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Hyaluronan and hyaluronidase, which is better for embryo development?

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ABSTRACT

Our aim was to examine size-specific effects of Hyaluronan (HA) on preimplantation embryo development. We investigated the effects of Hyalovet (HA, 500–750 kDa; the size produced by HA synthase-3, which is abundant in the oviduct), or HA treated with Hyaluronidase-2 (Hyal2; also expressed in the oviduct that breaks down HA into 20 kDa fragments). In experiment 1 (*in vivo*), oviducts of synchronized and superovulated ewes (n = 20) were surgically exposed on Day 2 post-mating, ligated, and infused with either Hyalovet, Hyalovet + Hyal2, Hyal2, or PBS (control). Ewes were killed 5 days later for recovery of embryos and oviductal epithelial cells (OEC). Blastocyst rates were significantly higher in Hyal2 and Hyalovet + Hyal2 oviducts. Hyaluronidase-2 infusion resulted in higher blastocyst cell numbers and hatching rates. This was associated with increased *HSP70* expression in OEC. In contrast, Hyalovet resulted in the lowest development to blastocyst stage and lowest hatching rates, and decreased *IGF2* and *IGFBP2* expression in OEC. *IGF1* and *IL1α* expression were not affected. In experiment 2, to rule out indirect effects of oviductal factors, ovine embryos were produced and cultured with the same treatments *in vitro* from Day 2 to 8. Hyaluronidase-2, but not Hyalovet, enhanced blastocyst formation and reduced inner cell mass apoptosis. Hyalovet inhibited hatching. In conclusion, the presence of large-size HA (500–750 kDa) in the vicinity of developing embryos appears to disturb the oviductal environment and embryo development *in vivo* and *in vitro*. In contrast, we show evidence that breakdown of HA into smaller fragments is required to maximize embryo development and blastocyst quality.

1. Introduction

The oviduct provides the appropriate conditions required for the process of zona maturation, capacitation, fertilization, and early stages of early embryo development. Distinctive differences in the oviductal transcriptome [1] and proteome [2] between follicular and luteal phases of the reproductive cycle in human and animal models are regulated by the hormonal environment of the oviduct. This in turn interacts with and influences gametes and embryos during early development. The secretory proteome of the oviduct was also shown to change in response to gametes [3] illustrating bidirectional cross-talk. A vast number of oviductal factors that support oocyte and embryo development have been described [4]. Among those factors, hyaluronic acid (Hyaluronan, HA) is of special interest.

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Hyaluronan is a major component of the extracellular matrix. It is synthesized by hyaluronic acid-synthases (HAS1, HAS2, and HAS3) [5] in the form of linear polymer that extrudes through the membrane during its synthesis to the outside of the cell [6]. The molecular weight of HA is determined by the isoform of HAS, which also determines its functions (for review, see [7,8]). Hyaluronic acid-synthase 1 and HAS2 produce large-sized HA (up to 2000 kDa), whereas HAS3 produce a lower molecular-weight HA (100–1000 kDa). The functions of low molecular weight HA are mainly mediated through HA receptors, most commonly, CD44 [9] and is reported to be involved in cell proliferation, survival, and differentiation, as well as cell-cell and cell-matrix interaction [7,10].

Ovulated cumulus oocyte complexes (COCs) reach the oviduct embedded in a large-sized HA-rich matrix of expanded cumulus cells [11]. In addition, HA is synthesized by the oviduct and embryos; HA could be detected in the oviductal fluid of cyclic heifers and cows, and has been shown to be at highest concentration on the day of ovulation [12]. Transcripts for *HAS2* and *HAS3* have been found in the oviduct of several animal species [13–15]. It was noticed that *HAS3* expression was higher in the isthmus compared to ampulla [13,15] suggesting that a gradient decrease in the molecular size of HA is required during embryo development in the oviduct. In addition, *HAS2* messenger RNA (mRNA) has been detected in all stages of preimplantation human embryos [16]. In cattle, we have demonstrated that *HAS2* and *HAS3* are expressed at all stages of early embryo development (from the 2-cell to the blastocyst stage). We have found that *HAS2* mRNA expression tends to decrease with the progression to the blastocyst stage, whereas the level of *HAS3* expression did not change [17].

At the local tissue and cellular level, HA undergoes high rate of turnover, which includes HA depolymerization by Hyaluronidases (Hyals). Hyaluronidase 2 and Hyal1 are the major mammalian hyaluronidases in somatic tissues [18]. They act sequentially to degrade high molecular weight HA to tetrasaccharides. Hyaluronan fragments of 20 kDa are generated at the cell surface by Hyal2, transported intracellularly by internalization (which is evident in bovine embryos [19]), and then further digested by Hyal1 to produce HA tetrasaccharides [20]. The HA fragments generated during HA depolymerization by Hyals are biologically active molecules that have important functions associated with embryo growth and survival, including stimulation of cytokine production, growth factor receptor expression, and protection against apoptosis [8]. Hyaluronan have been shown to regulate expression of factors such as insulin-like growth factors (IGFs) [21], heat shock proteins [22], and interleukins, which are known to be important for early embryo development [4]. We have recently shown that bovine oviductal epithelial cells (OECs) express *Hyal2*, and the level of expression in the isthmus is much higher than that in the ampulla [17]. We have also shown that developing embryos express Hyal2 starting at the morula and blastocyst stages [17] suggesting their dependence on the oviductal secretions for endogenous HA breakdown. The presence of HA and its turnover in the vicinity of the oocyte during fertilization and the developing embryo suggest a biological role of HA in regulating these processes; however, this role is not well defined. We have demonstrated that *in vitro* supplementation of cleaved bovine embryos with Hyal2 significantly increased their development to blastocyst stage and increased embryonic cell numbers [17]. However, the differential effects of HA and its fragments during early embryo development have not been previously studied.

Building on the fundamental knowledge generated in our laboratory about the role of HA and Hyal2 during early embryo development *in vitro* [17,23], our objective in the present study was to compare size-specific effects of HA and its fragments on preimplantation embryo development. We compared the effects of large-size HA (at the molecular weight produced by HAS3, 500–750 kDa), or fragmented HA (HA treated with Hyal2) versus vehicle control. Hyaluronidase 2 alone was also tested as an extra control to examine the effect of depolymerization of endogenous HA produced by oviduct and/or embryos. The effects on early embryo development were observed. In addition, the expression patterns of selected candidate genes expressed in the oviduct and known to affect embryo development were investigated in the OECs in each treatment group. Simultaneously, direct effects of the previously mentioned HA and Hyal2 treatments on embryo development were confirmed using *in vitro*–produced ovine embryos.

2. Materials and methods

2.1. Experimental animals

The *in vivo* study was conducted at the Royal Veterinary College, after obtaining approval from the local ethical committee and authorization from the UK Home Office in compliance with Animal Scientific Procedures Act (1986). A total of 20 proven fertile, non-pregnant, 2-year old Welsh-mountain ewes, were used for this study during their breeding season. After a period of acclimatization, ewes were scanned to confirm the absence of pregnancy. Ewes were synchronized to a common estrus in five groups of four ewes every other day using vaginal sponges (Chronogest, Intervet, UK) for 12 days. Ewes were superovulated using a single injection of 700 IU pregnant mare serum gonadotropin (PMSG, intervet) and 230U Follitropin (Bioniche Animal Health, Belleville ON) 2 days before sponge removal to maximize the number of embryos recovered and increase the statistical power of the *in vivo* study. Receptal (8 μg Buserelin, GnRH agonist) was injected 24 hours after sponge removal to synchronize ovulations. Ewes in each synchronization group were then hand-mated by one of two proven fertile rams. Two days after mating, ewes were anesthetised, and reproductive tract was exposed by laparotomy. The number of corpora hemorrhagica (indicating number of ovulations) on each ovary was recorded, and oviductal treatments were infused.

2.2. Oviductal infusions

Oviducts were ligated with suture material (Silk) at the utero-tubal junction. A blunt needle attached to a syringe