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ABSTRACT

The present article critically reviews the recent data in the literature concerning the methods of quantitation of round cells in semen and in particular of white blood cells. The most reliable methods for the differentiation of round cells in semen are the peroxidase cytochemistry with the use of benzidine or o-toluidine and immunocytochemistry with the use of antibodies. The effect of increased numbers of white blood cells in semen on parameters of semen analysis as well as most of the sperm function tests is not yet clear. The clinical significance of such a finding is discussed along with the influence that it will have on fertility and the outcome of in vitro fertilization.
Key words: Peroxidase cytochemistry, semen, white blood cells

INTRODUCTION

When assessing a semen analysis the number and type of round cells present should be taken into account along with "conventional" parameters, i.e. number, motility and morphology of spermatozoa (1). Round cells in semen can be distinguished into a) white blood cells (WBCs) and b) immature germ cells. Differentiation between these two cell types is considered of upmost importance for diagnostic and therapeutic purposes. For example, an increased number of WBCs in combination with other criteria may be indicative of a genital tract infection (20). On the other hand, an increased number of immature germ cells may be a sign of abnormal spermatogenesis (3).

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Over the past few years immature germ cells have been the focus of attention due to the use of spermatids for in vitro conception. Round spermatid injection (ROSI) and round spermatid nuclear injection (ROSNI) were pioneered in Japan (4). These methods were the only therapeutic approach for some cases of azoospermia with spermatids taken from the testes (5) or the ejaculate (6). Identification of immature germ cells present in the ejaculate or the testicular tissue can be achieved with the use of antibodies (7) or it can be based on the morphological features of this cell category (8). Enriched fractions of spermatids can also be collected using cell separation techniques (8,9).

The injection of spermatids in the oocyte to achieve pregnancy has still obstacles to overcome (10), some of which are the correct identification as well as good quality of the cells isolated (11). The pregnancy rate after the use of round spermatids is disappointingly low (11) whereas the

use of elongated spermatids gives acceptable pregnancy rates (12). This is not the only reason why the use of round spermatids warrants further attention. Very recent data have shown that the maturation of spermatozoa can only be arrested at the stage before meiosis (13). Large scale studies are needed to clarify the picture and decide on the future use of ROSI as a therapeutic approach.

White blood cells in semen

There are two groups of WBCs in semen, those with granular cytoplasm and those with agranular cytoplasm. The former includes polymorphonuclear granulocytes, eosinophils and basophils, while the latter include monocytes and lymphocytes. Despite the presence of all above mentioned cells in semen samples, granulocytes - in particular polymorphonuclear granulocytes- are the predominant cell type (50-60%), macrophages follow (20-30%) and finally lymphocytes account for a very small proportion (2-5%). Other cell types are rare in semen samples (14-17). In a very interesting study (14) the number and type of cells present in the semen of large numbers of fertile and infertile men were counted using immunocytochemistry with antibodies specific for each cell type. This study revealed significant differences between the two groups of men. The number of WBCs in infertile men was significantly higher in comparison to the fertile group (median value: 1,035,000 per ejaculate versus 170,000 per ejaculate). The same was true concerning granulocytes (median value: 537,000 per ejaculate for the infertile group versus 100,000 per ejaculate for the fertile group). Significant increases were also observed in infertile men concerning the number of macrophages, B-lymphocytes and T-lymphocytes (14).

An issue widely discussed in the literature concerns the highest number of WBCs that can be considered "normal" for fertile semen. The WHO laboratory manual (18) considers 1 million/ml as the highest normal WBC's number. Most specialists have accepted this limit as a norm (3,14). In cases of accessory genital gland infection, the number of WBCs is usually higher than the limit set in most studies, although there are a few papers that find no increase in the number of WBCs in infertile men with infection (16,17). An increased WBC number may be indicative of infection and it can affect the penetration of spermatozoa into the oocyte but it cannot be considered as the only indicator of the fertilizing potential of a semen sample (19).

Methods for the determination of white blood cells in semen

It is generally recognized nowadays that round cells in semen samples must be counted accurately and distinguished carefully into WBCs and immature germ cells (20). Many methods have been developed to this purpose and the most commonly used ones are discussed below.

Bryan Leishman staining: This staining method was developed in 1976 by Couture (21). It uses a-naphthol to differentiate between WBCs and immature germ cells. The method is simple, but may lead to an overestimation of the number of lymphocytes and underestimation of the number of granular cells, so it is not routinely used.

Elastase determination Elastase is an enzyme secreted by stimulated granulocytes. Its concentration in the seminal plasma can be measured with the use of Enzyme-Linked Immunosorbent Assay (ELISA) (22). This method correlates well with the immunohistochemical method (23) but not with the histochemical method of peroxidase stain (24). This discrepancy between the two methods can be explained by the fact that peroxidase staining measures a substance within the cytoplasm, whereas the enzyme elastase in secreted extracellularly. Elastase determination is a costly method demanding specialized equipment and for this reason it is not routinely used when studying WBCs in semen.

Papanicolaou staining: This is a widely used method for the evaluation of sperm morphology. The differentiation in this method is based on the shape and size of cell nuclei. However, cell classification is not always accurate with this method as polymorphonuclear granulocytes can be

taken for spermatids with lobular nuclei, and lymphocytes for monocytes (25).

Immunocytochemical method: Many monoclonal antibodies developed since 1980 can today be used to accurately identify all cell types, as there are antigens specific for each cell type (15, 26-28). This is the most accurate method available to date, but its high cost limits its use to research purposes, as it is too expensive to be used routinely.

Peroxidase cytochemistry: The original method developed by Endzt (29) was based on the benzidine compound for the differentiation between cell types. Later, Nahoum et al substituted o-toluidine for benzidine (30). The distinction between WBCs and immature germ cells is made possible because both benzidine and o-toluidine turn brown intracellularly due to the effect of peroxidase on hydrogen peroxide. As the method is both accurate and easy for an experienced scientist to perform and its cost relatively low, it is widely used routinely (20).

White blood cells in semen and sperm parameters

Many attempts have been made to answer the question of whether and to what extent WBCs - in particular polymorphonuclear granulocytes - can affect the quality of a semen sample. A number of studies have been conducted leading to varying results in an attempt to reach a conclusive answer (2,15,16,31,32). Older papers showed a decrease in number and motility of spermatozoa in infertile patients with leukocytospermia (15,31). Concerning the morphology of spermatozoa a reduction of normal forms was observed along with the decrease of their number and motility (32,33). These older findings have not been confirmed by recent studies (16,17) which involve large numbers of infertile men with leukocytospermia. On the contrary, most reports seem to agree that leukocytospermia has a negative effect on sperm function tests (34-39). The most commonly used tests are the following:

1. Zona-free hamster egg penetration test (HEPT).
This test assesses the ability of spermatozoa for capacitation, acrosome reaction and fusion with the oolemma, which may not be present in acrosome-reacted sperm. In leukocytospermic samples this ability of spermatozoa was found reduced (34,35). These findings were experimentally confirmed by incubating semen samples with WBCs isolated from peripheral blood (36,37).

2. Nuclear chromatin decondensation test (NCD).
This test assesses the capacity of nuclear chromatin to decondense after the influence of certain chemicals. A recent study showed a reduced capacity of nuclear chromatin to decondense in leukocytospermic semen samples (36).

3. Hypo-osmotic swelling (HOS).
This test examines the ability of the sperm membrane for osmoregulation. Leukocytospermia has a negative effect on this test (38), but there is no general agreement on the issue (39).

In conclusion, it seems that leukocytospermia has a negative effect on sperm function tests that are related to the function of nuclear chromatin, the plasma membrane capacitation and acrosome reaction of spermatozoa.

White blood cells in semen and fertility

A number of studies have shown that the semen of infertile men contains higher numbers of WBCs (14,40,41). There are, however, papers with contradictory findings, i.e. a decrease in the number of WBCs in the semen of infertile men as compared to semen of fertile men (26,42). As already mentioned WHO suggests 1 million/ml as the highest number of WBCs in a normal semen sample. This generally accepted number is quite arbitrary and has, consequently, been questioned by several researchers (23,32,43,44). A particularly interesting study was conducted by Harrison et al (45) concerning this issue. They evaluated the number of WBCs in the semen of men who had fathered a child in the last twelve months. The numbers of WBCs found in these samples ranged from O.5 - 16.5 million/ml. This

indicates that quite a high percentage of men who were definitely leukocytospermic, according to the WHO limit, were in fact fertile. This discrepancy puzzles researchers and there is a clear need to redefine relevant norms. It is also widely accepted that there is a need for an objective count of round cells in semen. WBCs should also be differentiated from immature germ cells using either the immunocytochemistry or the peroxidase cytochemistry method. This would ensure a clear differentiation between granular and agranular cells (peroxidase cytochemistry) or lead to the accurate identification of all cell types (immunocytochemistry).

White blood cells in semen and IVF outcome

Does the number of WBCs in semen - in particular that of polymorphonuclear granulocytes - affect the outcome of IVF? A number of reports seem to suggest that there is a negative effect of WBCs on the outcome of IVF (46). Another study also confirms this negative effect and concludes that the effect is particularly detrimental if the number of WBCs exceeds 6 million/ml (47). As there is no change in conventional semen parameters, it can be concluded that an increased number of WBCs affects functional aspects of the spermatozoa, such as capacitation, hyperactivation and the acrosome reaction, thus preventing fertilization (48).

Reactive oxygen species and cytokines

WBCs affect the fertilizing potential of the semen sample possibly through the action of the molecules that they secrete mainly reactive oxygen species (ROS) and cytokines. ROS are molecules such as the superoxide ion and the hydroxyl radical, which cause cell dysfunction. Cytokines are also a diverse group of molecules with various functions on cellular mechanisms. The presence of both groups was documented in semen over the past few years with yet undefined roles or modes of action.

ROS in the ejaculate are produced by WBCs (49-51) as well as by morphologically atypical spermatozoa (52). They can be measured in the seminal plasma with the use of chemiluminescence (49) or spectrophotometry (53). Increased amounts of ROS in semen have already been correlated with infertility (54,55). A number of sperm parameters as well as sperm function testing are affected by the presence of ROS (56-58).

ROS initiate the peroxidation of the unsaturated fatty acids of the sperm membrane, which alters fluidity affecting the membrane fusion necessary for fertilization (59). As a result increased ROS in seminal plasma can affect sperm function tests in vitro (60). They can also influence the outcome of in vitro fertilization and could be used as a predictive index (48).

The presence of cytokines in semen was first reported by Hill and his coworkers (61) and since then many research groups have attempted to clarify the role of cytokines in fertility. It has been reported that the levels of interleukin-2 in seminal plasma of infertile patients are decreased (62) whereas interleukin-11 levels are increased especially in the presence of urogenital infections (63). Patients with varicocele or infection have been found to have increased interleukin-6 levels in seminal plasma (64). Cytokines can also affect the motility of spermatozoa (65, 66) although this finding has not been confirmed by others (67, 68).

It becomes evident that consensus regarding the role of both ROS and cytokines in the ejaculate and the effect that they might have on the fertilizing potential of a semen sample has not yet been reached. There are many parameters that one has to consider such as the antioxidant systems of seminal plasma and synergy between different cytokines. Further research in the future will give definite answers.

What is the actual role of white blood cells in semen?

Lately, systematic research has been initiated into the actual role of WBCs in semen (69,70). Aitken and Baker (71) in a recent article

humorously wonder whether WBCs in semen are passengers, terrorists or good Samaritans. In other words, the conventional view that the presence of WBCs in semen is definitely detrimental is being questioned. Contradictory data are accumulating with time (17,44). The conclusion so far is that assessment should take into account not only the population of polymorphonuclear granulocytes but also the immature germ cells, which may be responsible for negative effect, observed on certain sperm parameters (71).

In conclusion, although leukocytospermia is indicative of infection, it does not necessarily have a negative effect on the fertilizing capacity of a semen sample. However, it must be emphasized that an accurate count as well as differentiation of round cells in semen is strongly recommended, as this will be a valuable tool for both diagnostic and therapeutic decisions. That is particularly true when applying the latest methods of ROSNI and ROSI using round spermatids isolated from semen and then injected into oocytes (4,6), where counting and differentiation of round cells becomes imperative.

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