The effect of GM-CSF on development and aneuploidy in murine blastocysts

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BACKGROUND: Growth factors and cytokines are present in small quantities in the oviduct and uterus and some are synthesized by the growing embryo. Granulocytes–macrophage colony-stimulating factor (GM-CSF) is known as an important regulator, which enhances cell proliferation and reduces apoptosis in developing blastocysts, during normal fetal and placental development. The purpose of this study is to investigate whether adding GM-CSF to the culture media affects blastulation or the chromosomal status of mouse embryos.

METHODS: Murine embryos were cultured in vitro from the 2-cell stage until the blastocyst stage in the presence of different concentrations of GM-CSF of 0 ng/ml (control), 1, 2, 5 and 10 ng/ml. The development of each embryo was noted and the embryos were then spread for fluorescence in situ hybridization (FISH) using locus-specific probes (LSI) for chromosomes 2, 11 and 16 in all embryos.

RESULTS: No difference in the blastulation potential was noted with the addition of 1 and 2 ng/ml of GM-CSF compared with the controls, but there was a significant decrease (P < 0.001) in the blastulation rate in the 5 and 10 ng/ml concentrations. The rate of mosaicism/aneuploidy noted in all GM-CSF groups (1, 2, 5 and 10 ng/ml) was slightly higher than in the control group (0 ng/ml GM-CSF) but the differences were not significant. In the mosaic embryos from the GM-CSF cultured groups, the percentage of aneuploid cells was statistically higher than in the control group.

CONCLUSIONS: GM-CSF exerted a negative impact on blastocyst development at higher concentrations. GM-CSF did not affect the rates of mosaicism/aneuploidy, but did increase the percentage of aneuploid cells within the mosaic embryos. Adding GM-CSF to the culture media for clinical use requires further studies either on human or animal models to evaluate its long-term effects.

Key words: GM-CSF / embryo development / aneuploidy / FISH / growth factors

Introduction

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine normally expressed in the female reproductive tract. It is produced by the epithelial cells of structures such as the ovaries, fallopian tubes and the endometrium, under the influence of hormones such as estrogen (Chiavegato et al., 1995). In humans and mice, the GM-CSF receptor has been detected in embryos from the fertilized oocyte until the blastocyst stage (Sjoblom et al., 2002). It is known for its importance in the development of blastocysts and in normal fetal and placental development, due to its anti-apoptotic and mitogenic effects (Sjoblom et al., 1999; Sjoblom et al., 2005; Richter, 2008). The level of GM-CSF fluctuates during the menstrual cycle. It rises from the time of fertilization up until the time of implantation, after which progesterone reduces the secretion of this cytokine (Robertson and Seamark, 1992; Tremellen et al., 1998). Robertson et al. (2001) reported that mice which were null for GM-CSF produced blastocysts with a slower developmental potential and lower cell numbers (up to 18% less) than normal mice, thus highlighting the importance of this cytokine in preimplantation development. The same group reported that culturing both mutant null and normal blastocysts in media containing GM-CSF resulted in an increase in both the size of blastocysts and in the number of cells (Robertson et al., 2001).

Supplementing media with GM-CSF has been shown to reduce apoptosis by up-regulating molecules such as Bcl-2, which suppress apoptotic pathways (Wislez et al., 2001; Behr et al., 2005). Sjoblom et al. (2002) demonstrated a reduction by half in the number of apoptotic cells in blastocysts following in vitro culture with GM-CSF. In addition, metabolism was found to be influenced by the cytokine, since culturing embryos in vitro with GM-CSF led to an increase in glucose uptake (Robertson et al., 2001). Media containing hemopoietic cells, and thus containing GM-CSF, have been shown to promote the...
development of mouse zygotes into blastocysts (Robertson and Seamark, 1990). Pig embryos also follow the same trend when cultured with GM-CSF, and 2-cell human embryos that were cultured in media containing recombinant GM-CSF, doubled their chances of forming blastocysts and produced an inner cell mass (ICM) that was larger in size (Imakawa et al., 1993; Sjoblom et al., 1999). This increase in cell numbers in the ICM could be explained by a lower incidence of apoptosis allowing the survival of more cells, including cells with chromosomal abnormalities.

Few studies have examined the effects of growth factors in the culture media on the chromosome constitution of embryos in IVF. Agerholm et al. (2010) used FISH to look at chromosomes 13, 16, 18, 21, 22, X and Y in human embryos that were cultured in Embryo-Assist™ media (Medicult) to which 2 ng/ml of GM-CSF was added. They reported that culturing with GM-CSF did not increase chromosome abnormalities compared with the controls (66.7 versus 65.2% respectively). Also, no significant difference was seen in the rate of fertilization or the rate of early cleavage between the two groups. However, further studies are needed, particularly since a culture medium (EmbryoGen) containing 2 ng/ml GM-CSF was recently launched by Origio (Harper et al., 2012).

The purpose of this study was to investigate whether adding GM-CSF (1, 2, 5 and 10 ng/ml) to the culture media has any effect on murine preimplantation embryos, both in terms of their chromosomal constitution and their ability to reach the blastocyst stage.

### Materials and Methods

#### Embryo collection and culture

MF1 mouse (~5–7 weeks, Charles River, UK) were used in this study. The superovulation protocol and embryo procurement were accomplished as described in Sabhnani et al. (2011). Post-mating, the females were sacrificed and 2-cell stage embryos (48 h post-hCG) were flushed from the oviduct using HEPES-buffered potassium simplex optimization medium (H-KSOM) (Summers et al., 2000; Sabhnani et al., 2011). Thereafter, these embryos were divided randomly by simple randomization into five groups; one group was cultured in potassium simplex optimization medium (H-KSOM) (Summers et al., 2000; Sabhnani et al., 2011). Hereafter, these embryos were divided randomly by simple randomization into five groups; one group was cultured in potassium simplex optimization medium containing 1 g/l BSA (bovine serum albumin) (KSOM, Millipore) only and was used as a control at 37°C and 5% CO2. Four other groups were cultured in the same media supplemented with concentrations of 1, 2, 5 and 10 ng/ml of recombinant mouse GM-CSF (R&D system, Abingdon, UK) which was previously dissolved in phosphate-buffered saline (PBS) according to the manufacturer instructions. The selection of GM-CSF concentrations was made after a review of relevant studies on the effect of GM-CSF on the developmental potential of embryos from different species (Sjoblom et al., 1999; Behr et al., 2005; Karagenc et al., 2005; Sjoblom et al., 2005; Agerholm et al., 2010). All of the embryos (control or treated) were cultured, under the same conditions using the same incubator, for 3 days until they reached the blastocyst stage (Day 5: 120 h post-hCG), with a daily developmental assessment. The protocol was carried out in three different replicates.

#### Embryo fixation and slide preparation

On Day 5, whole embryos (blastocysts) were spread on poly-lysine slides to prevent the loss of nuclei as described previously (Coonen et al., 1994; Harper et al., 1994). Subsequently, the nuclei locations were recorded using an England finder (Graticules, UK). Control slides for FISH were prepared using mouse kidney cells (MF1 strain). The preparation of the slides was performed as previously described (Sabhnani et al., 2011).

### FISH analysis

FISH analysis was carried out as previously described (Coonen et al., 1994; Harper et al., 1994), but with some amendments. The procedure was implemented as two rounds of FISH using dual-color ready-to-use locus-specific probes (LSI) for chromosomes 2 (2qH3) and 11 (11qE2) in the first round and chromosome 16 (16qC4) in the second round (Kreatech, Netherlands). To evaluate the efficiency of each probe, they were applied on control slides (kidney) where the numbers of signals for each probe were counted in 200 interphase nuclei per slide for each FISH procedure.

The first round of FISH was accomplished as described in Sabhnani et al. (2011) with the LSI probe for chromosomes 2 and 11 that binds to the AurKa gene on chromosome 2 and to the TK gene on chromosome 11 close to the telomere.

Observation and slide analysis were made under an epifluorescence microscope (Olympus Optical, BX40F4, Japan) connected to a Photo metrics cooled charged camera utilizing Smartcapture II software (Digital Scientific, UK). The visualization was accomplished using a 10× objective, 100× objective and different color filters which made it possible to distinguish between several excitation and emission bands, and thus between several fluorochromes, allowing observation of different probes. Scoring criteria were applied as previously described (Mantzouratou et al., 2007).

The second round of FISH was performed with the LSI probe for chromosome 16, which binds to the DSCR gene. The coverslips were removed, slides were incubated in PBS followed by incubation in 4× saline sodium citrate (SSC)/0.5% Tween 20 for 10 min each on a rocking plate and dehydrated in 70, 90 and 100% ethanol for 3 min each. The same steps of slide denaturation, probe denaturation, overnight incubation, post-hybridization washes, and slide visualization and scoring as used for the first round were applied in the second round.

### Classification of embryos

The guidelines listed previously (Delhanty et al., 1997) were followed so that each embryo was classified based on the chromosomal constitution of the nuclei; (i) normal embryos were those with unvaryingly diploid signals in >90% of the nuclei; (ii) if an abnormality was present in at least 90% of the nuclei analyzed, the embryo was considered uniformly abnormal; (iii) the presence of two different cell lines was identified as mosaicism; (iv) diploid mosaic embryos were those which had mostly diploid nuclei (>50%) but with some being haploid, aneuploid or tetraploid; (v) abnormal mosaic referred to embryos with a majority of mosaic nuclei (>50%) but with a few normal nuclei and (vi) embryos showing different chromosomal constitutions in all of their nuclei were deemed fully chaotic; otherwise they were relatively diploid/aneuploid or partially chaotic.

### Statistics

For each group, the blastulation rate (successful conversion of the morula to a blastocyst containing a blastocele cavity) and mean cell count were calculated. The number of aneuploid/mosaic and diploid embryos was recorded. The aneuploidy rate for each group was calculated by dividing the number of aneuploid/mosaic embryos by the total number of embryos analyzed and multiplying by 100. The diploid rate was calculated in a similar manner. All of the treatment groups were compared with the untreated (control) group. Data were analyzed with SPSS software version 19 using the Pearson χ2 test. For cell numbers, there was an indication that the variation was substantial; therefore we have used the non-parametric Kruskal–Wallis one-way analysis of variance. To avoid spuriously significant results due to multiple testing, the result was considered as significant only if the P-value < 0.01.
Results

Effect of GM-CSF on blastulation of murine embryo in vitro

The rates of blastulation and aneuploidy were studied in 283 murine embryos. The numbers and percentages of embryos that reached the blastocyst stage in all GM-CS groups including the control (0 ng/ml) are illustrated in Table I and Fig. 1.

No difference in the blastulation potential was noted with the addition of 1 and 2 ng/ml of GM-CSF compared with the control group. On the contrary, there was a significant decrease (P < 0.001) in the rate of blastulation in the 5 and the 10 ng/ml groups.

Total cell enumeration was carried out for every blastocyst in all groups (0, 1, 2, 5 and 10 ng/ml) during slide visualization and analysis with the fluorescent microscope, and the average cell number was calculated per group (93 ± 1.1, 84 ± 7.9*, 93 ± 1.8, 71 ± 11.7* and 73 ± 11.5* for 0, 1, 2, 5 and 10 ng/ml, respectively). Statistical analysis of the average cell count indicated a significant difference (P < 0.001) in all GM-CSF groups compared with the control except for the 2 ng/ml group in which the analysis gave a non-significant result (P = 0.07).

Effect of GM-CSF on aneuploidy

A total of 215 blastocysts were analyzed using FISH and 213 gave conclusive results: 48/48 from the control group (no growth factor added), 48/48 from the 1 ng/ml group, 52/53 in the 2 ng/ml group, 34/34 blastocysts in the 5 ng/ml group and 31/32 in the 10 ng/ml group. The efficiency of the procedure (i.e. embryos giving results/embryos examined) was 99% (213/215). Overall, 6576 nuclei were examined and versus 6295 conclusive results were obtained (96%). Control kidney cells were run with each FISH procedure in order to calculate the efficiency of each run. The average was taken for the whole experiment, resulting in 93% with normal/diploid signals. Therefore, 7% was taken as background and any embryo showing up to that percentage of abnormal cells was classified as normal.

There were 33 embryos from the control group (69%) which were chromosomally normal for the chromosomes analyzed (2, 11 and 16), while the number (and proportion) of chromosomally normal embryos in the 1, 2, 5 and 10 ng/ml groups were 30 (63%), 33 (63%), 18 (53%) and 20 (65%), respectively (Table II). Mosaicism was the most common chromosomal abnormality observed in the aneuploid embryos with different types: diploid/aneuploid, diploid/chaotic and abnormal mosaic (Table II and Fig. 2). Two triploid embryos were found in the 5 ng/ml group and two chaotic embryos were found in the 1 ng/ml group.

There was a slight increase in the proportion of aneuploid/mosaic embryos following the addition of GM-CSF (1 ng/ml; 37%, 2 ng/ml; 37%, 5 ng/ml; 47% and 10 ng/ml; 35%), but the statistical analysis did not show a significant difference (Fig. 2).

The proportion of aneuploid cells (± SD) in the mosaic embryos of the control group was 12.5 ± 2.5%, while in the GM-CSF groups (1, 2, 5 and 10 ng/ml) the proportions were 20 ± 6.1, 15 ± 3.7, 16 ± 4.4 and 18 ± 4.4%, respectively. Statistical analysis of the percentage of aneuploid cells in the mosaic embryos in the control group versus all the GM-CSF groups demonstrated a significant difference (P < 0.01).

Discussion

GM-CSF is known to stimulate cell numbers in developing blastocysts, and in normal fetal and placental development, through either anti-apoptotic or mitogenic effects (Sjoblom et al., 1999; Sjoblom et al., 2005; Richter, 2008). The purpose of this study was to investigate, using FISH and morphological assessments, whether the addition of growth factors to the culture media affects the blastulation rate or chromosomal status of mouse preimplantation embryos.

The results reported in the current experiments showed that adding GM-CSF to the culture media did not have any significant positive impact on the blastulation rate or cell number. On the contrary,

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Table I  The number and percentage of embryos reaching the blastocyst stage after culturing with and without GM-CSF.

<table>
<thead>
<tr>
<th>GM-CSF concentration (ng/ml)</th>
<th>0 (control)</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total embryos cultured</td>
<td>55</td>
<td>55</td>
<td>61</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>Embryos reaching blastocyst stage</td>
<td>48</td>
<td>48</td>
<td>53</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>Blastulation rate (%)</td>
<td>87</td>
<td>87</td>
<td>87</td>
<td>61*</td>
<td>57*</td>
</tr>
<tr>
<td>Cell count (mean ± SD)</td>
<td>93 ± 1.1</td>
<td>84 ± 7.9*</td>
<td>93 ± 1.8</td>
<td>71 ± 11.7*</td>
<td>73 ± 11.5*</td>
</tr>
</tbody>
</table>

*Significantly different from the control (P < 0.01).

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Figure 1  The percentage of embryos that reached blastocyst stage after 3 days culture with and without GM-CSF. The five bars represent the five different concentrations of GM-CSF. *Significantly different from the control (P < 0.01).
higher concentrations of GM-CSF (5 and 10 ng/ml) had a significantly negative impact on the blastulation rate and cell count. This is a similar pattern to that reported by Behr et al (2005) where low concentrations of GM-CSF (1 and 2 ng/ml) did not promote the blastulation rate of preimplantation mouse embryos and when the GM-CSF concentration was increased to 20 ng/ml in the culture media, embryonic development seemed to be inhibited. This effect could possibly be explained by the negative regulation of GM-CSF receptors when GM-CSF is present at high concentrations (Behr et al., 2005).

Other studies have shown variable effects of culturing embryos in GM-CSF. Desai et al. (2007) showed no significant differences in the blastocyst development or hatching of cryopreserved mouse embryos post-thaw with addition of GM-CSF (2 ng/ml). Their only significantly positive effect of GM-CSF was in modulating apoptosis and continued cell survival but there was no increase in the overall cell number (Desai et al., 2007).

Karagenc et al. (2005) examined the effect of adding GM-CSF (0, 2, 4, 8 and 16 ng/ml) on the development and differentiation of mouse embryos using two different mouse strains (F1 and CF1) and two different types of media (simple and sequential) under different conditions of culturing in the presence and absence of a protein source. They found no marked effect of supplementing the media with GM-CSF using different concentrations either on the potential for embryo development (at any developmental stage) or on the cell number in the trophectoderm (TE) or ICM of blastocysts or even in the number of apoptotic cells. The only stimulatory effect of GM-CSF was observed on the TE and ICM cell number in the absence of protein source (i.e. under deficient conditions) but again there was no increase in the number of embryos that reached the blastocyst stage (Karagenc et al., 2005).

Behr et al. (2005) tested the effect of a range of concentrations of GM-CSF (0.0625–2 ng/ml) on mouse embryo development and found that the best effect was reported in the 0.125 ng/ml group, and all other concentrations from 0.25 to 2 ng/ml did not promote blastocyst development. A study by Papayannis et al. (2007) did not report any increase in the percentage of embryos reaching the blastocyst stage or in the rate of re-expansion when GM-CSF (2 ng/ml) was added prior to freezing.

Some studies however do report significant effects of adding GM-CSF to mouse embryo culture. Sjoblom et al. (2005) reported an increase in the rate of blastulation of mouse embryos cultured in a media supplemented with 2 ng/ml of GM-CSF compared with the control group where no GM-CSF was added (99 versus 95%, P < 0.0001) but there was no significant improvement in the implantation rate.

Robertson and Seamark (1990) has shown, with GM-CSF-deficient mice, that GM-CSF is not fundamental for a successful pregnancy. However, Robertson et al. (2001) observed that murine embryo development was faster with 2 ng/ml of GM-CSF, yet the addition of GM-CSF did not influence the percentage of 8-cell embryos reaching the blastocyst stage. These investigators did find an increase in the glucose uptake, which promotes metabolic activity and probably stimulates cell division. Nevertheless, increased metabolism could possibly be a marker of poor embryonic health according to Henry Leese’s hypothesis of the ‘quiet embryo’ which states that the embryo with a ‘quiet’ metabolism has a higher viability rate than that with an ‘active’ metabolism (Leese, 2002). In addition, it has been found that glucose uptake is affected by blastocyst quality such that blastocysts with poor quality have high glycolytic activity (Lane and Gardner, 1996).

One publication on the effect of GM-CSF on human blastocyst development showed that a 50% increase in blastocyst formation was obtained 14 h earlier with reduced apoptosis (Sjoblom et al., 1999). This effect could result from the use of a different type of medium in that study (with different nutrient components), or perhaps due to the use of human frozen-thawed embryos where

Table II Distribution of chromosomal abnormalities in blastocysts from embryos cultured in different concentrations of GM-CSF.

<table>
<thead>
<tr>
<th>GM-CSF concentration (ng/ml)</th>
<th>Total embryos</th>
<th>Diploid, n (%)</th>
<th>Mosaic</th>
<th>Total abnormal embryos, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diploid/aneuploid</td>
<td>Diploid/chaotic</td>
</tr>
<tr>
<td>0</td>
<td>48</td>
<td>33 (69)</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>30 (63)</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>33 (63)</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>18 (53)</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>31</td>
<td>20 (65)</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Diploid/aneuploid was the most common form of mosaicism that was seen in all the GM-CSF groups including the control. There were no statistical differences in the occurrence of the various types of mosaicism.

Figure 2 The incidence of diploid and aneuploid (mosaic) embryos in each group after adding GM-CSF to the culture media.
the stress induced by the thawing procedure might contribute to the observed impact of GM-CSF on embryo development.

This present study is the first to examine the effect of adding four different concentrations of GM-CSF (1, 2, 5 and 10 ng/ml) on the rate of aneuploidy/mosaicism of murine blastocysts. The rate of aneuploidy noted in all GM-CSF groups (1, 2, 5 and 10 ng/ml) was slightly higher (37, 37, 47 and 35%, respectively) than in the control group (31%), however, the difference was not statistically significant. Our finding is comparable to that of a study that examined the percentage of aneuploidy using FISH to scan chromosomes 13, 16, 18, 21, 22, X and Y after culturing human zygotes with 2 ng/ml of GM-SCF until Day 3. These researchers showed no significant difference either in the cell count or in the rate of aneuploidy between the control (no GM-CSF added) group (65.2%) and the group cultured with GM-CSF (66.7%; Agerholm et al., 2010). The same study did not report any significant improvement in the rates of fertilization or early cleavage rate or in the total number of normally developed embryos (Agerholm et al., 2010).

An interesting finding is that the proportion of the aneuploid cells in the mosaic embryos of the GM-CSF group was significantly higher than in the control group. This could be due to the lower incidence of apoptosis that allows more cells, including chromosomally abnormal cells, to survive. In reality, the addition of the GM-CSF neither improved nor worsened the stock of embryos with regard to chromosomal constitution, but it did increase the percentage of the aneuploid cells within the mosaic embryos. However, looking at only three chromosomes could be a limitation for the current study as nuclei which were diploid for these chromosomes may have other abnormal chromosomes. In subsequent studies of human PGD embryos, there was no significant increase in the overall rates of aneuploidy when array comparative genomic hybridization was used to look at all of the chromosomes compared with studies where only two chromosomes were analysed (Delhanty et al., 1993; Fragouli et al., 2010).

Currently, most of the IVF clinics are moving toward extending embryo culturing to the blastocyst stage to select the most viable embryo with the highest implantation and developmental potential as a means to select and transfer one single viable embryo. Origio (MediCult) has announced headline results from a multicenter, double-blind, randomized study accomplished at 13 centers on the efficiency of their new media that contains 2 ng/ml of GM-CSF (EmbryoGen), stating that GM-CSF may improve the implantation efficiency of their new media that contains 2 ng/ml of GM-CSF (Origio, 2011). More specifically, their data show a significant improvement in the rates of fertilization and 2-cell stage embryo flushing and collection. Origio has involved in the FISH analysis and slides scoring. K.J. was involved in study design, data analysis, drafting of the manuscript and final approval of the version to be published.

### Authors’ roles

A.E. contributed to study design, embryo collection and cultures, daily developmental assessment, embryo spreading, FISH analysis, slides scoring, data analysis, drafting of the manuscript and final approval of the version to be published. K.G. contributed to embryo procurement (female mice superovulation, mating with males, females sacrificing and 2-cell stage embryo flushing and collection). K.K. was involved in the FISH analysis and slides scoring. J.H. was involved in study design, data analysis, drafting of the manuscript and final approval of the version to be published.

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### Conflict of interest

None declared.

### References


