Eurasia Specialized Veterinary Publication

International Journal of Veterinary Research and Allied Science
ISSN:3062-357X

2022, Volume 2, Issue 2, Page No: 61-68

Available online at: www.esvpub.com/

Synergistic Effects of Heparin and Progesterone on In Vitro Fertilization Rates of Bovine Sperm Bound to Isthmus-Derived Oviduct Cell Aggregates

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ABSTRACT

Following mating, only a subset of spermatozoa begin an extensive migration toward their final objective—the fertilization of the oocyte. Within the oviduct, these sperm are temporarily retained, attaching to the upper membrane of epithelial cells, forming a "sperm nest." This reservoir performs several crucial tasks: maintaining sperm contact with epithelial cells, filtering viable and structurally sound spermatozoa, and ultimately prolonging their functional lifespan. The present work investigated the fertilization competence of sperm adhered to cellular aggregates obtained from three oviductal sections (infundibulum, ampulla, and isthmus). Additionally, it examined how heparin and/or progesterone (P4) influence the in vitro fertilization efficiency of sperm preincubated with isthmic aggregates. In vitro fertilization was defined by the cleavage of an oocyte into two or more blastomeres. The sperm attached to isthmic aggregates showed the highest fertilization percentage (48.09%) compared with those associated with infundibular (36.90%) or ampullary (37.61%) tissue. Moreover, treating mature COCs with heparin (40 µg/mL) combined with P4 (80 nM) produced a joint stimulatory effect, raising the in-vitro fertilization rate of sperm attached to isthmic aggregates to 63.33%, in contrast with 42.61% in the absence of cell aggregates. Overall, sperm interaction with isthmic cell clusters enhances bovine sperm fertilizing efficiency, and the joint presence of heparin and progesterone further potentiates this effect.

Keywords: Bovine, Oviduct, Cellular aggregates, Fertilization, Heparin, Progesterone

Received: 21 May 2022
Revised: 16 August 2022
Accepted: 19 August 2022

How to Cite This Article: Laurent J, Lefèvre A, Moreau C. Synergistic Effects of Heparin and Progesterone on In Vitro Fertilization Rates of Bovine Sperm Bound to Isthmus-Derived Oviduct Cell Aggregates. Int J Vet Res Allied Sci. 2022;2(2):61-8. https://doi.org/10.51847/7sn1X7QyAd

Introduction

To achieve successful fertilization, sperm must overcome several physiological barriers before reaching the oocyte. Compared to the majority that fail to enter the oviduct, sperm reaching the isthmus are generally characterized by superior morphology and greater zona pellucida-binding capacity [1]. The interaction between sperm and oviductal cells has been modeled in vitro across several species, including cattle [2], pigs [3], and horses [4]. The oviduct provides a temporary environment for sperm retention between insemination and fertilization through a finely tuned biological system. Experimental evidence suggests that both the epithelial lining and oviductal fluid help sustain sperm activity in cattle, likely by offering metabolizable substrates [5].
Research has shown that adhesion between spermatozoa and epithelial cells increases sperm survival [4], enhances fertilizing capability [6], maintains zona pellucida affinity, and prolongs sperm viability [7]. The oviductal reservoir selectively keeps morphologically intact, non-capacitated sperm while minimizing chromatin modification [8]. Such adhesion not only supports sperm longevity but also enriches spermatozoa with improved fertilization capacity [9]. Additionally, culture systems using epithelial cell-conditioned medium [10] or direct sperm–cell co-cultures [11] demonstrate beneficial impacts on motility.

Different in-vitro models have been adopted to investigate sperm–oviduct interactions, such as epithelial monolayers [12] and tissue explants [13]. A variety of supplements have been added to these systems, influencing both sperm endurance and fertilization performance [14]. Among these, progesterone and heparin have received particular attention for their roles in regulating sperm–cell binding and capacitation. Studies in pigs revealed that nanogram concentrations of progesterone (P4) stimulate sperm release from epithelial monolayers, leading to improved in-vitro fertilization outcomes [14]. Heparin has been shown to initiate hyperactivation [15], induce capacitation in cattle [16] and horses [17], and promote sperm detachment from epithelial layers [18]. In this research, cell aggregates were chosen instead of monolayers to better represent the spatial interaction between sperm and oviductal cells. The goals were twofold: (1) to assess fertilization performance of sperm adhering to cell aggregates from distinct oviductal areas (infundibulum, ampulla, and isthmus); and (2) to determine the contribution of heparin and/or progesterone to the in-vitro fertilization rate of sperm preincubated with isthmic cell aggregates.

Materials and Methods

All media components and reagents for preparing oviductal tissue were purchased from Sigma Aldrich (St. Louis, MO, USA) unless noted otherwise.

Experiment 1: influence of sperm preincubation with cell aggregates from various oviductal sections on in-vitro fertilization rate

This experiment examined how exposing bovine sperm to cell aggregates derived from specific oviductal regions affects the in-vitro fertilization outcome **(Figure 1)**.

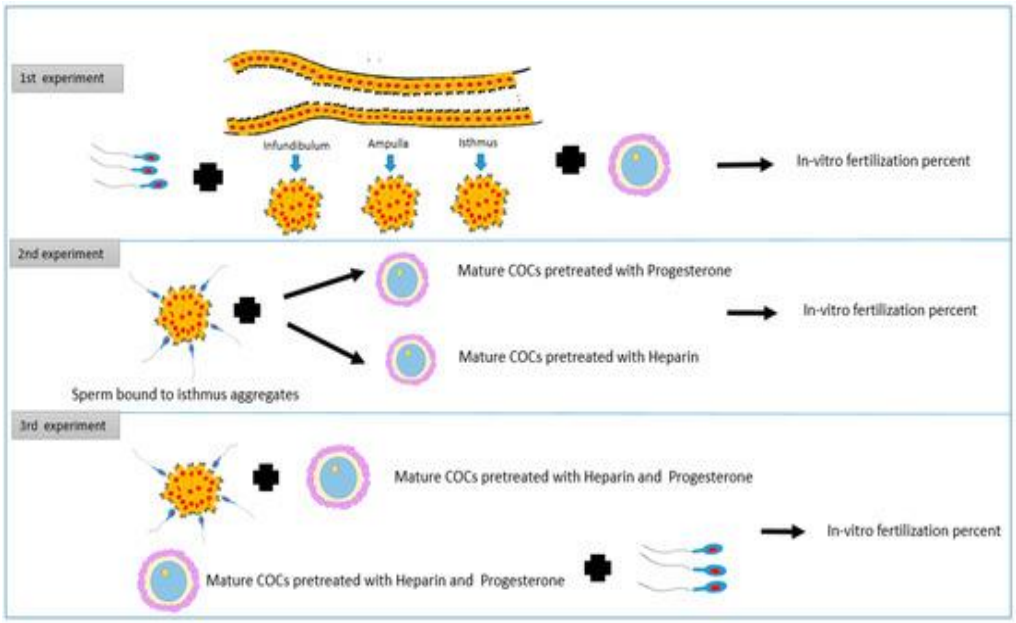
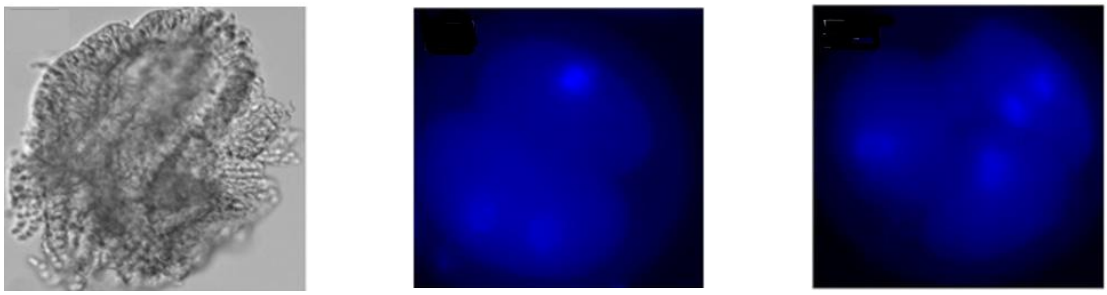


Figure 1. Overview of the three experimental phases: the first tested sperm preincubation with aggregates from different oviduct sections on fertilization results; the second analyzed the impact of adding mature COCs treated with either heparin or progesterone to sperm preincubated with isthmic aggregates; and the third explored the combined influence of both heparin and progesterone on fertilization when applied under the same conditions.

A total of 663 oocytes were used. Epithelial tissues were isolated from the infundibulum, ampulla, and isthmus, processed in dmTALP medium, and assembled into aggregates. Frozen-thawed bovine semen was allowed to interact with these aggregates for 30 minutes at 39 °C**.** Subsequently, mature COCs were introduced to each group, along with a control containing free sperm only. All sets were incubated for 8–12 hours under identical environmental conditions **(**39 °C**,** 5% CO_2**,** 95% humidity**)** to facilitate fertilization. The resulting fertilization rates were recorded across all treatments **(Figure 2)**.



a) b) c)

Figure 2. (a) Oviductal cell aggregates range from 100–150 µm in diameter. (b ,c) display embryos at the 3-cell and 4-cell stages, respectively (×400 magnification).

Experiment 2: effect of supplementing sperm preincubated with isthmus-derived cell aggregates with mature COCs exposed to heparin or progesterone on in-yitro fertilization efficiency

This trial examined how exposing mature COCs to heparin (40 µg/mL) or progesterone (80 nM) alters fertilization outcomes when combined with sperm that had been previously associated with epithelial aggregates from the isthmus. A total of 914 oocytes were utilized.

In Group 1, untreated mature COCs were combined directly with free sperm. Groups 2 and 3 included mature COCs pre-exposed to progesterone (80 nM) and heparin (40 µg/mL)**,** respectively, before being mixed with free sperm. For Groups 4 and 5, sperm were first incubated with isthmus cell aggregates and then supplemented with mature COCs that had been pretreated with either heparin (40 µg/mL) or progesterone (80 nM)**.** All treatments used the same sperm density, and fertilization was allowed to occur for 8–12 hours under identical environmental parameters **(**5% CO_2**,** 39 °C**,** 95% relative humidity**).** Fertilization percentages were then calculated for each group. In this experiment, only isthmus-sourced epithelial aggregates were employed, as indicated by results from the earlier trial and under equivalent handling conditions **(Figure 3)**.

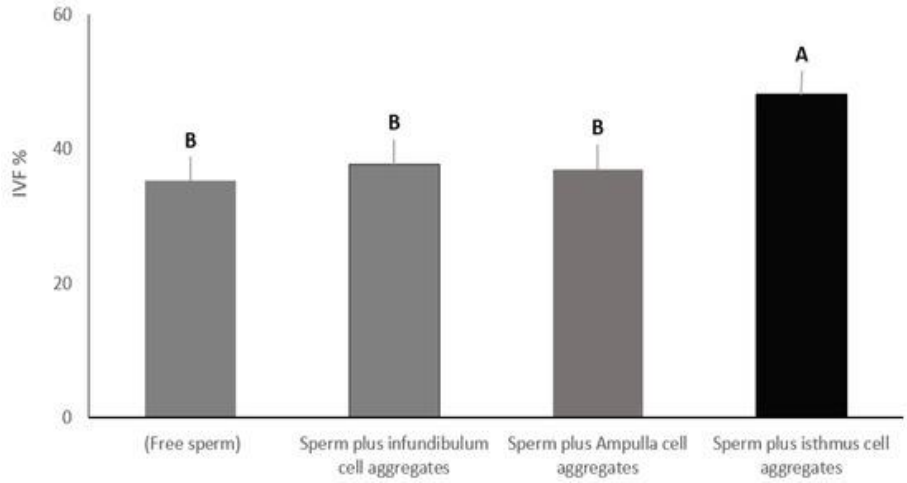


Figure 3. Aggregates from the infundibulum**,** ampulla**,** and isthmus were separately combined with sperm, followed by the addition of mature COCs for co-culture during IVF. Columns bearing distinct letters differ significantly ($p < 0.01$).

Experiment 3: combined influence of heparin and progesterone on fertilization when added to sperm previously co-cultured with isthmus aggregates

This phase evaluated whether simultaneous exposure to both heparin and progesterone (P4) influences in vitro fertilization success. The total number of oocytes used was 589.

In Group 1, untreated mature COCs were incubated with free sperm. Group 2 contained COCs exposed to both heparin (40 µg/mL) and progesterone (80 nM**)** together with free sperm. Group 3 included sperm first co-cultured with isthmus cell aggregates and then introduced to COCs pretreated with the same heparin–progesterone combination. The sperm concentration remained uniform for all treatments. Each group underwent fertilization

for 8–12 hours under the same incubation settings **(**5% CO_2**,** 39 °C**,** 95% humidity), and subsequent fertilization rates were determined.

Recovery of oviduct epithelial cells

Oviducts were sourced from normal pubertal females at a local abattoir and kept on ice during transport. The infundibulum, ampulla, and isthmus were dissected, rinsed in PBS, and their epithelial linings carefully stripped using a glass slide positioned at roughly 45°. The collected sheets were transferred into PBS and centrifuged for 1 minute at 84× g in 15 mL Falcon tubes. The supernatant was removed, and the cells were repeatedly pipetted (10 strokes with a 1000 µL tip) to disperse them. The volume was adjusted to 15 mL with PBS and centrifuged again for 1 minute at 84× g. The resulting pellet was gently fragmented by passing it 10 times through a 22-gauge syringe needle. The suspension was brought to 12 mL with dmTALP medium and distributed into 100 mm culture dishes in three equal parts. Incubation was carried out for 90 minutes at 39 °C to promote re-aggregation, and spherical clusters ranging from 100 to 150 µm were selected for sperm-binding tests [19].

Sperm preparation

Semen pooled from three bulls was used. Straws were thawed in a 39 °C water bath for 1 minute. To evaluate binding to epithelial cells from various oviduct regions, sperm were suspended in dmTALP buffer (pH 7.4) containing 100 mM NaCl, 3.1 mM KCl, 1.5 mM $MgCl_2$, 2.1 mM $CaCl_2$, 0.29 mM KH_2PO_4, 25 mM HEPES, one mM sodium pyruvate, 21.6 mM lactate, 6 mg/mL BSA, 100 U/mL penicillin, and 100 mg/mL streptomycin.

For motile sperm selection, 50 µL of thawed semen was layered under 2 mL of medium and incubated for 1 hour at 39 °C to allow swim-up. The top 1 mL fraction was pooled, transferred to sterile 15 mL tubes, and centrifuged at 600× g for 5 minutes [20].

Quantifying sperm binding to oviduct aggregates

Aggregates measuring 100–150 µm were rinsed twice in 100 µL droplets of dmTALP medium. Each sperm droplet received 20 aggregates and a final sperm concentration of 1.6×10^6 cells/mL. All tests were carried out in triplicate. Sperm and aggregates were co-incubated at 39 °C for 4 hours to allow adhesion. Loose sperm were washed away through gentle pipetting. Bound sperm were visualized and documented using a Zeiss Axioskop microscope [21]. For subsequent IVF procedures, the aggregates were washed again to remove loosely attached sperm and re-suspended in fresh medium.

Oocyte processing

Oocytes were cultured in four-well Petri dishes, each containing 150 µL of TCM-199 medium enriched with 10% fetal calf serum, 50 µM cysteamine, and 50 µg/mL gentamycin sulfate. Each drop contained roughly 10–15 oocytes, which were covered with mineral oil and incubated for 22 hours under 5% CO_2, 39 °C, and 95% humidity conditions [22].

In-vitro fertilization (IVF) and embryo culture (IVC)

Mature COCs were pre-exposed to heparin (40 µg/mL) and/or progesterone (80 nM) in dmTALP medium. Fertilization was carried out by co-culturing sperm and COCs for 8–12 hours at 5% CO_2, 39 °C, and 95% humidity. After fertilization, oocytes were washed with TCM-199 to remove attached sperm and vortexed for 2–4 minutes in 1 mL tubes. Sets of 10–20 oocytes were transferred to 100 µL droplets of pre-equilibrated TCM-199 containing 10% fetal calf serum and 50 µg/mL gentamycin. After 48 hours, pronuclear formation and cleavage were evaluated, and embryo counts were recorded using an inverted microscope [23, 24].

Nuclear staining of embryos

The nuclear labeling of embryos followed the approach described by Siqueira and Hansen [25]. Embryos were successively transferred between microdroplets to eliminate culture residues and washed twice in 100 µL PBS containing 1 mg/mL polyvinylpyrrolidone (PVP). Fixation was performed in 100 µL paraformaldehyde solution [4% (w/v) in PBS, pH 7.4] for 1 hour at 22 °C. After fixation, embryos were rinsed three additional times with 100 µL PBS supplemented with PVP and immersed in a 50 µL droplet of Hoechst 33,342 (1 µg/mL) for 10 minutes, followed by two washes. Subsequently, they were mounted in a minimal volume on poly-l-lysine–coated slides and air-dried for 15 minutes at ambient temperature. The slide area containing embryos was marked

underneath with a diamond pen, and 2–16 µL of antifade reagent was applied to that location. Slides were covered, kept in darkness for 2 hours to dry, and examined under a fluorescence microscope for nuclear observation and counting **(Figures 2b and 2c)**.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Microsoft Excel (Windows version 2019). Tukey's post-hoc test was applied for mean comparison, and statistical differences were considered significant when $p < 0.05$. The model included the following fixed factors: oviduct segment, sperm–explant co-incubation, and treatments with heparin and/or progesterone.

Results and Discussion

Experiment 1: influence of sperm preincubation with oviduct cell aggregates from different segments on in-vitro fertilization rate

To determine how sperm pre-exposure to cells from different oviduct sections affects IVF efficiency, four groups were tested. Group 1 contained untreated sperm (control). Group 2 included sperm combined with infundibulum cell aggregates, group 3 with ampulla cell aggregates, and group 4 with isthmus cell aggregates. Identical sperm concentrations were used across groups. Sperm–cell co-incubation was performed at 39 °C for 4 hours to permit sperm binding, after which mature COCs were introduced and co-cultured for 8–12 hours (5% CO_2, 39 °C, 95% humidity). The number of oocytes exhibiting two-cell cleavage or pronuclear formation was recorded. The IVF rate in the isthmus aggregate group (48.09%) was markedly higher ($p < 0.01$) than in the control (35.23%), ampulla (37.61%), and infundibulum (36.90%) groups. No notable differences appeared among the latter three groups **(Figure 3)**.

Experiment 2: effect of adding heparin- or progesterone-treated mature COCs to sperm preincubated with isthmus cell aggregates on in-vitro fertilization

This trial investigated how heparin (40 µg/mL) or progesterone (80 nanomolar) influences the fertilization rate of sperm pre-exposed to isthmus cell aggregates. Group 5 achieved the highest fertilization percentage (59.28%) compared to all others ($p < 0.01$). Additionally, group 4 (40%) showed significantly greater fertilization than group 3 (36.42%), group 2 (39.04%), and group 1 (35%). No statistical variation was found among groups 1–3 **(Figure 4)**.

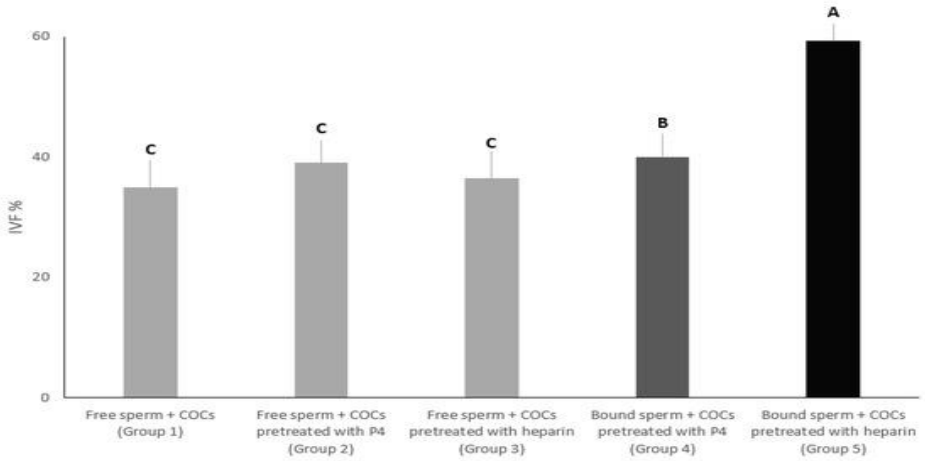


Figure 4. Group 1—untreated mature COCs with free sperm; Groups 2 and 3—mature COCs pretreated with 80 nM progesterone or 40 µg/mL heparin, respectively, with free sperm; Groups 4 and 5—sperm co-incubated with isthmus cell aggregates, followed by addition of mature COCs pretreated with heparin (40 µg/mL) or progesterone (80 nM), respectively. Groups denoted by different letters differ significantly ($p < 0.01$).

Experiment 3: effect of adding mature COCs pretreated with both heparin and progesterone to sperm preincubated with isthmus cell aggregates on in-vitro fertilization

This experiment assessed whether exposing sperm pre-incubated with isthmus cell aggregates to COCs treated with both heparin and progesterone modifies fertilization outcomes. Group 1 involved untreated COCs and free sperm. Group 2 contained COCs treated with heparin (40 µg/mL) and progesterone (80 nM) plus free sperm. Group 3 consisted of sperm pre-incubated with isthmus aggregates and COCs treated with both agents (same concentrations). All groups were cultured for 8–12 hours under identical conditions (5% CO_2, 39 °C, 95% humidity). Group 3 displayed the greatest fertilization rate (63.33%), significantly higher than group 2 (42.61%) and group 1 (34.28%) ($p < 0.01$). Group 2 also exceeded group 1 significantly ($p < 0.05$) **(Figure 5)**.

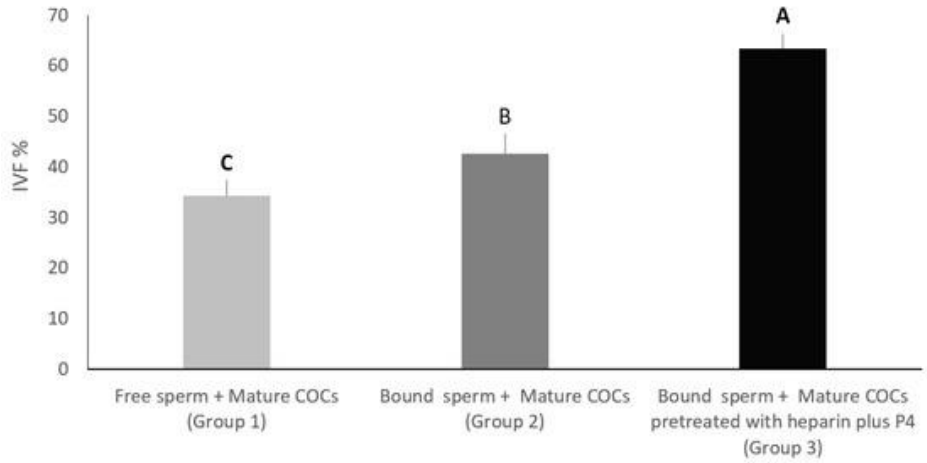


Figure 5. Group 1—untreated mature COCs with free sperm; Group 2—mature COCs treated with 40 µg/mL heparin plus 80 nM progesterone and free sperm; Group 3—sperm pre-incubated with isthmus cell aggregates plus COCs treated with both compounds. Distinct letters indicate significant variation ($p < 0.05$).

The purpose of this study was to examine the fertilization potential of sperm that adhered to cellular aggregates isolated from distinct oviductal regions (infundibulum, ampulla, and isthmus), both without and with the presence of progesterone (80 nanomolar) and/or heparin (40 µg/mL). The findings indicated that sperm attached to aggregates derived from the isthmus produced a significantly greater in vitro fertilization (IVF) rate than those associated with ampulla or infundibulum tissue. This variation in fertilization efficiency likely reflects the stronger binding preference of bovine sperm for isthmus-derived aggregates. This observation aligns with results by Elsokary [13], who demonstrated that buffalo sperm exhibit their greatest affinity for isthmus-sourced cellular aggregates.

Moreover, attachment to isthmus cell aggregates appears not only to regulate the proportion of unbound sperm but also to reduce the risk of polyspermic fertilization, consistent with the findings of Timothy [26]. Similarly, association with isthmus-derived cells may help sperm maintain reduced intracellular Ca^{2+} levels, postpone capacitation, and extend viability [27]. There remains discussion in the literature about which experimental system best models sperm–oviduct interactions. Rather than employing monolayer cultures, the present work utilized oviduct cell aggregates, which more closely approximate the three-dimensional architecture of the native epithelium and are simpler to obtain and manipulate. In contrast, monolayer cultures, maintained for several days, tend to flatten and assume a fibroblast-like morphology [28], with their sperm-binding characteristics diminishing after extended culture periods [29]. Given that capacitated sperm survive only briefly, the modulation of capacitation timing within the isthmus reservoir likely ensures the availability of competent sperm at the site of fertilization.

Our results further revealed that treating mature cumulus–oocyte complexes (COCs) with progesterone (80 nM) or heparin (40 µg/mL) significantly enhanced IVF rates, but only when sperm were pre-bound to isthmus cell aggregates. When isthmus cells were absent, the addition of either compound alone did not yield a comparable improvement, indicating that sperm–isthmus interaction is essential for achieving elevated fertilization outcomes in cattle. The IVF rate rose markedly when COCs were pretreated with both progesterone and heparin before exposure to sperm attached to isthmus aggregates. Additionally, COCs treated simultaneously with both agents yielded higher fertilization rates than untreated controls. The observed enhancement may be attributed to heparin's well-known role in promoting capacitation and facilitating sperm detachment from isthmus cell aggregates,

consistent with Ardon (2016) [30]. Comparable improvements in bovine IVF success with heparin concentrations around 40 µg/mL have also been reported [31]. Sperm-binding glycans are believed to be associated with annexins [32], which contain heparin-binding motifs; thus, heparin might promote sperm release by competing for these sites on either sperm membranes or oviductal cells [33, 34].

Furthermore, preincubation of COCs with progesterone (P4) enhanced the fertilization capacity of sperm previously bound to isthmus aggregates. This improvement may be attributed to P4's ability to induce sperm release from binding sites. Similar P4-mediated release mechanisms have been documented in porcine sperm attached to isthmus cell aggregates [35] and in sperm interacting with SuleX-coated beads [36]. In pigs, P4 has also been shown to trigger sperm detachment from oviductal monolayer cultures and to increase IVF rates [12]. According to Saint-Dizier [37], progesterone at physiological levels is critical in releasing sperm from bovine oviduct epithelial cells (BOEC). Such release events are accompanied by the shedding of binder-of-sperm proteins (BSPs) and heightened membrane fluidity—processes associated with the onset of capacitation. Moreover, sperm freed from BOEC under the influence of P4 display pronounced alterations in membrane phospholipid and protein organization.

Conclusion

In summary, sperm attachment to isthmus-derived cell aggregates can enhance the fertilizing potential of bovine sperm in vitro. Additionally, during the fertilization stage, the combination of mature COCs pretreated with progesterone (80 nanomolar) and heparin (40 µg/mL) together with sperm bound to isthmus cell aggregates produces a synergistic effect, resulting in a marked increase in the in-vitro fertilization efficiency of bovine sperm.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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