Blastocyst embryo transfer is associated with a sex-ratio imbalance in favor of male offspring

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Objective: To evaluate the sex ratio of offspring born after blastocyst transfers.
Design: Retrospective data analysis.
Setting: A large assisted reproductive technology center.
Patient(s): We included 1,284 offspring from 937 deliveries during the period August 2003–August 2005.
Intervention(s): Tabulation and statistical analysis of all births resulting from fresh IVF cycles. The sex of resulting offspring was compared in both day 3 and blastocyst transfers for all births and for singleton deliveries. In addition, the sex of children conceived with the use of autologous oocytes and donor oocytes was evaluated.
Main Outcome Measure: Sex ratio of offspring born following embryo transfers (ETs) after day 3 of culture and sequential blastocycst culture.
Result(s): The overall sex ratio was significantly shifted toward males when blastocyst transfers were performed. Blastocyst transfers with only the use of autologous oocytes resulted again in a significantly higher proportion of male offspring. An even greater proportional difference was encountered in singleton offspring from donor oocytes. However, significance was not reached because of the limited number of offspring in the subgroup.
Conclusion(s): This is the first individual-center report of a significant sex-ratio imbalance after the sequential media culture of blastocysts. The large imbalance in singleton births associated with the use of donor oocytes, although not significant, is cautionary in regard to the use of elective single ETs. Observation and publication of phenomena such as the effects of extended culture on the sex ratio of live-borns will allow us a better understanding of early differences in sexual dimorphism of the embryo, and will allow us to counsel our patients more appropriately. (Fertil Steril® 2007;87:519–23. ©2007 by American Society for Reproductive Medicine.)

During the process of in vitro fertilization (IVF), the selection of human embryos for transfer is most often based on morphological criteria and the cleavage and development rate. It was demonstrated that, in animal species, the fastest-growing embryos are more commonly male (1, 2). The same relationship was found in human embryos (3), in which we encounter a greater likelihood of male offspring in cleavage-stage embryos (days 2 and 3 after retrieval) with a greater number of cells.

The advent of high-quality blastocyst culture techniques, using sequential media, has led many centers to use blastocyst culture to assist in embryo selection. Transferring blastocyst-stage embryos has enhanced implantation rates, allowing high-order multiple births to be decreased when the number of embryos transferred is reduced (Duke et al., unpublished data). It was demonstrated that male embryos maintained in culture to the blastocyst stage of development had significantly greater numbers of cells on day 2 than female embryos, and that this difference persisted throughout further in vitro development (4).

Received March 14, 2006; revised and accepted June 29, 2006.
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Blastocyst grading for embryo transfers (ETs) usually occur on day 5 of culture at 120–124 hours after retrieval, and is based on the degree of blastocoel expansion and the cellularity of both the inner cell mass (ICM) and trophectoderm (5). Since the most advanced blastocyst is often selected for ET, it is plausible that the sex ratio will be altered in favor of males. We set out to investigate whether blastocyst-selection criteria in our laboratory would affect the male-to-female sex ratio, compared to our day 3 ETs.

MATERIALS AND METHODS

We retrospectively analyzed all offspring born from August 2002 to August 2005 as a result of fresh IVF cycles performed at our center. As required by law for the Centers for Disease Control Reproductive Reporting Guidelines, the gender of all offspring resulting from IVF cycles is ascertained from each couple. A broad-based retrospective institutional review board waiver was obtained for this study. The sex of all offspring resulting from blastocyst ETs was compared to day 3 ETs. In addition, the sex of all children born from autologous oocytes as opposed to donor oocytes was evaluated.

Singleton births resulting from blastocyst ETs were analyzed in comparison to those singletons resulting from day 3

0015-0282/07/$32.00
doi:10.1016/j.fertnstert.2006.06.058

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ETs. Further assessment according to the origin of oocytes used was also performed for singleton deliveries.

Blastocyst grading for embryo selection occurs on day 5 of culture. The percentage of blastocyst formation was determined, and each blastocyst was assigned a score by using a modified system based on the classification originally reported by Gardner and Schoolcraft (6). Briefly, blastocysts were given a numerical score from 1–6 on the basis of their degree of expansion and hatching status, as follows: 1, an early blastocyst with a blastocoel whose volume is less than half that of the embryo; 2, a blastocyst with a blastocoel whose volume is half of, or greater than half of, that of the embryo; 3, a blastocyst with a blastocoel completely filling the embryo; 4, an expanded blastocyst with a blastocoel volume larger than that of the early embryo, with a thinning zona; 5, a hatching blastocyst with the trophectoderm starting to herniate though the zona; and 6, a hatched blastocyst, in which the blastocyst has completely escaped from the zona.

For blastocysts with expansions graded as 3–6, the development of the ICM was assessed as follows: A, many cells, tightly packed; B, several cells, loosely grouped; C, few cells, slightly disorganized; or D, very few disorganized and uneven cells. The trophectoderm was assessed as follows: A, many cells forming a cohesive epithelium; B, a few cells forming a loose epithelium; C, very few large cells; or D, very few cells of an uneven nature.

Statistical analysis for the sex ratio was performed with the chi-square test. One-way analysis of variance (ANOVA) was used to analyze blastocyst expansion, and for grading of the ICM and trophectoderm. Analysis was performed with Analyse-It® Software, Ltd (Leeds, England, UK) for Microsoft Excel 2000. Statistical significance was set at $P<.05$.

RESULTS

In total, 937 patient deliveries were included in this analysis. The 603 singleton pregnancies, 321 twin pregnancies, and 13 triplet pregnancies were analyzed. The overall number of offspring was 1,284, of whom 674 resulted from day 3 ETs, and 610 resulted from blastocyst ETs. The overall female-to-male (F:M) ratio for offspring resulting from day 3 ETs was significantly higher than the ratio for offspring resulting from blastocyst ETs ($P=.023$) (Table 1).

When analyzing the sex ratio according to the number of offspring born per delivery, we found a sex-ratio imbalance for singleton deliveries toward males of 51.5% when day 3 ETs were performed, compared to 63.7% when blastocysts were transferred. The F:M ratio for singleton births was 1:1.06 for day 3 ETs, compared to 1:1.76 for blastocyst ETs ($P=.003$; odds ratio [OR], 1.654). In multiple deliveries, the F:M ratio was similar between day 3 ETs and blastocyst ETs ($P=.666$; OR, 1.081) (Table 1).

When analyzing all births using only autologous oocytes, the sex of offspring resulting from blastocyst ETs was significantly higher in the proportion of male offspring, compared to offspring resulting from day 3 ETs ($P=.025$). A similar trend in the ratio was encountered for offspring resulting from donor oocytes when transfers were performed at the blastocyst stage. However, statistical significance was not attained because of the limited number of deliveries in this subgroup (Table 1).

Analysis of singleton births from the autologous oocyte subgroup also demonstrated that blastocyst ETs resulted in a significantly higher number of male offspring, compared to day 3 ETs ($P=.014$; OR, 1.608). Although an even more pronounced trend toward a higher number of singleton male

TABLE 1

Sex ratio according to type of delivery.

Deliveries (n)	Offspring (n)	Day 3 ETs, female (%)/ male (%)	Blastocyst ETs, female (%)/ male (%)	*P* value, OR, and 95% confidence interval
All deliveries (n = 937)	1,284	329 (48.8)/345 (51.2)[a]	258 (42.3)/352 (57.7)[a]	.0233, 1.301, and 1.044–1.622
Autologous	1,060	288 (48.8)/302 (51.2)[a]	196 (41.7)/274 (58.3)[a]	.0246, 1.333, and 1.044–1.702
Donor	224	41 (48.8)/43 (51.2)	62 (44.3)/78 (55.7)	.6036, 1.200, and 0.6972–2.064
Singleton (n = 603)	603	160 (48.5)/170 (51.5)[a]	99 (36.3)/174 (63.7)[a]	.0033, 1.654, and 1.192–2.296
Multiple (n = 334)	681	169 (49.1)/175 (50.9)	159 (47.2)/178 (52.8)	.6660, 1.081, and 0.8003–1.460

[a] $P<.05$.

Luna. Sex-ratio imbalance in blastocyst transfers. Fertil Steril 2007.

TABLE 2

Singleton pregnancies.

Total offspring	Autologous female (%)/ male (%)	*P* value, OR, and 95% confidence interval	Donor female (%)/ male (%)	*P* value, OR, and 95% confidence interval
Day 3				
330	136 (48.4)/145 (51.6)[a]	0.0139, 1.608, and 1.115–2.318	24 (49.0)/25 (51.0)	.1698, 1.833, and 0.8557–3.925
Blasts				
273	77 (36.8)/132 (63.2)[a]		22 (34.4)/42 (65.6)	

[a] $P<.05$.

Luna. Sex-ratio imbalance in blastocyst transfers. Fertil Steril 2007.

offspring with blastocyst ETs was encountered in the offspring of recipients who used donor oocytes, with a F:M ratio of 1:1.90, the difference was not significant because of the limited number of infants in this subgroup ($P=.1698$; OR, 1.833) (Table 2).

We further analyzed blastocyst grading (expansion, ICM, and trophectoderm). Embryos of patients who had 100% implantation with same-sex offspring were analyzed according to gender. In total, 131 blastocysts were analyzed, of which 74 were male and 57 were female. Although female embryos demonstrated a slightly greater expansion and ICM grading, the difference did not approach significance. Male embryos had a slightly higher trophectoderm grading than female embryos. However, this finding was not significant (Figs. 1, 2). The transfer of embryos with a grade 3 expansion score resulted only in male offspring.

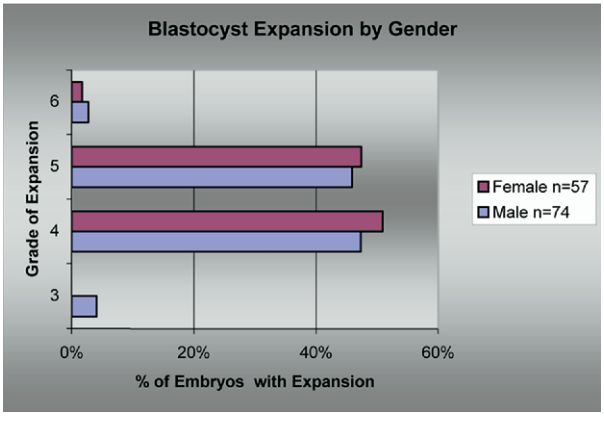
In addition, the gender of 126 offspring resulting from cryopreserved and thawed ETs was analyzed. The sex ratio of offspring resulting from these cycles was not significantly altered ($P=.649$; OR, 0.75).

DISCUSSION

It was demonstrated in animal studies that the fastest-cleaving embryos tend to be male. However, several large, retrospective studies in humans failed to replicate these results (7–10). Anderson et al., in a retrospective analysis, reported that no significant difference was found in the sex ratio

FIGURE 1

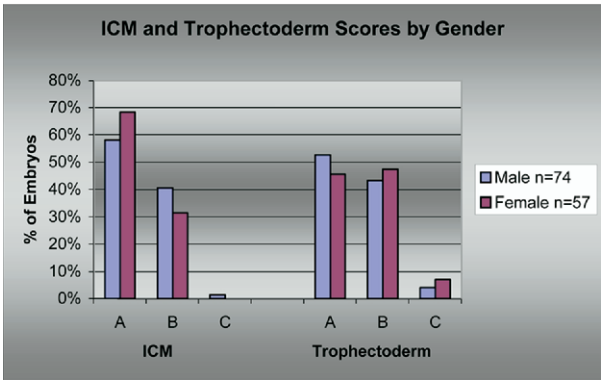
Blastocyst expansion according to gender. Blastocyst expansion is not significantly different between female and male embryos. Only male embryos resulted from blastocysts with an expansion score of 3 ($P=.7306$).



Luna. Sex-ratio imbalance in blastocyst transfers. Fertil Steril 2007.

FIGURE 2

Inner cell mass (ICM) and trophectoderm grading according to gender. More female embryos presented a higher ICM score than did males. However, significance was not attained ($P=.1895$). Trophectoderm grading was higher in male embryos, although a lack of significance is observed ($P=.3412$).



Luna. Sex-ratio imbalance in blastocyst transfers. Fertil Steril 2007.

relative to the day of transfer, and considered these results attributable to their selection criteria based on cellular morphology, including observation of embryos with the healthiest ICM and trophectoderm formation (11). Two additional small studies retrospectively analyzed their own outcome data, and found only a nonsignificant trend toward more male offspring after human blastocyst transfers (2, 12).

The lack of power, in individual studies, to show an altered sex ratio with blastocyst transfer has been described (13). Statistical significance was encountered only after including data from both published and unpublished study populations in a meta-analysis, computing a F:M ratio of 1:1.34 (594/797, or 42.7%/57.3%) for blastocyst ETs, compared to 1:1.04 (932/977, or 48.8%/51.2%) for day 3 ETs ($P=.0001$).

The only two studies (an abstract and a peer-reviewed report) that encountered a significant shift toward male offspring after ETs involved the coculture of blastocysts, a technique no longer permitted in the United States (14,15). Kausche et al. described a small shift in the sex ratio toward males (16), perhaps because these authors performed transfers on day 6 of embryo culture, allowing for the slower-developing blastocysts to expand more fully and reach a similar developmental grade to the faster-developing blastocysts on day 5.

Our study entails the first retrospective analysis to demonstrate a significant sex-ratio imbalance toward male offspring for patients who have a blastocyst ET with the use of sequential media culture. For patients who have a blastocyst ET, the sex ratio is significantly altered for all offspring born (1:1.36) in favor of males, and the ratio is even more significantly altered when analyzing only singleton births (1:1.76). Although we did not find a significant difference when evaluating oocyte donor cycles, the trend toward a sex-ratio imbalance in this group is also very evident (1:1.90 in favor of males). This finding suggests that higher-quality oocytes may have a faster developmental potential when fertilized, and thus male embryos are selected more predominantly.

At our center, preference for transfer is given to blastocysts showing the most expansion of the blastocoel cavity, with signs of hatching and good cellularity of the ICM and trophectoderm on day 5. We believe that selection criteria in favor of faster-developing and more expanded embryos, with presumably the greatest implantation potential, are predominantly male after 120–124 hours of culture after retrieval. Because of these significant findings, we analyzed blastocyst quality relative to expansion, ICM, and trophectoderm according to gender in patients who demonstrated a 100% implantation rate and had same-sex offspring in cases of twin or triplet pregnancies. Interestingly, we found that the difference in blastocyst grading between female and male embryos was not significant. Although this analysis was limited in power by the number of blastocysts analyzed, no subsets of differences were noted between male and female embryos. Ideally, a prospective study in which trophectoderm biopsies are performed, or Y-chromosome-expressed proteins in extended culture media are analyzed after blastocyst exposure, is required to determine the association between morphological criteria and gender.

An important goal for reproductive endocrinologists is to reduce the number of embryos transferred, to eliminate the high rate of complications associated with multiple pregnancies. In fact, many centers have adopted single-blastocyst transfers in their practice. This study clearly demonstrates that fresh blastocyst ET is associated with a higher proportion of male offspring ($P<.03$), and we believe that this is because male embryos have a faster preimplantation developmental rate than do female embryos. Most of our patients underwent ET with more than one embryo, which obviously enhances the chance for a more balanced sex ratio. However, this was not seen in the singleton offspring that resulted. In fact, a significant shift toward male offspring was most apparent when analyzing singleton pregnancies (1 female: 1.76 male) ($P=.003$). Therefore, we feel that the elective selection of the single, most fully expanded blastocyst would potentially lead to the selection of more male embryos, and would even further imbalance the sex of children born than what was demonstrated in this study.

Our findings suggest that it is not blastocyst culture itself that significantly alters the sex ratio, but perhaps the selection criteria that favor the faster-developing and most expanded embryos on day 5 of embryo culture, even though this was not confirmed when analyzing the embryo morphology of those patients with 100% implantation, because of the lack of power.

It is possible that the morphological selection criteria that bias the embryologists' choice of blastocysts for transfer may overlap with morphological characteristics that predict sex. Given these findings, and trying to confirm that our selection criteria are the cause of the sex-ratio imbalance, we analyzed the gender of offspring resulting from cryopreserved ETs, assuming that nontransferred, cryopreserved embryos would be more frequently female. However, when analyzing these cases, we did not find significance in the difference in the gender of offspring from frozen-blastocycst ETs.

Perhaps other reasons for our significant findings, such as immunological factors or X-chromosome imprinting errors, may be involved that would select against female embryos at the preimplantation stage of development, or may be responsible for the spontaneous loss of female embryos after implantation.

Perhaps, when performing an elective single ET at the blastocyst stage, we should consider our selection criteria more fully, or aim toward the use of computerized embryo modeling. This may allow for optimization of embryo selection purely for implantation potential, hopefully without the resultant imbalance of sex ratio observed here. Observation

and publication of phenomena such as the effects of extended culture on the sex ratio of live-borns will allow us to better understand early differences in sexual dimorphism, and allow us to counsel our patients more appropriately.

REFERENCES

1. Avery B, Madison V, Greve T. Sex and development in bovine in vitro-fertilized embryos. Theriogenology 1991;35:953–63.
2. Valdivia R, Kunieda T, Azuma S, Toyoda Y. PCR sexing and development rate differences in preimplantation mouse embryos fertilized and cultures in vitro. Mol Reprod Dev 1993;35:121–6.
3. Tarin J, Bernabeu R, Baviera A, Bonada M, Cano A. Sex selection may be inadvertently performed in in-vitro fertilization-embryo transfer programs. Hum Reprod 1995;10:2992–8.
4. Ray P, Conaghan J, Winston R. Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization. J Reprod Fertil 1995;104:165–71.
5. Gardner D, Lane M, Stevens J, Schlenker T, Schoolcraft W. Blastocyst score implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 2000;73:1155–8.
6. Gardner D, Schoolcraft W. In vitro culture of human blastocyst. Towards reproductive certainty: infertility and genetics beyond 1999. Carnforth: Parthenon Press, 1999:378–88.
7. Rodriguez H, Bustillo M, La Palme J, Riley E, Eisermann J, Thompson K. Clinical outcomes with transfer of blastocyst or cleavage stage embryos: high order pregnancies, newborn weights, and sex ratio. Fertil Steril 2001;76(Suppl 3):S170.
8. Wilson M, Hartke K, Kiehl M, Rodgers J, Brabec C, Lyles R. Integration of blastocyst transfer for all patients. Fertil Steril 2002;77:693–6.
9. Fanchin R, Righini C, Olivennes F, Lejuene V, Volante M, Frydman R. Female and male human embryo growth rates are similar before the eight cell stage. Am J Obstet Gynecol 1998;178:45–9.
10. Ng E, Claman P, Leveille M, Tanphaichitr N, Compitak K, Suwajanakorn S, et al. Sex ratio of babies is unchanged after transfer of fast versus slow-cleaving embryos. J Assist Reprod Genet 1995; 12:566–8.
11. Anderson M, Graham J, Tucker M, Krecko T, Levy M, Widra E. Comparison of gender and birth weight following day three and blastocyst transfers. Fertil Steril 2001;76(Suppl 3):S179.
12. Meintjes M, Crider-Pirkle S, Ward D, Rodriguez J, Chantilis S, Madden J. Sex ratio and birthweight outcome when considering blastocyst expansion, multiple gestation, and facility. Fertil Steril 2001;76(Suppl 3):S147.
13. Milki A, Jun S, Hinckley M, Westphal L, Giudice L, Behr B. Comparison of the sex ratio with blastocyst transfer and cleavage stage transfer. J Assist Reprod Genet 2003;20:323–6.
14. Mercader A, Gamiz P, de los Santos M, Remohii J, Pellicer A, Simon C. Sex ratio after day 2, day 3, or blastocyst transfer. Fertil Steril 2001;76(Suppl):S261–2.
15. Menezzo Y, Chouteau J, Torello M, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. Fertil Steril 1991;72:221–4.
16. Kausche A, Jones G, Trounson A, Figueiredo F, MacLachlan V, Lolatgis M. Sex ratio and birth weights of infants born as a result of blastocyst transfers compared with early cleavage stage embryo transfers. Fertil Steril 2001;76:688–93.