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Hyaluronan supplementation enhances *in vitro* maturation and embryo yield in sheep

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Abstract
This study evaluated the impact of hyaluronan supplementation on the *in vitro* maturation (IVM) and developmental competence of sheep embryos. Oocytes collected from abattoir-sourced ovaries were cultured in IVM media with or without hyaluronan. Maturation was assessed via cumulus cell expansion, followed by fertilization using cauda epididymal sperm. Presumptive zygotes were then cultured in synthetic oviduct fluid (SOF), with or without hyaluronan. Results showed significantly higher maturation rates in hyaluronan-treated oocytes compared to controls (88.45% vs 78.30%, $P< 0.01$). Similarly, cleavage rates and embryo yields were significantly improved in the hyaluronan-supplemented group (89.64% vs 55.79%, $P< 0.01$). These effects are likely mediated by hyaluronan's role in cumulus expansion, sperm capacitation, fertilization and early embryogenesis. In conclusion, hyaluronan supplementation enhances both oocyte maturation and embryonic development *in vitro*, offering a promising strategy for improving reproductive efficiency and genetic conservation in sheep.

Keywords: Hyaluronan, zygotes, *in vitro* maturation, morula, embryo

Introduction
Sheep (*Ovis aries*) are among the most globally significant livestock species, playing a vital role in food production, sustaining rural livelihoods and serving as important models in biomedical and reproductive research due to their physiological and genetic parallels with humans (McNatty *et al.,* 2003) [13]. Despite their wide ranging contributions, the genetic diversity of many domestic sheep breeds is increasingly at risk. Factors such as intensive selection for productivity traits, environmental degradation and shifts in agricultural practices have contributed to the ongoing erosion of genetic variation (Taberlet *et al.,* 2008) [19]. Safeguarding this genetic diversity is crucial for ensuring the adaptability and resilience of sheep populations in the face of emerging diseases, climatic fluctuations and evolving market pressures. Genetic variability also forms the foundation for long-term breeding program sustainability and global food security (Notter, 1999) [14]. Consequently, the conservation of ovine genetic resources has been acknowledged as a global priority (Paramio and Izquierdo, 2016) [16]. *In vitro* embryo production (IVEP) technologies have emerged as indispensable tools in this context, facilitating the preservation, multiplication, and distribution of valuable genotypes particularly from rare or endangered breeds (Cognie *et al.,* 2003) [13].
One promising strategy to enhance the efficiency of IVEP involves the use of hyaluronan (HA), a high-molecular-weight glycosaminoglycan abundantly found in the extracellular matrix of various tissues (Laurent and Fraser, 1992) [11]. In reproductive physiology, HA is an essential component of the cumulus-oocyte complex (COC), synthesized by granulosa cells in response to gonadotropic stimulation (Eppig, 1982) [6]. It plays diverse and critical roles, including promoting cumulus expansion, supporting oocyte cytoplasmic and nuclear maturation, aiding sperm capacitation, and enhancing early embryonic development (Gutnisky *et al.,* 2007; Hess *et al.,* 1999) [7, 9]. Additionally, HA influences a range of cellular processes such as cell adhesion, proliferation, migration and programmed cell death through interactions with specific surface receptors like CD44 and RHAMM (Entwistle *et al.,* 1996; Knudson and Knudson, 1993; Thomas *et al.,* 1992; Borland *et al.,* 1998) [10, 20, 2]. Emerging evidence indicates that supplementing culture media with HA can significantly improve oocyte maturation, blastocyst development and overall embryo quality across multiple species,

including sheep (de Figueiredo *et al.*, 2011; Abeydeera, 2002; Palasz *et al.*, 2006) [5, 1, 15]. In human assisted reproduction, the use of HA-enriched transfer media has also been associated with improved implantation and pregnancy outcomes compared to standard albumin-based media (Simon *et al.*, 2003).

Moreover, HA supplementation has been reported to enhance embryonic ultrastructure, modulate the expression of genes involved in development and stress responses and reduce apoptosis rates (Palasz *et al.*, 2006) [15]. These promising results support the continued investigation of HA's role in ovine IVEP systems. Optimizing HA use within embryo culture protocols may enhance embryo yield and viability, thereby advancing efforts in the conservation and sustainable management of sheep genetic resources.

Materials and methods

Ovaries and testes from local sheep were collected at slaughterhouses and transported to the laboratory under sterile conditions. Oocytes were retrieved using the slicing technique and only those classified as Grade A, B or C based on morphological criteria were selected for further processing. After several washes in handling medium, the selected oocytes were placed in 50 µL droplets of *in vitro* maturation (IVM) medium under mineral oil. The droplets were incubated for approximately 24 hours in a humidified atmosphere containing 5% CO_2 at 38.5 °C. Oocyte maturation was assessed by evaluating the degree of cumulus cell expansion under a stereomicroscope.

Experimental design

Experiment 1: Effect of hyaluronan on *in vitro* maturation of sheep oocytes

This experiment aimed to evaluate the influence of hyaluronan supplementation on the i*n vitro* maturation (IVM) of sheep oocytes. Grade A, B, and C cumulus-oocyte complexes (COCs) were collected and subjected to four washes in TCM-199 supplemented with 10% fetal sheep serum (FBS; GIBCO, Invitrogen, USA), followed by a final wash in maturation medium. The IVM medium consisted of TCM-199 supplemented with 10% FBS, 1 µg/mL follicle stimulating hormone (Folltropin), 0.02 IU/mL luteinizing hormone (LH), 1 µg/mL estradiol and hyaluronan. Groups of 10 -15 COCs were transferred into 50 µL droplets of the maturation medium in 35 mm Petri dishes. To prevent evaporation, droplets were overlaid with sterile mineral oil that had been pre-equilibrated in a CO_2 incubator for 2 hours at 38.5 °C with 5% CO_2 in air. The COCs were cultured for 24 hours under the same incubation conditions. Maturation was evaluated based on cumulus cell expansion, which was classified into three degrees. Oocytes exhibiting grade one or two cumulus expansion were considered mature. Each experimental group was replicated six times.

Experiment 2: Effect of hyaluronan on *in vitro* embryo development

This experiment investigated the effect of hyaluronan supplementation in the embryo culture medium on cleavage and subsequent development. Cauda epididymides were collected from local rams and sperm-rich fluid was flushed into a 60 mm Petri dish containing 1 mL of pre-equilibrated sperm-TALP (Tyrode's Albumin Lactate Pyruvate) medium. Progressive motile spermatozoa were isolated using the swim-up technique. For fertilization, 75 µL droplets of IVF-TALP medium supplemented with 10 µg/mL heparin were prepared in 35 mm Petri dishes, overlaid with sterile mineral oil, and pre-equilibrated at 38.5 °C in 5% CO_2. Matured COCs were transferred into the IVF droplets (10-15 oocytes per droplet) and motile sperm were added to achieve a final concentration of 2×10^6 sperm/ml. The gametes were co-incubated for 24 hours at 38.5 °C in a humidified atmosphere of 5% CO_2.

Following fertilization, presumptive zygotes were washed three times in embryo culture (IVC) medium to remove residual spermatozoa and debris. Zygotes were then transferred (10 -15 per droplet) into 50 µL droplets of a two-step synthetic oviduct fluid (SOF) medium, either supplemented with hyaluronan or used without supplementation (control). All droplets were pre-equilibrated and overlaid with mineral oil. Embryos were cultured for six days under standard incubation conditions (38.5 °C, 5% CO_2). Cleavage was assessed at 48 hours post-insemination, and embryo development was monitored every 24 hours up to Day 6. On Days 2 and 5, each droplet received a 10 µl feeding of fresh IVC medium. Embryo development stages including 2-cell, 4-cell, 8-16 cell, morula and compact morula were recorded. Each treatment group was replicated six times.

Statistical analysis

Data were analyzed following the procedures outlined by Snedecor and Cochran (1994). Mean percentages and standard errors (mean % ± S.E.) were calculated for oocyte grades and developmental stages of embryos. The differences in developmental rates between treatment groups were analyzed using the Student's t-test. Statistical significance was determined at the 1% level ($P < 0.01$).

Results and Discussion

Optimizing *in vitro* embryo production (IVEP) protocols particularly through refinement of culture media composition can significantly enhance embryonic development and survival, thereby improving the efficacy of genetic conservation strategies. A key requirement for successful sheep embryo production is the availability of a large number of high-quality oocytes. In this study, the slicing technique was employed for oocyte recovery, as it enables the release of oocytes not only from surface follicles but also from deeper cortical stroma, thus increasing retrieval efficiency (Das *et al.*, 1996; Table 1) [4].

Maturation rates, assessed via cumulus cell expansion (Table 2), were significantly higher in the group supplemented with hyaluronan compared to the control group ($88.45 \pm 1.81\%$ vs $78.30 \pm 1.67\%$, $P < 0.01$). Hyaluronan (HA), a key component of the cumulus-oocyte complex (COC), is synthesized by granulosa cells in response to gonadotropin stimulation (FSH and LH). Its biological roles in reproduction are multifaceted, contributing to cumulus expansion, sperm capacitation, fertilization, and early embryogenesis. These effects are largely mediated by HA's interaction with surface receptors such as CD44, RHAMM, and ICAM-1.

The structural configuration and concentration of HA in the culture environment form a three-dimensional gel-like matrix that enhances cell signaling by increasing the availability and efficacy of growth factor binding. These interactions are crucial for successful oocyte maturation and early embryonic development. In contrast, insufficient HA-CD44 interactions may impair oocyte competence, leading to suboptimal fertilization and developmental potential. Mucification of the COC, characterized by mucus production, appears to play an essential role in matrix stabilization and selective sperm penetration by facilitating sperm orientation and capacitation.

Additionally, HA is a constituent of oviductal and uterine fluids in various species, including cattle and its receptor CD44 has been detected in preimplantation-stage sheep embryos.

Previous work by Yokoo *et al.* (2007) showed that HA-CD44 interaction during cumulus expansion reduces Connexin 43 (Cx43) expression in granulosa cells, thereby interrupting cAMP transfer to oocytes. The resulting drop in intra-oocyte cAMP levels triggers activation of the maturation-promoting factor (MPF), which in turn initiates germinal vesicle breakdown (GVBD) and meiotic resumption. This mechanism underlines HA's regulatory role during oocyte maturation.

While Marei *et al.* (2012) [12] reported that high HA concentrations may be detrimental, our findings support their conclusion that lower concentrations (e.g., 100 µg/mL) enhance nuclear maturation. In the present study, low-dose HA supplementation during maturation resulted in significantly higher cleavage rates (Table 3), with embryos derived from HA-supplemented media achieving an average cleavage rate of 89.64 ± 1.80% compared to 55.79 ± 1.84% in controls.

These results align with findings by Stojkovic *et al.* (1999) [18], who demonstrated improved sheep embryo development with HA enriched culture media. Embryos cultured in HA supplemented media were morphologically comparable to *in vivo* derived embryos and exhibited higher trophectoderm (TE) cell counts. This suggests HA's potential to enhance embryonic structural integrity and developmental competence.

Beyond early development, HA contributes to tissue remodeling processes essential for implantation and morphogenesis. It has been shown to regulate cell migration, proliferation and differentiation key events in embryogenesis and homeostasis (Tootle *et al.,* 1997) [21]. In mice, HA plays a vital role in blastocyst attachment and trophectoderm outgrowth, with its absence linked to degeneration of the inner cell mass (ICM) and loss of trophoblast proliferation (Hamashima *et al.*, 1982) [8].

HA's interaction with hyaladherins and membrane-bound receptors such as CD44 orchestrates cellular behavior, influencing adhesion, differentiation and intercellular communication. Elevated HA levels during estrus and ovulation further underscore its importance in pre-fertilization and early embryonic events.

In assisted reproductive technologies (ART), HA has been explored as an alternative to serum albumin in culture media. Simon *et al.* (2003) reported its safe use in human embryo culture, showing no adverse effects on frozen-thawed embryo development. Furthermore, HA may serve as a cryoprotective agent, preserving embryo viability during freezing and thawing procedures.

Taken together, the present study and supporting literature suggest that hyaluronan supplementation when used at appropriate concentrations can significantly improve oocyte maturation and embryo development in sheep. Its multifactorial biological functions offer promising avenues for enhancing IVEP outcomes and contributing to the conservation and sustainable utilization of ovine genetic resources.

Table 1: Oocyte number, quality and recovery rate from sheep ovaries using the slicing method

No. of ovaries used	Different Grades of oocytes recovered					
	Grades	**A**	**B**	**C**	**D**	**Total**
165	No. of oocytes	210	254	135	66	665
	Percentage	31.58	38.20	20.30	9.92	100
	Number per ovary (Mean ± SE)	1.31±0.10	1.59±0.15	0.83±0.05	0.41±0.30	4.14±0.29

Table 2: Effect of hyaluronan on *in vitro* maturation of sheep oocytes

***In vitro* maturation medium**	**No. of immature oocyte cultured**	**No. of oocytes matured on cumulus cells**
With Hyaluronan	302	267 (88.45±1.81)[a]
Without Hyaluronan	297	232 (78.30±1.67)[b]

Values with different superscripts within the same column differ significantly (P<0.01)

Table 3: Influence of hyaluronan on *in vitro* culture of sheep embryos

Group	**No. of oocytes used**	**Cleavage rate**	**Number that developed to (Mean percentage ± SE)**		
			4 cells	**8 cells to 16 cells**	**Morula**
With Hyaluronan	267	239(89.64±1.80)[a]	212(79.48±1.59)[a]	196(73.36±1.71)[a]	153(57.70±3.25)[a]
Without Hyaluronan	232	129(55.79±1.84)[b]	106(45.89±5.06) b	87(37.64±4.31) [b]	68(29.36±3.48) [b]

Values with different superscripts within the same column differ significantly (P<0.01)

Summary

This study assessed hyaluronan supplementation in *in vitro* culture systems on sheep embryo development. Oocytes from abattoir sourced ovaries were matured and fertilized *in vitro,* then cultured with or without hyaluronan. Maturation rates were higher with hyaluronan (88.45% vs 78.30%), as were embryo yields (57.70% vs 29.36%). These results indicate that hyaluronan enhances oocyte competence and embryo production, making it a valuable additive for improving sheep IVEP efficiency and supporting genetic conservation in small ruminants.

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Author's Contribution

Not available

Conflict of Interest

Not available

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