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Factors affecting the cryopreservation of oocytes and embryos in buffalo (*Bubalus bubalis):* A review

Fatores que afetam a criopreservação de oócitos e embriões em búfalos (*Bubalus bubalis*): Uma revisão

Factores que afectan la criopreservación de ovocitos y embriones en búfalos (*Bubalus bubalis*): Una revisión

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Abstract
Cryopreservation of oocytes and embryos is a supplementary technique for *in vivo* and *in vitro* embryo production. In the last decade, the technique has been improved but did not achieve a satisfactory commercial level. Also, the results were variable. The pregnancy and live birth rates were 16 to 52% and 5.3 to 26.9% after implantation of vitrified embryos, respectively (from the year 1993 to 2020). In final consideration, the quality of oocytes and embryos is the most important factor to obtain better results after cryopreservation. The addition of antioxidant and lipid-depleting agents to the medium is a factor that improves cryogenic tolerance. Additionally, two-step vitrification is a reliable method for cryopreservation. Studying epigenetic effects and introducing lipidomic, transcriptomic and proteomic technologies can help improve this technology.
Keywords: Cryoinjuries; Embryo quality; Semen; Sexed embryos; Vitrification.

Resumo
A criopreservação de oócitos e embriões é uma técnica complementar para a produção de embriões *in vivo* e *in vitro*. Na última década, a técnica foi aprimorada, mas não atingiu um nível comercial satisfatório, com resultados variáveis. As taxas de prenhez e nascidos vivos são de 16 a 52% e 5,3 a 26,9% após implantação de embriões vitrificados, respectivamente. Em consideração final, a qualidade de oócitos e embriões é o mais importante fator para obter

melhores resultados após a criopreservação. A adição de agentes antioxidantes e depletores de lipídios ao meio é um fator que melhora a tolerância criogênica. Além disso, a vitrificação em duas etapas é um método confiável para criopreservação. O estudo dos efeitos epigenéticos e a introdução de tecnologias lipidômicas, transcriptômicas e proteômicas podem ajudar a solucionar oss grandes desafios impostos pela criopreservação, aprimorando essa tecnologia.
Palavras-chave: Lesões criogênicas; Qualidade do embrião; Sêmen; Sexagem de Embriões; Vitrificação.

Resumen
La criopreservación de ovocitos y embriones es una técnica complementaria para la producción de embriones *in vivo* e *in vitro*. En la última década, la técnica se ha mejorado pero no alcanzó un nivel comercial satisfactorio. Además, los resultados fueron variables. Las tasas de preñez y nacidos vivos fueron del 16 al 52% y del 5,3 al 26,9% después de la implantación de embriones vitrificados, respectivamente (del año 1993 al 2020). En definitiva, la calidad de los ovocitos y embriones es el factor más importante para obtener mejores resultados tras la criopreservación. La adición de antioxidantes y agentes hipolipemiantes al medio es un factor que mejora la tolerancia criogénica. Además, la vitrificación en dos pasos es un método confiable para la crioconservación. El estudio de los efectos epigenéticos y la introducción de tecnologías lipidómicas, transcriptómicas y proteómicas pueden ayudar a mejorar está tecnologia.
Palabras clave: Criolesiones; Calidad del embrión; Semen; Embriones sexados; Vitrificación.

1. Introduction

Cryopreservation of cumulus-oocyte-complexes (COCs) and embryos is an important technique for saving and transferring high-quality genetics worldwide (Hufana-Duran et al., 2004). The main goals of this technique are to keep cells with minimal damage and recover the capacity of vital processes after thawing or heating. Worldwide, slow freezing and vitrification are used to cryopreserve embryos in humans and animals (Gupta et al., 2017). However, in buffalo, these structures still suffer damage during processing (Attanasio et al., 2010; Khalil, Gabr, Shamiah, El-Haif, & Abdel-Khalek 2014; Mahmoud, Scholkamy, & Darwish, 2015). In addition, studies reveal that several factors can interfere with the viability of cryopreservation and post-thawing of COCs and buffalo embryos, in addition to the technique itself. As an example, the survival and blastocyst rate of the vitrified immature COCs were 71 to 96% and 0 to 15.4%, respectively; and for matured COCs were 66 to 98.5% and 0 to 13.6%, respectively for different protocols of vitrification during IVEP in buffalo (Parnpai, Liang, Ketudat-Cairns, Somfai, & Nagai, 2016).

In this context, it is important to know the available options, adopt them or explore other options to get better and consistent results. This review aimed to point out the factors that influence the cryopreservation of oocytes and embryos and to improve this technique in buffalo species.

2. Methodology

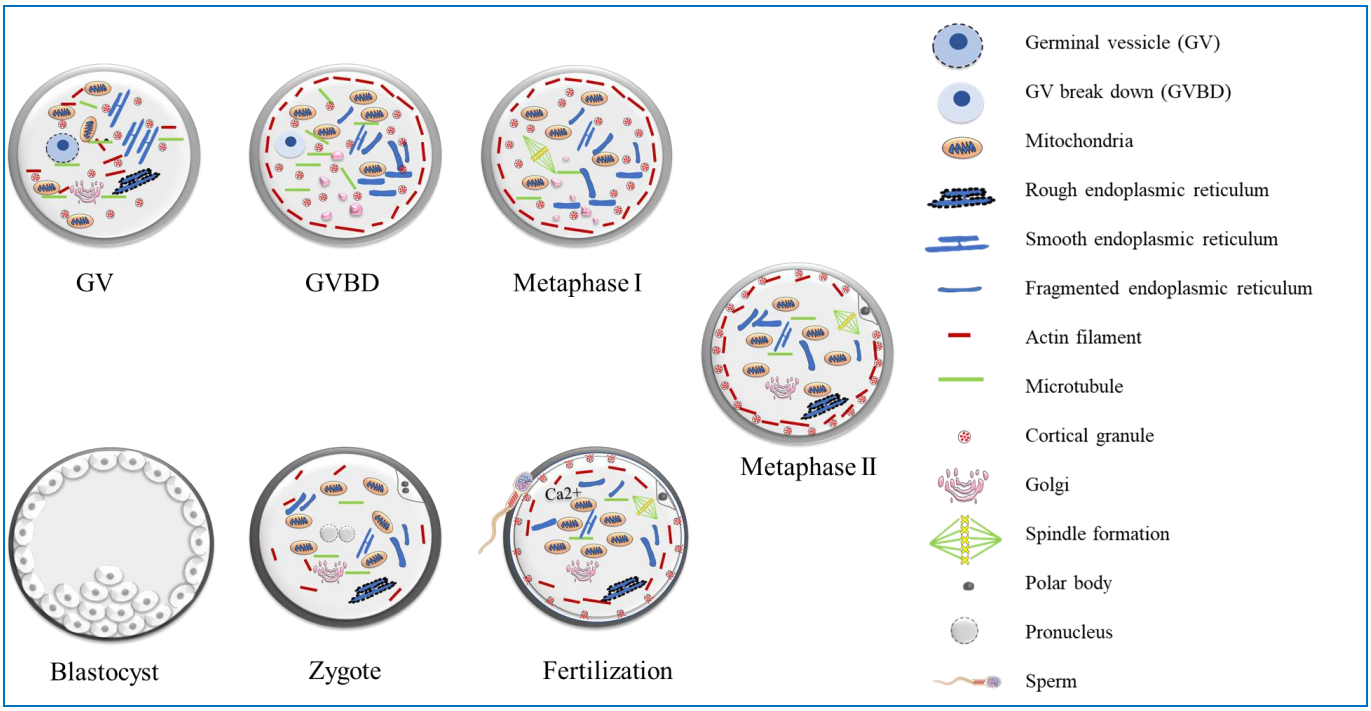
This review was written based on scientific publications in journals, books, etc. The figures were designed based on scientific publications related to the subject. A total of 63 references were used from the year 1993 to 2021. These references were searched in the following databases: Google, Researchgate, Science Alert, Science direct and Pubmed, using the following keywords: buffalo oocytes/COCs, buffalo embryos, cryopreservation, vitrification, slow freezing and pregnancy rate after transfer cryopreserved or vitrified embryos.

3. Results and Discussion

3.1 Basic cryobiology of oocytes and embryos

The difficulties associated with cryopreservation of oocytes and embryos are related to inherent structural and physiological characteristics (Barrera et al., 2018). Thus, before discussing the impact of cryopreservation on female gametes and embryos, it is important to understand some of the morphological events occurring in these structures (Figure 1).

Figure 1- Illustrated normal movement of organelles during the development from germinal vesicle to zygote stage of the embryo. The mitochondria are near the germinal vesicle and located more peripherally during the GV stage, afterwards stages more towards the centre. Also, the number of mitochondria increased Metaphase I (MI) onward stages or as a requirement. Ribosomes, Golgi apparatus and endoplasmic reticulum are more active and distributed in the ooplasm. They produce more proteins during the GV stage than GVBD or MII stage. During or afterwards the MII stage, these organelles are located more towards the periphery. The actin filament moves toward the periphery during GVBD onward stage. The cortical granules are arranged periphery during the MII stage and released during the fertilization process.



Source: Prepared by the authors with adaptations from Ferreira et al. (2009); Mondadori et al. (2010); Gustina, Hasbi, Karja, Setiadi, & Supriatna (2017); Reader, Stanton, & Juengel (2017).

The maturation of oocytes is an important event for the further development of embryos after fertilization. This event involves sequential molecular changes from the germ vesicle (GV) to metaphase-II (MII) stage. During maturation, mitochondria are the driving force of cells and move towards ATP-demanding processes in oocytes. Furthermore, their number increases according to their need or oocyte development (Kirillova, Smitz, Sukhikh, & Mazunin, 2021). Similarly, structural and biochemical changes occur in the endoplasmic reticulum (ER) during oocyte maturation, which is important for intracellular calcium regulation during fertilization.

During the metaphase-I (MI) stage of oocytes, maximum protein synthesis and ribosome storage occur for further embryonic development. Cortical granules (CG) derived from the Golgi apparatus are distributed throughout the ooplasm during the GV stage. In the MII phase, the GCs are distributed to the inner side of the oolemma and during fertilization, the GC will prevent polyspermy, altering the structure of the oolemma and hardening the zona pellucida. Furthermore, the cytoskeleton system of oocytes plays an important role in maturation. It helps in chromosome movement, nucleus migration, spindle formation and cytokinesis during cell division (Reader, Stanton, & Juengel, 2017).

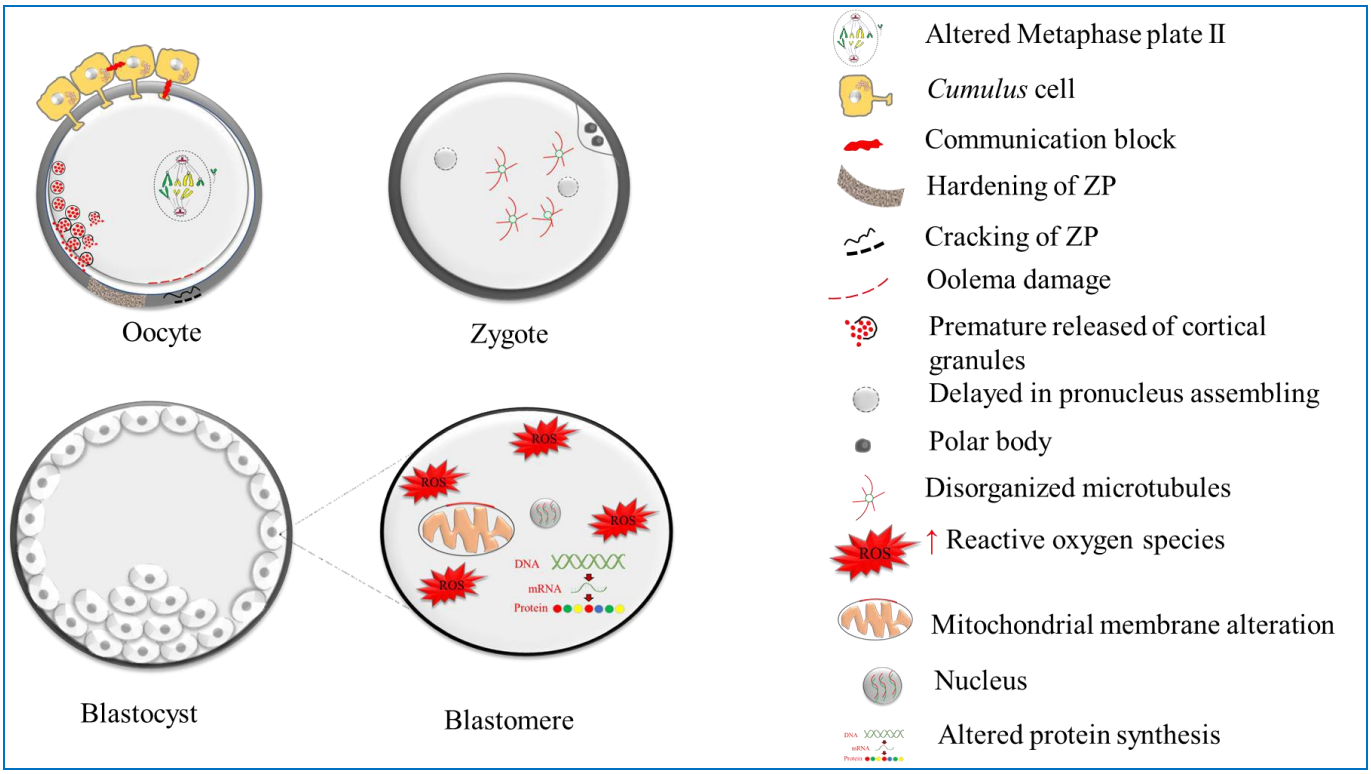
During the process of fertilization, the sperm binds to oocytes. After binding, it penetrates the oocytes and releases its genetic material, followed by the male pronucleus is formed. During the same time, the release of cortical granules takes place. This event changes the physiology of zona pellucida or hardening and prevents polyspermy (Hochi, 2016). In addition, the

plasma membrane potential of oocytes is changed and increases of calcium ions inside of oocytes due to influx through plasma membrane or internal release (Stein, Savy, Williams, & Williams, 2020). On another side, the female pronucleus is formed and release the second polar body. The two pronuclei come together and make a single-cell embryo. The embryonic cells continue to multiply and make blastocyst. At this stage, embryos can be cryopreserved or transferred directly to recipients.

In buffalo, the oocytes have some specific characteristics as compared to cattle. The mitochondria are present uniformly in the oocytes, GCs are located inside the plasma membrane of oocytes. Sometimes the oval mitochondria are also observed in the cytoplasm of oocytes. In addition, an important characteristic of oocytes and embryos is more number lipid droplets than cattle (Sharma, Jerome, & Purohit, 2014).

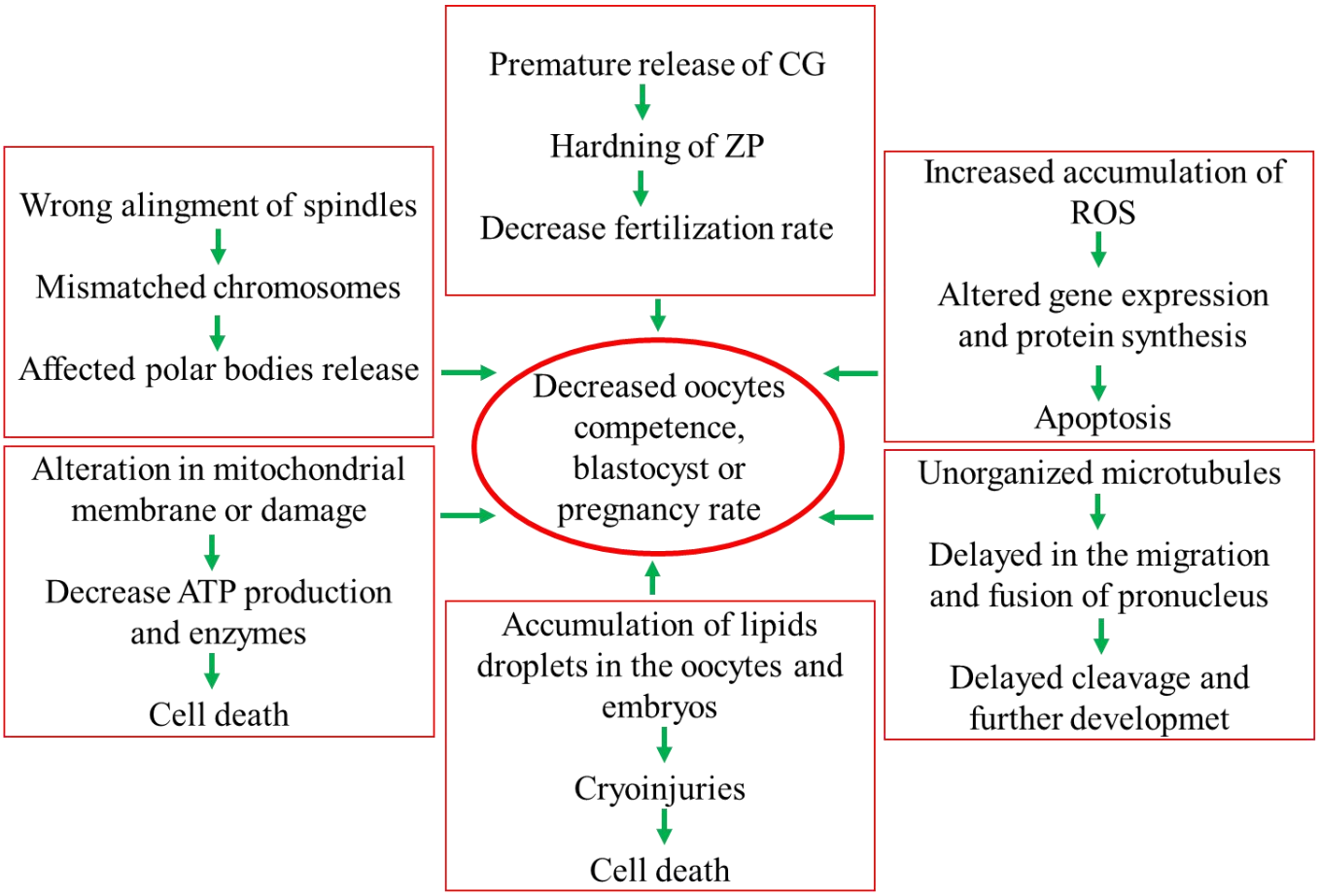
The process of cryopreservation damages various organelles of the immature or mature oocytes and blastocysts. Consequently, occur compromises the various morphological events involved in the process of oocytes maturation and embryo development. A basic idea of how all these changes affect the overall result is presented in figures 2 and 3.

Figure 2- Illustrated cryoinjury of oocytes, zygotes and blastomeres during the cryopreservation procedure. In oocytes, interruption of the cumulus cells communication, damage to the oolemma, rupture and hardening of the zona pellucida, alteration to metaphase II plate formation, premature release and aggregation of cortical granules. In embryos, disorganization of microtubules and delays pronucleus assembling during zygote formation. In addition, altered protein synthesis, increased reactive oxygen species (ROS). **↑:** Increased, **ZP:** Zona pellucida.



Source: Prepared by the authors with adaptations from Boonkusol, Faisaikarm, Dinnyes, & Kitiyanant (2007); Gautam, Verma, Palta, Chauhan, & Manik (2008); Abd-Allah (2009); Hochi (2016); Parnpai, Liang, Ketudat-Cairns, Somfai, & Nagai (2016); Tharasanit & Thuwanut (2021).

Figure 3- Illustrated how cryoinjuries affect the developmental competence of oocytes and embryos.



Source: Prepared by the authors with adaptations from Boonkusol, Faisaikarm, Dinnyes, & Kitiyanant (2007); Gautam, Verma, Palta, Chauhan, & Manik (2008); Abd-Allah (2009); Prentice & Anzar (2010); Parnpai, Liang, Ketudat-Cairns, Somfai, & Nagai (2016).

3.2 Factors affecting the cryopreservation of oocytes and embryos in buffalo

3.2.1 Immature and mature COCs

The developmental competence of COCs is affected by cryopreservation processes, compared to control its maturation and blastocyst rates are very low (Liang et al., 2012). The best results of survival, maturation and blastocyst rates of COCs after cryopreservation observed from different studies are presented in table 1. Although, the developmental competence is better for the cryopreserved mature oocytes than immature (Sharma & Loganathasamy 2007). In addition, after cryopreservation, the matured oocytes with or without cumulus cells do not affect the further development after fertilization (Gasparrini et al., 2007). After cryopreservation of COCs, the leakage, rupture, shrinkage and partial or complete denudation of cell content, zona pellucida, cytoplasm and COCs, respectively, are common (Gautam, Verma, Palta, Chauhan, & Manik, 2008; Abd-Allah, 2009). Other microstructure changes such as reduced microvilli in the perivitelline space, accumulated lipid droplets and vesicles seen with oolema, clusters of mitochondria peripheral to the ooplasm and decreased cristae in some mitochondria (Boonkusol, Faisaikarm, Dinnyes, & Kitiyanant, 2007).

Table 1 - Survival, maturation and blastocyst rates for cryopreserved immature and mature COCs in buffalo (best results of different groups in a study).

Type of COCs	Survival rate % (n)	Maturation rate % (n)	Blastocyst rate % (n)	References
IM	98.4 (100/104)	31.5 (28/88)	----	Dhali, Manik, Das, Singla, & Palta (2000)
IM	97.1 (33/34)	21.0 (53.8)	3.2 (2/62)	Wani, Maurya, Misra, Saxena, & Lakhchaura (2004a)
IM	----	42.5 (20/47)	----	Wani, Misra, & Maurya (2004b)
IM	80.7 (420/520)	82.1 (345/420)	15.4 (65/420)	Abd-Allah (2009)
IM	80.6 (157/194)	41.5 (46/110)	----	Mahmoud Scholkamy, Ahmed, Seidel, & Nawito (2010)
IM	79.0	32.0 (35/110)	1.4 (3/208)	Liang et al. (2012)
M	98.5 (130/132)	----	7.0 (6/109)	Gasparrini et al. (2007)
M	96.0 (182/185)	----	5.4 (10/182)	Gautam, Verma, Palta, Chauhan, & Manik (2008)
M	84.0 (100/119)	----	7.8 (8/111)	Attanasio et al. (2010)
M	97.0 (126/130)	----	8.0 (10/126) *	Liang, Phermthai, Nagai, Somfai, & Parnpai, (2011)

Note: **IM:** Immatured. **M:** Matured. *****Parthenogenetic embryos. Source: Authors.

3.2.2 Vitrification methods and cryodevices

The oocytes and embryos can be cryopreserved by one step or stepwise vitrification methods. In one step vitrification, the loaded cryo-straw is plunged directly into liquid nitrogen. In contrast, in two-stage vitrification, the loaded cryo-straw is placed first on the surface of liquid nitrogen inside the cryocan for approximately 20-30 seconds. After that, it is plunged in a prelabeled goblet inside the cryocan. In buffalo, stepwise vitrification is a reliable method for immature oocytes and embryos. It increases the speed of cryopreservation, post-thaw survival rates and a decrease in surface tension of straws. Also, it reduces the loss and damage of embryos by stabilizing the wall of straw during cryopreservation, preventing the mixing of different segments within the straw and the explosion of the straw during the thawing process (Abd-Allah, 2011).

Different cryodevices such as conventional straw, hemi-straw and spatula (cryotop) has been used for the vitrification. The cryotop device is a reliable alternative, inexpensive and easy to assemble for vitrification of immature (Khalil, Gabr, Shamiah, El-Haif, & Abdel-Khalek 2014) or matured buffalo oocytes and embryos. Cryotop devices may minimise the toxic and osmotic effect of cryoprotectant and cryo-injuries (Khalil, Gabr, Shamiah, El-Haif, & Abdel-Khalek 2014). It may be due to in cryotop device the volume of vitrified solution staying less than 0.1 µl during the vitrification process (Liang et al., 2012).

3.2.3 Cryoprotectants

A cryoprotectant is a chemical substance that can be permeable or non-permeable depending upon its molecular weight. It protects the cells from cryoinjuries by preventing ice crystal formation during cryopreservation. Studies have been observed that the cryoprotectants used in combinations with appropriate concentration give better results in buffalo for oocytes

or embryos (Manjunatha et al., 2009; Mahmoud, Scholkamy, & Darwish, 2015). It may be due to the toxic concentration of cryoprotectant can be reduced by using it in combination (Mahmoud, Scholkamy, & Darwish, 2015).

Different concentration of ethylene glycol (EG), dimethyl sulphoxide (DMSO) and sucrose has been used for vitrification. In buffalo, a better result was observed with 20% of EG and DMSO in a two-step equilibration and with addition of with 0.5 M sucrose in the second solution during the vitrification of *in vitro* matured oocytes (Attanasio et al., 2010).

3.2.4 Semen

The cryotolerance efficiency of *in vitro* produced buffalo embryos is not altered by the bull, regardless of breeds for *in vitro* fertilization (Soliman et al., 2018). Likewise, the use of conventional or sexed semen does not interfere with the ability of full-term embryos to develop after cryopreservation (Liang et al., 2008). Thus, other factors influenced the outcome of pregnancy and calving rate in buffalo after the transfer of cryopreserved embryos. These may be the medium, culture conditions (Kasiraj, Misra, Mutha Rao, Jaiswal, & Rangareddi, 1993) and the cryo-sensitivity of these embryos (Techakumphu et al., 2001).

3.2.5 Sex of Embryos

In cattle, the survival rate after cryopreservation is influenced by the sex of the embryo. Male embryos have better survival rates due to faster development (Nedambale, Dinnyés, Yang, & Tian, 2004), a higher number of cells and a lower apoptosis rate (Ghys et al., 2016) compared to female embryos.

In buffalo, according to one study the survival and death rates of male vitrified-thawed embryos are more ($P<0.05$) than female embryos (Mahmoud, Scholkamy, & Darwish, 2015). On the other hand, studies did not observe a significant difference for the percentage of male and female embryos development to term (Table 2) (Liang et al., 2008; Saliba et al., 2020). However, a more female sex ratio was observed by Liang et al. (2008) and Saliba et al. (2020). Therefore, these data are not sufficient to give a definitive statement about the survivability of particular sex in buffalo species (Table 2).

Table 2 - Viability and development up to term of female and male cryopreserved embryos in buffalo.

Experimental groups	Viability % (n)		Live birth (n)		Aborted (n)		References
	Male	Female	Male	Female	Male	Female	
Unsexed semen	----	----	2	3	2	0	Liang et al. (2008)
Sexed semen	----	----	0	4	1	0	
Unsexed semen	86.2 (25/29) [a]	13.8 (4/29) [b]	----	----	----	----	Mahmoud, Scholkamy, & Darwish (2015)
Unsexed semen	----	----	7	10	----	----	Saliba et al. (2020)

Note: [ab]superscripts within column differed significantly at $P < 0.05$. Source: Authors.

3.2.6 Developmental stages of embryos

In cattle, studies have found that the viability of the cryopreserved embryo at the expanded blastocyst stage is superior to the early blastocyst stage (Silveira, Marques, Silva, & Leão, 2020) or morula and hatched blastocyst (Jainudeen, Wahid, & Hafez, 2016). Additionally, other studies claim that bovine embryos that show more advanced developmental kinetics at day 7 of culture are more likely to survive after transfer to recipients (Sanches et al., 2013; Muñoz et al., 2014). This is because good quality embryos with fewer apoptotic cells grow faster. Furthermore, the down-regulation of apoptotic and up-regulation of the IFNT gene are responsible for the implantation of these embryos (Barnwell et al., 2016).

In buffaloes, according to various studies (Table 3), vitrification of embryos at different stages of development did not

influence the pregnancy rate and birth rate (Hufana-Duran et al., 2004; Hufana-Duran et al., 2008). However, the time of exposure to cryoprotectant solution (2 vs 3 minutes) during cryopreservation (Mahmoud, Scholkamy, & Darwish, 2015) interferes with embryonic survival. Generally, cryopreservation damages the cell membrane and causes DNA fragmentation (Sudano et al., 2012). These cryo-lesions are responsible for decreasing the total number of cells and changing the ratio between inner cell mass and trophectoderm cells (Sudano et al., 2012). Thus, survival chances are higher for good quality embryos, highlighting that embryo quality is a more important factor than embryonic stages at the time of cryopreservation (Hufana-Duran et al., 2004; Mahmoud, Scholkamy, & Darwish, 2015).

Table 3- Pregnancy and birth rate after transfer of *in vitro* or *in vivo* produced cryopreserved embryos in buffalo.

Stage of embryos	Method of cryopreservation	Pregnancy rate % (n)	Birth rate % (n)	References
Mor to BL*	Slow freezing	28.2 (11/39)	23.1 (9/39)	Kasiraj, Misra, Mutha Rao, Jaiswal, & Rangareddi (1993)
Mor to BL*	----	5.9 (1/17)	Aborted	Techakumphu et al. (2001)
Mor to ExBL	Vitrification	16.3 (9/55)	10.9 (6/55)	Hufana-Duran et al. (2004)
ErB to ExBL	Vitrification	----	26.9 (7/26)	Hufana-Duran et al. (2008)
BL	Vitrification	15.4 (6/39)	12.8 (5/39)	Liang et al. (2008)
TMor to HBL	Vitrification	52.6 (20/38)	5.3 (2/38)	Boccia et al. (2013)
BL	Vitrification	37.1 (26/70)	----	Saliba et al. (2013)
BL	Vitrification	29 (11/37)	----	Gamarra et al. (2015)
BL	Vitrification	23.8 (5/21)	19.0 (4/21)	Presicce (2017)
BL	Vitrification	37.1 (26/70)	24.3 (17/70)	Saliba et al. (2020)

Note: **TMor:** Tight morula. **Mor:** Morula. **ErBL:** Early blastocyst. **BL:** Blastocyst. **ExBL:** Expanded-blastocyst. **HBL:** Hatched-blastocyst. * *in vivo* produced embryos. Source: Authors.

3.2.7 *In vitro* conditions

Components of the *in vitro* culture medium affect the developmental and cryopreservation competence of oocytes and embryos (Xu et al., 2019).

Supplementation of acetyl-l-carnitine (ALC) (Reader, Cox, Stanton, & Juengel, 2015; Xu et al., 2019) to the *in vitro* maturation medium and β-mercaptoethanol (β-ME) (Moussa et al., 2019) to the medium of embryonic culture (IVC) improves the cryotolerance of vitrified oocytes and buffalo embryos, respectively. According to Sepand et al. (2016), ALC and β-ME protect cells from oxidative damage. ALC helps in the growth and division of mitochondria (Cassano et al., 2010). Also, it improves the quality of oocytes and their developmental competencies (Reader, Cox, Stanton, & Juengel, 2015). However, the exact mechanism of β-ME is not known how it improves the developmental competence of vitrified embryos. The supplementation of β-ME to the IVC media may modify the implantation related gene expression of vitrified-thawed embryos (Moussa et al., 2019).

According to Lowther, Weitzman, Maier, & Mehlmann (2009), cells with more lipid contents are more cryo-sensitive. Thus, supplements with lipid-depleting agents (forskolin) or antioxidants have been used to improve the efficiency of the cryopreservation technique (Silva et al., 2021) in buffalo (Panyaboriban, Tharasanit, Chankitisakul, Swangchan-Uthai, & Techakumphu, 2018) and other species (Paschoal et al., 2014).

3.3 Recent development and future perspective

The offspring obtained from cryopreserved COCs or embryos have been observed many changes, including morphological, structural and gene expression patterns. Zhu et al. (2020) observed alteration in the protein expression in the brain tissue of mouse offspring that produced after the transfer of cryopreserved embryos. Recently, offspring of mice derived from cryopreserved embryos show changes in lipid and glucose metabolism (Qin et al., 2021). In buffalo calves, there is no information about these changes. Also, there is no information about reproductive potential and other productive aspects such as milk and meat composition.

Studying epigenetic effects along with lipidomic, transcriptomic and proteomic helps to understand and overcome these problems (Estudillo et al., 2021; Silva, Lima, & Figueiredo, 2021). Thus, with the adoption of omics techniques, it will be possible to understand the cryogenic effect in buffaloes at the molecular level, as observed in other species (Shirazi et al., 2016; Barrera et al., 2018).

Transfer of vitrified embryos in natural estrus has also been found to provide a better pregnancy outcome than synchronized estrus in recipient buffaloes (Hufana-Duran et al., 2004). Perhaps this fact may be related to the premature luteolysis that occurs during estrus synchronization (Pereal & Inskeep, 2008). In this context, it will be interesting to develop an estrus synchronization protocol for recipients, avoiding physiological changes that may compromise the results.

4. Final Considerations

Finally, it was considered that two-stage vitrification and the use of more than one cryoprotectant give better results during the cryopreservation process. Also, the outcome of this technique is not affected by semen (fresh, cryopreserved and sexed) used during *in vitro* fertilization and cryo-thaw embryos (male or female). However, the quality of embryos during cryopreservation is a factor that influences the results. Considering the culture mediums, the enrichment of antioxidant and delipidating agents improves the cryo-tolerance of oocytes and embryos.

Despite advances in cryopreservation of buffalo oocytes and embryos, these are still not satisfactory for commercial purposes. Therefore, more research on the different topics is needed.

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