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Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer

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The effect of macromolecules on mouse embryo development and viability after culture in sequential media was investigated. It was found that high rates of viable blastocysts could be obtained in the absence of any macromolecule. Blastocyst cell numbers were increased when bovine serum albumin was present in the culture medium, although this benefit was not manifest after blastocyst transfer. Rather, the highest rates of implantation and fetal development after blastocyst transfer were observed when hyaluronan was the macromolecule in the culture media. Subsequent analysis revealed that the beneficial effects of hyaluronan were due to its presence in the transfer medium. As the highest cell numbers and hatching rates obtained in this study occurred when both serum albumin and hyaluronan were present in the same medium, it is proposed that embryo culture media should contain both serum albumin and hyaluronan, while the transfer medium need only contain hyaluronan.

Key words: albumin/macromolecule/mouse embryo/PVA/viability

Introduction

Over the past few years, there has been a resurgence of interest in the culture of mammalian embryos. Extensive research has culminated in a number of new and more effective media for the embryos of several mammalian species including the human (reviewed by Gardner, 1994; Bavister, 1995; Gardner and Lane, 1997). The research that led to such new media was primarily focused on the role of ions, amino acids, and carbohydrates. In contrast, there has been relatively little work on the role of different macromolecules in embryo culture media, with serum albumin being the primary macromolecule in most media. However, although albumin is the most abundant macromolecule in the female reproductive tract (Leese, 1988), serum albumin is not a pure substance, and has been shown to vary from source to source and even from lot to lot (Kane, 1983; Batt *et al*., 1991; McKiernan and Bavister, 1992). One identified contaminant of commercial preparations of serum albumin is citrate (Gray *et al*., 1992). The use of a biological source for a medium component not only makes it almost impossible to truly standardize culture conditions but also raises the potential for viral and prion contamination.

In attempts to standardize culture conditions, synthetic macromolecules such as polyvinylalcohol (PVA) have been suggested as alternatives to replace the albumin in embryo culture medium (Bavister, 1981; Bavister, 1995). While PVA has been used extensively for successful culture of the hamster embryo in culture (Bavister, 1981; Bavister, 1995), substitution of serum albumin with PVA in culture systems for other species such as the cow has been questioned (Thompson *et al*., 1998). In contrast, glycosaminoglycans (GAGS) are found at high concentrations in the fluid of the female reproductive tract of several mammalian species (Lee and Ax, 1984; Yanagishita, 1994; Laurent *et al*., 1995; Zorn *et al*., 1995; Legge *et al*., 1996). One of these GAGS hyaluronan, a linear polysaccharide of alternating D-glucuronic acid and N-acetyl-D-glucosamine residues, increases in concentration at the time of implantation in the mouse embryo (Carson *et al*., 1987). Furthermore, both human (Campbell *et al*., 1995) and bovine (Valcarcel *et al*., 1999) embryos have the surface receptor for hyaluronan, CD44, throughout development from the oocyte to the blastocyst stage. Importantly, Wheatley and colleagues observed a strict temporospatial regulation of CD44 expression in post-implantation mouse embryos at the sites of hyaluronan-mediated morphogenesis, indicating a role for receptor–ligand interaction (Wheatley *et al*., 1993). In addition, hyaluronan also has an important role in the maintenance of human sperm motility (Sbracia *et al*., 1997). It is therefore most plausible that hyaluronan has a role in early embryo development.

The aim of this study was to examine the role of hyaluronan in sequential culture media and to compare this with the effects of other macromolecules. Part of this study was presented in Abstract form at the annual ESHRE meeting in Edinburgh, 1997.

Materials and methods

Media

Embryo culture media used in this study were DM2 and DM3 (Table I), which were derived from the media developed by Lane and Gardner [Lane and Gardner, 1995, (DM1); Gardner and Lane, 1996]. Media were prepared weekly from stock solutions and stored at 4°C. The medium used for collection and embryo manipulations was a HEPES-buffered modification of DM2, in which 20 mmol/l $NaHCO_3$ was replaced with 20 mmol/l HEPES (H-DM2) at pH 7.35. The medium for embryo transfer was a HEPES-buffered modification of DM3 at pH 7.35 (H-DM3). All salts were of Analar grade (BDH,

Table I. Composition of sequential mouse embryo culture media DM2 and DM3

Component (mmol/l)	DM2	DM3
NaCl	98.4	93.4
KCl	4.78	4.78
KH_2PO_4	1.19	1.19
$CaCl_2.2H_2O$	1.71	1.71
$MgSO_4.7H_2O$	1.19	1.19
$NaHCO_3$	25.0	25.0
Sodium pyruvate	0.37	0.37
Sodium lactate (L-isoform)	4.79	4.79
Glucose	3.40	3.40
Glutamine	1.0	1.0
Non-essential amino acids	All	All
Essential amino acids	None	All
EDTA	0.01	None
Penicillin	0.06 g/l	0.06 g/l
Streptomycin	0.05 g/l	0.05 g/l
Phenol red	0.01 g/l	0.01 g/l

Poole, UK). Sodium pyruvate, sodium lactate, amino acids, HEPES, taurine, EDTA, and antibiotics were cell culture grade (Sigma Chemical Co. St Louis, MO, USA).

Embryos

Embryos were collected from CF1 female mice. Multiple ovulations were induced by an i.p. injection of 5 IU of pregnant mare's serum gonadotrophin (Folligon, Intervet, Victoria, Australia) followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG, Chorulon, Intervet). After the second injection, females were placed with males of the same strain to produce truly outbred embryos. Zygotes were collected at 21 h post-HCG in H-DM2 and denuded by a brief (less than 1 min) incubation with hyaluronidase (0.5 mg/ml, Sigma). Embryos were washed twice in H-DM2 and once in the appropriate culture medium before being placed in culture. Four different macromolecules were chosen; bovine serum albumin (BSA, Life Technologies, Victoria, Australia), hyaluronan (extracted from rooster comb with an average molecular weight of 3×10^6, Pharmacia, Sweden), PVA (Sigma) and dextran (Sigma). Macromolecule-free medium served as a control.

Embryo culture

Embryos were cultured in groups of 10 in 20 μl drops of medium under mineral oil (Lane and Gardner, 1992). All embryos were cultured at 37°C for 48 h in DM2 to the 8-cell stage, followed by culture for a further 48 h in DM3 to the blastocyst stage in 5% CO_2, 7% O_2, and 88% N_2.

Determination of inner cell mass and trophectoderm cell number

Embryo cell number and allocation to the inner cell mass (ICM) and trophectoderm (TE) were determined using differential nuclear staining with the dyes propidium iodide and bisbenzimide (Hardy *et al*., 1989). Blastocysts were incubated in 0.5% pronase in H-DM1 to dissolve the zona pellucida. Embryos were then washed in H-DM1 and incubated in picrylsulphonic acid (Sigma) for 10 min at 4°C before a further wash and 10 min incubation in 0.1 mg/ml of Anti-DNP BSA (ICN Technologies, Costa Mesa, CA, USA) at 37°C. Following incubation with the antibody, embryos were again washed in H-DM1 and incubated in a 1:5 dilution of guinea-pig serum (ICN Technologies) containing 25 μg/ml of propidium iodide (Sigma) in H-DM1 for 7 min. Embryos were subsequently placed in 25 μg/ml bisbenzimide (Hoechst 33258, Sigma) in ethanol overnight at 4°C. The following morning, differential staining of nuclei was determined under UV light using a fluorescence microscope.

Embryo transfer

Blastocysts were transferred to pseudopregnant F1 (C57BL/6 x CBA/Ca) female mice (–1 day asynchronous). Treatments were allocated to each uterine horn using random numbers. Five blastocysts were transferred to each uterine horn. Embryos were transferred to the uterus in the H-DM3 supplemented with the same macromolecule as the culture medium in which they were developed. On day 15 of pregnancy, implantation rates, fetal development, and normalcy were assessed. Fetal age was determined using a scoring system (Lane and Gardner, 1994).

Statistical analysis

Data for embryo morphology and viability were assessed using linear-logistic regression in which the error distribution was assumed to be binomial. Replicate of experiment was fitted as a factor. The null hypothesis was tested using the log-likelihood ratio statistic. Data for cell number, ICM and TE cell numbers were assessed using analysis of variance. Between-treatment differences were assessed using the Bonferroni procedure for multiple comparisons. A P value of 0.05 was considered significant.

Results

Effect of hyaluronan concentration on embryo development in culture

Zygotes were cultured in DM2/DM3, supplemented with increasing concentrations of hyaluronan. Hyaluronan concentrations between 0.125 and 2.0 mg/ml resulted in morula/blastocyst and blastocyst development equivalent to development in medium supplemented with BSA (Table II). However, 4.0 mg/ml hyaluronan resulted in significantly fewer embryos developing to both the morula/blastocyst ($P < 0.05$), blastocyst ($P < 0.01$) and hatching blastocyst ($P < 0.01$) stages compared with culture with BSA (Table II).

Blastocyst cell numbers, and allocation to the ICM and TE were similar for embryos cultured with hyaluronan concentrations between 0.125 and 1.0 mg/ml and blastocysts cultured with BSA (Table II). Culture with 2.0 and 4.0 mg/ml hyaluronan resulted in blastocysts with significantly lower blastocyst cell numbers compared to culture with BSA (Table II). Hyaluronan at 4.0 mg/ml also significantly reduced development of the ICM, and TE (Table II).

Effect of different macromolecules on embryo development in culture

There was no difference in morula/blastocyst and blastocyst development between embryos cultured with no macromolecule, hyaluronan, or BSA. However, significantly fewer embryos cultured with dextran or PVA formed morula/blastocyst or blastocysts (Table III). Blastocyst hatching was significantly increased by culture with both 0.5 mg/ml hyaluronan and BSA compared with all other treatments (Table III).

Blastocyst cell numbers were highest in embryos cultured in 0.5 mg/ml HA and BSA (79.4 ± 3.2), and significantly higher than 0.5 mg/ml hyaluronan alone. Lowest cell numbers were observed when embryos were cultured in PVA (Table

Table II. Effect of hyaluronan concentration of CF1 mouse zygote development after 96 h of culture

Hyaluronan (mg/ml)	Morula/ blastocyst (%)	Blastocyst (%)	Hatching (%)	Total number of blastocyst cells	Number of ICM cells	Number of TE cells
0.125	74	68	32	63.8 ± 5.5[a]	14.8 ± 1.2	49.1 ±5.6[e]
0.25	86	66	12	60.7 ± 6.3	13.8 ± 1.5	44.9 ± 5.9[f]
0.5	80	70	20	64.2 ± 3.6[b]	17.7 ± 1.3[d]	46.5 ± 3.6[g]
1.0	75	59	16	52.5 ± 3.1	14.0 ± 1.3	38.8 ± 3.1
2.0	62	57	17	42.9 ± 4.7[abc]	12.7 ± 1.4	29.9 ± 4.3
4.0	44*	27**	2**	19.7 ± 2.6**	7.0 ± 1.6[d]	13.2 ± 2.2[efgh]
BSA	88	71	26	60.2 ± 3.5[c]	13.9 ± 1.5	46.2 ± 2.7[h]

$n = 60$ embryos per treatment.
Cell numbers are mean ± SEM.
BSA present at 4 mg/ml.
Significantly different from all other hyaluronan concentrations and BSA; *$P < 0.05$; **$P < 0.01$.
Like pairs are significantly different; [a,c,f]$P < 0.05$; [b,d,e,g,h]$P < 0.01$.

Table III. Effect of different macromolecules on CF1 mouse zygote development

Hyaluronan	Morula/ blastocyst (%)	Blastocyst (%)	Hatching (%)	Total number of blastocyst cells	Number of ICM cells	Number of TE cells
HA (0.25 mg/ml)	85	69	30	72.8 ± 3.2[a]	17.8 ± 0.9	57.4 ± 3.2
HA (0.5 mg/ml)	82	73	27	66.1 ± 3.1[b]	16.9 ± 0.9	54.2 ± 3.5
HA (0.25 mg/ml) + BSA	82	70	27	66.2 ± 2.4[c]	17.7 ± 0.8	52.6 ± 2.2
HA (0.5 mg/ml) + BSA	96	88	52¶	79.4 ± 3.2[bcdef]	18.6 ± 0.9	63.0 ± 3.3[jkl]
BSA (4 mg/ml)	91	71	30	76.2 ± 3.3[ghi]	19.5 ± 0.8	57.5 ± 3.1
Dextran (4 mg/ml)	66†	58†	14	58.3 ± 3.1[dg]	16.3 ± 1.0	43.9 ± 2.9 [j]
PVA (0.1 mg/ml)	68†	56†	8††	56.2 ± 4.3[aeh]	15.2 ± 1.4	44.8 ± 4.4[k]
No macromolecule	82	68	24	60.1 ± 3.7[fi]	18.1 ± 1.6	43.8 ± 2.9[l]

$n = 60$ embryos per treament.
Cell numbers are mean ± SEM.
†Significantly different from HA (0.5 mg/ml) together with BSA (4 mg/ml); $P < 0.05$.
¶Significantly different from other treatments; $P < 0.05$.
††Significantly different from all other treatments except dextran; $P < 0.05$.
Like pairs are significantly different; [a,b,c,i]$P < 0.05$; [d,e,f,g,h,j,k,l]$P < 0.01$.

III). There was no significant difference in ICM cell number over all treatments. However, TE cell numbers were increased by culture with 0.5 mg/ml HA + BSA compared with culture with no macromolecule, dextran, or PVA (Table III).

Effect of macromolecules on blastocyst viability

Culture with hyaluronan ($P < 0.01$), hyaluronan together with BSA ($P < 0.01$), or PVA or dextran ($P < 0.05$), significantly increased implantation rates compared to culture with BSA or no macromolecule. However, fetal development was only significantly increased by culture with hyaluronan or hyaluronan together with BSA (Table IV). Fetal weight was not affected by the presence of macromolecules. It was notable that almost a quarter of fetuses derived from embryos cultured in the presence of dextran were retarded by up to 24h. Furthermore, 12% of the fetuses derived from embryos cultured with PVA were grossly abnormal, exhibiting skeletal and tissue degeneration.

Table IV. Effect of macromolecules on blastocyst viability

Macromolecule	Implantation (%)	Fetus (%)	Fetus/ implantation (%)	Fetal weight (mg)
HA (0.5 mg/ml)	82**	56*	67	275 ± 10
HA + BSA (0.5 + 4.0 mg/ml)	86**	51*	60	252 ± 14
BSA (4 mg/ml)	53	36	67	256 ± 10
Dextran (4 mg/ml)	70*	48	68†	233 ± 9
PVA (0.1 mg/ml)	70*	38	61‡	256 ± 13
No macromolecule	56	44	79	268 ± 10

n = at least 40 embryos per treatment.
Fetal weights are mean ± SEM.
Significantly different from no macromolecule and BSA; *$P < 0.05$; **$P < 0.01$.
†21% of fetuses were retarded by up to 24 h.
‡12% of fetuses were abnormal.

Effect of hyaluronan in the transfer medium on fetal development

Zygotes were cultured in medium without any macromolecule to the blastocyst stage. Blastocysts were randomly allocated for transfer in medium either without macromolecule or supplemented with 0.5 mg/ml hyaluronan. Blastocysts were incubated in either medium for 5 min prior to transfer. The presence of 0.5 mg/ml hyaluronan in the transfer medium alone significantly increased both implantation rates and fetal development rates, compared with no macromolecule in the

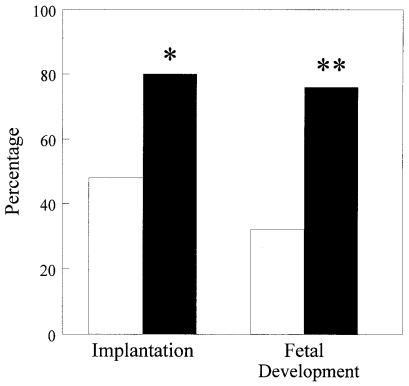

Figure 1. Effect of hyaluronan in the transfer medium on subsequent outcome. Embryos were cultured from the zygote to the blastocyst stage in sequential media lacking protein or macromolecule. Blastocysts were then equilibrated in medium H-DM3 either with or without 0.5 mg/ml hyaluronan. Open bars, no macromolecule; solid bars, 0.5 mg/ml hyaluronan. $n = 25$ embryos per treatment. Significantly different from no macromolecule; $^{*}P < 0.05$; $^{**}P < 0.01$.

transfer medium (Figure 1). Fetal weight was not affected by the presence of hyaluronan in the transfer medium (280 mg with hyaluronan and 283 mg without).

Discussion

This study demonstrated that mouse zygotes from an outbred strain can be cultured in physiologically based sequential media to the blastocyst stage, resulting in high rates of fetal development in the absence of any protein or macromolecule. In fact the addition of BSA to the medium in this study did not affect development to the blastocyst stage in culture or subsequent viability after transfer. While addition of hyaluronan to the culture medium did not affect development to the blastocyst stage, it did lead to significantly increased implantation rates and fetal development rates for these blastocysts following transfer. Subsequently, the beneficial effect of hyaluronan was attributed to its presence in the transfer medium.

Hyaluronan at low concentrations (0.5 mg/ml) has been demonstrated to increase the development of porcine and bovine embryos in culture (Miyano *et al*., 1994; Stojkovic *et al*., 1999). While hyaluronan could be used as the sole macromolecule, an interaction between hyaluronan and albumin in the culture medium on embryo development in culture was evident. Specifically, trophectoderm cell number and hatching were highest when both macromolecules were present in the culture medium. As both hyaluronan and albumin are present at high concentrations in the female reproductive tract (Carson *et al*., 1987; Leese, 1988) this observation highlights the importance of emulating the physiological environment when designing culture media for the mammalian embryo. It is evident that albumin has a role to play in embryo development in culture, even though it conferred no apparent benefit post-transfer. Therefore a source of albumin that is not isolated from mammalian material, but rather produced in a recombinant form is highly desirable. Preliminary results from our laboratory indicate that recombinant albumin is a promising source for

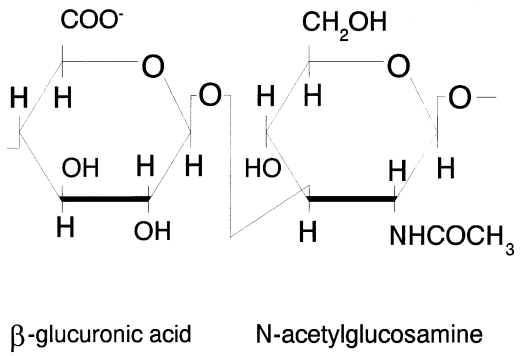

Figure 2. Chemical structure of hyaluronan.

albumin supplementation for mammalian embryo culture media (Gardner and Lane, unpublished observations).

Addition of hyaluronan to the culture medium in the presence of BSA not only resulted in the highest rates of blastocyst development and blastocyst cell number, subsequent implantation rates and fetal development were increased. However, interestingly, addition of hyaluronan to the transfer medium only (blastocysts were cultured in the absence of any macromolecule) significantly increased the rate of implantation and fetal development compared to transfer medium without macromolecule. In the mouse uterus, there is a fivefold increase in the concentration of hyaluronan on the day of implantation. This increase was not observed in mated but non-pregnant mice (Carson *et al*., 1987; Zorn *et al*., 1995). Furthermore, there was no corresponding increase in the concentration of other glycoproteins indicating that the increase was specific to hyaluronan (Carson *et al*., 1987). The observed increase in implantation and fetal development when hyaluronan was added to the transfer medium are consistent with a role in embryo and endometrium interaction. There are several possible means by which hyaluronan could facilitate implantation: hyaluronan has been shown to increase cell–cell adhesion and cell–matrix adhesion (Turley and Moore, 1984), and so may function during the initial stages of apposition and attachment of the blastocyst and endometrium. However, even though the human embryo may specifically bind hyaluronan, it is plausible that the beneficial effects of hyaluronan may be manifest through other indirect routes. For example, hyaluronan can promote angiogenesis by both its degradation products (West *et al*., 1985) and by interaction with epidermal growth factor (EGF) (Sato *et al*., 1991). The latter point is of particular interest given the stimulatory effect of EGF on implantation in the mouse (Paria *et al*. 1991). Hyaluronan may also confer some degree of viral protection and anti-immunogenic properties (Laurent and Fraser, 1992). Alternatively, the beneficial effect of hyaluronan in the transfer medium may be a physical phenomenon, by facilitating rapid diffusion of the contents of the transfer medium (the embryo) into the fluid of the uterus. As uterine fluid is a viscous solution, the transfer of a relatively aqueous solution, such as culture medium with albumin, to the uterine lumen will result in the slow dispersal of the medium and embryo with the luminal contents. In contrast, the transfer of an embryo in a hyaluronan solution will facilitate dispersal of the embryo into the luminal environment.

An additional advantage of hyaluronan is that although it is a glycosaminoglycan, unlike other glycosaminoglycans it is not covalently linked to a core protein, and can therefore be considered a polysaccharide (Figure 2). This therefore removes both the problems of variation and contamination, as hyaluronan can be synthesized and isolated in a pure form. Initial studies have shown that pure hyaluronan, obtained from a bacterial source, in the culture medium results in increases in mouse embryo development in culture and implantation rates post-transfer (D.K.Gardner and M.Lane, unpublished observations), indicating that pure hyaluronan is as effective as that refined from the rooster comb.

Although the addition of BSA resulted in blastocysts with high cell numbers, the resultant implantation rates were low, being equivalent to those embryos cultured and transferred in the absence of a macromolecule.

Addition of dextran to the culture medium at 4 mg/ml also resulted in significantly reduced development to the blastocyst stage compared with no macromolecule. Dextran was used as it is a polyhydroxyl compound, which alters the solvent conditions of aqueous solutions to be more akin to the fluid of the reproductive tract, in a similar way to that in which α- and β-globulin have been proposed to act (Pool and Martin, 1994). However, neither of these compounds is present in the female reproductive tract and cannot therefore be deemed as physiological. However, similar to PVA, blastocysts cultured with dextran had an increased viability compared to culture without macromolecule. Interestingly, however, 21% of the fetuses resulting after transfer were significantly delayed by around 24 h. Nevertheless it is possible that the single concentration used in this study was too high, and for optimum results it may be necessary to titrate the dextran concentration in the medium, and that at lower concentrations dextran may confer benefit to the embryos in culture.

Addition of PVA to the medium at the reported optimum concentration (Bavister, 1981; 1995) as the sole macromolecule resulted in significantly reduced blastocyst formation and hatching compared to development without a macromolecule. Interestingly, implantation rates of resultant blastocysts in this study were increased by culture with PVA compared to culture without macromolecule, although fetal development rates were equivalent. It was found, however, that two of the resultant fetuses (12%) were abnormal. This observation necessitates further investigation.

In conclusion, in the appropriate sequential culture media containing amino acids, mouse blastocysts do not require any protein to develop into viable fetuses after transfer. The inclusion of hyaluronan (refined from rooster comb) in the transfer medium significantly increased implantation and fetal development, implicating a role for this glycosaminoglycan in the implantation process. It should be noted that this effect might not necessarily apply to human embryos. Although it is possible to culture the mammalian embryo to the viable blastocyst stage in the absence of any macromolecule, it would seem prudent to include macromolecules in the culture media for the following reasons: the presence of a macromolecule (i) greatly facilitates embryo manipulation, (ii) enables the binding and stabilizing of growth factors produced by the embryo, and (iii) enables the binding of potential toxins such as low levels of transition metals, thereby making culture conditions more robust. With the advent of recombinant albumin and fermented hyaluronan, such as that already available and used in ophthalmology, it is now possible to standardize culture media composition, while keeping the culture system physiological. Before the widespread introduction of such culture and transfer media in human assisted reproduction technology, prospective clinical trials are warranted.

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