART). Apollo Medicine, 6(3): 212–221. doi:10.1016/s0976-0016(11)60530-9

Son, W.Y., Yoon, S.H., Yoon, H.J., Lee, S.M., Lim, J.H. (2003). Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum Reprod, 18: 137−139

Souza, J. F., Oliveira, C. M., Lienou, L. L., Cavalcante, T. V., Alexandrino, E., Santos, R. R., … Dias, F. E. F. (2017). Vitrification of bovine embryos followed by in vitro hatching and expansion. Zygote, 26(01):99–103. doi:10.1017/s0967199417000570

Souza, J.F., Oliveira, C.M., Lienou., L.L., Cavalcante, T.V., Alexandrino, E., Santos, R.R., Rodrigues, A.P.R., Campello, C.C., Figueiredo, J.R., Dias, F.E. F. (2018).Vitrification of bovine embryos followed by in vitro hatching and expansion', Zygote, 26 (1): 99–103, Cambridge University Press. doi:10.1017/S0967199417000570

Takagi, A .Y., Shimizu, A. M., Kato, A. T., Danguri, A .A., Sakamoto. A. M.(2004). Vitrification of Mouse Morulae by A New Method: Pullulan Film-Straw Vitrification. Reproduction Fertility and Development, 16(1-2): 183

Varago, F.C., Saliba, W.P., Alvim, M.T.T., Vasconcelos, A.B., Oliveira, C.H., Stahlberg, R., Albuquerque, M. (2006). Vitrification of in vitro produced zebu embryos. Anim. Reprod, 3, 353–8.

Villamil, P.R., Lozano, D., Oviedo, J.M., Ongaratto, F.L., Bó, G.A. (2012). Developmental rates of in vivo and in vitro produced bovine embryos cryopreserved in ethylene glycol-based solutions by slow freezing or solid surface vitrification. Anim. Reprod, 9, 86–92.

Zhou, G.-B., Zhu, S.-E., Hou, Y.-P., Jin, F., Yang, Q.-E., Yang, Z.-Q., … Tan, H.-M. (2005). Vitrification of Mouse Embryos at Various Stages by Open-Pulled Straw (OPS) Method. Animal Biotechnology, 16(2):153–163. doi:10.1080/10495390500263831