THE RECENT STATE OF EMBRYO PRODUCTION TECHNIQUES IN SHEEP BREEDING - A REVIEW

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SUMMARY

Conventionally, embryos can either be produced *in vivo* or *in vitro,* both procedures aimed at improving farm animals' reproductive performance. The former being the easiest or less invasive, while the latter is the most cumbersome, has many advantages and potential to rapidly foster genetic improvement. However, the efficiency and prospects of *in vitro* embryo production (IVEP) techniques in sheep are limited by so many factors. Among these include the reproductive system›s anatomy, oocytes and embryo quality, and cytoplasmic lipid droplets. This review aimed to highlight some of the procedures/steps involved in embryo production in sheep and recent developments in the techniques for breeding purposes. It covers the two EP methods; *in vivo* (estrus synchronization of the donor and the recipient ewes, multiple ovulation and embryo transfer, insemination of the donor ewe and recovery of oocytes/embryos) and *in vitro* (grading and selection of best quality oocyte, *in vitro* oocyte maturation, *in vitro* fertilization, *in vitro* culture of embryos, selection of best quality embryos for transfer, mature *in vitro* embryo transfer (MIVET) and juvenile *in vitro* embryo transfer (JIVET)). The use of short-term protocol (5-7 days) of estrus synchronization with fixed-time artificial insemination by a laparoscopic method can result in a high pregnancy rate, reduce time and cost of EP in ewes than with the conventional method. The JIVET and nonsurgical embryo recovery techniques and a transfer would enable the commercialization of IVEP and transfer in sheep. More intense researches are needed on the strategies of improving the viability of *in vitro* produced sheep embryos. There is also the need to ascertain whether the oocyte source (abattoir and laparoscopic ovum pick-up) affects the sheep oocytes› quality and developmental competence.

ÖSSZEFOGLALÁS

Mujitaba, M. A. - Vass, N. - Bodó, Sz. - Angyal, E.: AZ EMBRIÓELŐÁLLÍTÁSI TECHNIKÁK AKTUÁLIS HELYZETE A JUHTENYÉSZTÉSBEN - IRODALMI ÁTTEKINTÉS

Az állattenyésztési gyakorlatban az *in vivo* és *in vitro* embrió-előállítást alkalmazzák. Mindkét módszer javítja a gazdasági haszonállatok reprodukciós teljesítményét. Az *in vivo* embrióprodukció a legkönnyebben kivitelezhető és kevésbé invazív, míg az *in vitro* embrióelőállítás bonyolult eljárás, amely számos előnye mellett meggyorsítja a genetikai előrehaladást. Ugyanakkor juh fajban az *in vitro* embrió-előállítás hatékonyságát és a technikában rejlő lehetőségeket számos tényező befolyásolja: az anyajuhok nemi szerveinek anatómiája, a petesejt és az embriók minősége, a citoplazmában fellelhető lipidcseppek száma. A review közlemény célja egyrészt olyan eljárások kiemelése, melyek a juh embrióprodukció részét képezik, másrészt azoknak a legújabb tudományos eredményeknek a bemutatása, amelyek a tenyésztők érdeklődésére is számot tarthatnak. Juhoknál kétféle embrió-előállító eljárásról beszélhetünk: *in vivo* (a donor és recipiens anyajuhok ivarzásszinkronizálása, többszörös ovuláció és embriótranszfer, a donor anyák termékenyítése, a petesejtek/embriók kinyerése) és *in vitro* (a petesejtek osztályozása és a legjobb minőségű petesejt kiválasztása, *in vitro* oocyta érlelés, *in vitro* termékenyítés, *in vitro* embrió tenyésztés, a legjobb minőségű embriók kiválogatása az átültetéshez, érett *in vitro* embrió előállítás (MIVET), juvenilis *in vitro* embrió előállítás (JIVET)). Anyajuhokban az 5-7 napos ivarzásszinkronizálási protokoll és a fix időpontban végzett laparoszkópos mesterséges termékenyítés alkalmazása magasabb vemhesülési arányt eredményezhet, mint a hagyományos módszer, sőt, költség- és időhatékonyabb is. A JIVET technológia, valamint a nem-sebészi embriókinyerési és átültetési technológia alkalmazása a közeljövőben az IVEP és embrió átültetés térhódítását teheti lehetővé a juhtenyésztésben. Azonban az *in vitro* előállított juh embriók életképességének javításához további kutatások szükségesek. Fontos lenne azt a kérdést is tisztázni, hogy a petesejt forrása (vágóhíd, laparoszkópiás ovum pick-up) befolyásolja-e a petesejt minőségét és fejlődési kompetenciáját.

INTRODUCTION

Embryo production (EP) from the donor and embryo transfer (ET) to a surrogate opened a new way to produce several progenies from a superior female within the required possible time. The conventional biotechnological approach of EP and ET requires to hormonally superovulate the selected donor females and at the appropriate time (depending upon species and breed of animals) induced to artificial insemination (AI) (*Gupta et al*., 2018). Conventionally, embryos can either be produced *in vivo* or *in vitro, b*oth procedures aimed at improving farm animals' reproductive performance. The former being the easiest or less invasive while the latter is the most cumbersome, though, has many advantages. *In vitro embryo production* (IVEP) involves a sequence of events which include; oocytes *in vitro maturation* (IVM)*, in vitro fertilization* (IVF), and *in vitro culture* of the resultant embryos to the blastocyst stage (*Hyttel et al*., 2019). The technique allows for the production of offspring from selected genetically superior females that would not be able to reproduce using AI, multiple ovulation and embryo transfer (MOET), or recently using juvenile in vitro embryo transfer (JIVET) (*Menchaca et al.,* 2018). According to the European Embryo Transfer Association cited by *Paramio and Izquierdo*, (2014), when compared to a cow, research and commercial embryo activities in small ruminant (SR) are low in the European Union.

Similarly, International Embryo Transfer Association also reported that there exist few IVEP studies in SR compared to other livestock species. Ovine EP, particularly *in vitro,* is still associated with many challenges that include low efficiency majorly due to poor quality embryos, leading to poor viability after the transfer/cryopreservation process. The technique is still relevant and vital, especially in increasing genetic progress by shortening generation interval compared to the natural breeding system. Addressing such problems would virtually enable breeders to exploit the best out of this technique. This review›s principal aim was to highlight some of the procedures/steps involved in sheep EP and recent developments recorded in the techniques for breeding purposes. It covers EP methods (*in vivo* and *in vitro*).

EMBRYO PRODUCTION METHODS

In EP, one of the initial steps is the selection of good quality spermatozoa for the IVF. Conventionally, it involves selecting a frozen-thawed sperm based on a *percoll* separation method; in Hungary, work showed that sperm with a high viability and chromosome integrity could be obtained by *percoll* separation than by swim-up method (*Gordon,* 2017). One of the advantages of EP techniques is that embryos are more resistant than gametes when subjected to high body temperatures due to thermal stress. Thus, the pregnancy rates are better in ET than in AI throughout the year (*Sanches et al.,* 2018). Embryo development is influenced by several factors: maternal, paternal, breed, age, individual variation, follicle, oocyte diameter, and environment (*Camargo et al.,* 2018).

In vivo embryo production

In vivo EP activities in SR started as early as the 1930s. Since then, the great majority of embryo recovery (ER) and ET attempts were performed by surgery procedures (*Gordon*, 2017). Recently, a novel and noninvasive ER and ET procedure was invented in SR and had been proven to be successful (*Fonseca,* 2016). The *in vivo* produced embryos are of more outstanding quality than their *in vitro* counterparts because of higher survival, implantation, and birth rate (*Zhu et al*., 2018).

Estrus synchronization of the donor and the recipient ewes

Reproduction in SR can be controlled by administering exogenous hormones to modify the physiological chain of events involved in the estrous cycle. The manipulation of the estrus cycle can concentrate the insemination and births and induce cyclicity, shorten the interval between births, schedule births for a favorable season of the year with available feed, and use genetically improved animals (*Ramos and Silva*, 2018). Estrus can be synchronized in cyclic or anoestrus ewes by using either prostaglandin or progesterone/progestagens (a synthetic hormone with a similar action to progesterone). In sheep, PGF2α (≥15 mg) or cloprostenol (125 mg) is effective after day 5 of the cycle (estrus is day 0). Estrus may be synchronized by two doses of prostaglandin, 7-9 days apart. Controlled internal drug release (CIDR) intra-vaginal plastic device impregnated with progesterone (300 mg) can also be used. The CIDR uses for 7 days, and a luteolytic dose of PGF2α is administered 1 day before or at device removal. The estrus response is high within 72-84 h (*Romano,* 2019).

The latest development in estrus synchronization (ES) and AI is the short-term protocols for fixed-time artificial insemination (FTAI). It consists of exposure to exogenous progesterone (usually in a CIDR-type) for 5-7 days, associated with a dose of equine chorionic gonadotropin (eCG) and PGF2α at the time of device removal. This system reduced the exposure period to progesterone from 10-14 days to 5-7 days and the detrimental effects of the decline in fertility due to low progesterone concentration experienced in the 10-14 days protocol (*Menchaca et al.*, 2018). Estrus, LH peak, and ovulation occur approximately 30, 40 and 60 h after a device removal, respectively (*Vilariño et al*., 2010). For first-use devices, FTAI should be performed on the morning of day 8 (46-50 h after device removal) by the cervical route or in the afternoon (52-56 h) by the intrauterine route. For second-use devices, FTAI could be performed by both insemination routes in the morning or afternoon without affecting fertility (*Menchaca et al.,* 2018). *Menchaca et al.* (2018) reported a higher pregnancy rate 43.5% in short-term (6 days) than 37.8% in long-term (14 days) protocol in multiparous ewes that were inseminated by laparoscopic method. This suggests that the short-term protocol would shortly become the best method of choice for breeding purposes in SR.

Multiple ovulation and embryo transfer

The MOET program is based on ES and superovulation of donor ewes, followed by AI and collection of embryos with uterine lavage, and subsequent transfer to

recipient ewes or cryopreservation. The technique offers rapid multiplication of the number of offspring from the genetically superior ewe, increases selection pressure, and reduces generation interval (GI), making the technique an instrument of genetic progress (*Ramos and Silva,* 2018). Donor ewes are treated with progestagens/prostaglandin and gonadotropins so that estrus and ovulation are control and to enhance large production (10-20) and release of ova from the ovary. Doses of the hormones used usually range between 500-800 IU of PMSG and/or 120-140 mg of FSH, while 40-50 μg of GnRH is mostly used to increase ovulation synchrony (*Cottle,* 2010). The GnRH is administered two days before removing the progesterone device. At the same time, FSH injections need to be continued for a total of 4-6 shots, and animals are allowed to breed naturally or by AI. After 6-7 days of embryo development, morula/ blastocyst stage embryos need to be flushed and preserved or transfer to the surrogate (*Gupta et al.,* 2018). Protocols using vaginal progesterone pessary for 7-8 days can achieve similar results (*Driancourt,* 2001). Despite advances in the technique recorded in recent years, there is still ovarian response variability, which remains the major limitation in MOET programs (*Pinto et al.,* 2018). The super-stimulatory treatment (ST) and the difference in the composition of commercially available FSH preparations are seen as some of the causes (*Cognié et al.,* 2003). However, the ovarian response to ST also depends on factors such as follicular condition, genetics, season, and nutritional status of animals. Between 20 and 40% of the treated females do not respond to the ST (*Brasil et al.,* 2016). The onset of estrus variability and the LH peak after hormonal treatment and the lack of synchrony at the onset of superovulation are among the problems leading to failed fertilization (*Amiridis and Cseh,* 2012). Therefore, ST should consider ewes' ovary status at CIDR insertion (size of antral follicles and presence of luteal structures). It should begin on Days 5 or 9 of CIDR treatment in ewes without corpus luteum and ovaries bearing medium-sized follicles (*Bartlewski,* 2019).

Insemination of the donor ewe

AI techniques enable ram to inseminate many more ewes that it could be possible by natural mating. Before collecting semen, ram should be performance tested and proven superior before using their semen (*Cottle*, 2010). In sheep, conventional AI protocols require daily estrus detection (ED) with teasers› aid, which is quite labor-intense. For this reason, the traditional AI had now been replaced by the use of FTAI, which is a labor-effective option because ED becomes unnecessary (*Olivera-Muzante et al*., 2011). *Miranda et al.* (2018) highlighted that FTAI allows the synchronization of lambing periods, organization of lambs into batches suitable for meeting market demands, an earlier onset of puberty, and improved conception rates regardless of whether the estrus is observed or not. Usually, the treatment for FTAI in sheep is based on progesterone-releasing devices and eCG or GnRH administration at device removal, with an acceptable pregnancy rate (*Menchaca et al*., 2017). The eCG or GnRH are used to reduce the interval between sponge withdrawal and estrus and to improve the efficiency of ES and ovulation during the breeding season (*Fornazari et al.* 2018). *Menchaca et al.* (2017) noted an earlier appearance of estrus behaviors and subsequent preovulatory LH peaks

and ovulation in animals receiving CIDR-eCG than in the CIDR-GnRH. Based on eCG protocol, *De et al*. (2015) observed estrus response of 79.4% (374/471) and lambing rate of 60.42% (226/374), *Miranda et al.* (2018) reported a 55.4% lambing rate, while *Tekim* (2019) recorded 71.4% estrus response and 100% kidding rate in goats. Similarly, *Rekik et al*. (2016) reported that the use of flourogestone acetate for 14 days and 300 IU eCG administered at sponge removal resulted in a 70.4% lambing rate.

Recovery of oocytes/embryos

The ER in SR can be performed using surgical, laparoscopic, or trans-cervical methods (*Fonseca et al.,* 2016).

Surgical embryo recovery technique

Surgical/Laparotomy technique allows exact counting of corpora lutea and evaluation of total structures recovery rate. However, disadvantages are the relatively high cost of equipment, stress to the animal due to the manipulation of the exteriorized reproductive tract that can cause adhesions, and progressive reduction in the success of ER rates (*Fonseca et al.,* 2016). As a result, the technique is not mostly used nowadays by most farms.

Laparoscopic ovum pick-up

Laparoscopic ovum pick-up (LOPU) is generally regarded to be an effective and minimally invasive procedure for the oocyte/ER in SR (*Gordon,* 2017). Slaughterhouses represent a low-cost and abundant source of oocytes useful for research projects, oocytes from live animals are required for commercial application of IVEP. For this purpose, follicular aspiration by LOPU is mandatory in SR, providing approximately 10-14 oocytes per female in each session (*Baldassarre et al.,* 2002; *Teixeira et al.,* 2011) cited by *Menchaca* (2018). Follicular aspiration of live animals needs to be associated with ovarian stimulation, usually achieved by using a single dose of FSH and eCG 36 h before LOPU (*Baldassarre et al.*, 1996; *Gibbons et al.,* 2007). *Baldassarre* (2002) reviewed follicle aspiration by LOPU in SR and identified some important key steps which include; donor selection (it should be superior or high genetic merit), donor treatment (synchronized and hormonally-primed for LOPU) and lastly follicular aspiration (animal need to have fasted for 24 h for food and 12 h for water and the procedure should be performed under anesthetic condition). The technique is very flexible and does not interfere with the donors' productive or reproductive career yet allows the production of more embryos than the conventional superovulation (*Gordon,* 2017). Sometimes, it leads to fewer adhesions and, therefore, a donor could be collected more than seven times. However, this method still requires special equipment and highly trained personnel. Thus, regardless of the excellent efficiency described, this technique did not become popular (*Fonseca et al.,* 2016).

The non-surgical embryo recovery technique

Embryos can be recovered from sheep by nonsurgical embryo recovery (NSER) technique. It was first reported in SR in the 1980s. This technique's anesthetic protocols are much simpler, and animals may remain in a standing position under sedation combined with epidural block and local cervical anesthesia. To dilate the uterine cervix for transcervical embryo flushing and deposition. Several drugs have been used successfully with combined treatment using estradiol benzoate, d-cloprostenol (both administered 16 h before embryo flushing), and oxytocin (20 min before cervical penetration), providing the most optimal results in cycling and superovulated ewes. Recipient animals do not require any hormonal drug to induce cervical dilation. Embryos were transferred directly by a cervical route, which yields an excellent result (*Fonseca et al*., 2019) cited by *Bartlewski* (2019). The transcervical recovery method was successful in 61% of ewes receiving either cloprostenol or misoprostol, whereas no catheter passage was achieved in control-ewes.

Similarly, in Dorper ewes, animals receiving 200 mg misoprostol by vaginal route 5 h before ER reached 95% of cervical transposition, compared with 0% for control-ewes. On average, the technique was accomplished in 30 min and allowed recovery of six embryos per ewe, demonstrating its feasibility for Dorper ewes (*Fonseca et al*., 2016). The reduced or non-adhesion formations are pointed as the main advantage of this technique, suggesting that successive collections are more feasible in NSER than in laparotomy. Conversely, the introduction of a catheter through the cervix, mainly in sheep, and the incapability of rectal manipulation of the tract are the main difficulties for NSER procedures (*Fonseca et al.*, 2016).

In vitro embryo production

The results of a meta-analysis study conducted by *Zhu et al*. (2018) indicated that slaughterhouse had been the primary resource for cumulus oocytes complexes for IVEP among many countries around the globe. The IVEP can produce more offspring from genetically superior animals than with the use of conventional MOET. This because it is capable of avoiding most of the causes of failure in MOET (*Baldassarre*, 2002). In addition to selective breeding, IVEP also permits; production of offspring from genetically superior senile or prepubertal females, species conservation programs and represents a valuable research tool in developmental biology and in the study of human infertility treatments (*Menchaca et al*., 2018). In sheep, the technique is still inefficient; approximately only 70-90% of immature oocytes undergo maturation, from prophase I to metaphase II out of which, 50-80% undergo fertilization and cleave to a two-cell stage at 24 to 48 h after insemination; only 20-50% of immature oocytes ever reach the blastocyst stage, on day 7 to 8 post-fertilization (*Zhu et al*., 2018). *Viana* (2017) reported that in bovine, the numbers of transferred in vitro produced embryos (IVP) exceeded that of their in vivo-derived (IVD) counterparts. Unfortunately, the reverse is still the case in ovine. Data collected from six different world regions; Africa, Asia, Europe, North America, Oceania, and South America, on the total number of ovine IVD and IVP transferrable embryos indicated that 18652 were IVD and only 66 were IVP. For transferred embryos, 12571 were IVD, out of which 10 were frozen, and only

55 were IVP and were all transferred fresh (*Viana*, 2017). This calls for the need for more intense research on this subject for possible improvement. The IVEP includes oocyte collection/recovery (previously discussed), grading and selection of best oocyte with more cumulus oophorus, IVM of selected oocytes, IVF of a matured oocyte, selection of best quality embryo at the morula stage, and then finally, transfer of the produced embryos to the recipient ewes (*Gupta et al*., 2018).

Grading and selection of best oocyte

The ability of oocytes to undergo maturation and support embryonic development varies; this is referred to as developmental competence (DC) or oocyte quality (OQ) (*Reader et al*., 2017). The oocyte source in goats was observed to have marked effects on oocyte maturation, viability, and developmental competence of in vitro produced embryos. The LOPU-sourced were reported to be of more outstanding quality than the abattoir-sourced oocytes (*Rahman et al*., 2009). Age of donor was also reported (*O'Brien et al*., 1996; 1997; *Ledda et al*., 1997; *Ptak et al*., 1999; *Kochhar et al*., 2002; *Ptak et al*., 2006) to affects the OQ (*Table 1*.).

The oocyte is known to be a unique and highly specialized cell responsible for creating, activating, and controlling the embryonic genome, as well as supporting basic processes, such as cellular homeostasis, metabolism, and cell cycle progression in the early embryo (*Mtango et al.,* 2008) cited by *Hoshino,* (2018). It is essential, therefore, to assess the fertility potentials and guarantee the necessary health qualities of the oocytes since the DC of an embryo is principally dictated by the health and OQ (*Reader et al.,* 2017; *Hoshino,* 2018). Usually, for IVF and intracytoplasmic sperm injection, oocyte selection is based on morphological parameters related to the cumulus cells, polar body, and cytoplasm (*Hoshino,* 2018; *Wani et al.,* 2013). *Wani et al.* (2013) graded oocytes into three categories based on the number of cumulus cells and uniformity of the oocytoplasm, which include;

a. Good: Oocytes with many complete layers of cumulus cells and uniform cytoplasm.

b. Fair: Oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm.

c. Poor: Oocytes with few or no cumulus cells.

Only good and fair oocytes should be used for better embryo DC. A potential technique that can be used to determine OQ is intracellular temperature imaging with a fluorescent polymer thermometer to evaluate the oocytes› thermal profile. Moreover, OQ can be determined by assessing molecular markers (microRNAs) and certain morphological factors that are related to OQ like; first polar body morphology, meiotic spindle, cumulus cells, and mitochondria (*Hoshino*, 2018). *Reader et al.* (2017) reported that follicle and oocyte size, mitochondria, lipid droplets, oocyte vesicles, and cortical granules could be examined to assess OQ. Similarly, OQ can be assessed by staining procedure using brilliant cresyl blue to measure glucose-6-phosphate dehydrogenase activity. By the end of maturation, the authors concluded that oocytes with a greater volume or number of evenly distributed mitochondria, lipid droplets and vesicles, and cortical granules located immediately below the oolemma have greater DC.

Table 1.

Comparison of developmental competence of oocytes of prepubertal and adult sheep

Parameters (%) (1)						
S/N	Oocyte source (2)	Type of media used (3)	Fertilization rate (4)	Cleavage rate (5)	Blastocyst rate (6)	Source (7)
1	Pre-pubertal (8)	Medium-199 (Earle's salt, L-G, 2200 mg/l SB, 25 mM HEPES, Gibco, BRL, Grand Island, NY) +0.3 mM P+0.3 mM G+10% FBS(v/v)+ 10 μg/l FSH+10 μg/l LH	71.7	81.9	15.4[a]	*O'Brien et al.* (1996; 1997)
	Adult (9)		70.0	82.0	34.1[b]	
2	Pre-pubertal (8)	TCM-199(10% v/v FBS, 10 mg/l FSH and 10 mg/l LH)	64.0[a]	72.0	20.0[a]	*Ledda et al.* (1997)
	Adult (9)		72.0[b]	73.0	49.0[b]	
3	Pre-pubertal (8)	B-b TCM-199 (275 mosm) supplemented with 2 mM G, 100 μg/ml C, 0.3 Mm SP, 10% FBS, 5 μg/ml FSH (Ovagen), 5 μg/ml LH and 1 μg/ml E_2	NA	NA	22.9[a]	*Ptak et al.* (1999)
	Adult (9)		NA	NA	35.8[b]	
4	Pre-pubertal (8)	TMC-199+10% FCS under oil	69.0	63.2[a]	17.8[a]	*Kochhar et al.* (2002)
	Adult (9)		72.7	69.25[b]	26.8[b]	
5	Pre-pubertal (8)	B-b TCM-199 (2 mM G, 100 μg/ml C, 0.3 mM SP, 10% FBS, 5 μg/ml FSH (Ovagen), 5 μg/ml LH and 1 μg/ml E_2	NA	75.5[a]	12.0[a]	*Ptak et al.* (2006)
	Adult (9)		NA	81.0[b]	36.0[b]	

B-b: Bicarbonate-buffered, BSA: Bovine Serum Albumin, C: Cysteamine, CB: Cytochalasin B, E_2: Estradiol, FBS: Fetal Bovine Serum, FSH: Follicle Stimulating Hormone, G: Glutamine, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, LH: Luteinizing Hormone, NA: Not Available, P: Pyruvate, SB: Sodium Bicarbonate, SP: Sodium Pyruvate, TCM: Tissue Culture Media (10)
Values in the same column between two oocyte sources with different superscripts are significantly different (11)

1. táblázat Prepubertás korú és felnőtt anyajuhokból származó petesejtek fejlődési kompetenciájának összehasonlítása

paraméterek (1); petesejt-forrás (2); felhasznált médium típusa (3); fertilizációs arány (4); osztódási arány (5); blastocysta arány (6); forrás (7); prepubertás (8); felnőtt (9)
B-p: Bikarbonát-pufferelt, BSA: szarvasmarha szérum albumin, C: Ciszteamin, CB: citokalazin B, E_2: ösztradiol, FBS: fetális borjú savó, FSH: follikulus stimuláló hormon, G: glutamin, HEPES: 4-(2-hidroxi-etil)-piperazin-1-etán-szulfonsav, LH: luteinizáló hormon, NA: nem áll rendelkezésre, P: Piruvát, SB:nátrium-bikarbonát, SP: nátrium piruvát, TCM: szövettenyésztő médium (10).
A két különböző petesejt-forráshoz tartozó, de egyazon oszlopban szereplő értékek között akkor van szignifikáns különbség, ha a felső indexben eltérő betű szerepel (11).

In vitro oocyte maturation

Maturation is the most critical stage of IVEP; this is majorly due to immature oocytes' heterogeneous nature (*Paramio and Izquierdo*, 2014). The quality of the embryo to be produced depends mainly on the OQ. High-grade oocytes can only be achieved by using the right maturation media and maturation processes. For immature oocytes to become fertilizable they must undergo; cytoplasmic and nuclear maturation, extrude the first polar body, and have entered metaphase II (*Cognié et al*., 2003). As stated by *Zhu et al*. (2018), the most commonly used media for the maturation of oocytes is the traditional medium-Tissue Culture Medium-199 (TCM-199), supplemented with various serum at 10% including fetal bovine serum (FBS) (13 of 25 laboratories), sheep serum (5 of 25), bovine serum albumin (5 of 25), follicular fluid (1 of 25). FSH and LH combinations or 17β-estradiol are the most common hormones added to the maturation media and maintained under 38.5-39 °C, 5% CO_2 for 20-24 h.

In vitro fertilization

Here, both the oocytes and sperm cells are involved; therefore, their quality determines their fertilization ability and overall success of the process. Moreover, both gametes' ability to fertilize is time-dependent, which is limited and generally referred to as the „fertile span" (FS) beyond which they cannot fertilize. The FS is variable even in the same individual and is greatly determined by certain factors breeds, season, donor's age, donor nutrition, gamete quality, culture or preservation conditions including pH value, osmolality, compounds, gases, and so on (*Zhu et al*., 2018). Sometimes, FS may vary depending on OQ; poor quality or aged oocytes may have shorter FS. Therefore, timing is a vital factor that affects the success of IVF. Matured oocytes and spermatozoa must be co-incubated together within their FS to ensure that a greater proportion of the matured oocytes can be fertilized by capacitated and appropriate spermatozoa (*Zhu et al*., 2018). Besides, IVF success depends on proper oocyte maturation, sperm selection, sperm capacitation, and IVF media (*Paramio and Izquierdo*, 2014). The most common fertilization medium for sheep oocytes is SOF medium supplemented with 1-2 μg/mL heparin + 2-20% either FBS or SS, respectively. Besides other conditions, the blastocyst rate of oocytes IVF with 2% serum ranges from 20.0% to 59.2%, whereas that with 20% serum, it ranges from 24.1% to 42%. After insemination, the IVF drops are incubated at 38.5 °C in a humidified atmosphere incubator with 5% CO_2 in the air for 15 to 20 h (*Baldassarre, 2002*). Semen can be sex-sorted before the IVF process is carried out (*Paramio and Izquierdo*, 2016). Sex is determined in farm animals by sex chromosomes content of sperm; females are produced by gamete containing X-chromosomes and males by those carrying the Y-chromosomes. The former is larger and has more DNA than the latter, 4.2% and 3.5%, respectively (*Gordon*, 2017). Different methods are now available for semen sexing, including albumin gradient, percoll density gradient, free-flow electrophoresis, identification of H-Y antigen, or Flow-cytometry (*Boro et al*., 2016). With the flow-cytometry technique or cell sorting machine, semen from many mammalian species can be sexed at about 90% accuracy without

damaging them. This development permitted the production of many offspring of the desired sex (*Boro et al*., 2016).

In vitro culture of embryos

Among all the stages involved in the IVEP, the culture stage is longest (6-8 days) and also has a marked influence on the embryo developmental competence (EDC), viability, pregnancy rate, fetal development, and birth weight (*Zhu et al*., 2018). Ovine zygotes are cultured in SOF supplemented with amino acids (AA) and BSA. This combination is the standard or conventional culture medium for sheep embryos production. Keeping oxygen concentration at 5 % (5% O_2, 5% CO_2, and 90% N_2) reduces oxidation incidence. The blastocyst is formed on day 6-8 of culture. The culture medium is usually changed after every 48 h, or applied with two-step culture, namely; embryos are cultured in SOF+ AA+ BSA on the first three days, and on day 4, they are transferred into a SOF+ AA+ glucose+ bovine/ovine serum albumin and cultured in the medium up to day 8 (*Zhu et al*., 2018). *Fernández-Gonzalez et al*. (2014) observed that blastocyst culture in FBS's presence significantly affects some imprinting genes' mRNA expression. In 2013 an entire serum-free ready-to-use media suite for all the steps, maturation, fertilization, and culture, was made commercially available by IVF Bioscience, UK, combining synthetic serum replacements and BSA (*Hyttel et al*., 2019).

Selection of best quality embryos for transfer

Embryos can be assessed for quality by placing them under a magnifying glass. According to their morphological aspect, their quality is estimated due to the time after insemination and the pellucid membranes' integrity that surrounds the embryo (*Rizos et al.,* 2002). Embryos grading is mostly focused on the state of development and morphology, and only embryos graded as «excellent» or "good" will allow good results of cryoconservation (*Brem and Wiener*, 1990). *Jones et al.* (2001) reported some of the criteria that can be adopted to optimize the selection of embryo development, including; the rate of embryo development, blastocyst development, pronuclei expression and nucleoli orientation, ovarian/follicular vascularity, noninvasive assessment of metabolic products of embryos during development, pre-implantation genetic diagnosis, and morphological evaluation. *Geber et al.* (2002) identified the following criteria for assessing EDC in the human being;

a. Developmental rate**:** embryos must be assessed in culture, during the 1-cell, cleaving, and morula/blastocyst stages, and classified according to quality. On day 2, the first cleavage division occurs. After this, embryos double their number of blastomeres each day. Thus embryos have an expected 2-4 cells on day 2, 6-10 cells on day 3, become morula at day 4, and blastocysts at day 5 and 6. Embryos with odd numbers of blastomeres probably have cells with slower division than others.

b. Morphology: best embryos are those that are having thinner zonae and bla stomeres of similar size without fragments. Thicker zonae might impair hatching,

while greater degrees of fragmentation and blastomere irregularity gives a poor prognosis of pregnancy.

A combination of all these criteria to select the most likely embryos to implant might improve pregnancy rates.

Mature in vitro embryo transfer and juvenile in vitro embryo transfer

Transferrable embryos can be produced in vitro after ovum pick-up from super-stimulated donor ewes. The ewes can either be matured, in which case the technique is termed „mature in vitro embryo transfer" (MIVET), or immature/prepubertal ewe that is as young as 3 weeks old „juvenile in vitro embryo transfer" (JIVET) (*Cottle*, 2010). This technique offers a great opportunity for rapid genetic improvement through reduction of GI to as low as 6 months. Moreover, it also circumvents the problems of variation in fertilization rate and the super-stimulatory response observed in MOET (*Cottle,* 2010). However, juvenile embryos were observed to have lower viability following cryopreservation or transfer to recipient ewes. This was presumably, due to lower volume fraction and size of cortical granules in juvenile oocytes than adult oocytes following IVM, which may be related to the increased rate of polyspermy reported in oocytes from juvenile animals (*O'Brien et al.,* 2000). There is a need for intense studies on the other causes of poor DC/low viability of juvenile oocytes after maturation and/or cryopreservation.

Embryo transfer

ET can be used to expand the population of a particular breed or elite strain of sheep in demand; a further consideration is in import and export of sheep in the form of frozen embryos rather than animals on the hoof (*Gordon*, 2017). One of the most challenging aspects of assisted reproductive technologies is determining which embryos are most suitable for transfer into the uterus. The two most important factors that need consideration here are; the choice of the embryo with the best DC (previously discussed) and the risk of multiple pregnancies associated with the number of embryos transferred (*Jones et al.*, 2001). Usually, two embryos are transferred to each recipient ewe via laparoscopic insertion into the uterine horns ipsilateral to the ovary containing corpus luteum. Moreover, the recipient needs to be the same breed as the donor, and their estrus and ovulation be synchronized simultaneously (*Cottle,* 2010).

The most significant obstacles limiting the widespread use of the ET technique in sheep include:

a. Considerably limited available data on pedigree and performance

b. A lack of simple and cost-effective methods of dispersing superior gene in female sheep (i.e., laborious and non-standardized superovulatory protocols); and

c. The unique ewes' anatomical structure of the reproductive tract; this precludes the simple transcervical passage of an insemination/ET apparatus (*Bartlewski,* 2019).

Addressing those mentioned above and other problems associated with ET in sheep could improve the efficiency and farmers' acceptability of the technique.

CONCLUSION

The techniques of IVEP in sheep breeding can offer overwhelming promising results on livestock improvement in the future. Techniques like JIVET help accelerate the rate of genetic gain by shortening GI to as low as 6 months. The recent development of short-term protocol (5-7 days) of ES with FTAI using laparoscopic insemination method, if adopted, can increase the success rate and reduce the cost and time of EP. The use of a combination of different oocyte and embryo quality indicators could improve ovine IVEP. More researches are needed on the strategies of enhancing the viability of *in vitro* produced sheep embryos. There is a need to ascertain whether the oocyte source (abattoir and LOPU) affects the DC of sheep oocytes.

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REFERENCES

Amiridis, G. - Cseh, S. (2012): Assisted reproductive technologies in the reproductive management of small ruminants. Anim. Repr. Sci., 130. 152-161.

Baldassarre, H. - Furnus, C. - De Matos, D. - Pessi, H. (1996): *In vitro* production of sheep embryos using laparoscopic folliculocentesis: alternative gonadotrophin treatments for stimulation of oocyte donors. Theriogenology, 45. 707-717.

Baldassarre, H. - Wang, B. - Kafidi, N. - Keefer, C. - Lazaris, A. - Karatzas, C. (2002): Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. Theriogenology, 57. 275-284.

Bartlewski, P. M. (2019): Recent advances in superovulation in sheep. Rev. Bras. Reprod. Anim., 43. 126-128.

Boro, P. - Naha, B. C. - Madkar, A. - Prakash, C. (2016): Sexing of semen in bulls: A mini review. Int. J. Appl. Res., 2. 460-462.

Brasil, O. O. - Moreira, N. H. - Santos, G. - Silva, B. D. - Mariante, A. S. - Ramos, A. F. (2016). Superovulatory and embryo yielding in sheep using increased exposure time to progesterone associated with GnRh agonist. Small Rum. Res., 136. 54-58.

Brem, G. - Wiener, G. (1990): Future biotechnological possibilities in preserving animal germplasm. FAO Animal Production and Health Paper (FAO).

Camargo, L. S. d. A - Viana, J. H. M. - Sá, W. F. d. - Ferreira, A. d. M. - Ramos, A. d. A. - Vale Filho, V. (2018): Factors influencing in vitro embryo production. Anim. Reprod., 3. 19-28.

Cognié, Y. - Baril, G. - Poulin, N. - Mermillod, P. (2003): Current status of embryo technologies in sheep and goat. Theriogenology, 59. 171-188.

Cottle, D. J. (2010): International sheep and wool handbook.

De, K. - Kumar, D. - Sethi, D. - Gulyani, R. - Naqvi, S. M. K. (2015): Estrus synchronization and fixed-time artificial insemination in sheep under field conditions of a semi-arid tropical region. Trop. Anim. Health Prod., 47. 469-472.

Driancourt, M. (2001): Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology, 55. 1211-1239.

Fernández-Gonzalez, R. - Moreira, P. - Bilbao, A. - Jimenez, A. - Perez-Crespo, M. - Ramirez, M. A. - Rodriguez De Fonseca, F. - Pintado, B. - Gutierrez-Adan, A. (2004): Long-term effect of *in vitro* culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behaviour. Proceedings of the National Academy of Sciences of the United States of America. 16. 101. 5880-5885.

Fonseca, J. F. - Souza-Fabjan, J. M. G. - Oliveira, M. E. F. - Leite, C. R. - Nascimento-Penido, P. M. P.-Brandão, F. Z. - Lehloenya, K. C. (2016): Nonsurgical embryo recovery and transfer in sheep and goats. Theriogenology, 86. 144-151.

Fonseca, J. F. - Oliveira, M. E. F. - Brandão, F. Z. - Batista, R. I. - Garcia, A. R .- Bartlewski, P. M. -Souza-Fabjan, J. M. (2019): Non-surgical embryo transfer in goats and sheep: the Brazilian experience. Reprod. Fert. Develop., 31. 17-26.

Fornazari, R. R. - Mateus, Ó. - Correia, T. M. - Quintas, H. - Maurício, R. - Conradi, A. - Francisco, L. F. -Álvaro, A. - Valentim, R. (2018): Estrus synchronization and artificial insemination with fresh and chilled semen in Assaf ewes. Agricult. Sci., 9. 8-22.

Geber, S. - Sales, L. - Sampaio, M. A. (2002): Laboratory techniques for human embryos. Reprod. Biomed. Online, 5. 211-218.

Gibbons, A. - Bonnet, F. P. - Cueto, M. - Catala, M. - Salamone, D. - Gonzalez-Bulnes, A. (2007): Procedure for maximizing oocyte harvest for in vitro embryo production in small ruminants. Reprod. Domest. Anim., 42. 423-426.

Gordon, I. (2017): Reproductive technologies in farm animals. CABI International, UK, 307.

Gupta, A. - Chaudhary, M. - Goyal, R. K. - Yadav, V. - Chandra, S. - Sinha, S. (2018): Recent advances in reproductive biotechnologies in small ruminants. J. Entomol. Zool. Stud., 6. 62-66.

Hoshino, Y. (2018): Updating the markers for oocyte quality evaluation: intracellular temperature as a new index. Reprod. Med. Biol., 17. 434-441.

Hyttel, P. - Pessőa, L. J. B. - Dittlau, K. S. - Freude, K. - Hall, V. J. - Fair, T. - Assey, R. J. - Laurincik, J. - Callesen, H. (2019): Oocytes, embryos and pluripotent stem cells from a biomedical perspective. Anim. Reprod., 16. 508-523.

Jones, G. M. - Trounson, A. O. - Vella, P. J. - Thouas, G. A. - Lolatgis, N. - Wood, C. (2001): Glucose metabolism of human morula and blastocyst-stage embryos and its relationship to viability after transfer. Reprod. Biomed. Online, 4. 124-132.

Ledda, S. - Bogliolo, L. - Calvia, P. - Leoni, G. - Naitana, S. (1997): Meiotic progression and development competence of oocytes collected from juvenile and adult ewes. J. Reprod. Fertil., 109. 73-78.

Kochhar, H. - Wu, B. - Morris, L. - Buckrell, B. - Pollard, J. - Basrur, P. - King, W. (2002): Maturation status, protein synthesis and developmental competence of oocytes derived from lambs and ewes. Reprod. Domest. Anim., 37. 19-25.

Menchaca, A. - dos Santos-Neto, P. C. - Cuadro, F. - Souza-Neves, M. - Crispo, M. (2018): From reproductive technologies to genome editing in small ruminants: An embryo's journey. Anim. Reprod., 15(Suppl1). 984-995.

Menchaca, A. - No, S. P. - Cuadro, F. (2017): Estrus synchronization treatments in sheep: brief update. Rev. Bras. Reprod. Anim., 41. 340-344.

Miranda, V. O. - Oliveira, F. C. - Dias, J. H. - Júnior, S. F. V. - Goularte, K. L. - Sá Filho, M. F. - de Sá F. - Ocilon, G. - Baldassarre, H. - Vieira, A. D. - Lucia Jr, T. (2018): Estrus resynchronization in ewes with unknown pregnancy status. Theriogenology, 106. 103-107.

Mtango, N. R. - Potireddy, S. - Latham, K. E. (2008): Oocyte quality and maternal control of development. International Rev. Cell Mol. Biol., 268. 223-290.

O'Brien, J. - Catt, S. - Ireland, K. - Maxwell, W. - Evans, G. (1997): In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. Theriogenology, 47. 1433-1443.

O'Brien, J. - Dwarte, D. - Ryan, J. - Maxwell, W. - Evans, G. (1996): Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. Reprod. Fertil. Dev., 8. 1029-1037.

O'Brien, J. - Dwarte, D. - Ryan, J. - Maxwell, W. - Evans, G. (2000): Comparison of in vitro maturation, in vitro fertilization, metabolism and ultrastructure of oocytes from prepubertal and adult pigs. Reprod. Domest. Anim., 35. 101-107.

Olivera-Muzante, J. - Gil, J. - Fierro, S. - Menchaca, A. - Rubianes, E. (2011): Alternatives to improve a prostaglandin-based protocol for timed artificial insemination in sheep. Theriogenology, 76. 1501-1507.

Paramio, M. - Izquierdo, D. (2014): Current status of in vitro embryo production in sheep and goats. Reprod. Domest. Anim., 49. 37-48.

Paramio, M. - Izquierdo, D. (2016): Recent advances in in vitro embryo production in small ruminants. Theriogenology, 86. 152-159.

Pinto, P. - Bragança, G. - Balaro, M. - Arashiro, E. - Dos Santos, G. - de Souza, G. - Souza-Fabjan, J. - Da Fonseca, J. - Brandão, F. (2018): Colour-Doppler ultrasound imaging as a laparoscopy substitute to count corpora lutea in superovulated sheep. Reprod. Domest. Anim., 53. 266-269.

Ptak, G. - Loi, P. - Dattena, M. - Tischner, M. - Cappai, P. (1999): Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. Biol. Reprod., 61. 1568-1574.

Ptak, G. - Matsukawa, K. - Palmieri, C. - Salda, L. D. - Scapolo, P. A. - Loi, P. (2006): Developmental and functional evidence of nuclear immaturity in prepubertal oocytes. Hum. Reprod., 21. 2228-2237.

Rahman, A. N. M. A. - Abdullah, R. B. - Wan-Khadijah, W. E. (2009): Effects of oocyte source on the developmental competence of in vitro matured goat oocytes fertilized by the intracytoplasmic sperm injection technique. Turk. J. Vet. Anim. Sci., 33. 323-331.

Ramos, A. F. - Silva, B. D. (2018): Hormonal protocol in small ruminants. In T. G. Bergstein-Galan, & T. G. Bergstein-Galan (Ed.), Reprod. Biotech. Farm Anim., 138 - 154. Avid Science.

Reader, K. - Stanton, J. - Juengel, J. (2017): The role of oocyte organelles in determining developmental competence. Biology, 6. 35.

Rekik, M. - Haile, A. - Abebe, A. - Muluneh, D. - Goshme, S. - Ben Salem, I. - Hilali, M. E. - Lassoued, N. - Chanyalew, Y. - Rischkowsky, B. (2016): GnRH and prostaglandin-based synchronization protocols as alternatives to progestogen-based treatments in sheep. Reprod. Domest. Anim., 51. 924-929.

Rizos, D. - Ward, F. - Duffy, P. - Boland, M. P. - Lonergan, P. (2002): Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev., 61. 234-248.

Romano, J. E. (2019). Hormonal control of estrus in goats and sheep. USA: Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA. Retrieved 10 03, 2019,from: https://www.msdvetmanual.com/management-and-nutrition/hormonal-control-of-estrus/hormonal-control-of-estrus-in-goats-and-sheep.

Sanches, B. V. - Zangirolamo, A. F. - da Silva, N. C. - Morotti, F. - Seneda, M. M. (2018): Cryopreservation of in vitro-produced embryos: challenges for commercial implementation. Anim. Reprod., 14. 521-527.

Teixeira, P. P. M. - Padilha, L. C. - Oliveira, M. E. F. - Motheo, T. F. - da Silva, A. S. L. - Barros F. F. P. C. Coutinho, L. N. - Flőres, F. N. - Lopes, M. C. S. - Bandarra, M. B. - Silva, M. A. M. - Vasconcelos, R. O. - Rodrigues, L. F. S. - Vicente, W. R. R. (2011): Laparoscopic ovum collection in sheep: Gross and microscopic evaluation of the ovary and influence on ooctye production. Anim. Reprod. Sci., 127. 169-175.

Tekim, K. (2019): Cervical insemination with frozen thawed semen in goats at different breeding age. Kocatepe Veterinary Journal, 12. 357-362.

Viana, J. (2017): Statistics of embryo production and transfer in domestic farm animals. Embryo Technology Newsletter, 36. 8-25.

Vilariño, M. - Rubianes, E. - Van Lier, E. - Menchaca, A. (2010): Serum progesterone concentrations, follicular development and time of ovulation using a new progesterone releasing device (DICO®) in sheep. Small Rum. Res., 91. 219-224.

Wani, A. - Khan, M. - Sofi, K. - Malik, A. - Lone, F. - Bhat, F. (2013): Effect of follicular size on in vitro maturation, fertilization and culture of sheep embryos. Iran. J. Vet. Res., 14. 299-309.

Zhu, J. - Moawad, A.R. - Wang, C. - Li, H. - Ren, J. - Dai, Y. (2018): Advances in in vitro production of sheep embryos. Int. J. Vet. Sci. Med., 6. 15-26.

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