Sex-related differences in the developmental rate of in-vitro matured/in-vitro fertilized ovine embryos

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Sex determination of in-vitro matured/in-vitro fertilized ovine embryos cultured in synthetic oviduct fluid (SOF) medium was performed by the polymerase chain reaction amplification of specific Y DNA sequences so as to test the influence of sex on developmental growth during the preimplantation period. At 144 h post-insemination, embryos with a blastocoele were classified as the fast-developing group, whereas those showing a blastocoele only after this length of time were classified as the slow-developing group. At 144 h post-insemination, fast-developing embryos were cultured separately and some were classified according to the size of their blastocoele. At the end of culture (207 h post-insemination), all embryos were classified according to both their developmental stage and their morphological quality. The male:female sex ratio of fast-developing embryos was significantly higher than the expected ratio of 50%. More males were observed at the most advanced developmental stage at both 144 and 207 h post-insemination. The proportion of males did not differ between the good- and poor-quality groups, although a skewed sex ratio was observed with embryos of better quality at the most developed stage. In conclusion, embryos at the most developed stage were predominantly male and were derived mainly from the fast-developing group, raising the possibility of a deviation in the sex ratio after the transfer of in-vitro matured/in-vitro fertilized ovine embryos.

Key words: sex ratio/sheep embryo/XX–XY difference

Introduction

The occurrence of a sexual dimorphism well before the onset of gonadal differentiation has contributed to the focusing of studies on the sex ratio at the early stages of mammalian development as well as on the determination of the mechanisms involved. The influence of sex on the rate of early embryo development was first observed in the mouse by Tsunoda et al. (1985), being confirmed for both preimplantation (Burgoyne, 1993; Valdivia et al., 1993) and somite-stage mouse embryos (Seller and Perkins-Cole, 1987). The differential growth of male and female embryos was also observed in both in-vitro- and in-vivo-produced bovine embryos (Avery et al., 1989a,b, 1991, 1992; Marquant-Le Guienne et al., 1992; Xu et al., 1992; Yadav et al., 1993; Carvalho et al., 1995). In porcine species, male concepti have been shown to be larger than female concepti on day 10 of gestation (Cassar et al., 1994). Pergament et al. (1994) studied the correlation between the average number of cells in embryos transferred on day 2 after insemination and sex at birth in 36 singleton human pregnancies. They reported that more males were born when the mean number of cells per embryo was greater than four at the time of transfer.

Increased numbers of cells in mammalian preimplantation male embryos have been reported previously (Xu et al., 1992; Burgoyne, 1993; Yadav et al., 1993; Cassar et al., 1995; Peippo and Bredbacka, 1995). In addition, some metabolic parameters have been shown to be increased in male embryos (Tiffin et al., 1991; Ray et al., 1995). There are no reports concerning the sex ratio or sex-related differences in growth rate of ovine embryos, but because of the increased use of the in-vitro production, manipulation and culture of embryos of this species, the possibility of such effects should be investigated. In our study, the sex of in-vitro matured and fertilized ovine embryos cultured in synthetic oviduct fluid (SOF) medium under an atmosphere of 5% O2, 5% CO2, 90% N2 was determined by using the sensitive and accurate technique of polymerase chain reaction (PCR), which has been used successfully to determine the sex of embryos of several mammalian species (Herr et al., 1990; Bredbacka et al., 1991; Peura et al., 1991; Avery et al., 1992; Thibier and Nibart, 1992; Kirkpatrick and Monson, 1993; Machaty et al., 1993; Valdivia et al., 1993; Carvalho et al., 1995; Grisart et al., 1995; Peippo et al., 1995; Ray et al., 1995).

Here we show that male embryos grow faster than female embryos, leading to a significant sex ratio deviation according to the stage of development of the ovine embryos.

Materials and methods

In-vitro embryo production

Procedures used for in-vitro maturation (IVM) and in-vitro fertilization (IVF) were those described previously (Bernardi and Delouis, 1995). Briefly, sheep ovaries were collected at the slaughterhouse and cumulus–oocyte complexes were obtained by aspiration of follicles with a diameter of 2-6 mm. Oocyte maturation was performed in four-well dishes (Nunc, Roskilde, Denmark) for 24 h at 38.5°C in a humidified atmosphere under 5% CO2 in 500 µl TCM 199–HEPES medium (Sigma, St. Louis, MO, USA) supplemented with 2X10^6 granulosa cells/ml, 10% fetal calf serum (FCS, Gibco, Paisley, UK), 110 µg/ml sodium pyruvate, 1 µg/ml 17β-oestradiol (Sigma), 0.1 µg/ml follicle stimulating hormone (pFSH, Stimufol; Rhône
Mérieux, Lyon, France) and 0.02 μg/ml luteinizing hormone (pLH; Stimufol; Rhône Mérieux). After maturation, cumulus cells were partially removed and oocytes were transferred to 1 ml of fertilization medium with Percoll (Sigma)-selected spermatozoa (1 × 10^7/ml). Frozen–thawed spermatozoa pooled from four Ile de France rams were used throughout the experiment.

Presumptive zygotes (indistinguishable from unfertilized ova) were removed from the fertilization medium 16–17 h after insemination, washed in TCM 199–HEPES and cultured in 500 μl SOF medium at 38.5°C under an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. The composition of the SOF medium was that reported by Tervit et al. (1972), with the modifications of Takahashi and First (1992), except for glucose which was maintained at 1.5 mM. Culture was continued until 207 h post-insemination. The medium was renewed at 65 and 144 h post-insemination. SOF medium was supplemented with 3.2 mg/ml bovine serum albumin (Sigma) until 65 h post-insemination and with 10% FCS for the remaining period of culture.

At 144 h post-insemination, embryos showing a blastocoel cavity were classified as fast-developing and were cultured separately from those forming a blastocoeal cavity after 144 h post-insemination; the latter embryos were classified as slow-developing. The culture of both groups was continued until 207 h post-insemination. Some embryos (103/133) belonging to the fast group were scored individually at 144 h post-insemination and were subdivided into three categories as follows: BI, initial blastocyst (the blastocoeal cavity occupies up to 50% of the embryo); BL, blastocyst (blastocoeal cavity occupies up to 90% of the embryo); and BX, expanded blastocyst (blastocoeal fully expanded, diameter of the blastocyst increases, trophoblast and inner cell mass (ICM) are juxtaposed to the zona pellucida which is very thin). At the end of the culture period (207 h post-insemination), all blastocysts were scored as expanded (BX), hatching (BH; a small or great portion of the embryo is herniated through the zona pellucida) or hatched (BE; blastocyst has completely escaped from the zona pellucida, its shape is spherical or slightly elongated). Blastocysts were also assessed morphologically and classified into four quality grades: grade I = excellent quality, distinct cellular outlines and embryonic structures (ICM, trophoblast and blastocoeal), and the absence of intracytoplasmic vesicles, cellular debris or extruded cells; grade II = good quality, similar to those of grade I but with some slight imperfections; grade III = fair quality, the presence of imperfections such as intracytoplasmic vesicles, granulations on the cell surface and cellular debris in the blastocoeal or extruded cells to a moderate extent; and grade IV = poor quality, the absence of defined cellular outlines and/or embryonic structures and the presence of pronounced imperfections.

**Sex determination**

Blastocysts were lysed in a 0.5 ml Eppendorf tube with 30 μl of a buffer containing 50 mM KCl, 2.5 mM MgCl₂, 0.1% Triton X-100, 200 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) and 15 mM Tris–HCl, pH 8.9. Before lysis, expanded and hatching blastocysts were exposed to an acid Tyrode’s solution, pH 2.5, for 3–5 min so as to remove the zona pellucida and prevent possible contamination with DNA from spermatozoa attached to the zona pellucida (Herr and Reed, 1991). All the blastocysts were rinsed individually three times in phosphate-buffered saline before being submitted to the lysis treatment. Because the slight presence or absence of PCR amplification signals was observed initially with grade IV BX and BH, probably as a result of their reduced number of viable cells, the volume of lysis buffer was reduced to 20 μl so as to increase the DNA concentration in samples and thus improve the efficacy of the PCR amplification. Tubes containing lysed blasto-

Statistical analysis

A χ² analysis in a 2 × 2 contingency table was performed to compare sex ratios between different treatments, while a χ² goodness of fit was used to compare sex ratios with an expected ratio of 50:50.

**Results**

From a total of 226 blastocysts, 219 (97%) showed PCR amplification for autosomal primers. Of these, 122 (56%) also showed amplification of the Y-specific sequence. Figure 1 illustrates the pattern obtained by agarose gel electrophoresis after co-amplification of the Y-specific and autosomal sequences.

Table I shows the results obtained after determining the sex of the embryos from the fast- and slow-developing groups. Sex ratios for these two groups were significantly different (P < 0.01). Furthermore, the male:female sex ratio of the fast-developing group was significantly higher (P < 0.01) than the expected ratio of 50%. Although >50%, the overall male:
female sex ratio (56%) did not differ significantly from the expected ratio of 50% (P < 0.05).

The results of the sex ratio analysis distributed according to the stage of development 144 h post-insemination are shown in Table II. The highest male:female sex ratio was observed at the most developed stage (BX) and the lowest at the less advanced stage (BI). The sex ratios of those embryos belonging to these two stages differed significantly (P < 0.02). A proportion of males significantly (P < 0.01) >50% was observed only at the most advanced stage of development (BX).

When the sex ratio was analysed according to the developmental stage at 207 h post-insemination (Table III), a proportion of males significantly >50% was again observed at the most advanced stage (P < 0.02). If considered separately, a skewed sex ratio towards males was observed with hatched blastocysts derived from the fast-developing group (P < 0.02), whereas a skewed sex ratio towards females was observed with hatching blastocysts derived from the slow-developing group (P < 0.05). However, a reduced number of blastocysts (21) was present in the latter group. Although >50% (65 and 60% for BH and BX respectively), the proportions of males observed in stages BH and BX, both from the fast-developing group, were not significantly different from the expected ratio of 50%, probably because of the reduced number of blastocysts of these categories available 207 h post-insemination. Indeed, it was observed that a greater proportion of embryos from the fast-developing group reached the hatched blastocyst stage (83%; 111/133) than those from the slow-developing group (52%; 45/86).

Table I. Sex ratio of ovine embryos derived from in-vitro maturation/in-vitro fertilization and subsequent culture in synthetic oviduct fluid medium

<table>
<thead>
<tr>
<th>Time of blastocyst formation</th>
<th>No. of embryos</th>
<th>Sex ratio (male:female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sexed</td>
<td>Females (%)</td>
<td>Males (%)</td>
</tr>
<tr>
<td>Fast&lt;sup&gt;a&lt;/sup&gt;</td>
<td>133</td>
<td>50 (37.6)</td>
</tr>
<tr>
<td>Slow&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86</td>
<td>47 (54.7)</td>
</tr>
<tr>
<td>Total</td>
<td>219</td>
<td>97 (44.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Group of embryos that reached the blastocyst stage at 144 h post-insemination.
<sup>b</sup>Group of embryos that reached the blastocyst stage after 144 h post-insemination.
<sup>c</sup>Different letters within columns indicate male proportions significantly different (P < 0.01).
<sup>d</sup>Groups in which the sex ratio differs significantly from 50% (P < 0.01).

Table II. Sex ratios of various developmental stages present at 144 h post-insemination for ovine embryos derived from in-vitro maturation/in-vitro fertilization and subsequent culture in synthetic oviduct fluid medium

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>No. of embryos</th>
<th>Sex ratio (male:female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sexed</td>
<td>Females (%)</td>
<td>Males (%)</td>
</tr>
<tr>
<td>BX</td>
<td>57</td>
<td>17 (29.8)</td>
</tr>
<tr>
<td>BL</td>
<td>22</td>
<td>10 (45.5)</td>
</tr>
<tr>
<td>BI</td>
<td>24</td>
<td>14 (58.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expanded blastocyst; <sup>b</sup>Blastocyst; <sup>c</sup>Initial blastocyst.
<sup>d</sup>Different letters within columns indicate male proportions significantly different (P < 0.02).
<sup>e</sup>Sex ratio significantly different from the expected 50% (P < 0.01).

A greater proportion of blastocysts with a non-transferable quality (grade IV) was observed in the slow- (52%) than in the fast-developing group (22%). A statistical analysis of the sex ratio according to the morphological quality 207 h post-insemination was also performed (Table IV). In an attempt to reproduce the results of a theoretical situation of embryo transfer, blastocysts of transferable quality (grades I–III) were assembled for analysis. No difference was observed in the proportions of males between the good- and poor-quality groups (P > 0.05). When only the most advanced stage (BE) was taken into consideration, the male:female sex ratio of blastocysts with a transferable quality was significantly >50% (P < 0.02). When the three stages (BE, BH and BX) were considered together, a tendency for a shift in the sex ratio towards males (P < 0.08) was again observed within better quality blastocysts (grades I–III).
Discussion

The results of this study show that male embryos derived from IVM/IVF and cultured in vitro developed faster than female embryos. In our IVM/IVF procedure, only embryos that reached at least the expanded blastocyst stage were considered as being normally developed at 207 h post-insemination. Embryos reaching the initial blastocyst or the blastocyst stage at the end of the culture period were thus not sexed. This aspect, together with the fact that embryos showing no PCR amplification were derived mainly from the slow-developing group, could explain the slightly elevated overall sex ratio observed.

Observed differences in the time of the first cleavage of bovine embryos (Yadav et al., 1993), in cell number and in the pyruvate metabolism of human embryos on day 2 (Ray et al., 1995) indicate that sexual differences arise at an earlier stage of development during the first or second cleavage divisions. Differences in the timing of fertilization were excluded by Valdivia et al. (1993), at least for mouse embryos, because they observed that 100% of the oocytes were penetrated by spermatozoa within 1 h post-insemination.

Differential growth seems to be maintained at later stages, because it was reported for somite-stage mouse embryos (Seller and Perkins-Cole, 1987) and for pre-attachment pig embryos (Cassar et al., 1994) both after in-vivo development. On the other hand, metabolic differences initially observed with in-vitro-produced human embryos (Ray et al., 1995) were not present on the last day of culture (day 6), the time at which male blastocysts had hatched (or even collapsed) than female blastocysts. In our study, we observed that >50% of the overall developed embryos were already hatched 24 h before the end of the culture period. In addition, the proportion of males in the most advanced stage was 10% lower at 207 h post-insemination if compared with that observed at 144 h post-insemination. Therefore it is possible that a long-term culture could limit the growth of more advanced embryos, whereas less advanced embryos are able to achieve morphological or metabolic profiles similar to those shown by embryos initially more advanced, thus reducing or even suppressing sex-related differences.

It has been observed that embryo viability after micro-manipulation, freezing or culture may be sex-dependent, with female embryos surviving adverse conditions less often (King et al., 1992; Yadav et al., 1993; Carvalho et al., 1995) or showing a reduced number of cells compared with males (Peippo and Bredbacka, 1995). Grisart et al. (1995) suggested that the absence of differential growth in bovine embryos could be accounted for by the use of improved culture conditions. In our study, the proportion of males in the good-quality group was no different from that in the poor-quality group, indicating that culture conditions had no selective influence on embryo quality. However, we cannot know if possible differential effects could have been reduced following a prolonged period in culture, as postulated above. Therefore it is necessary to perform additional studies at an earlier time during culture and also using in vivo-produced embryos.

Various mechanisms can be implicated in the occurrence of a sexual dimorphism before the development of gonads. Sex-related metabolic differences may be involved, as a cause or effect, because both bovine and human male embryos showed an increased metabolic rate compared with female embryos (Tiffin et al., 1991; Ray et al., 1995). In contrast, Tiffin et al. (1991) suggested that the slightly greater glucose uptake observed in female mouse embryos compared with males (Gardner and Leese, 1987) may possibly be accounted for by a greater glucose incorporation into glycogen by females, which was not taken into account in measurements performed on bovine embryos. Even if the effect of glycogen accumulation on the developmental arrest of mouse embryos has been alleviated by replacing glucose with fructose in the culture medium (Ménézo and Khatchadourian, 1990), a greater glycogen accumulation by females has not yet been confirmed. A double dose of the glucose-6-phosphate dehydrogenase X-linked enzyme is likely to be implicated, causing faster glucose metabolism via the pentose-phosphate pathway in female bovine embryos (Tiffin et al., 1991), but it is not clear whether this can be related to a slower rate of development.

A possible effect of Y-linked genes promoting the growth of male embryos, as suggested previously (Avery, 1989; Burgoyne, 1993; Zwinger et al., 1993), has been strengthened recently by the presence of Y-linked transcripts, including Sry and Zfy, as early as the zygote stage and through to the blastocyst stage in mouse and human embryos (Zwingman et al., 1993; Ao et al., 1994; Cao et al., 1995; Fiddler et al., 1995). Although translational activity for Sry and Zfy at early stages has not yet been determined, the expression of male-specific H-Y antigen, demonstrated on embryos of several mammalian species as early as the 8-cell stage (White et al., 1987a,b,c), shows that not only transcriptional but also translational differences exist between male and female preimplantation embryos. Furthermore, a role for the H-Y antigen in the regulation of embryonic growth and development was postulated by Heslop et al. (1989).

Mittwoch (1977) has suggested that the expression of H-Y antigen could be related to the enhanced growth rate of embryonic gonads of the heterogametic sex at a critical stage of development, prerequisite for its differentiation. In this context, she postulated that a fast growth rate for male mammalian embryos should be a necessary condition which increases the probability of promoting the normal development of the male gonad (Mittwoch, 1993).

Skjervold (1979) observed a drop in the sex ratio from single to multiple births in sheep and suggested that this difference may be the result of a differential loss of predominantly male fetuses, an assumption supported further by others (Bondioli et al., 1989; Berg et al., 1992; Burgoyne, 1993). If this hypothesis is correct, XY embryos should initially be represented in greater numbers than XX embryos. Nevertheless, there is no evidence suggesting this because the overall sex ratio of embryos showing differences in the morphology, size and number of cells (Avery et al., 1991, 1992; Xu et al., 1992; Cassar et al., 1994, 1995; Carvalho et al., 1995) was not skewed significantly towards males. A sex-selective fetal mortality seems to be inconsistent with the facts that no excess
of males was reported among spontaneous abortions with normal karyotype in the first trimester of human pregnancy (Hassold et al., 1980; Guernieri et al., 1987) and that the sex ratio of bovine fetuses of gestational age, varying from 7 to 22 weeks, was not different from that observed at calving (Leibo and Rall, 1990). Furthermore, fast- and slow-cleaving mouse embryos showed no difference in their developmental ability after transfer (Tsunoda et al., 1985; Valdivia et al., 1993).

However, the possibility of the occurrence of a deviation in the sex ratio after the transfer of in-vitro-matured/in-vitro fertilized ovine embryos cannot be ruled out, especially if more advanced embryos are transferred preferentially at ~6 days post-insemination.

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