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human reproduction LETTER TO THE EDITOR

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Sir,

We read with interest the paper by Aitken *et al.* (2010). In their study, the percentages of TUNEL were compared in selected [with density-gradient centrifugation (DGC)] versus unselected and in live versus total sperm. A further aim of the study was to define a diagnostic threshold value by comparing donors and patients. The study was conducted by flow cytometry without any strategy to exclude M540 bodies. M540 bodies are non-sperm elements that occur in semen, especially from subfertile men (Muratori *et al.*, 2004; Marchiani *et al.*, 2007). Indeed, M540 bodies are located in the same FSC/SSC (Forward/Side Scatter) region as sperm (Muratori *et al.*, 2004, 2005). Hence, the gate on FSC/SSC does not imply that 'only spermatozoa were assessed' (Aitken *et al.*, 2010). In our opinion, omitting to exclude M540 bodies will have significantly affected the results of the paper, as following:

(1) The percentages of TUNEL in unselected semen are expected to be greater than those reported by Aitken *et al.* (2010), as previously demonstrated (Muratori *et al.*, 2008a,b).

(2) M540 bodies in semen samples are reduced by selection with DGC (Muratori *et al.*, 2008a). Thus, the percentages of TUNEL in selected samples are less affected by them. Consequently (see also Point 1), the differences in the percentages of TUNEL in total sperm between selected and unselected samples are expected to be greater than those reported by Aitken *et al.* (2010).

(3) The LIVE/DEAD Fixable Dead Cell Stain also labels M540 bodies (Marchiani *et al.*, 2010). Therefore, M540 bodies are scored as 'dead cells' and the percentages of live sperm appear decreased with respect to the real amount. Hence, the percentage of live and TUNEL positive sperm in unselected samples may be underestimated. On the other hand, since the M540 bodies amount decreases with DGC (Muratori *et al.*, 2008a), the reported percentages of live sperm labeled by TUNEL in selected samples are expected to be closer to the real values.

In light of this, a difference in occurrence of M540 bodies in selected versus unselected samples, rather than the loss of dead cells during selection (Aitken *et al.*, 2010) might explain why DNA fragmentation in live sperm is greater after selection in the patient group. This issue appears relevant in light of the use of DGC in assisted reproductive techniques and deserves further investigation.

(4) Donors show better semen quality (Aitken *et al.*, 2010) thus the amount of M540 bodies is expected to be smaller, and less affecting the measures, than in patient group (Muratori *et al.*, 2004; Marchiani *et al.*, 2007). This might explain the reported lack of increase of live TUNEL positive sperm after selection in donors at variance with the patient group. In addition, the exclusion of M540 bodies might help to achieve a threshold that more sharply separates fertile donors from infertile men.

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