The effect of glycosaminoglycans on rat gametes *in vitro* and the associated signal pathway

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Abstract

The effects of adding the extracellular glycosaminoglycans (GAGs), hyaluronic acid (HA) and chondroitin sulphate (CS) to rat *in vitro* fertilisation (IVF) media were assessed. Metaphase II (MII) oocytes were also incubated in GAG-supplemented modified rat 1-cell embryo culture medium (mR1ECM + BSA) for 3 days. Cytoplasmic fragmentation was significantly reduced in mR1ECM + BSA with HA (39.0–48.0%) compared with the control (82.0%). In IVF experiments, neither HA (8.0–30.8%) nor CS (9.7–42.5%) improved fertilisation rates compared with controls fertilised in M16 (47.2%) or enriched Krebs–Ringer bicarbonate solution (61.5%). RT-PCR and Western blot were used to probe for CD44 mRNA and protein in Sprague–Dawley gametes and cumulus cells. CD44 was identified in cumulus cells, suggesting a role for oocyte maturation and cumulus expansion. The CD44 protein was also present on caudal epididymal spermatozoa that were highly stimulated by CS *in vitro* implicating a role in fertilisation for CS and CD44.

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Introduction

Glycosaminoglycans (GAGs) are negatively charged polysaccharide chains of alternate lengths that have a moderate diversity of form (Ruoslahti & Yamaguchi 1991, Kim *et al.* 2001). GAGs are synthesised by many somatic cell types and have functions in cell-to-cell adhesion (Kosaki *et al.* 1999), following migration, proliferation (Camenisch *et al.* 2002) and (Itano *et al.* 2002, Legg *et al.* 2002) differentiation (Hamashima 1982). They are extensively distributed within the extracellular matrix of many tissues (Camaioni *et al.* 1993, Bose & Masellis 2005). Hyaluronic acid (HA) or hyaluronan is a non-sulphated, linear GAG that comprises alternating units of D-glucuronic acid and N-acetylglucosamine linked by β 1-3 and β 1-4 glycosidic bonds (Yokoo *et al.* 2002a, Toyokawa *et al.* 2005). Chondroitin sulphate A (CS) is made of a repeating D-glucuronic acid and N-acetylgalactosamine disaccharide with the same glycosidic linkages as HA and is sulphated (Sakai *et al.* 2007). HA (Kano *et al.* 1998, Kimura *et al.* 2002, Kim *et al.* 2005) and CS (Jensen & Zachariae 1958, Lee & Ax 1984, Kano *et al.* 1998) are GAGs that have been observed to have roles in reproductive physiology.

HA is abundant in the uterine, oviductal and follicular fluids of murine (Sato *et al.* 1987), porcine (Archibong *et al.* 1989), bovine (Lee & Ax 1984) and human (Suchanek *et al.* 1994, Hamamah *et al.* 1996, Ohta *et al.* 2001) species.

Furthermore, HA has been proposed to be an essential player of macaque spermatozoa capacitation and fertilisation (Cherr *et al.* 1999). CS has been identified in human (Hamamah *et al.* 1996) and porcine (Ax & Ryan 1979, Yanagishita *et al.* 1979) follicular fluid. Hydrolysis of rat granulosa cells with chondroitinase ABC revealed that CS is the major GAG of rat granulosa cells (Yanagishita & Hascall 1979).

The synthesis of HA occurs at the cell membrane by HA synthases (HAS; Watanabe & Yamaguchi 1996, Spicer *et al.* 1997b, Weigel *et al.* 1997). There are three isoenzymes that facilitate this process: HAS1 (Watanabe & Yamaguchi 1996), HAS2 (Salustri *et al.* 1989, 1992) and HAS3 (Spicer *et al.* 1997a). HAS1 protein has been identified in equine cumulus cells (Marchal *et al.* 2003). HAS2 mRNA transcripts have been detected in bovine oocyte–cumulus complexes (OCC; Schoenfelder & Einspanier 2003) and murine (Salustri *et al.* 1989, Fulop *et al.* 1997) and porcine cumulus cells (Kimura *et al.* 2002). mRNA transcripts for HAS3 are expressed in bovine OCC (Schoenfelder & Einspanier 2003) and porcine metaphase II (MII) oocytes (Kimura *et al.* 2002). Additionally, HAS3 protein has been identified in equine cumulus cells (Marchal *et al.* 2003).

There have been six glycosyltransferases identified that regulate the synthesis of CS (Sakai *et al.* 2007) within the Golgi apparatus (Silbert & Freilich 1980). These are CS synthase-1 (CSS-1; Kitagawa *et al.* 2001), CSS-2 (Yada *et al.*

2003a), CSS-3 (Yada *et al.* 2003b), CS *N*-acetylgalactosaminetransferase-1 (CSGalNAcT-1; Gotoh *et al.* 2002b, Uyama *et al.* 2002) and CSGalNAcT-2 (Sato *et al.* 2003) and CS glucuronyltransferase (CSGlcAT; Gotoh *et al.* 2002a). CSS have been reported to stimulate cumulus expansion and HAS activity in mice (Eppig 1981).

The primary receptor for HA is the cell surface glycoprotein CD44, which also functions as a receptor for CS (Culty *et al.* 1990). CD44 is thought to interact with HA via a highly conserved transmembrane region (Isacke 1994, Entwistle *et al.* 1996, Liu *et al.* 1998, Shi *et al.* 2001). This receptor has been identified on a wide range of cells that include epithelial cells (Alho & Underhill 1989), macrophages (Green *et al.* 1988a, 1988b) and lymphocytes (Lesley *et al.* 1993). The CD44 receptor has also been identified in bovine OCC (Schoenfelder & Einspanier 2003) and pre-implantation embryos (Furnus *et al.* 2003), porcine cumulus cells (Kimura *et al.* 2002, Yokoo *et al.* 2002a, 2002b, 2007), germinal vesicle-intact and MII oocytes and cleaved parthenotes (Toyokawa *et al.* 2005).

The use of extracellular HA and CS for *in vitro* maturation (IVM), *in vitro* culture (IVC) and *in vitro* fertilisation (IVF) media is documented in mammalian species, including canine (Kawakami *et al.* 2000), porcine (Kano *et al.* 1998), bovine (Lenz *et al.* 1983, Lee *et al.* 1986, Jang *et al.* 2003, Therien *et al.* 2005) and human (Eriksen *et al.* 1994, Hamamah *et al.* 1996) but not the rat. These studies reported significant influences on spermatozoa (increased motility and incidence of acrosome exocytosis), oocytes (elevated IVF rates) and pre-implantation embryos (improved rates of development in IVC) in GAG-supplemented media.

Here, we report on the ability of GAGs, HA and CS in a rat IVF system to improve overall fertilisation outcomes and potentially suppress spontaneous activation. Furthermore, we report on the expression of CD44 in Sprague–Dawley gametes and MII cumulus cells.

Results

Incubation of MII oocytes in GAG-supplemented mR1ECM + s-BSA

To examine the influence of the GAGs on MII oocytes, mR1ECM+BSA was supplemented with HA and CS. Denuded oocytes were subsequently incubated over a 72-h time frame with inspection every 24 h. Figure 1 displays the changes to MII cytoplasmic cohesion over a 72-h period in standard mR1ECM+BSA (control) or supplemented with CS or HA at 250 or 500 µg/ml. All denuded MII oocytes had a normal-appearing morphology at the start of the experiment. After 24 h, the proportion of morphologically normal oocytes was high (>79%) for all media groups (Fig. 1). At 24 h, parthenotes represented only a small, non-significant proportion of oocytes in all groups.

At 48 h, the number of MII oocytes with a normal appearance was significantly higher in HA-500 (Fig. 1E, $P<0.0001$; 47%) and HA-250 (Fig. 1D, $P<0.0001$; 36%).

Additionally, a significant increase was observed for CS-250 (Fig. 1B, $P<0.05$; 26%). This effect correlated with the significantly lower numbers of fragmented oocytes (Fig. 1D and E, 36%; 51%, $P<0.001$ – 0.0001) compared with the control (Fig. 1A, 79%). There was a significant number of cleaved parthenotes present in CS-500 (Fig. 1C, 25%; $P<0.001$) compared with the mR1ECM control (Fig. 1A).

By 72 h, there were significantly fewer fragmented oocytes for mR1ECM+BSA supplemented with HA (Fig. 1D and E, 36–39%, $P<0.0001$) compared with the control (Fig. 1). Any positive effect of CS by this time had ended (Fig. 1B and C). Numbers of two-cell parthenotes were significantly higher in CS-500 (Fig. 1C, 20%; $P<0.05$) and higher for all GAG-supplemented media (Fig. 1B–E, 8–16%) compared with the control (Fig. 1A, 6%).

Spermatozoa motility

At the onset of co-incubation, all dishes were inspected to ensure that spermatozoa appeared healthy to the naked eye. CS-supplemented media contained greater numbers of visually motile caudal epididymal spermatozoa. While not as active, spermatozoa in all other media still presented a very healthy motility prior to insemination (Table 1).

At the end of co-incubation, CS-complemented media contained higher numbers of motile spermatozoa. While all spermatozoa were now sluggish, this effect was the most reduced in media with CS. Spermatozoa in controls were predominantly dead at this time point, whereas HA-supplemented media had some activity.

IVF of MII oocytes in GAG-supplemented medium

A control IVF experiment was run alongside IVF-GAG experiments to confirm normal oocyte and spermatozoa function (Table 2). All experiments were conducted with cumulus-intact oocytes as in previous IVF experiments. Thus, unlike the incubation experiments, MII oocytes were not denuded. The control outcomes observed were reasonably consistent with past laboratory experiments. EKRB (65.8%) and M16 (54.8%) had substantially higher numbers of fertilised embryos than mR1ECM+BSA (36.7%; Table 2).

GAG supplementation significantly decreased fertilisation rates in M16-HA (13.5%; $P<0.001$), EKRB-CS (14.3%; $P<0.05$), mR1ECM-CS (9.7%; $P<0.01$) and mR1ECM-HA (8.0%; $P<0.001$; Table 3) compared with the controls (M16=47.2% and EKRB=61.5%). The media, M16-CS (42.5%) and to a lesser extent EKRB-HA (30.8%), displayed levels of fertilisation similar to their respective control media (Table 3). Rates of polyspermic fertilisation were higher than previously observed in IVF experiments but rates were generally similar to the controls.

Figure 1 Fragmentation suppression in denuded, zona pellucida (ZP) intact rat MII oocytes incubated in mR1ECM+BSA (310 mOsm) with/without GAG supplementation at 37 °C in a 5% CO₂ atmosphere for 72 h. Samples ($n=100$) were compared with the control value ($n=100$) every 24 h over the 3-day period; * $P\leq 0.05$, ** $P<0.001$, *** $P<0.0001$ indicate statistical significance to the standard mR1ECM-BSA control for the respective rows. (A) mR1ECM+BSA (control); (B) mR1ECM+BSA (CS 250 µg/ml); (C) mR1ECM+BSA (CS 500 µg/ml); (D) mR1ECM+BSA (HA 250 µg/ml) and (E) mR1ECM+BSA (HA 500 µg/ml).

Secondary binding of spermatozoa to the embryo

Examination of Hoechst-stained embryos and oocytes revealed no prevalence of secondary binding by spermatozoa among the various groups (data not shown). The presence of GAGs did not seem to influence this trait of IVF in any specific manner.

mRNA expression of the HA receptor CD44 in Sprague–Dawley gametes and cumulus cells

RT-PCR revealed a CD44 mRNA transcript present in cumulus cells collected from MII oocytes obtained from superovulated immature female rats (Fig. 2). The band

obtained corresponded to the predicted amplicon size (1090 bp). Both MII oocytes and caudal epididymal spermatozoa showed no presence of the CD44 transcript. All samples were also probed with primers for adenyl cyclase III (ACIII) as a control gene to confirm that mRNA was obtained from the extraction procedure. The ACIII PCR product was present for all samples and the liver positive control (Fig. 2).

CD44 protein expression in Sprague–Dawley gametes and MII oocyte cumulus cells

The CD44 mAb showed the presence of a protein band (~50 kDa) present in both caudal epididymal

Table 1 *In vitro* fertilisation (IVF) medium composition.

	mR1ECM + BSA	EKRB	M16
Stock A			
NaCl	110.0 mM	119.4 mM	94.66 mM
KCl	3.2 mM	4.8 mM	4.78 mM
MgSO ₄ ·7H ₂ O	–	1.2 mM	1.19 mM
MgCl ₂	0.5 mM	–	–
Na lactate (C ₃ H ₅ NaO ₃)	10.0 mM	21.0 mM	23.28 mM
Glucose (C ₆ H ₁₂ O ₆)	7.5 mM	5.0 mM	5.56 mM
Stock B			
NaHCO ₃	25.0 mM	25.0 mM	25.0 mM
Stock C			
Na pyruvate (C ₃ H ₃ O ₃ Na)	0.5 mM	0.25 mM	0.33 mM
Stock D			
CaCl ₂	2.0 mM	1.0 mM	1.71 mM
Stock P			
KH ₂ PO ₄	–	1.2 mM	1.19 mM (stock A)
MEM essential amino acids	1:100 (v:v)	–	–
MEM non-essential amino acids	1:200 (v:v)	–	–
BSA	4 mg/ml	3 mg/ml	4 mg/ml

spermatozoa and cumulus cells obtained from ovulated MII oocytes (Fig. 3). The presence of a CD44 protein in the sperm sample was contradictory to the RT-PCR result (Fig. 2) that did not detect an mRNA transcript. Faint signals were present in the liver controls.

Discussion

In this study, the GAGs HA and CS were able to suppress fragmentation in denuded MII oocytes. HA was more effective at this than CS implicating a role for HA and CD44. RT-PCR and Western blot of MII oocytes did not detect CD44 mRNA transcripts or an expressed CD44 protein. However, CD44 was identified in cumulus cells obtained from MII oocytes obtained by superovulation. CD44 expression was influenced by equine chorionic gonadotrophin in porcine cumulus cells during IVM (Kimura *et al.* 2002). Furthermore, it is documented that follicle stimulating hormone and luteinizing hormone (LH) increase CD44 expression in bovine

Table 2 *In vitro* fertilisation (IVF) outcomes for cumulus-intact, ZP intact rat metaphase II (MII) oocytes inseminated with pre-incubated rat caudal epididymal spermatozoa (7.5×10^5 sp/ml, 6.0 h) in standard IVF media (310 mOsm) at 37 °C in a 5% CO₂ atmosphere for 11.5 h.

Control	M16 % (n)	EKRB % (n)	mR1ECM + BSA % (n)
n	100 (31/31)	100 (38/38)	100 (30/30)
Unfertilised	16.1 (5/31)	10.5 (4/38)	46.6 (14/30)*,
Normal fertilisation	54.8 (17/31)	65.8 (25/38)	36.7 (11/30)
Polyspermic	29.0 (9/31)	23.6 (9/38)	16.7 (5/30)

* $P \leq 0.05$ indicates statistical significance to M16 for the row.

|| $P < 0.001$ indicates statistical significance to EKRB for mR1ECM + BSA.

OCC (Schoenfelder & Einspanier 2003). Chorionic gonadotrophin is known to induce an endogenous LH surge in superovulated female rats soon after its administration (Goh *et al.* 1992). This suggests that HA and CD44 have a role to play in regulating the integrity of oocyte cytoplasm during cumulus expansion and post-ovulation as seen by the presence of a CD44 mRNA transcript in cumulus cells obtained from superovulated MII oocytes.

After granulosa cell (GC) differentiation into mural GCs and oocyte-bound cumulus cells and the subsequent LH surge, expansion of cumulus cells is essential to the final stages of maturation (Dekel & Phillips 1979, Kimura *et al.* 2002, Yokoo *et al.* 2007). During this process of expansion, HA is actively synthesised and accumulates in the cumulus matrix (Dekel & Phillips 1979, Eppig 1979, Salustri *et al.* 1990). The ability of HA to suppress oocyte fragmentation has been observed in murine (Sato *et al.* 1987), bovine (Sato *et al.* 1988) and porcine (Sato *et al.* 1994) species. The incubation experiments in this study implicate a role for the CD44-HA signal pathway in oocyte maturation and/or maintenance prior to fertilisation. The ability of HA and CS to suppress oocyte fragmentation *in vitro* supports a role for HA within the extracellular matrix of cumulus cells.

IVF experiments in media supplemented with HA or CS did not improve fertilisation rates above those of controls, suggesting that there is a limit to their usefulness *in vitro*. Indeed, it seemed that the two GAGs promoted spontaneous activation of MII oocytes. Resumption of meiosis in MII oocytes is controlled by maturation promoting factor protein that acts to regulate MAP kinase (MAPK) via activation of an oocyte exclusive kinase. The active c-mos kinase promotes MAP kinase (MEK) phosphorylation of MAPK and its activation (Lu *et al.* 2002, Josefsberg *et al.* 2003).

CD44 has been observed to induce proliferation in colon cancer cells (Singh *et al.* 2006) and mouse thymoma EL4 cells via activation of MAPK (Marhaba *et al.* 2005). Furthermore, there is a large body of evidence to show that HA and CD44 are active in many signal pathways (Kobayashi *et al.* 2002, Roscic-Mrkic *et al.* 2003, Bourguignon *et al.* 2005, 2006, Lugli *et al.* 2006, Shi *et al.* 2006). The primer sequence (Kon *et al.* 2006) for CD44 in RT-PCR targeted the transcript for the whole protein. There are over 30 exons present in the entire CD44 sequence that through alternative mRNA splicing generates numerous isoforms (Screaton *et al.* 1992, Tölg *et al.* 1993, Bajorath 2000). There is a reasonable likelihood that one of these may be present in rat oocytes that could function to induce parthenogenesis *in vitro* due to extracellular HA or CS stimulation. This hypothesis would support the effect of HA and CS on rat MII oocytes and requires further investigation.

The CD44 protein detected in spermatozoa and cumulus cells was ~50 kDa in mass. The apparent standard for CD44 is a protein in the range of 85–90 kDa (Lesley & Hyman 1998, Yokoo *et al.* 2002b). The amino

Table 3 *In vitro* fertilisation (IVF) outcomes for cumulus intact, ZP intact rat metaphase II (MII) oocytes inseminated with pre-incubated rat caudal epididymal spermatozoa (7.5×10^5 sp/ml, 6.0 h) in M16 (310 mOsm) with/without 500 µg/ml glycosaminoglycan (GAG)-supplementation at 37 °C in a 5% CO₂ atmosphere for 11.5 h.

Capacitation and co-incubation	M16 control % (n)	EKRB control % (n)	M16 (CS) % (n)	M16 (HA) % (n)	EKRB (CS) % (n)	EKRB (HA) % (n)	mR1ECM+ BSA (CS) % (n)	mR1ECM+ BSA (HA) % (n)
n	53	39	40	37	14	26	31	25
Unfertilised	28.3 (15/53)	5.1 (2/39)*	27.5 (11/40) [§]	86.5 (32/37) ^{‡,¶}	64.3 (9/14) ^{*,¶}	34.6 (9/26)	83.9 (26/31) ^{‡,¶}	92.0 (23/25) ^{‡,¶}
Normal fertilisation	47.2 (25/53)	61.5 (24/39)	42.5 (17/40)	13.5 (5/37) ^{‡,¶}	14.3 (2/14) [§]	30.8 (8/26)	9.7 (3/31) ^{‡,¶}	8.0 (2/25) ^{‡,¶}
Polyspermic	24.5 (13/53)	33.3 (13/39)	30.0 (12/40)	0.0 (0/37) ^{‡,¶}	21.4 (3/14)	34.6 (9/26)	6.5 (2/31) [§]	0.0 (0/25) ^{*,}

* $P \leq 0.05$, [†] $P < 0.001$, [‡] $P < 0.0001$ indicate statistical significance to M16 for the respective rows. [§] $P \leq 0.05$, ^{||} $P < 0.001$, [¶] $P < 0.0001$ indicate statistical significance to EKRB for the respective rows.

acid sequence for CD44 predicts a band of 40 kDa, yet bands ranging from 73 to 88 kDa were detected in porcine cumulus cells (Yokoo *et al.* 2002b). The level of CD44 glycosylation is thought to be responsible for the apparent differences in mass (Borland *et al.* 1998).

The CD44 mRNA transcript was also absent in caudal epididymal spermatozoa yet immunoblotting detected the protein. It is probable that the CD44 protein is bound to spermatozoa during maturation as are other proteins (Cuasnicu *et al.* 1990, Cohen *et al.* 2000). Furthermore, in the presence of extracellular CS, sperm motility was greatly enhanced in human (Eriksen *et al.* 1994, Hamamah *et al.* 1996) and canine (Kawakami *et al.* 2000) spermatozoa motility. CD44 is a receptor for CS (Culty *et al.* 1990) which has been identified in human follicular fluid (Hamamah *et al.* 1996) and canine oviductal and uterine fluids (Kawakami *et al.* 2000). Additionally, CS is reported to be the primary GAG within rat granulosa cells (Yanagishita & Hascall 1979). The presence of CS within uterine or oviductal fluids or the OCC matrix may act to stimulate spermatozoa so that they can reach the zona pellucida.

In conclusion, CD44 is present in rat cumulus cells and caudal epididymal spermatozoa, suggesting roles in cumulus expansion, oocyte maturation and motility stimulation. The protein detected suggests a low level of glycosylation or a specific isoform. Further investigation is required to assess the mRNA transcripts for CD44 isoforms in MII oocytes and pre-implantation embryos. The identification of HAS is also required.

Materials and Methods

All reagents were purchased from the Sigma–Aldrich Chemical Company unless otherwise specified. BSA, protein supplement, was the Albumax I liposaccharide-rich variety (Gibco, Invitrogen). Cultureware was obtained from Becton-Dickinson (BD; Melbourne, Australia) unless otherwise specified.

Animals

Rats were obtained from the Monash University Central Animal Services (MUCAS, Clayton, Victoria, Australia) outbred Sprague–Dawley (OBSD) – specific pathogen-free (spf) colony.

Rats were housed at the Block B animal house facility, Monash Medical Centre in standard rat sized boxes (one male per box, five females per box) with high top cage lids. Rats were kept under a standard 12 h light:12 h darkness cycle (0800:2000 h) and provided water and food *ad libitum*. The holding room temperature was maintained at 22 °C with an average humidity of 50%. This study was approved by the Monash Medical Centre Animal Ethics Committee.

Media

All media used in these experiments were made from a specific salt solutions with additional compounds added at different concentrations (Table 1). The rat-specific medium, modified rat 1-cell embryo culture medium, mR1ECM+BSA (Oh *et al.* 1998), was used for all MII oocyte incubations and as an IVF medium. In addition the media, namely enriched Krebs–Ringer bicarbonate (EKRB; Bendahmane *et al.* 2002) and M16 (Whittingham 1971) were used in IVF experiments. The osmolarity of all IVF media was set at 310 mOsm for all experiments.

Caudal epididymal spermatozoa collection and vitro capacitation

Spermatozoa were obtained from the caudal epididymides of sexually mature (3–4 months old) OBSD males. Rat MII oocytes were fertilised following standard IVF methodology (Toyoda & Chang 1974) with some minor modification. The caudae were washed in pre-warmed calcium (Ca²⁺)/magnesium (Mg²⁺)-free Dulbecco's PBS (DPBS) and were immediately placed into

Figure 2 Expression of CD44 mRNA transcripts in Sprague–Dawley gametes and MII oocyte cumulus cells. Lanes 2 and 9, rat liver (positive control); lanes 3 and 10, MII oocyte; lanes 4 and 11, cumulus cells; lanes 5 and 12, caudal epididymal sperm; lanes 6 and 13, RT blank (negative control); lanes 7 and 14, PCR blank (negative control); lanes 1, 8 and 15, Generuler Base pair Standard.

Figure 3 Expression of CD44 protein in Sprague–Dawley gametes and MII oocyte cumulus cells Lane 2, MII oocyte; lane 3, caudal epididymal sperm; lane 4, cumulus cells; lanes 5 and 6, positive control, lanes 1 and 7, protein molecular weight standard.

standard mR1ECM+BSA, EKRb or M16 for 5 min to facilitate sperm dispersal. A sperm count was conducted to produce a working concentration of 7.5×10^5 spermatozoa per ml. Dishes were subsequently returned to the incubator for a 6-h incubation period to facilitate capacitation (37 °C, 5% CO₂).

MI I oocyte collection

MI I-stage oocytes were obtained from sexually immature (24-days old) females superovulated with pregnant mare's serum gonadotrophin (10–15 i.u. i.p.) at 0 h and human chorionic gonadotrophin (hCG; 10–15 i.u.) at 48 h by i.p. injection. OCC were immediately transferred to dishes for specific experiments.

Denuding of MI I oocytes

To prepare MI I oocytes for incubation in GAG-supplemented mR1ECM+BSA, the surrounding cumulus matrix was removed. OCC were placed into HEPES-buffered mR1ECM+BSA supplemented with hyaluronidase (100 i.u./ml) for 5 min, after which the oocytes were washed in clean HEPES-buffered mR1ECM+BSA. Oocytes were subsequently transferred to mR1ECM+BSA with/without GAGs.

Incubation of MI I oocytes in GAG-supplemented mR1ECM+BSA

Oocytes were placed into a 400 µl drop of pre-equilibrated mR1ECM-BSA (37 °C, 5% CO₂). Standard mR1ECM+BSA was the control medium for incubation experiments. The mR1ECM+BSA was supplemented with HA or CS at 250 or 500 µg/ml. All dishes were placed into an incubator (37 °C, 5% CO₂) for 72 h and their status checked every 24 h.

IVF of MI I oocytes in GAG-supplemented media

OCC designated for IVF were immediately transferred to their respective insemination medium after collection. The insemination dish with spermatozoa (3.0×10^5 in 400 µl) and OCC present was returned to the incubator (37 °C, 5% CO₂ in air) for an 11.5-h co-incubation period.

Primer sequence

To probe samples for CD44 mRNA transcripts, the forward (F) and reverse (R) sequences described by Kon *et al.* (2006) were used. These sequences would yield an amplicon 1095 bp in size:

F: CCCGAATTCATGGACAAGGTTTGGTGGCA
R: CCCGAATTCCTACACCCCAATCTTCATAT

To confirm the presence of mRNA during RT, adenylyl cyclase III was selected to act as a control gene as its presence had previously been identified in rat caudal epididymal spermatozoa (Wade *et al.* 2003). Additionally, it was probable that this particular splice variant would be present in rat MI I oocytes and liver. These sequences would yield an amplicon 290 bp in size:

F: GACAACCGGGATAGAGCTGG
R: CTGCTGTCAGTGCCATTGAGC

mRNA isolation

The isolation of mRNA from caudal epididymal spermatozoa and MI I oocytes was achieved with the Dynal Dynabead mRNA purification kit (Invitrogen). Oocytes were collected in 5 µl mRNA lysis buffer (485.5 µl DEPC water, 25 µl dithiothreitol (DTT), 4 µl IGEPAL (MP Biomedicals Inc., Melbourne, Australia) and 12.5 µl RNaseout (Invitrogen) and immediately snap frozen in liquid nitrogen (N_{2(l)}) and stored at –80 °C until required. Spermatozoa was collected as described above on the day of RT-PCR and kept in DPBS for use.

The mRNA extracted from rat liver to act as a positive control was done by the Trizol (Invitrogen) method to the specifications of the manufacturer.

RT-PCR

RT of mRNA samples to produce cDNA was done in a RT solution; 19 µl Ultrapure DNase/RNase-free distilled water, 8 µl 5× buffer (Superscript III kit, Invitrogen), 2 µl random primers (1:10 dilution; Invitrogen), 2 µl of 10 mM dNTP (Fisher Biotech, Perth, Western Australia), 2 µl of 0.1 mM DTT (Superscript III kit, Invitrogen) and 2 µl RNaseout. Immediately prior to RT, 2 µl Superscript III was added to the sample. The rat liver control RT to act as a positive control was obtained by the Superscript II method to the specifications of the manufacturer.

PCR of samples was performed in a PCR solution; 33.8 µl DNase/RNase-free distilled water, 5 µl of 2 mM dNTP, 5 µl of 10× PCR buffer, 3 µl of 1.5 mM MgCl₂, 1 µl forward primer (1:32.3 dilution), 1 µl reverse primer (1:32.3 dilution), 0.2 µl Taq polymerase (#TAQ-3, Fisher Biotech) and 1 µl sample cDNA.

Protein extraction

Spermatozoa were collected as described above in DPBS and the solution transferred to a Centricon YM-100 centrifugal filter device (Millipore, Melbourne, Australia) and the manufacturer's instructions were followed to remove salts and isolate spermatozoa. The spermatozoa pellet was transferred into 400 µl mammalian protein extraction reagent (M-PER; Pierce, Sydney, Australia) with 5 µl Calbiochem Protease Inhibitor Cocktail set III. The spermatozoa sample was constantly shaken for 4 h at RT.

MII oocytes were collected and denuded as described in Leibovitz L-15 tissue culture medium (Gibco, Invitrogen) supplemented with 1 mg/ml PVA. Rat liver was homogenised and protein extracted in the same manner as spermatozoa with M-PER, snap frozen in N_{2(l)} and kept at -80 °C until required for electrophoresis.

Electrophoresis and electrotransfer

Protein samples were separated on a 12% bis-acrylamide gel in a Bio-Rad Mini-Protean 3 cell as specified by the manufacturer. To prepare samples for probing with a CD44 MAB (mAb), the 12% gel was prepared according to instructions for the Immobilon transfer polyvinylidene difluoride membrane (Millipore).

Western blot

Following electrotransfer of proteins, the membrane was blocked and blotted with mouse anti-ratCD44 mAb (Phar-Mingen, BD Biosciences, Sydney, Australia) following the methods for the LI-COR Odyssey infrared imaging system. The primary antibody was used at a ratio of 1:500 in a Tris-buffered saline solution with 0.2% Tween-20.

Hoechst 33258 staining

To evaluate sperm penetration and pronuclei formation post-insemination, oocytes were fixed in 4%-paraformaldehyde for a minimum period of 12 h at 4 °C. Oocytes were subsequently transferred to 15 µg/ml bisbenzimidazole (Hoechst 33258) in DPBS for 15 min to permit labelling.

Statistical analysis

χ^2 analysis was performed by comparing experimental groups with the control group for the numbers of fertilised and polyspermic embryos and unfertilised oocytes. A value of $P \leq 0.05$ was considered to be statistically significant.

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