

Reply of the Authors:

We are very happy that Dr. Ricci and his colleagues found our article (1) interesting. Our aim was to examine the relationship between sperm chromatin integrity and maturity, with sperm membrane changes detected by annexin V assay. We evaluated DNA damage by use of sperm chromatin structure assay (SCSA), toluidine blue staining, annexin V–propidium iodide (PI) assay, and sperm DNA cytometry for detecting the percentage of apoptotic cells (PI-positive cells) (2). We agree with Dr. Ricci and colleagues that each assay has some limitations; for example, the PI-negative population may contain some necrotic cells that may fail to stain with PI. All viability markers for staining the DNA may not permeate the cell membrane except at certain stages of membrane integrity. In such instances, early or dead cells may not allow the PI and other DNA probes to reach the cell nucleus.

Significant improvement in sperm motility after preparation of sperm by density gradient does not indicate the absence of membrane or nuclear damage that may subsequently affect the sperm quality. Objective parameters such as the percentage of late apoptotic sperm in the annexin–PI staining assay, the %DFI (DNA fragmentation index) in SCSA, or the toluidine blue assay failed to show statistically significant improvement. These observations confirm that something more than the methodology may be involved. Also there may be variations in the sperm preparation both among the patients and from the healthy donors (3).

We also agree with their comment that cell debris must be taken into consideration when running flow cytometry analyses. Advancement in flow cytometry technology has allowed dual annexin V–PI staining for flow cytometry with greater ability to exclude cell debris. Adequate instrument settings, the acquisition setting, compensation, and negative and single dye control as well as an experienced analyzer using sophisticated software for correct data analysis and interpretation are critical factors for flow cytometry analysis. Certain dyes such as 6-carboxyfluorescein diacetate (CFDA) or SYBR green may be used to identify the viable fraction along with PI staining (4).

Finally, we agree with Dr. Ricci and colleagues that the differentiation between late apoptotic (PI positive/annexin V positive) and necrotic (PI positive/annexin V negative) cells is not practical because late apoptosis is an irreversible stage of apoptosis. Annexin V does not permeate to the plasma membrane; it can only bind to the externalized phosphatidylserine (PS) portion. Cell debris may occur as cell damage progresses and the plasma membrane fragments. Many assays have been recently introduced for assessing viability, such as

annexin V–PI, Yo-Pro-1–PI, SYBR green–PI, Syto 16–PI, or Syto-7-AAD. Syto16 is superior when combined with 7-AAD than PI because of the narrower emission spectrum of 7-AAD compared with PI. In our laboratory, we use a combination of Yo-Pro1 and PI (5). Finally, we also agree with their conclusion and suggest that the most reliable method is the one that has been standardized by the laboratory, based on available equipment with correct interpretation of its results and limitations.

Again, we would like to thank Dr. Ricci and his colleagues for affording us the opportunity to address these important points.

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