Increased polymorphonuclear granulocytes in seminal plasma in relation to sperm morphology

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Much controversy surrounds the clinical significance of an increased concentration of white blood cells (WBC) in the male ejaculate. The World Health Organization’s classification of leukocytospermia is a concentration >1x10^6 WBC/ml. The aim of this study was to assess the association of varying concentrations of leukocytes to sperm morphology evaluated by strict criteria. Semen samples were collected from a total of 79 patients. Round cells on the initial semen analysis were stained for identification of polymorphonuclear granulocytes (PMN) as the largest group (50-60%) of white blood cells using the Endtz Method commercially produced as Leucoscreen™. Diff Quick Staining Kit was used for sperm morphology assessment and 200 spermatozoa were assessed per slide. Data were evaluated using two cut-off criteria, at 0.5x10^6 WBC/ml and 1x10^6 WBC/ml. Mann–Whitney U-values at both < and >0.5x10^6/ml PMN (P < 0.001) and at < and >1.0x10^6/ml PMN (P < 0.015) showed differences between percentage normal forms. Spearman’s rank correlation coefficient for PMN concentration showed a negative correlation (P < 0.01) with percentage normal sperm morphology and positive correlation for midpiece abnormalities (P < 0.04). These data support the hypothesis that PMN have a role in the increase of abnormal spermatozoa, particularly those with midpiece abnormalities, by as yet unknown mechanisms.

**Key words:** leukocytospermia/male infertility/polymorphonuclear granulocytes/sperm morphology

**Introduction**

Spermatozoa and a population of round cells comprised mainly of immature germ cells and leukocytes are the main components of human semen [World Health Organization (WHO), 1992]. It has been stated that leukocytes are found in almost every human ejaculate (El-Demiry et al., 1987), and that it may also be associated with inflammation of the genital tract, caused by the presence of bacteria (Comhaire et al., 1980). However, controversy surrounds the role of leukocytes in the human reproductive tract.

Immunohistochemical studies report that polymorphonuclear granulocytes (PMN) are the dominant white blood cell (WBC) type in human semen (Auroux et al., 1985; Eggert Kruse et al., 1992; Tomlinson et al., 1993; Aitken et al., 1994; Wolff, 1995), and these represent ~50-60% of all WBC in semen. Macrophages/monocytes represent 20-30% and T-lymphocytes represent ~2-5%. PMN have the ability to form large amounts of reactive oxygen species (ROS) (Aitken et al., 1990; Kessopoulou et al., 1992).

Leukocytospermia, defined by a concentration of WBC >1x10^6/ml (WHO, 1992), has been diagnosed by methods using immunohistochemistry, cytchemistry, and morphological techniques (Endtz 1974; Barratt et al., 1990; Wolff et al., 1992). The peroxidase activity of PMN is detected by the Endtz method/peroxidase stain (Endtz, 1974). Although this technique is limited because it can only detect PMN (Wolff et al., 1992), it has been recommended for clinical purposes by the WHO (WHO, 1992; Wolff et al., 1992; Politch et al., 1993; Shekkariz et al., 1995). Its advantages are that it is easy to perform, rapid and inexpensive (Wolff et al., 1992; Wolff, 1995; Shekkariz, 1995), compared to more expensive and labour-intensive methods such as monoclonal antibodies (Wolff and Anderson, 1988; Barratt et al., 1990; Tomlinson et al., 1992; Aitken et al., 1994; Keissling et al., 1995). The peroxidase-positive leukocytes detected by the Endtz method are granulocytes, which are the major source of ROS (Auroux et al., 1985; Wolff et al., 1988; Eggert Kruse et al., 1992; Tomlinson et al., 1993; Aitken et al., 1994; Wolff et al., 1995). Shekarriz et al. (1995) concluded that the Endtz test could also be used as an indicator of excessive ROS formation by WBC in semen using chemiluminescence and found a positive correlation between WBC concentrations and ROS detected.

The effects of WBC on seminal parameters, especially their correlation to sperm morphology, remains controversial. Some authors have reported deteriorating sperm morphology with increased leukocyte concentration (Berger et al., 1982; Eggert Kruse et al., 1992; Gonzales et al., 1992; Yanushpolsky et al., 1996). However, Fedder et al. (1993) and Van Der Ven et al. (1987) reported that a high number of leukocytes showed no significant association with abnormal sperm morphology. Hughes et al. (1981) and Smith et al. (1990) have observed sperm fragments within digestive vacuoles of phagocytic cells, thus indicating sperm destruction. Recent studies have suggested that there is no association between an increase in morphologically abnormal spermatozoa and an increase in leukocytes (Tomlinson et al., 1992; Keissling et al., 1995).
In this study we have used strict criteria to correlate the relationship between PMN and sperm morphology in our patients.

**Materials and methods**

**Semen samples**

Semen samples were obtained from 79 patients attending NURTURE (Nottingham University Research and Treatment Unit in Reproduction) and the National Health Service fertility clinic at the Queen’s Medical Centre, Nottingham, UK. These were obtained by masturbation, collected into sterile plastic containers and left to liquefy at 37°C for 30 min before analysis. These samples were then subjected to semen analysis including cell concentration, sperm motility and round cell measurements in a Makler Counter (Sefi Medical Instruments, Hafza, Israel).

**Morphology assessment**

Sperm morphology assessments were performed on smears stained by Diff Quik (Baxter Dade Diagnostics, UK) using ×1000 magnification. The number of abnormalities in 200 spermatozoa was recorded and scored using the strict criteria as described by Hall et al. (1995a,b) and Kruger et al. (1987). The morphology evaluation was carried out by two observers, unaware of the results of the PMN concentration.

**Quantitation of polymorphonuclear granulocytes**

The presence of PMN (polymorphonuclear granulocytes) in the semen samples was assessed by the Endtz test, commercially produced as LeucoscreenTM (Microm UK Ltd, Oxon, UK).

The working solution was prepared by adding 30 μl hydrogen peroxide to 1 ml of benzidine cyanosine solution. Equal volumes of working solution and the liquefied semen sample were mixed on a clean glass slide. After allowing 2 min for mixing, a coverslide was placed on the sample and the slide was assessed at X400 magnification. Peroxidase-positive PMN stained dark brown whereas all other cells stained pink.

The total number of all cells (excluding sperm cells) was counted on the Makler counter and their concentration was determined. Upon carrying out the Endtz method a ratio of positive to negative cells was determined for 500 spermatozoa. The ratio was then used to calculate the total PMN concentration (peroxidase positive cells) from the total number of cells originally calculated on the Makler counter.

**Statistical analysis**

Statistical analysis was performed using the Mann–Whitney U-test and Spearman’s rank correlation coefficient with the assistance of Excel™ and Spreadware Menu™ computer packages. The medians were calculated for the varying morphological criteria and the difference between the medians was tested by the Mann–Whitney U-test. To determine how each of the morphological characteristics related to the PMN concentration in the ejaculate, the Spearman rank correlation coefficient was calculated.

**Results**

Statistical analyses were performed on concentrations < and > 0.5×10⁶/ml and < and > 1×10⁶/ml WBC. The former value was the threshold recommended by Endtz (1974), the latter by Kolvasi et al. (1991), Wolff et al. (1992) and the WHO (1992).

The median percentage normal sperm morphology for < and > 0.5×10⁶/ml PMN and the median percentage normal sperm morphology for < and >1.0×10⁶/ml PMN was calculated. The median percentage normal sperm morphology for <0.5×10⁶/ml PMN was 8% (n = 46; range 0–17.5%) and for >0.5×10⁶/ml, 3% (n = 33; range 0–20.5%) (P < 0.001). The median percentage normal sperm morphology for <1.0×10⁶/ml was 7.5% (n = 61; range 0–20.5%) and for >1.0×10⁶/ml was 3% (n = 18, range 0–11.5%) (P < 0.015). When the difference between medians was tested for < and > 0.5×10⁶/ml PMN using the Mann–Whitney U-test, z = −2.997 (P < 0.001) and for < and > 1.0×10⁶/ml, z = −2.425 (P < 0.015).

Table I shows the mean, SD, SEM, range, r and P values for each morphological criteria. The mean value for PMN concentration was 0.998×10⁶/ml (range 0–8.8×10⁶/ml ± 1.68 SD, 5th and 95th percentiles calculated as 0.779–1.217×10⁷/ml PMN). The mean value for percentage normal forms was 7.21% with a range of 0–20.5% ± 5.01 SD. Calculation of Spearman rank correlation coefficient for percentage normal forms versus PMN concentration was r = −0.276 (P < 0.01) and for midpiece abnormalities r = +0.225 (P < 0.04). The intra- and interobserver coefficients of variation of the cell concentrations and sperm morphology were <5%.

**Discussion**

In this study we found a highly significant difference between median percentage normal forms for < and > 0.5×10⁶/ml PMN (P < 0.001) and for < and > 1.0×10⁶/ml PMN (P < 0.015). There was also an overall significant negative correlation between PMN and percentage normal sperm morphology (P < 0.01) and a positive correlation with increasing percentage of midpiece abnormalities (P < 0.04).

<table>
<thead>
<tr>
<th>Normal</th>
<th>Amorphous heads</th>
<th>Megalo heads</th>
<th>Small heads</th>
<th>Elongated heads</th>
<th>Duplicated heads</th>
<th>Coiled tails</th>
<th>Multiple tails</th>
<th>Midpiece abnormality</th>
<th>Loose heads</th>
<th>Cytoplasmic droplets</th>
<th>Head piece defects</th>
<th>Midpiece defects</th>
<th>Tail piece PMN concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.209</td>
<td>47.066</td>
<td>2.424</td>
<td>7.703</td>
<td>3.166</td>
<td>0.626</td>
<td>4.117</td>
<td>0.543</td>
<td>13.393</td>
<td>6.538</td>
<td>7.215</td>
<td>39.912</td>
<td>37.936</td>
</tr>
<tr>
<td>SD</td>
<td>5.015</td>
<td>11.732</td>
<td>1.949</td>
<td>4.428</td>
<td>2.068</td>
<td>1.131</td>
<td>4.523</td>
<td>1.028</td>
<td>4.6</td>
<td>4.239</td>
<td>4.948</td>
<td>11.812</td>
<td>11.806</td>
</tr>
<tr>
<td>SEM</td>
<td>0.564</td>
<td>1.322</td>
<td>0.219</td>
<td>0.498</td>
<td>0.233</td>
<td>0.127</td>
<td>0.509</td>
<td>0.116</td>
<td>0.518</td>
<td>0.477</td>
<td>0.557</td>
<td>1.329</td>
<td>1.328</td>
</tr>
<tr>
<td>Range</td>
<td>0–20.5</td>
<td>10.5–67.5</td>
<td>0–8.5</td>
<td>0–21.5</td>
<td>0–20.5</td>
<td>0–3</td>
<td>0–23.5</td>
<td>0–7</td>
<td>7.5–32</td>
<td>0–23.5</td>
<td>0–18</td>
<td>17–66</td>
<td>3–58</td>
</tr>
<tr>
<td>r</td>
<td>−0.276</td>
<td>0.095</td>
<td>−0.091</td>
<td>0.137</td>
<td>−0.03</td>
<td>0.187</td>
<td>0.095</td>
<td>0.168</td>
<td>0.225</td>
<td>−0.037</td>
<td>0.085</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>P</td>
<td>P &lt; 0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.04</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = not applicable.
This study provides support for previous observations that leukocytes may be linked to a decreased percentage of normal spermatozoa (Berger et al., 1992; Eggert Kruse et al., 1992; Gonzales et al., 1992; Agarwal et al., 1994; Yanushpolsky et al., 1996); also that there is a significant decrease in normal percentage sperm morphology in leukocytospermic samples. Although Fedder et al. (1993) and Van Der Ven et al. (1987), reported that a high number of leukocytes showed no significant association with abnormal morphology and Tomlinson et al. (1992) suggested morphologically abnormal spermatozoa undergo phagocytosis by leukocytes, strict morphological criteria were not used. Furthermore immunohistochemistry was used which may have compromised their analysis because it detects all subpopulations of leukocytes, including T-lymphocytes and macrophages, whereas in this study only the specific relationship between morphology and PMN was considered.

Aitken and Baker (1995) challenged the alternative view that a role for PMN was phagocytosis of abnormally shaped spermatozoa. They felt that leukocytes were seriously outnumbered by spermatozoa (by \( > 3000:1 \)) and that there would be little opportunity for phagocytosis in the epididymis where selection would presumably occur. What they did not consider was the potentially adverse effect of increasing PMN concentration on normal sperm morphology.

However, Wolff (1995) noted that granulocytes seem to be contributed largely by the prostate and seminal vesicles, which suggests that normal spermatozoa may undergo phagocytosis or ROS damage after maturation. Also, because there is an absence of seminal plasma when the spermatozoa are maturing in the epididymis, there is a suggestion that there are no protection mechanisms in the seminal plasma such as superoxide dismutase affecting the maturation process (Kolvaski et al., 1992). Recently Aitken et al. (1995) also concluded that low concentrations of leukocytes in the human ejaculate i.e. 10th percentiles of 0.00 and 0.24 \( \times 10^4/\text{ml} \) and also in the absence of seminal plasma, can impair sperm function, although strict morphological criteria were not discussed in this study (Aitken et al., 1995).

So far no conclusive evidence from this study exists to explain the significantly increased percentage of midpiece abnormalities with increased PMN concentration. However, Rao et al. (1989) and Aitken et al. (1993) concluded from their study that morphological abnormalities of the midpiece of human spermatozoa were caused by lipid peroxidation induced by malondialdehyde. Although Tomlinson et al. (1993) showed no significance when considering increasing PMN concentration and midpiece defects, their study did not separate the PMN concentration from the total and did not consider strict morphological criteria. The PMN concentration only was considered and not the leukocyte population; furthermore, strict morphological criteria are considered in this current study.

In conclusion, it is suggested from our patient data that increased PMN concentrations are related to decreased percentages of normal forms of spermatozoa, and a significant increase in the percentage midpiece abnormalities was related to an increased PMN concentration. Future work should therefore attempt to determine the cofactors contributing to the decline in percentage normal forms with increased PMN concentrations, a biological threshold for fertility and a rationale for the apparent specific effect on midpiece abnormality.

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