RESEARCH ARTICLE

Enhancing Pregnancy Outcomes in Cattle: Effect of Hyaluronan-Enriched Embryo Transfer Medium on *In Vitro* Produced Embryos

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Abstract

Suboptimal pregnancy rates following the transfer of *in vitro* produced (IVP) bovine embryos remain a significant constraint in livestock genetic improvement programs. This study evaluated whether supplementing embryo transfer media with hyaluronan (HA), a key biomolecule in implantation, could improve pregnancy outcomes. Bovine IVP embryos were allocated to four treatment groups for transfer: two commercial media (ET-HEPES; IMV holding) and two in-house prepared media (with 0.5% HA; without HA). Key outcomes measured were *in vitro* hatching rate after a 24 h of incubation simulating transport, early embryonic death (EED) rate, and pregnancy rate at day 60 post-transfer in 120 recipient cattle (n=30 per group). The in-house medium with 0.5% HA yielded the highest pregnancy rate (20.0%), which was numerically superior to ET-HEPES (16.67%), IMV holding media (13.33%), and the non-supplemented in-house medium (3.33%). Furthermore, the non-supplemented medium resulted in a markedly higher EED rate (83.33%) compared to the HA-supplemented (33.33%) and commercial media groups. It is concluded that supplementing in-house embryo transfer media with 0.5% hyaluronan enhances pregnancy outcomes and represents a cost-effective and biologically superior alternative to standard commercial media for bovine embryo transfer programs.

Key words: Bovine, Embryo Transfer, Hyaluronan, *In Vitro* Embryo Production, Pregnancy Rate.

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Introduction

India stands as the world's leading milk producer, with a total output of 239.20 million tonnes in 2023-24, supported by the largest global bovine population of 303.76 million. Over the last few decades, per capita milk availability has increased significantly, yet the average milk yield per animal remains considerably below international standards. This productivity gap is attributed to factors including suboptimal genetic potential and environmental challenges. To bridge this gap, assisted reproductive technologies (ARTs) have become indispensable tools. While artificial insemination (AI) has been foundational for disseminating superior bull genetics, embryo transfer technology (ETT) and *in vitro* embryo production (IVEP) offer powerful pathways to amplify the genetic contributions of elite females, thereby accelerating genetic gain within the national herd (Faizah *et al.,* 2018).

The global adoption of IVEP has surged dramatically. In 2022, *in vitro* produced (IVP) embryos accounted for 80.4% of all transferable cattle embryos worldwide, reflecting a major paradigm shift away from *in vivo* derived embryos (Viana, 2023). This technology allows for the rapid multiplication of desirable traits from genetically superior donors, complementing modern genomic selection programs that identify elite animals at a young age (Taylor *et al.,* 2018). However, the full potential of IVEP is consistently hampered by a critical bottleneck: suboptimal pregnancy rates and high instances of early embryonic mortality following transfer (Hansen, 2020). Pregnancy retention with IVP embryos is often lower than anticipated, with early embryonic losses accounting for up to 40% of pregnancy failures in dairy cattle (Diskin *et al.,* 2016). This persistent challenge is attributed to a combination of embryonic, maternal, and environmental

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factors, as well as inherent differences between the *in vivo* and *in vitro* developmental environments (Hansen, 2020).

Successful establishment of pregnancy hinges on the intricate process of implantation, which requires a synchronized molecular dialogue between the embryo and a receptive uterine endometrium (Pedersen *et al.,* 2018). This process involves a complex interplay of cell-adhesion molecules, such as integrins, and the downregulation of anti-adhesive molecules on the endometrial surface (Aplin, 1997). A key facilitator of this interaction is hyaluronan (HA), a high-molecular-weight glycosaminoglycan naturally abundant in follicular, oviductal, and uterine fluids (Fu *et al.,* 2018). Secreted during the mid-proliferative and late secretory phases of the estrous cycle, HA performs multiple functions critical for implantation. Its high viscosity helps prevent embryo expulsion from the uterus, while its biochemical properties promote cell adhesion, migration, and tissue hydration (Heymann *et al.,* 2020). Crucially, the primary cell surface receptor for HA, CD44, is expressed on the bovine pre-implantation embryo, enabling the initial binding to the endometrium that precedes placentome formation (Aruffo *et al.,* 1990). The expression of CD44 is notably downregulated on the embryonic surface after implantation is complete, highlighting its specific role in the initial attachment phase (Campbell *et al.,* 1995).

Given that implantation failure is a primary cause of pregnancy loss with IVP embryos, and HA is biologically integral to the attachment process, supplementing embryo transfer media with HA presents a logical and targeted strategy to enhance success rates (Reena *et al.,* 2020). Therefore, the present study was designed to investigate the effect of a hyaluronan-enriched embryo transfer medium on embryo viability and pregnancy outcomes in cattle. The specific objectives were: (1) to compare the *in vitro* survivability of embryos transported in hyaluronan-enriched media versus commercial media; (2) to assess the impact of these media on early embryonic death rates; and (3) to compare the resulting pregnancy rates following embryo transfer.

Materials and Methods

The research was conducted at the Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, in collaboration with the R & D and Training facility on OPU-IVEP-ET at the National Dairy Development Board (NDDB), Anand, Gujarat (India). Recipient animals were sourced from the milk shed areas of Sabarkantha and Mehsana District Co-operative Milk Producers Unions Ltd of Gujarat.

Genetically superior donor cows (Gir, Sahiwal, and Holstein Friesian crossbreds) and sires (Gir, Sahiwal, and pure Holstein Friesian) were selected for the study based on their genetic merit and reproductive history.

In Vitro Embryo Production

OPU-IVEP procedures were carried out using the standard technique, with modifications adapted for Indian dairy cattle, following the standardized protocol established in the laboratory (Patil *et al.,* 2022). Briefly, transvaginal follicular aspiration was conducted under epidural anesthesia using an ultrasound-guided aspiration device. All follicles ≥3 mm in diameter were aspirated into collection tubes containing OPU medium. Immediately after aspiration, the tubes were sealed and transported to laboratory for further processing.

Immature oocytes were subjected to *in vitro* maturation (IVM) for 22 h in incubator with 5% CO_2 in air (O_2 concentration around 14-16%), 38.5 °C temperature and more than 90% relative humidity, followed by *in vitro* fertilization (IVF) for 18 h in incubator with the same climatic conditions. Presumptive zygotes (fertilized oocytes) were then cultured *in vitro* for up to 7 days in a benchtop incubator (38.5 °C, 5% CO_2, 5% O_2, 90% N_2, ≥90% RH).

Embryo Transfer Media

In the study, five different embryo transfer media were evaluated by transferring fresh embryos to the transfer media from the culture media at day 7 post-IVF, *viz.,* (i) ET-HEPES (Vitrogen), (ii) Embryo Holding Media (IMV Technologies), (iii) In House Media without Hyaluronan, (iv) In House Media with Hyaluronan (Concentration 0.5%) and (v) In House Media with Hyaluronan (Concentration 0.025%)

Embryo Survivability

In the study, the recipient animals were located far from the laboratory. Therefore, embryos were transported to the field using a transport incubator (WTA, LABMIX, Brazil). To simulate this condition, on day 7 post *in vitro* fertilization fresh embryos of grade 1 (blastocyst and expanded blastocyst) were loaded into 1.2 mL tubes containing 500 µL of transfer media from different groups, overlaid with 300 µL of embryo-tested oil. The tubes were then placed in an incubator at 38.5°C for 24 h with the caps tightly closed. After incubation, the embryos were examined to assess the hatching rate.

Recipient Animals and Embryo Transfer

A total of 120 cyclic and reproductively sound recipient heifers and cows with a body condition score (BCS) of 3.0-3.5 were selected from field. The recipient animals were randomly and equally divided into four groups each of 30 heads with respect to which embryo transfer medium i to iv was used. Considering poor hatching performance, the 0.025% HA medium was excluded from the subsequent embryo transfer trials.

Estrus synchronization was performed via a single intramuscular injection of 500 µg Cloprostenol sodium ($PGF_2\alpha$) two days prior to the donor's OPU session, aligning the recipient's cycle for a day 7 embryo transfer. Embryos were transported from the laboratory to the field locations in a transport incubator maintained at 38.5°C. Upon reaching

the location, the embryos were carefully loaded into 0.25 mL straws using an embryo loading device. Only grade 1 blastocyst and expanded blastocyst were selected for embryo transfer. Embryos were transferred non-surgically close to the tip of horn ipsilateral to the corpus luteum (CL) using a Cassou embryo transfer gun.

Pregnancy Diagnosis

Early pregnancy diagnosis was conducted on day 28 post-estrus using a rapid test kit (BovEasy, Prompt Equipment Pvt. Ltd., Ahmedabad) on whole blood samples. Final pregnancy status was confirmed via per-rectal palpation on day 60 post-estrus (Ciplak, 2024). The early embryonic death (EED) rate was calculated as the percentage of pregnancies lost between day 28 and day 60. The final pregnancy rate was defined as the percentage of recipients confirmed pregnant at day 60.

Statistical Analysis

Data on hatching rates, EED rates, and pregnancy rates were analyzed using one-way ANOVA. Statistical significance was set at $p<0.05$. All analyses were performed following the methods described by Snedecor and Cochran (2014).

Results and Discussion

Comparison of Survivability (Hatching) Rate of Embryos between Different Media

The ability of a transfer medium to maintain embryo viability during transport from the laboratory to the field is a critical determinant of success in large-scale embryo transfer programs. The 24 h *in vitro* incubation assay was designed to directly simulate these logistical conditions and assess the protective capacity of each medium. The results, summarized in Table 1, revealed significant differences in embryo hatching rates among the groups. The commercial medium ET-HEPES demonstrated the highest hatching rate of 87.30 ± 12.70%, which was significantly greater than that observed in the in-house media supplemented with 0.025% HA. This superior performance likely reflects a highly optimized formulation containing an ideal balance of energy substrates, amino acids, and buffering agents (HEPES) designed to support embryo metabolism and homeostasis outside of standard culture conditions (Hasler, 2009). Historically, media were often supplemented with serum, but this practice has been linked to developmental abnormalities such as Large Offspring Syndrome, leading to the widespread adoption of defined, serum-free formulations (Farin *et al.,* 2001; Rooke *et al.,* 2007).

Notably, the in-house medium supplemented with 0.5% HA achieved a hatching rate of 78.98± 02.30%, which was statistically comparable to ET-HEPES and numerically superior to the other media. In contrast, the lower concentration of 0.025% HA resulted in the lowest survivability (55.00±05.00%), suggesting that a biological threshold concentration of HA is necessary to confer its beneficial effects. These effects include maintaining the viscosity and colloidal osmotic balance of the medium and enhancing tissue hydration, which are crucial for protecting the embryo from physical and metabolic stress during handling and transport (Heymann *et al.,* 2020). The efficacy of the 0.5% HA concentration aligned with previous reports in both human and bovine ART, where similar concentrations have been shown to improve developmental outcomes (Gardner *et al.,* 1999; Reena *et al.,* 2020; Karadbhajne and More, 2022). Based on poor performance in this assay, the 0.025% HA medium was excluded from the subsequent embryo transfer trials.

Table 1: Comparison of *in vitro* hatching rates of bovine embryos incubated for 24 h in different transfer media (Mean ± SEM)

Media	No. of embryos	Mean hatching rate (%)
ET-HEPES	45	87.30 ± 12.70[a]
IMV holding	45	66.67 ± 06.67[ab]
In house media (without H)	45	66.21 ± 04.65[ab]
In house media (with H-0.5%)	45	78.98± 02.3[0a]b
In house media (with H-0.025%)	45	55.00 ± 05.00[b]

Values in the same column bearing different superscripts differ significantly ($p<0.05$).

Comparison of Early Embryonic Death (EED) Rate between Different Media

The study assessed the mean early embryonic death (EED) rates to compare the efficacy of different media formulations in supporting early embryonic survival and minimizing pregnancy loss between day 28 and day 60 post-transfer. The results summarized in Table 2, revealed significant differences among the groups.

The in-house media without hyaluronan showed a strikingly high EED rate of 83.33 ± 16.67%. This finding highlights the considerable challenge of using a basic in-house medium lacking supportive additive, as embryos transferred in this medium were far less likely to progress to successful pregnancies. The high EED rate underscores the critical role that media composition plays in early embryonic survival. In contrast, the commercial and HA-supplemented media performed markedly better. The EED rate for the in-house media with 0.5% hyaluronan was 33.33 ± 16.67%, a rate comparable to that of the IMV holding media (33.33 ± 21.08%). The ET-HEPES media group had a slightly higher EED rate of 37.50 ± 18.30%. The dramatically lower EED rates in these groups compared to the non-supplemented in-house media emphasize the importance of optimized formulations. These results aligned with previous studies highlighting that significant pregnancy losses following IVP embryo transfer occur during the first six weeks of gestation due to developmental anomalies (Ealy *et al.,* 2019). The unique properties of hyaluronan, including viscosity, lubrication, and cell adhesion, are critical for facilitating this attachment process. Moreover, hyaluronan regulates key biological functions such as cell migration, protein secretion, gene expression, and cell proliferation, all of which are vital for

successful implantation and early embryonic development, through these functions, hyaluronan contributes to successful implantation and early development, ultimately lowering the incidence of early embryonic loss (Stojkovic *et al.,* 2002). The inclusion of hyaluronan appears to mitigate early embryonic losses, likely by creating a more optimal microenvironment that supports cellular functions, embryo development, and successful implantation, thereby reducing early pregnancy failure (Nakagawa *et al.,* 2012).

Comparison of Pregnancy Rate between Hyaluronan-Enriched and Commercial Media

The study evaluated the mean pregnancy rates at day 60 post-transfer to determine how different media formulations affected the ability of embryos to establish and maintain pregnancies. The results, summarized in Table 3, indicated notable differences in pregnancy success among the media formulations. The in-house media supplemented with 0.5% hyaluronan showed the highest mean pregnancy rate of 20.0 ± 7.42%. This positive impact suggests that hyaluronan plays a significant role in enhancing embryo implantation and pregnancy establishment. This is consistent with previous findings where hyaluronan supplementation improved conception and pregnancy rates (Gardner *et al.,* 1999; Simon *et al.,* 2003; Nakagawa *et al.,* 2012; Reena *et al.,* 2020).

The commercial media groups yielded moderate results. ET-HEPES media resulted in a pregnancy rate of 16.67 ± 6.92%, while the IMV holding media yielded a rate of 13.33 ± 6.31%. These rates, while standard for the industry, were numerically lower than that achieved with the HA-supplemented in-house medium (20.0 ± 07.42%). The in-house media without hyaluronan demonstrated the lowest mean pregnancy rate of 3.33 ± 3.33%. This result highlights the critical need for supportive macromolecules in transfer media. Zhu *et al.* (2013) reported that primary trophoblasts produce high levels of hyaluronan, which promotes proliferation and invasiveness of trophoblasts while suppressing apoptosis via the PI3K/AKT and MAPK/ERK1/2 signaling pathways. Their findings also highlighted the crucial role of hyaluronan in implantation, placentation, and the maintenance of normal pregnancy. Hyaluronan has been shown to enhance blastocyst development and cell counts compared to media containing only BSA (Furnus *et al.,* 1998; Stojkovic *et al.,* 2002) and to improve implantation and fetal development rates (Gardner *et al.,* 1999). The results of the present study affirm these benefits, demonstrating that the inclusion of hyaluronan can transform a basic in-house medium into a highly effective formulation for improving pregnancy outcomes in cattle.

Conclusion

The findings of this study highlight the critical role of transfer medium composition in determining the success of bovine embryo transfer. The in-house medium supplemented with 0.5% hyaluronan not only preserved embryo viability under simulated transport conditions, comparable to a leading commercial medium, but also achieved the highest pregnancy rate with a markedly reduced incidence of early embryonic loss. These results suggest that supplementation with 0.5% hyaluronan provides a biologically superior, cost-effective alternative to currently available commercial media, with the potential to enhance the efficiency of ART programs and accelerate genetic gain in the Indian livestock sector. Nonetheless, the present findings are based on a limited number of embryo transfer in recipient animals, restricting the statistical strength of the conclusions. Therefore, further large-scale studies are warranted to validate these preliminary observations, optimize the concentration of hyaluronan, and investigate its synergistic interactions with other bioactive molecules. Such efforts could contribute

Table 2: Mean early embryonic death rate of embryos between hyaluronan-enriched media and commercial media (Mean ± SEM)

Media	No. of ET	No. early positive at 28 days	No. confirm positive at 60 days	Mean EED rate (%)
ET-HEPES	30	08	05	37.50 ± 18.30[a]
IMV holding	30	06	04	33.33 ± 21.08[a]
In house media (without H)	30	06	01	83.33 ± 16.67[a]
In house media (with H-0.5%)	30	09	06	33.33 ± 16.67[a]

Different superscripts (a) indicate no significant difference between the groups (p>0.05).

Table 3: Mean pregnancy rate (%) observed between hyaluronan-enriched media in comparison with commercial media (Mean ± SEM)

Media	No. of ET	No. confirm positive at 60 days	Mean pregnancy rate (%)
ET-HEPES	30	05	16.67 ± 6.92[a]
IMV holding	30	04	13.33 ± 6.31[a]
In house media (without H)	30	01	03.33 ± 03.33[a]
In house media (with H-0.5%)	30	06	20.0 ± 07.42[a]

Different superscripts (a) indicate no significant difference between the groups (p>0.05).

substantially to reducing embryonic losses and improving the overall success of OPU-IVEP-ET programs in cattle.

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