Reply: Effect of GnRH antagonists in FSH mildly stimulated intrauterine insemination cycles: a multicentre randomized trial

Sir,
We would like first to thank Drs. Niraj Mahajan, Kshitija Mahajan and Rajani Soni for their interest in our paper (Crosignani et al., 2007). In their brief comment, these authors have emphasized several important albeit still unclear aspects of controlled ovarian hyperstimulation (COH) and intrauterine insemination (IUI). Does double insemination provide any benefit? What is the most suitable protocol of COH? Are there factors that are able to reliably predict multiple pregnancies? Where there is a high risk for multiple births, is there any effective alternative to cancellation? Answers to these questions represent a challenging task, which at present is far from being resolved.

In this regard, we would like to underline the urgent need for large randomized clinical trials (RCT) for COH and IUI. Unfortunately, results from this type of study design are extremely scant. A major problem in designing RCTs in this field is related to the large sample size needed. Reasons for this necessity are essentially two-fold. First, due to statistical concerns, patients should be enrolled only for their first treatment cycle and the crossover study design (which requires a lower number of subjects) should be avoided (Daya, 2003). Second, given the relatively low rate of success of the procedure (10–12% per cycle), the number of couples to be recruited in randomized studies is remarkably large. Stating as clinically relevant an absolute increase of the pregnancy rate of 5% and setting the type I and type II errors at 0.05 and 0.20, respectively, the absolute increase of the pregnancy rate of 5% and setting the type I and type II errors at 0.05 and 0.20, respectively, the number of couples needed to be enrolled is >1000 for each study group (Mittendorf et al., 1995). A single center has undoubtly many difficulties in enrolling a sufficient number of cases in an acceptable period of time. As a consequence, only large multicenter RCTs may consent to draw reliable conclusions. The study we have presented is the result of a spontaneous and unfunded collaboration (Crosignani et al., 2007). It has been an enriching experience and we hope it might be followed as an example for those who are interested in clarifying the still unclear aspects of COH and IUI.

References


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Isolation of germ cells from leukemic cells

Sir,
We have read with great interest the article by Geens et al. (2007), titled ‘The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients’ published on Hum Reprod.

The authors concluded that fluorescence-activated cell sorting (FACS) was neither in a murine nor in a human model sufficient to completely deplete testicular tissue of malignant cells. Specifically, in the human model only one of 11 tumour-tissue suspensions were completely depleted of malignant cells. Their results contradict previous reports by our group (Fujita et al., 2005). We were able to restore fertility in sterile mice by transplantation of spermatogonial stem cells. We demonstrated that none of the recipients developed leukemia after transplantation of tumour-cell-depleted spermatogonial cells, isolated from leukemic mice.

As Geens et al. pointed out, one limitation of our study is the low number of mice (n = 12) included and we agree that further investigations to confirm our preliminary findings are warranted. Furthermore, we did not re-analyse the sorted fraction for presence of tumour cells. However, the fact that none of the recipients developed leukemia in our study as the most convincing clinical outcome variable, supports our hypothesis that FACS is sufficient to reliably separate tumour cells from spermatogonial stem cells.

However, we detected some limitations of the current study: positive selections with surface markers expressed on germ cells harbour the risks of contamination by leukemic cells since leukemic cells may adhere non-specifically to germ cells and may bypass the selection for CD49f. These adherent tumour cells would consequently be isolated as non-tumour cells in gate 1 (R1) using FACS analysis. Therefore, negative selection utilizing marker-expressed only on leukemic cells is preferred since this prevents the above mentioned mechanism of tumour cell contamination due to adherence of tumour cells to germ cells. Furthermore, CD49f (Stucki et al., 2001) is a frequently expressed surface antigen on human leukemia cells and the isolation of spermatogonial stem cells solely relying on positively for CD49f harbours a high risk of tumour cell contamination. If the enrichment of spermatogonial stem cells utilizing CD49f as selection criteria is desired, it would be essential to deplete for leukemia cells via negative selection first and in a second step enrich for spermatogonial stem cells via positive selection.

Furthermore, it seems that the limits of gate 2 (R2) were chosen too generously: the cutoff for the H-2Kb axis was 80 on and ~12 on CD49f axis. The spermatogonial stem cell fraction was sorted by utilizing R2 although R2 would contain some EL-4 cells when superimposed on Fig. 2A. Gate ‘R1’ was also set at an approximate intensity of 70 at the HLA-A–C axis in Fig. 4B. However, the non-R1 gate of Fig. 4B would still contain a fair amount of SB cells when superimposed on Fig. 4A. Therefore, by using the gates depicted in Figs. 2A and B and 4A and B, the contamination of spermatogonial stem cells by leukemic cells is an inevitable
consequence. As we suggested in our previous paper, it is of utmost importance to set the gate in a very restricted way to prevent contamination. It is well known that some subtypes of human leukemia cells express MHC-class I in low abundance which would result in a high risk of tumour cell contamination if isolation is based on only one (Fujita et al., 2006).

Our group isolated spermatogonial stem cells by setting gates for ‘MHC-class I-negative and CD45-negative region’ among the cell population of forward scatter (FCS) high and side scatter (SSC) low. We detected some leukemic cells in the MHC-class I-negative and CD45-negative region, if the additional gates, ‘FCS high and SSC low’, were not used. The enhancement of surface marker expression by interferon gamma would be necessary. Alternatively, additional surface antigens should be used to reliably separate spermatogonial stem and leukemia cells.

Overall the use of spermatogonial stem cell separation from malignant cells by FACS should not be discouraged. Rather additional studies in this regard are warranted. One future direction to circumvent this problem could be the development of in vitro proliferation of human spermatogonial stem cells: after the isolation of spermatogonial stem cells by FACS, an isolated single stem cell could form a colony, from which only one cell would be chosen for in vitro proliferation with no risk of tumour cell contamination.

References
Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H. The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the depletion of testicular cells, thereby allowing a faster and more efficient FACS where only cells that were negative for H-2Kb and at the same time positive for CD49f were used. However, the aim of this step was not to remove the malignant cells, but to enrich spermatogonial stem cells in the samples, thereby allowing a faster and more efficient FACS where only cells that were negative for H-2Kb and at the same time positive for CD49f were selected. If a malignant cell would adhere to a germ cell, this aggregation would be possible for both H-2Kb and CD49f and would not be selected. Even though positive selection for spermatogonial stem cells might not be necessary for the depletion of cancer cells, enrichment of these stem cells might be unavoidable in a clinical set-up, since the success of the spermatogonial stem cell transplantation technique is highly dependent on the number of stem cells injected (Dobrinski et al., 1999).

It is true that CD49f is frequently expressed by leukemic cells, but as mentioned in the article, for both cell lines used in our study, CD49f was not expressed (according to the gates used). In a clinical set-up, we would not advise to use the common marker for sorting suspensions of individual patients. Immunophenotyping of the malignant clone for each individual patient would be necessary to find the appropriate markers for reliable sorting.

We agree that the gates used in Figs 2A and B and 4A and B might comprise some malignant cells. However, these are not identical to the gates that we used in the experiments. Owing to technical restrictions, we were unable to transfer the co-ordinates of the gates from the computer where the analyses were done to the computer where the pictures were generated. The gates that were set during the sorting and analyses were generated using positive (labelled malignant cells) and negative (labelled testicular cells and unlabelled cells) controls.

We have read their comments with interest and we certainly agree that the separation of germ cells from cancer cells is a matter under debate.

In their letter, the authors state that the fact that none of the recipients (n = 12) in their study developed leukaemia proves that fluorescence-activated cell sorting (FACS) is sufficient to reliably separate tumour cells from testicular cells. We cannot agree with this assumption because, as can be seen in Table 2 of our article, not all mice developed tumours when only a low number of malignant cells was injected. The lower the number of cells injected, the lower chances of developing tumours, but even after injecting between 5 and 39 malignant cells, still more than 40% of mice developed malignancy. Moreover, after checking our data from the transplantations with the sorted fractions, we observed that out of 20 mice, the first 15 mice did not develop any malignancy but in the 16th mouse, a tumour was observed (data not presented as such in our paper). We therefore conclude that sorted cell fractions should be analysed extremely carefully to avoid even minimal contamination, since the exact number of cancer cells able to induce malignancy is unknown.

As we already mentioned in the discussion of our manuscript, non-specific aggregation of cancer cells to germ cells is possible (Jahnukainen et al., 2006). We therefore agree that it would not be a good option to try to separate germ cells from leukemic cells, solely relying on positive selection for germ cell markers. In the murine set-up, we used magnetic-activated cell sorting for positive selection of CD49f or α6-integrine expressing cells (germ cells). However, the aim of this step was not to remove the malignant cells, but to enrich spermatogonial stem cells in the samples, thereby allowing a faster and more efficient FACS where only cells that were negative for H-2Kb and at the same time positive for CD49f were selected. If a malignant cell would adhere to a germ cell, this aggregation would be possible for both H-2Kb and CD49f and would not be selected. Even though positive selection for spermatogonial stem cells might not be necessary for the depletion of cancer cells, enrichment of these stem cells might be unavoidable in a clinical set-up, since the success of the spermatogonial stem cell transplantation technique is highly dependent on the number of stem cells injected (Dobrinski et al., 1999).

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Reply: Isolation of germ cells from leukaemic cells

Sir,

We would like to thank Dr Fujita and colleagues for their letter to the editor regarding our recent article (Geens et al., 2007).