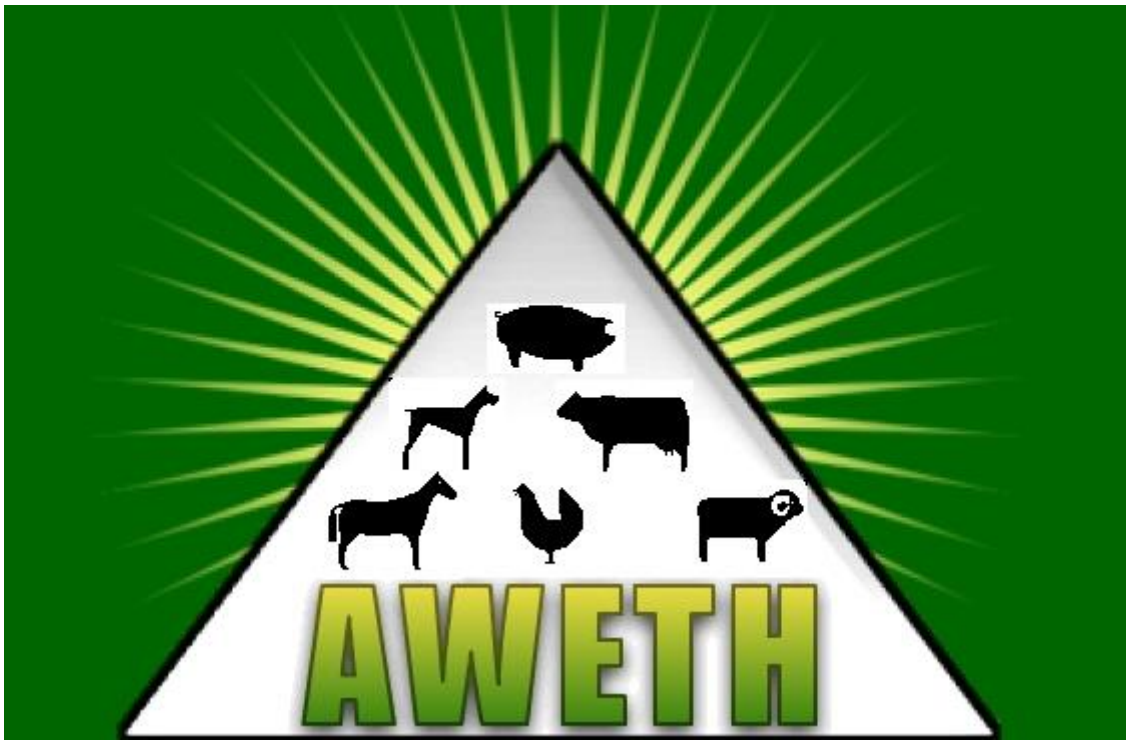
Animal welfare, etológia és tartástechnológia



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IN VITRO CONSERVATION OF GAMETES: THE WAY FORWARD TO CONSERVE THE GENETIC RESOURCES OF AUTOCHTHONOUS SHEEP BREEDS

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Abstract

Assisted reproductive technologies *(ARTs)* significantly improved livestock productivity and reproductive performance and enabled elite parents to produce thousands of offspring globally. However, indiscriminate use ARTs, lack of information and materials on autochthonous sheep breeds *(ASB)* resulted in a loss of their genetic resources *(GnR)*, thus shooting up the number of at-risk and extinct ASB globally. This problem is more in Europe and the Caucasus region, Africa, and Asia, according to the FAO 2005, 2017, and Sustainable Development Goals, 2022 reports. Considering the current economic challenges, *in vivo* gene conservation *(INGC)* is expensive and less sustainable. Therefore, an *in vitro* gene conservation *(IVGC)* can supplement the INGC for better efficiency and sustainability. The current review explored the pros and cons of ARTs on ASB's GnR, the need to conserve their GnR through IVGC, and the prospects of the IVGC on ASB's GnR conservation. Interestingly, Vitrification with reduced volume *(e.g., cryotop)* is the leading technique in oocyte cryopreservation, as it results in lower cryoprotectants toxicity, better oocyte viability, and pregnancy rates aside from being cheap and field-friendly than the other vitrification techniques. With oocyte and spermatozoa cryopreservation from the same breed, an extinct breed of interest can be regenerated in a single generation. However, oocyte cryopreservation holds a considerable prospect in IVGC, but the technique in sheep still needs improvements.

Key words: Sheep, Cryopreservation, Oocytes, Control Slow Rate Freezing, Vitrification, and Assisted Reproductive Technologies

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Introduction

Sheep contributes immensely to developing nations' economies or regions of extreme weather conditions and sub-fertile lands (*Amiridis and Cseh*, 2012). When combined with ARTs, their contributions will be overwhelming. However, wide commercial applications and the tremendous success of ARTs in sheep are restricted mainly to AI. It is primarily due to the naturally occurring anestrus period, variability in responses to super-ovulatory treatment, fertilization failure, and the necessity for surgery for recovery and transfer of gametes or embryos (*Amiridis and Cseh*, 2012). However, cryopreserving male and female gametes of the same breed can ensure the regeneration of the extinct breed population of interest in a single generation (*FAO*, 2012). However, oocyte cryopreservation (CP) is challenging due to its morphology *(plasma membrane and zona pellucida),* making water and cryoprotectants slowly move into and out of the oocyte (*Saragusty and Arav,* 2011). Moreover, metaphase II oocytes are very sensitive to CP due to their large size, low surface area to volume ratio, high water contents, and miotic spindles (*Lussing et al*., 2019). Despite their sensitivity, it is interesting that the first success in oocyte CP was recorded in the 1980s (*Lussing et al*., 2019). In sheep, high cytoplasmic lipid droplets and complex subcellular structures significantly contribute to sheep MII oocytes' sensitivity to freezing and thawing (Quan et al., 2017). Thus, oocyte CP and thawing procedures are not as developed and refined as the semen and embryo. The recent development of vitrification by cryotop markedly improved the success of oocytes CP in different species, including human beings. Vitrified human oocytes by cryotop resulted in good viability *(94.5 %)* and pregnancy rate *(41.5 %)* (*Kuwayama*, 2006). Thus, if extensively exploited and established in livestock, oocytes CP holds excellent prospects for GC. This review aims to highlight the different gametes retrieval methods and their CP techniques as essential tools for gene conservation (GC) of ASB. Furthermore, to draw the attention of sheep specialists/experts and policymakers on the need and prospect of IVGC of ASB.

The pros and cons of ARTs and the need for *in vitro* conservation of autochthonous sheep genetic resources

The flock size, success, and profitability of ARTs determine farmers' acceptability of a technique (*Sharkey et al*., 2001). To shorten generation intervals, using ARTs *(IVEP or JIVET)* becomes necessary for breeders as it is the fastest way. Similarly, with success recorded in improving the reproductive efficiency using ARTs, flock/herd size can be reduced while productive performance *(meat/milk/fur production*) and quality *(less cholesterol/less intramuscular fat/lean meat)* remain increasing (*Daly et al*., 2020), which is more environmentally friendly. It also allows inducing estrus out-of-season in seasonally sheep breeds, producing offspring of predetermined gender, GC of endangered breeds/species and control breeding which increases the overall profitability of an enterprise (*Amiridis and Cseh*, 2012).

Unfortunately, the indiscriminate use of ARTs led to the erosion of valuable GnR of ASB. According to the 2007 FAO reports, at least one livestock breed is becoming extinct each month, leading to the loss of its GnR forever; as a result, 148 Europe and the Caucasus sheep breeds have already become extinct. Moreover, North America, Europe and the Caucasus have the highest proportion of at-risk breeds globally (*FAO*, 2007;2015). Still, a recent report of 2022 Global SDG on ASB revealed a remarkable paucity of information and materials globally *(87.22 %)*, in North America and Europe *(92.25 %)*, and Sub-Saharan African region *(97.75 %)* (SDG, 2022). According to the 2022 DAD-IS local risk status report, almost all the Nigerian sheep breeds' local

risk statuses are unknown, and there is no data on the *in vivo* or *in vitro* conservation of such important genotypes (*http1*). Similarly, the population and a male-to-female ratio of Hungarian ASB are declining particularly; Alfoldi suta racka, Cikta, and Cigaja have already become extinct, endangered, and at risk, respectively (*http1*). It necessitates breed identification for proper record-keeping and the use of alternative conservation techniques like the IVGC. According to the *FAO* (2015) report, Africa, Asia, and Latin America are lagging in technology adoption. The authors of this review could not get exact data on the rate of ARTs adoption in the African sheep industry. But based on practical experience, ARTs adoption in the African sheep industry is minimal or zero. Therefore, indiscriminate mating/uncontrol breeding are the major problem that poses a more threat to the ASB GnR in Africa. Therefore, urgent steps are needed to address this devastating trend.

In vitro conservation of gametes of autochthonous sheep breeds

Gamete retrieval in male

Gametes retrieval from live males

a. *Retrieval using AV:* Semen collection by AV involves three (3) different steps/phases; i) Taming *(familiarizing animals to human presences/handling usually takes 4-6 weeks)*, training phase *(4-5 weeks),* and post-training phase *(Semen with the AV)* (Ambrosi et al., 2018). The device facilitates the collection of good quality semen samples.
b. *Retrieval using EEJ:* Gametes retrieval using this technique involves using a device called the electro-ejaculator. For details of the procedure, see Shipley et al. (2007). It is advisable to use sedatives or anesthesia before conducting the procedure (Abril-Sánchez et al., 2018). The most used devices are Bailey, Ruakura, and Lane probes (Shipley et al., 2007; http2). It is a simple procedure as it does not involve ram training (Leboeuf et al., 2000).
c. *Retrieval by TUMASG:* The procedure involves exteriorizing and holding the penis using sterile gauze and observing the three accessory sex glands *(bulbourethral glands, seminal vesicles, and ampullae)* with the aid of real-time transrectal ultrasound using a 7.5 MHz linear array probe. For details of the procedure, see Santiago-Moreno et al. (2013). It is a more welfare approach to collect semen in rams than the EEJ technique.

Post-mortem gametes retrieval

It involves collecting male genital organs or testes from a slaughterhouse/abattoir, after emasculation or immediately following the animal dead. For collection procedure details, see *Alvarez et al*. (2012). In sheep, the epididymal spermatozoa can be retrieved by; a). Slicing/mincing (*Kaabi et al.,* 2003) b). Incision methods (*Alvarez et al*., 2012) or c). Retrograde flow/flushing via the ductus deferens *(less contamination)* (*Bertol,* 2016). Good pregnancy rates have been reported using epididymal spermatozoa in sheep (87.5 %, 58.5 %, and 55.0 %) (*Ehlin et al*., 2006; *Rickard et al*., 2014; *Fernández Abella* et al., 2015), and other species. Therefore, it can be used to conserve the GnR of elite rams.

Gamete retrieval in females

Gametes can be retrieved regardless of their developmental *(primordial, pre-antral, or antral follicle)* and matured *in vitro* (*Saragusty and Arav*, 2011).

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Retrieval from live females (in vivo laparoscopic follicular aspiration): It involves using a technique called ovum pick-up (OPU) which can be achieved transabdominaly (Saragusty and *Arav*, 2011). Using the ideal aspiration elements, needle *(short: 18 or 20)*, tubes *(thin and intermediate: 1 and 2 mm)*, and correct aspiration vacuum pressure/flow rate *(10 and 20 ml/m)* improves the efficiency of the technique (*Rodríguez et al.*, 2006). The recent retrieval technique uses an endoscope, a pair of trocars *(inserted 15 cm cranial to udder),* and a specifically designed catheter to aspirate the oocytes along with the follicular fluid. The technique maintains good oocyte morphology and integrity, ensures repeated collection without much ovary adhesion, and is less traumatic to the animals (*Wieczorek et al.,* 2018).

Retrieval from postmortem females (*in vitro follicular retrieval):* Here the ovaries are sourced from either a slaughterhouse/abattoir or an immediately dead animal. For procedure details, see *Dadashpour Davaci et al.* (2014). The following methods are used to retried coocytes; a). Slicing *(using a sterile scalpel)* b). Aspiration *(aspiration pump fitted with 20-gauge needle at 10 ml H_2O/m flow rate)* c). Centrifugation *(incised the ovary surface and centrifuge at 750×g for 3 m in 3 ml of pre-incubated oocytes washing medium)* d). Puncture *(using an 18-gauge needle).* The aspiration technique gives a smaller number of high-grade oocytes than the other techniques, regardless of the season. Moreover, oocyte recovery by centrifugation yielded a significantly higher monospermic and blastocyst rate than the other techniques (*Dadashpour Davaci et al*., 2014).

Selection of ideal gametes and maturation for cryopreservation

Choosing the ideal sperm sample/ejaculate: it is usually achieved cheaply in certain sheep AI stations by observing a wave motion of freshly collected semen samples as an index of fertility (*David et al*., 2018). The ejaculates are categorized as good, moderate, or bad quality. Similarly, semen sample ideal for freezing must meet the following standards: volume *(1.0-1.5 ml)*, mass movement *(5 or 4),* concentration (*3.5-6.0 × 109/ml*), total motility *(>70%),* and morphological abnormalities *(<15%)* (Evans and Maxwell, 1987). However, in some instances poor-quality samples of elite males can be frozen to ensure GnR conservation.

Selecting ideal female gametes: Here, individual oocytes are selected based on their morphological characteristics as cumulus cells, cytoplasm, and polar body (Wani et al., 2013). The most used method is morphological, for details of morphological oocyte grading, see Wani et al. (2013). Careful selection of the ideal oocyte ensures better post-thaw viability and fertility results following IVF and transfer.

In vitro maturation of oocytes: Oocytes to be cryopreserved are usually matured *in vitro* and denuded off cumulus cells before vitrifying. The procedure mainly involves incubation using TCM-199 supplemented with 5 % FBS and antibiotics *(100 µg/ml Streptomycin and 100 IU/ml penicillin)* in an incubator set at; 38.5 ^{o}C, 5 % CO_2 and in the air for 21 h (Otoi et al., 1996). Oocytes' maturation is evident by the expansion of the cumulus cells and the extrusion of the first polar body.

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Cryoconservation of gametes

Cryopreservation of male gametes

Semen CP in rams is achieved either by vitrification *(not commonly used)* or freezing *(most widely/routinely used).* The freezing mainly takes two forms: pellets *(using a block of dried ice or solid carbon dioxide at -78.5 ^{o}C)* or straws *(most used, using liquid nitrogen at -196 ^{o}C)* (Evans and Maxwell, 1987). In sheep, semen is mostly cryopreserved in straws *(mostly 0.25 ml)*. It is achieved either manually *(static LN_2 vapor freezing)* (Evans and Maxwell, 1987) or using a programmable freezer (*Vozaf et al*., 2022). It is very important that the level of the LN_2 must be checked and refilled regularly during freezing and storage.

Cryopreservation of female gametes

Matured or immature oocytes can be cryopreserved for an extended period by either;

a. *Control slow rate freezing*: this technique involved equilibration of the oocytes for 10-15 m by exposing them to one or more solutions supplemented with 1-1.5 mol/l cryoprotectant *(usually glycerol or DMSO)* and non-permeating cryoprotectants like glucose, fructose, or sucrose (*Saragusty and Arav*, 2011). For details, see *Kasai and Mukaida* (2004).
b. *Vitrification:* involves using high initial concentrations of cryoprotectant and an ultra-rapid cooling rate to solidify the cell into a glass-like state without forming ice (*Banker et al*., 2017). The technique leads to fewer cyro-injuries, high success rate, simplicity, and doesn't require the use of a programmed freezer. However, a high concentration of permeating cryoprotectants may cause injury through the toxicity of the agents (*Banker et al*., 2017). Different techniques with reduced volume were developed recently like the cryotop, croloop, cryoleaf, plastic straw, among others (*Saragusty and Arav*, 2011). In pigs, vitrified germinal vesicle oocytes resulted in a better viability and developmental competence than the MII oocytes (*Wu et al*., 2006; *Egerszegi et al*., 2013). Good viability and cleavage rates were reported from vitrified-thawed oocytes in pigs: 87.1 % vs. 37.3 %; 85.4 % vs. 36.3 %; 56.8 % vs. 53.3 % (*Somfai et al*., 2014; *Egerszegi et al*., 2013; *Wu et al*. 2006) and sheep: 97.1 % vs. 32.9 %; 85.7 % vs. 57.5 % (*Sudiman et al.*, 2019; *Mardenli et al*., 2021) respectively.

Conclusion and recommendations

A drastic loss of ASB GnR is occurring globally, hence the need to explore other sustainable opportunities to supplement the *in vivo* GC. *In vitro* GC through gametes, CP holds a great prospect. Semen CP in sheep is well established, and the success rate is encouraging but this is not the case with oocytes. The oocytes' high sensitivity to chilling due to their large surface area-to-volume ratio and other complex cytoskeletal structures makes the success very low. Moreover, the CP technique leads to zona hardening *(due to the premature release of cortical granules)* and cytoskeletal damage, reducing the fertilization rate and developmental competence of the vitrified oocytes. Therefore, addressing the identified challenges would markedly increase the acceptability and success of the technique. Future studies should target improving cryopreserved oocytes' viability, fertilization, cleavage, pregnancy rates and if possible develop an LN_2-free conservation technique for better sustainability.

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