EFFECT OF ADDING *ALOE VERA* EXTRACT ON THE QUALITY OF BUFFALO OOCYTES USING TWO CRYOPRESERVATION METHODS

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ABSTRACT

This study was conducted to evaluate the impact of two types of cryopreservation and different concentrations of *Aloe vera* as an antioxidant (0,10% and 20%) on the viability and normal morphology of immature and *in vitro* mature buffalo cryopreserved oocytes. The *in vitro* mature and immature oocytes were kept in cryopreservation solutions for rapid and vitrification techniques. The results revealed that the proportion of buffalo oocytes found to be viable and normal morphology were significantly (P<0.01) higher produced when using a high concentration (20%) of *aloe vera* on *in vitro* mature buffalo oocyte which is79.11% and 59.02%; respectively. We conclude from the present study that the viability and normal morphology of post-thawed buffalo cryopreserved *in vitro* matured oocytes were significantly (P < 0.01) higher produced in using a high concentration of *aloe vera* than other concentrations of *aloe vera*, from the side while with other side the vitrification technique than a rapid technique.

Keywords: antioxidant, vitrification technique, rapid freezing technique, oocyte viable.

تأثير اضافة مستخلص الصبّار على نوعية بويضات الجاموس باستخدام طريقتين للحفظ بالتجميد

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المستخلص

أجريت الدراسة الحالية لتقييم تأثير نوعين من الحفظ بالتجميد إضافة الى تراكيز مختلفة من الصبّار كمضاد للأكسدة (سيطرة،10٪ و 20٪) على الحيوية والشكل الطبيعي لبويضات الجاموس الناضجة في المختبر وغير الناضجة. تم حفظ البويضات الناضجة في المختبر وغير الناضجة في محاليل الحفظ بالتجميد لتقنيات التزجيج والتجميد السريع، أظهرت النتائج أن نسبة بويضات الجاموس التي وجدت أنها حيوية وذات الشكل الطبيعي أعلى معنوياً (P <0.01) عند معاملة بويضات الجاموس الناضجة مختبريا مع تركيز عالي (20٪) من الصبار إذ بلغت 79.11٪ و 59.02٪؛ على التوالي. نستنتج من الدراسة الحالية أن الحيوية والشكل الطبيعي لبويضات الجاموس الناضجة مختبرياً بعد الاذابة كان أعلى بشكل واضح (P <0.01)عند استخدام التركيز العالي مقارنة مع التراكيز الأخرى للصبار، هذا من جانب بينما في الجانب الآخر، فإن تقنية التزجيج تفوقت على تقنية التجميد السريع.

الكلمات المفتاحية: مضاد الاكسدة، تقنية التزجيج وتقنية التجميد السريع، حيوية البويضات.

Received:14/9/2021 Accepted:30/1/2022

INTRODUCTION

One of the most important challenges facing Iraqi agriculture is protecting and improving national agricultural resources, including buffalo, to achieve sustainable food security (2). Different countries like the Mediterranean region, Latin America (Central and South America), and Asia consider domestic water buffalo (*Bubalus bubalis*) as one of the important livestock resources (27). The buffaloes that were resistant to diseases utilize unfortunate quality roughages and produce high-quality milk when compared to a cow (10). The term 'buffalo' refers to three species in the family Bovidae the African buffalo (*Syncerus caffer*) and the North American buffalo (*Bison bison*) that have yet to be domesticated. On the contrary, the Asian buffalo (*B. bubalis*) was domesticated around the same time in history as cattle for draft power, milk, and meat (37). These two types differed in their wallowing habits, physical features, and chromosome numbers, the swamp type consisted of 48 chromosomes, and the river type had 50 chromosomes (8). Reproductive traits are important in minimizing cost maximizing the net return, and determining production efficiency and genetic gain(14). The main problem with cooling or cryopreservation of oocytes is the low percentage of oocytes retaining the ability to undergo normal maturation and fertilization (19). Cryopreservation means exposure of the cells or tissues to subzero temperatures for extended periods using liquid nitrogen (-196 ºC), at low temperatures (10), to stop all biological activity and preserve physiological competencies and their viability for future use is called cryopreservation (16). The halfway technique between vitrification and slow freezing is rapid freezing. It is quicker than the slow-freezing technique and never involves using programmable machines. Also, requires lower concentrations of cryoprotectant agents (CPA) compared to vitrification (35). This technique uses the cryovials, which contain the oocytes, which are directly in contact with the vapor of LN2 (-80ºC) for 15 minutes. After that, samples must be plunged into LN2 tank (-196 ºC) (9). However, vitrification is a common method for cryopreservation of gametes and embryos. Successful oocyte vitrification has been achieved in several animal species, such as humans, but subsequent progress is still limited, especially in buffalo (23). However, during the freezing-thawing process, an imbalance between oxidizing factors and antioxidant molecules results in oxidative stress conditions there were different mechanisms to inhibit oxidative stress and reduce reaction oxygen species(ROS) damage during the freezing-thawing process, which is the use of antioxidants, Antioxidant substances in essential oils prevent oxidative damage of cell and reactive oxygen species (ROS) reacting with free radical, scavenging free radicals (12, 13, 19). *Aloe vera* (*Aloe barbadensis Miller*) is a perennial herbal succulent plant from Liliaceae family and is an important medicinal plant. It has been used in pharmaceutical, food, and its demand for cosmetic industries (6). The leaves of *Aloe vera* have two products, the bitter yellowish juice on the outside of the inner layer just before the external covering and the gel on the inner side of the leaf (7). It is cultivated widely in hot and dry climates in many countries, such as Africa and Mediterranean countries (6). *Aloe vera* is a rich source (containing over 200 bioactive) of compounds e.g. sugars, saponins, carotenoids, flavonoids, tannins, Anthraquinone, steroids, vitamins, minerals, enzymes, polysaccharides, alkaloids, phenolic compounds, phenols, and organic acids (7,14, 22, 29). A study by Hu *et al*. (15) showed that *Aloe vera* extracts had a stronger antioxidant activity than butylated hydroxytoluene (BHT) is a lab-made chemical that is added to foods and α-tocopherol(vitamin E). Also, Singh *et al*. (33) reported that *Aloe vera* enhances the body's natural defense against oxidative stress by elevating the level and activity of antioxidant enzymes. Another study by Debnath *et al*. (8) showed that *Aloe vera* extracts prevent lipid peroxidation, and DNA fragmentation by free radicals(which can abstract electrons from other compounds to attain stability). The antioxidant activity can be indicated in terms of the percentage of inhibition(13). The researchers stated that the antioxidant activity of this plant depends on its total phenolic content and the plant can be used as a good natural antioxidant source.

MATERIALS AND METHODS

This study was conducted at the Advanced Research Laboratory Unit, College of Medicine / Jabir Ibn Hayyan Medical University, Al-Najaf Al-Ashraf – Iraq, during the period from the 1st January 2020 to 11th April/ 2020.

Oocyte collection and classification

The buffalo ovaries recovered from the normal reproductive tract of adult buffaloes of unknown breeding history. Samples were collected from a local slaughterhouse in Al-Najaf Al-Ashraf Province. Both ovaries were collected directly from each buffalo after being slaughtered and kept in a thermos flask containing warm normal saline (0.9% NaCl) supplemented with antibiotics (100IU/mL penicillin and 100 µg/ml streptomycin). Ovaries were transported to a well-equipped laboratory unit, within 2 hours. In the laboratory, ovaries were washed with warmed normal saline solution (37.5 °C) three times to remove the clotted blood and reduce contamination on the ovarian surfaces (4,25). Oocytes were aspirated from all the visible follicles on the ovarian surface with 2 mm of more than diameter. Oocytes with follicular fluid were aspirated by using a 23-gauge hypodermic needle attached with a sterile disposable 3 mL syringe containing 0.3 mL of SMART culture medium supplemented with 5% bovine serum albumin (BSA) and 20 IU/ml heparin to prevent clotting within the follicular fluid. After oocytes were washed three times using the SMART medium, the cumulus-oocyte complexes (COCs) were classified under the inverted microscope into immature, mature, and atretic oocytes according to the presence of 1st polar body and some morphological features of oocytes (26, 29).

Preparing *aloe vera* solution

After obtaining the leaves of the *Aloe vera* plant from the nursery of the Directorate of Agriculture in Najaf, the leaves were washed thoroughly with normal saline three times to remove the impurities, after which a longitudinal incision is made in the outer layer of the leaf on one side, then the leaf was cut crosswise and the pieces were placed vertically in a vessel for liquid descent throughout five hours until the entire liquid was filtered.

Preparing cryopreservation solution

There are two cryopreservation solutions, The rapid solution was formed of the following:

A-Rapid solution 1 (G1) (Control)

- 7.5 mL Glycerol (15%) with 42.5 mL SMART (85%).

B-Rapid solution 2 (G2

- 45.0 mL (90%) [Glycerol (15%) with SMART (85%)]+ 5 mL from *Aloe vera* (10%).

C-Rapid solution 3 (G3)

- 40.0 mL (80%) [Glycerol (15%) with SMART (85%)] + 10 mL from *Aloe vera* (20%).

- Vitrification solution 1 (VS1)

1- 7.5 mL Glycerol (15%). 2- 42.5 mL (SMART culture medium up to 10 mL with 10% BSA).

Vitrification solution 2 (VS2)

1 - 7.5 mL Glycerol (15%). 2- 42.5 mL (SMART culture medium up to 10 mL with 10% BSA and 0.5 M of sucrose).

1 –7.5 mL Glycerol (15%). 2- 37.5 mL (SMART culture medium up to 10 mL with 0.5 M of sucrose and 10% BSA).

3- 5 mL (10 %) from *Aloe vera*.

- Vitrification solution 2 (VS2)

1 - 7.5 mL Glycerol (15%). 2- 32.5 mL (SMART culture medium up to 10 ml with 10% BSA and 0.5 M of sucrose).

3- 10 mL (20 %) from *Aloe vera*.

- Thawing solution (TS)

1- 1 M of sucrose (3.42 g / 10mL)

2- SMART culture medium up to 10ml with 10% bovine serum albumin.

Cryopreservation of oocytes:

Normal and viable oocytes (immature and *in vitro* mature) were transferred to 0.5 ml of the vitrification solution (VS1) at room temperature to equilibrate the oocytes for 5 minutes. Then, oocytes were placed into 0.5 mL of vitrification solution 2 (VS2) for 15 seconds, then the oocytes were loaded with a straw of VS2 and directly immersed into LN2. The straw was covered with a plastic tube in the LN2 to protect it during storage. The samples were pipetted into cryovials, the cryovials were left at room temperature for 10 minutes and then frozen by static-phase vapor cooling and plunged into liquid nitrogen vapor (10 cm above the level of liquid nitrogen; – 80 C°) for 15 minutes. The samples were then plunged into liquid nitrogen (–196C°) and

stored until required. Thawing was carried out at room temperature (9).

Thawing of Oocytes

For thawing, the straws and cryovials were taken out from the LN2 after two months and immersed in the thawing solution at 37 ºC for 1 minute. Samples were washed twice with SMART medium and tested for viability and morphology directly post-thawing. The thawed oocytes were considered abnormal when some changes occurred in shapes such as breakage of zona pellucida, uneven granulation, and leakage of oocyte contents (34).

Statistical analysis: The statistical analysis system (SAS) program (30) was used to determine the effect of two types of cryoprotectants in post-thawed oocytes. The analysis of variation (ANOVA) and Duncan's Multiple Range Test for viability were used to compare between means.

RESULTS AND DISCUSSION

The result noticed that a highly significant ($P < 0.01$) increase was achieved in the viability and normal morphology (%) of *in vitro* matured compared with immature cryopreserved oocytes, which appeared that the optimal result was performed in a high concentration of AV, which is 79.11% for viability, 59.02% for normal morphology (Table1, 2), which content the:

Table 1. Effect of the relationship between different concentrations of AV and types of oocyte on the percentage of viability of post-thawed buffalo cryopreserved oocytes

Concentration of AV	Type of oocyte	
	Immature	IVM
Control	50.41±1.41 F	64.85±1.44 D
Low(10%)	56.81±1.39 E	75.93±1.29 B
High(20%)	67.29±1.21 C	79.11±1.23 A

IVM: *in-vitro* matured, AV: *aloe vera*. Different letters in the table refer to significant ($P < 0.01$) differences

Table 2. Effect of the relationship between different concentrations of AV and type of oocyte on the percentage of normal morphology of post-thawed buffalo cryopreserved oocytes

Concentration of AV	Type of oocyte	
	Immature	IVM
Control	40.56±1.36 D	47.88±1.27 C
Low(10%)	45.73±1.46 C	53.67±1.36 B
High(20%)	53.03±1.20 B	59.02±1.23 A

IVM: in-vitro matured, AV: *aloe vera*. Different letters in the table refer to significant ($P < 0.01$) differences.

A highly significant ($P < 0.01$) effect was found in the viability and normal morphology of *in vitro* matured cryopreserved oocytes compared with immature when exposed immediately to LN2, which is 78.87 % and 58.35 % for viability and normal morphology of *in vitro* matured oocytes; respectively (Table 3,4).

Table 3. Effect of the relationship between the type of cryopreservation and oocyte on a percentage of viability of post-thawed buffalo cryopreserved oocytes

Type of Cryopreservation	Type of oocyte	
	Immature	IVM
Rapid tech.	63.94±1.14 D	72.48±1.14 B
Vitrification tech.	67.15±1.11 C	78.87±0.96 A

IVM: *in-vitro* matured. Different letters in the table refer to significant ($P < 0.01$) differences

Table 4. Effect of the relationship between the type of cryopreservation and oocyte on a percentage of normal morphology of post-thawed buffalo cryopreserved oocytes

Type of Cryopreservation	Type of oocyte	
	Immature	IVM
Rapid tech.	43.10±1.10 D	51.58±1.19 B
Vitrification tech.	47.38±1.07 C	58.35±0.96 A

IVM: *in-vitro* matured. Different letters in the table refer to significant (P < 0.01) differences.

The viability (%) and normal morphology (%) of cryopreserved oocytes, were significantly (P<0.01) increased produced when using a high concentration of AV and plunged immediately to LN2, which is 78.23% for viability and 60.62 % for normal morphology (Table 5,6).

Table 5. Effect of the relationship between different concentrations of AV with types of cryopreservation on the percentage of viability of post-thawed buffalo cryopreserved oocytes

Concentrations of AV	Type of Cryopreservation	
	Rapid tech.	Vitrification tech.
Control	57.09 ± 1.45 D	66.30 ± 1.35 C
Low(10%)	63.40 ± 1.30 C	72.23 ± 1.32 B
High(20%)	70.51 ± 1.29 B	78.23 ± 1.18 A

IVM: *in-vitro* matured AV: *aloe vera*. Different letters in the table refer to significant (P < 0.01) differences

Table 6. Effect of the relationship between different concentrations of AV with types of cryopreservation on a percentage of normal morphology of post-thawed buffalo cryopreserved oocytes

Concentrations of AV	Type of Cryopreservation	
	Rapid tech.	Vitrification tech.
Control	40.77 ± 1.46 E	46.93 ±1.32 D
Low(10%)	47.90 ± 1.41 CD	49.69 ± 1.33 C
High(20%)	54.78 ± 1.19 B	60.62 ± 1.24 A

IVM: *in-vitro* matured AV: *aloe vera*. Different letters in the table refer to significant (P < 0.01) differences

The current study revealed that a higher percentage of viability and normal morphology in samples cryopreserved with a concentration of 20% of *aloe vera* were yielded post-thawing as a result of several factors, including the types of cryoprotectant, and oocytes, and this reflects the dominance of concentration 20% of *aloe vera* in the conservation of cryopreserved oocytes. *Aloe vera* plant, due to some properties including anti-inflammatory, antimicrobial, anticancer, wound healing, neuroprotective, anti-diabetic, and antioxidant properties, is widely used as a therapeutic and medical agent(11, 17, 18). Therefore, Singh *et al*., (32) reported (59.0 % and 14.2 %) for live spermatozoa and abnormalities spermatozoa; respectively, a significant (P< 0.05) difference was assessed between the control and *Aloe vera* group when additive *Aloe vera* (5µl/ml) in cryopreservation of post-thaw cattle bull semen. Souza *et al*. (33) obtained a result that a higher concentration of *Aloe vera* gives higher sperm viability. Like that the result of Yong *et al*. (35) indicated that a higher concentration of *Aloe vera* gives better sperm

motility. Results of the present study indicated a good percentage of viability (78.23%) and normal morphology (60.62%) of post-thawed buffalo cryopreserved oocytes when using vitrification technique and high concentration (20%) of *aloe vera*. While, ensuring that during submerging, the oocytes were surrounded with liquid nitrogen and not vapor, giving a better heat exchange (24). So, the results of viability and morphology obtained in this study agreed with Mahmoud *et al.*(23) indicating the percentages of viable post-thawed buffalo vitrified oocytes were 72.8% and 27.0% for viability and abnormality oocytes; respectively. On the other hand, Al-Saadoon (1) showed no significant effect of vitrification and rapid cryopreservation on the morphology of immature oocytes, a higher percentage of viability is noticed at the post-thawing period for vitrified and rapid cryopreserved immature oocytes (91.11% and 84.44% respectively, and significant ($P<0.05$) differences in the percentage of normal and viable mature oocytes post thawing using vitrification and rapid cryopreservation 75.55% vs. 64.44%; respectively. Therefore, vitrification technologies applied to buffalo oocytes have become more successful as an alternative to slow cooling methods (22).= From the results of the present study, it is revealed that the percentage of post-thawing morphologically normal and viability vitrified *in vitro* mature buffalo oocytes were significantly higher ($p<0.01$) than that of vitrified immature with a high concentration (20%) of *aloe vera.*

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