

Effect of Macrophages in Semen on Sperm Quality

Gangxin Chen (✉ 286188243@qq.com)

Fujian Provincial Maternity and Children's Hospital <https://orcid.org/0000-0001-9393-2365>

Beihong Zheng

Fujian Provincial Maternity and Children's Hospital

Research

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Abstract

Background: This was a cross-sectional study in China, we analyzed the levels of macrophages(M ϕ) in semen. The study evaluated the influence of the levels of M ϕ in semen on sperm quality.

Methods: The subjects were 78 males between 25 and 35 years old. The samples were divided into a low group(M ϕ <6 \times 10⁵/ml) and a high group (M ϕ >6 \times 10⁵/ml). Evaluation included consideration of the influencing factors of male semen quality, macrophage concentration, sperm motility, morphology, membrane integrity DNA fragmentation index (DFI), anti-sperm antibodies (AsAb), IL-10, and IL-12 in semen.

Results: There was no difference in the physical or chemical indices of the semen, sperm concentration, AsAb, IL-10, or IL-12 between the two groups (P>0.05). The percentage of sperm forward motility (PR%), the rate of normal sperm shape, and the integrity of cell membranes in the low group were higher than those in the high group (P<0.05), while the percentage of sperm inactivity (IM%), the rate of sperm head deformity, the rate of deformity in the neck and middle segment, the sperm malformation index (SDI), the abnormal sperm index (TZI), and the sperm DFI in the low group were lower than those in the high group (P<0.05). The concentration of M ϕ in the semen was linearly correlated with sperm concentration, sperm PR%, IM%, sperm normal shape rate, head deformity rate, neck and middle deformity rate, SDI, TZI, sperm DFI, and sperm cell membrane integrity (P<0.05), but there was no linear correlation with IL-10 or IL-12 (P>0.05).

Conclusions: The concentration of M ϕ in semen had no significant correlation with semen volume or sperm concentration, but it did have a significant negative correlation with sperm motility, sperm morphology, cell membrane integrity, and DNA breakage rate. There was no significant correlation with the concentration of IL-10 or IL-12.

Background

Over the past 50 years, male fertility has declined. The quality of men's semen has reduced, and the concentration of sperm and the total number of sperm have decreased by more than 50% compared to counts from the past[1, 2]. At present, about 15% of all people of childbearing age suffer from infertility, and almost half of the infertility factors are related to men, with 34% unable to find the reason[3]. The general decline of male fertility has become a common problem facing all mankind.

Immune factors are important in relation to the decline of male fertility. There are a small number of immune cells (mainly neutrophils, M ϕ , and lymphocytes) in male semen, which contribute to immune defense and immune surveillance in the reproductive system[4]. The cells in semen other than sperm are called round cells[5]. In these round cells, if the concentration of leukocytes exceeds 1 \times 10⁶/ml, it may lead to a decline in sperm quality, but the effect of monocytes or M ϕ in semen on sperm quality is not clear[5]. M ϕ have the function of antigen presentation, mainly by secreting a variety of cytokines, enzymes, reactive oxygen species (ROS), and other substances, together with other immune cells, to

maintain the immune balance of the testis[6].In this research we analyzed the relationship between Mφ in semen and sperm quality parameters, so as to provide a reference basis for the diagnosis and treatment of male diseases. In addition, in the field of assisted reproductive technology, some patients experience repeated pregnancy failure, but the reasons are often unclear. The results from this study can be used to provide new ideas for the diagnosis and treatment of these patients.

Materials And Methods

Subjects

From 2018 to 2019 we collected 78 male semen samples from the Reproductive Center of the Fujian Provincial Maternity and Children's Hospital. This study was approved by the ethics committee of Fujian Provincial Maternity and Children's Hospital, under number 2019KY105. All donors provided informed consent in accordance with the regulations of the ethics committee. All methods were carried out in accordance with relevant guidelines and national regulations.

All participants were 20 to 35 years old. During each of their physical examinations, no organic lesions were found in reproductive organs such as the testis, epididymis, and prostate. Genital erection and sperm excretion were normal. No varicocele or endocrine disease was present in the participants, and each participant had a normal karyotype and a normal reproductive history. The semen was negative for mycoplasma, chlamydia, and gonorrhea. Other related factors affecting semen quality were excluded (BMI, orchitis, tobacco use, alcohol use, drug use, and poor health habits such as regularly staying up late).

Sample collection

Patients were asked to abstain from sexual activity for 2 to 7 days before semen extraction; after that, the masturbation method was used to obtain semen. The sample was collected in a sterile sperm collection cup and immediately placed in a 37 °C constant temperature incubator for liquefaction. We recorded the semen color, volume, liquefaction time, pH value, viscosity, and other physical and chemical parameters. We also recorded each patient's fertility history and physical examination findings.

Analysis of macrophages

The samples were stained with Pap staining according to the WHO guidelines,⁵ and the morphology of the Mφ in the semen was observed with standard macrophages (Cell Bank of Chinese Academy of Sciences). The concentrations of macrophages were detected by flow cytometry (BDCantoll, USA) with CD14 (Biolegend, USA). Three specific test tubes were used: Tube 1 contained upstream sperm as a blank control; tube 2 had a standard macrophage cell line as a positive control; and tube 3 contained a specimen. Following the instructions, we added CD14 antibody fluorescein isothiocyanate (CD14-FITC) in sequence and kept it in a dark room at the appropriate temperature for 20 minutes. Then we added 1000 μl of PBS, shook it well, washed it twice at 1500 r/min, centrifuged it for 5 minutes, shook it well, and

tested it on the machine. Next we used the software to make a dot plot of all the collected cells through CD14-FITC, and we used the FITC positive area as gate P1. According to the Solis[7], the samples were divided into a low concentration group ($M\phi < 6 \times 10^5/\text{ml}$) and a high concentration group ($M\phi > 6 \times 10^5/\text{ml}$).

Sperm kinetic analysis

According to the standards of the WHO guidelines[5], sperm kinetic analysis was performed using a Makler counting chamber (Haifa, Israel). The sperm concentration, progressive motility (PR%), non-progressive motility (NP%), and immotility (IM%) were detected, and the results were reviewed by computer automatic semen analyzer.

Sperm morphology analysis

According to the standards of the WHO guidelines[5], the samples were stained by Pap staining (Anhui Anke Biotechnology Co, Ltd, Anhui, China), and at least 200 sperm were observed in each sample. Normal sperm shape rate, head deformity rate, neck and middle segment deformity rate, major segment deformity rate, cytoplasmic residual droplet rate, the sperm malformation index (SDI), the abnormal sperm index (TZI) were evaluated according to the WHO criteria[5]. See **Figure 1**.

Sperm DFI analysis

The samples were stained by Sperm chromatin diffusion (SCD) staining. According to methods described in the literature[8-9]. we established that the semen had undergone fixation, acid denaturation, lysis, diffusion, dehydration, and staining, which are in keeping with the references in the manual for specific operations (Anhui Anke Biotechnology Co, Ltd, Anhui, China). At least 500 sperm were observed in each sample. A large halo on the sperm head indicated that the sperm DNA was intact, and no or only a small halo indicated that the sperm DNA was incomplete. We then calculated the DNA fragmentation index (DFI) of the sperm ($\text{DFI} = \text{number of sperm with positive DNA fragmentation rate} / \text{total number of observed sperm} \times 100\%$). See **Figure 1**.

Sperm cell membrane integrity analysis

The samples were subjected to Eosin-aniline black staining⁵. We mixed the staining solution (BRED Life Science, Shenzhen, China) and semen for 30 s and observed it under a microscope. At least 200 sperm were observed and the sperm survival rate was calculated. The head turned black or dark red to indicate abnormal sperm; they were colorless or light red to indicate normal sperm ($\text{sperm survival rate} = \text{number of spermatozoa without damage to the cell membrane} / \text{total number of observed sperm} \times 100\%$). See **Figure 1**.

Anti-sperm antibody analysis

An mixed antiglobulin response (MAR) reagent was used to detect anti-sperm antibodies on the surfaces of the sperm. Relevant operations were carried out in strict accordance with the instructions (BRED Life

Science, Shenzhen, China). Magnetic beads attached to the surfaces of the sperm represented a positive sperm antibody. When the number of immobilized sperm accounted for more than 50% of the total sperm, semen AsAb was positive[5]. as shown in **Figure 1**.

Cytokines analysis

According to the instructions of the ELISA kit (American Standard Biotechnology Co, Ltd, Jiangsu, China), the semen was centrifuged in a centrifuge (5000g/30min); the seminal plasma was sucked out; samples were added (calibrator, specimen); a wash plate was used; IL-10, IL-12 enzyme conjugate was added; color developing solution and stop solution were used; samples were colorimetric at 450 nm, and the concentration of IL-10 and IL-12 in semen was detected.

Statistical analysis

Statistical analysis of the data was performed by using the SPSS17.0 (IBM, Armonk, NY, USA). The measurement data were expressed as mean±standard deviation; the means were compared by use of an independent sample t-test; and the counting data were expressed by rate (%) and compared by a χ^2 test. The linear relationship between macrophage concentration and sperm quality was drawn by GraphPad Prism 6.07(San Diego, CA, USA), and the linear relationship was expressed as R². The significance level was 0.05.

Results

Analysis of macrophages

Some round cells which were similar to the standard macrophages could be seen in the semen (**Figure 2**). Pap staining showed that these cells had less cytoplasm; the nucleus was semi-elliptical; and the nucleus accounted for more than three-quarters of the whole cell (**Figure 2**). Among the 78 cases of semen, there were 34 with macrophages $<6 \times 10^5/\text{ml}$, and the mean value was $(2.17 \pm 1.76) \times 10^5/\text{ml}$; there were 44 cases with macrophages $>6 \times 10^5/\text{ml}$, and the mean value was $(9.45 \pm 2.47) \times 10^5/\text{ml}$ (**Figure 3**).

General situation analysis

There was no significant difference in age, abstinence time, or primary infertility rate between the two groups ($P > 0.05$). The semen liquefaction time of both groups was less than 30 min. There was no wire drawing phenomenon in the liquefied semen, and there was no significant difference in semen volume, pH, or any other physical or chemical parameters (**Table 1**).

Sperm kinetic analysis

The sperm concentration and NP% in the low concentration group were slightly higher than those in the high concentration group, but there was no significant difference between them ($P > 0.05$). The PR% of sperm in the low concentration group was higher than that of the high concentration group, and the IM%

of sperm in the low concentration group was lower than that of the high concentration group ($P < 0.05$ [Table 2, Figure 4]).

Sperm morphology analysis

The sperm stained with Pap clearly showed the morphological difference of each site, and there was a significant difference between the normal and abnormal morphology of sperm (Figure 2). The normal morphology rate of sperm in the low concentration group was higher than that of the high concentration group, and the rate of head deformity, neck and middle segment deformity, SDI, and TZI in the low concentration group were lower than those of the high concentration group ($P < 0.05$). There was no significant difference in the main segment deformity rate or cytoplasmic residual droplet rate between the two groups ($P > 0.05$ [Table 2, Figure 4]).

Sperm DFI analysis

After SCD staining, the head of the sperm could form halos of different thicknesses. When there was no halo or only a small halo around the sperm, the sperm DNA integrity was abnormal (Figure 1). The results showed that the sperm DFI of the low concentration group was significantly lower than that of the high concentration group ($P > 0.05$ [Table 2, Figure 4]).

Sperm cell membrane integrity analysis

The integrity of cell membranes could be clearly identified due to the eosin black staining of spermatozoa (Figure 1). The results showed that the sperm survival rate of the low concentration group was higher than that of the high concentration group ($P < 0.05$ [Table 2, Figure 4]).

Anti-sperm antibody analysis

Partial sperm agglutination could be seen in the semen with positive anti-sperm antibodies. The antibody-positive sperm adhered to the magnetic beads and had obvious immobilization (Figure 1). The results showed that the positive rate of anti-sperm antibodies in the low concentration group was lower than that of the high concentration group, but there was no significant difference between them ($P > 0.05$ [Table 2, Figure 4]).

Cytokines analysis

The concentration of IL-10 in semen was significantly higher than that of IL-12. There was no significant difference in the concentration of IL10 and IL12 between the two groups. There was no significant correlation between the concentration of macrophages and the concentration of IL-10 and IL-12 in semen (Table 2, Figure 4).

Macrophages and sperm quality parameters analysis

There was a significant correlation between macrophages in semen and most parameters of sperm kinetics, sperm morphology, the DFI of the sperm, and the survival rate of the sperm ($P < 0.05$), but there was no significant correlation between macrophages and the positive rate of anti-sperm antibodies ($P > 0.05$ [Figure 5]).

Discussion

There are a small number of macrophages in semen which play important roles in maintaining sperm quality[7]. They originate from the interstitial tissue of the testes and epididymis, accounting for 25% of the total testicular stromal cells[10]. When the testis is diseased or the “blood-testis” barrier is destroyed, the macrophages in the semen will increase significantly[4, 10]. When macrophages engulf sperm in the semen, it may be a sign that the immune response of the reproductive system is activated[10]. The increase of macrophages in semen lead to the decline of sperm quantity, sperm concentration, sperm motility, etc[10–13]. However, some people believe that an increase in macrophages will not reduce the quality of sperm, and the authors of some studies have shown that macrophages in semen are beneficial to improving male fertility[14, 15]. Such differences may be caused by the various identification methods of leukocyte subsets in semen and the different evaluation criteria for sperm quality. They may also be caused by the different proportions of monocytes and macrophages in semen. When the body is in a state of immunosuppression, the monocyte function is dominant. When the immune function is activated, monocytes will quickly transform into macrophages.

In physiological conditions, macrophages are in an immunosuppressive state, and when activated by related stimulating factors, they can differentiate into different subtypes[16, 17]. We know that there are many labeled antibodies for the monocyte-macrophage system[18–21]. In this study, CD14 was used as a labeled antibody to detect macrophages in semen, because none of the semen cells express CD14 except monocytes-macrophages. It was found that the concentration range of macrophages in semen was $0-15.5 \times 10^5/\text{ml}$, and the content of macrophages in the high group was nearly 5 times greater than that of the low group ($P < 0.05$). The low group contained macrophages at the rate of $0 \times 10^5/\text{ml}$ to $5.3 \times 10^5/\text{ml}$, with an average value of $2.17 \times 10^5/\text{ml} \pm 1.67 \times 10^5/\text{ml}$, and the high group contained macrophages at the rate of $6.1 \times 10^5/\text{ml}$ to $15.5 \times 10^5/\text{ml}$, with an average value of $9.45 \times 10^5/\text{ml} \pm 2.47 \times 10^5/\text{ml}$. This result was similar to the related results[11].

There was no significant difference in sperm concentration, semen volume, or other physicochemical parameters between the two groups, but there were significant differences in some kinetic parameters (PR%, IM%) between the two groups. The results were consistent with those of Kuzelova[12]. We know that semen is mainly composed of sperm and seminal plasma. Seminal plasma (mainly prostatic fluid and seminal vesicle fluid) accounts for more than 90% of semen volume, while sperm accounts for only about 5% of semen. Therefore, the function of prostate and seminal vesicles is the key to determining sperm concentration and semen volume. Spermatozoa are produced in the testis and stored in the epididymis. Sperm can only obtain energy under the action of modifiers in seminal plasma, thus

promoting mitochondrial ATP production, and sperm motility. This series of processes requires the participation of macrophages, so there is a certain correlation between sperm kinetic parameters and macrophages.

The content of macrophages in semen is closely related to the morphology of sperm, DFI, and the integrity of sperm cell membranes. The rate of normal sperm morphology in the high concentration group was lower than that of the low concentration group. The rate of head deformity and neck and middle end deformity of spermatozoa, the SDI and TZI was higher than those in the low concentration group. There was a significant linear correlation between macrophages in sperm and sperm DFI, and a significant negative correlation between macrophages and sperm membrane integrity, which was consistent with the results of the relevant reports[11, 22, 23]. Macrophages in semen come from testicular interstitium, which is an indispensable helper cell in spermatogenesis. They interact with Sertoli cells such as testicular podocytes and Leydig cells to maintain the microenvironmental stability of testicular interstitium[24, 25]. Macrophages mainly perform immune regulation by antigen presentation, the secretion of interleukins, nitric oxide, tumor necrosis factor, 25-hydroxycholesterol, etc[24].

When foreign antigens appear in semen, immune cells can produce an immunoglobulin that can specifically recognize and bind the corresponding antigen. This immunoglobulin is defined as an anti-sperm antibody. The results from this study show that there were fewer AsAb-positive samples in the lower concentration group of macrophages (2.9% vs 9.1%), but there was no significant statistical difference ($P > 0.05$), which is different from that of related studies[7]. This was mainly due to the fact that we excluded the high-risk population of AsAb in the initial screening of the sample.

Cytokines are mainly secreted by activated immune cells, which can combine with target cells to mediate immunity and inflammation. IL-12, secreted by macrophages, is mainly involved in immune surveillance and it secretes IL-10, which is mainly involved in immune down-regulation[26, 27]. The results showed that there was no significant correlation between the content of macrophages and the content of IL-10 and IL-12 in the two groups, which means that the direction of the immune action of the macrophages was independent of the number of cells. The concentration of IL-10 in semen in both groups was higher than that of IL-12, which means that regardless of the concentration of macrophages in the semen, its main role was to mediate the inflammatory response of the immune system. The immunosuppressive effect of macrophages on semen was significantly stronger than that of immune monitoring.

Regarding the mechanism of monocytes-macrophages in semen affecting sperm quality, the following may be of significance: (1) The reduction of macrophages in the testes will cause sperm formation in the testicular spermatogenic tubules to be blocked or the maturation to be hindered[28]. (2) Cytokines produced by macrophages in semen can activate other immune cells. It cooperates with these immune cells and ultimately affects the quality of sperm[24]. (3) Macrophages in semen can secrete ROS, and excess ROS can cause adverse effects on sperm capacitation, acrosome reactions, sperm kinetics, morphology, and sperm DNA[4, 29, 30]. and (4) Macrophages can indirectly mediate the function of

neutrophils. The increase of neutrophils in semen has a certain correlation with the occurrence of male teratogenicity[31, 32].

In summary, there are certain correlations between the macrophages and sperm kinetics, morphology, sperm cell membrane integrity, sperm DFI, and other parameters. The results from this study show that macrophages in semen are important with regard to sperm quality. At low concentrations, macrophages can protect sperm from foreign antigens, but if concentrations are too high, sperm quality will be impaired.

Abbreviations

Mφ

Macrophages; DFI:DNA fragmentation index; AsAb:Anti-sperm antibodies; IM:Immotility; NP:Non-progressive motility; PR:Progressive motility; SDI:Sperm malformation index ; TZI:The abnormal sperm index; WHO:World Health Organization; ROS:Reactive oxygen species.

Declarations

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Authors' contributions

GangxinChen: Project development, Experimental design, Data Collection, Data analysis, Manuscript writing; Beihong Zheng: Manuscript editing.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the ethics committee of Fujian Provincial Maternity and Children Hospital. (YCXM2019-105)

Consent for publication

All authors provided final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions.

Competing interests

The authors declare that they have no competing interests.

Author details

Center for Reproductive Medicine, Fujian Provincial Maternity and Children Hospital, affiliated hospital of Fujian Medical University, Daoshan Road 18, Fuzhou 350001, Fujian, China.

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Tables

Table 1
Comparison of condition and physical and chemical parameters of semen

Related parameters	Low group (n = 34)	High group (n = 44)	t/ χ^2	P-value
M ϕ (10^5 /ml)	2.17 \pm 1.76	9.45 \pm 2.47	-15.17	0.00
Age (years)	30.29 \pm 4.88	30.57 \pm 3.68	-0.28	0.77
BMI	19.67 \pm 1.04	19.95 \pm 1.14	-1.10	0.27
Primary infertility rate (%)	79.4 (27/34)	79.5 (35/44)	0.00	0.98 ^a
Abstinence time (day)	3.85 \pm 0.99	4.27 \pm 1.26	-1.59	0.11
Semen volume (ml)	3.24 \pm 0.91	3.52 \pm 1.16	-1.14	0.25
pH	7.46 \pm 0.19	7.40 \pm 0.42	1.34	0.18
^a χ^2 Test				

Table 2 Comparison of sperm quality parameters between the two groups

Sperm quality parameters	Low group (n = 34)	High group (n = 44)	t/ χ^2	P-value
Sperm concentration (10^6 /ml)	54.14 \pm 27.23	44.45 \pm 26.55	1.58	0.11
PR%	43.94 \pm 10.30	29.29 \pm 16.22	4.85	0.00
NP%	4.29 \pm 1.62	3.56 \pm 1.73	1.88	0.06
IM%	51.50 \pm 10.63	66.84 \pm 15.92	-4.84	0.00
Normal morphology rate (%)	8.52 \pm 3.37	5.68 \pm 3.14	3.83	0.00
Head deformity rate (%)	88.44 \pm 4.76	92.52 \pm 4.82	-3.72	0.00
Neck deformity rate (%)	16.05 \pm 5.49	19.13 \pm 5.63	-2.41	0.01
Main segment deformity rate (%)	6.44 \pm 4.87	6.75 \pm 4.26	-0.29	0.76
Slurry drop rate (%)	1.38 \pm 0.65	1.45 \pm 0.97	-0.39	0.69
SDI	1.10 \pm 0.11	1.19 \pm 0.13	-3.28	0.00
TZI	1.20 \pm 0.10	1.26 \pm 0.12	-2.44	0.01
DFI (%)	12.97 \pm 3.69	20.86 \pm 8.39	-5.10	0.00
Sperm membrane integrity (%)	74.52 \pm 8.61	67.86 \pm 8.88	3.32	0.00
AsAb positive rate (%)	2.9 (1/34)	9.1 (4/44)	0.40	0.52 ^a
IL-10 (pg/ml)	464.17 \pm 60.53	465.63 \pm 57.21	-0.10	0.91
IL-12 (pg/ml)	81.41 \pm 13.09	80.87 \pm 10.90	0.19	1.84
^a χ^2 Test				

Figures

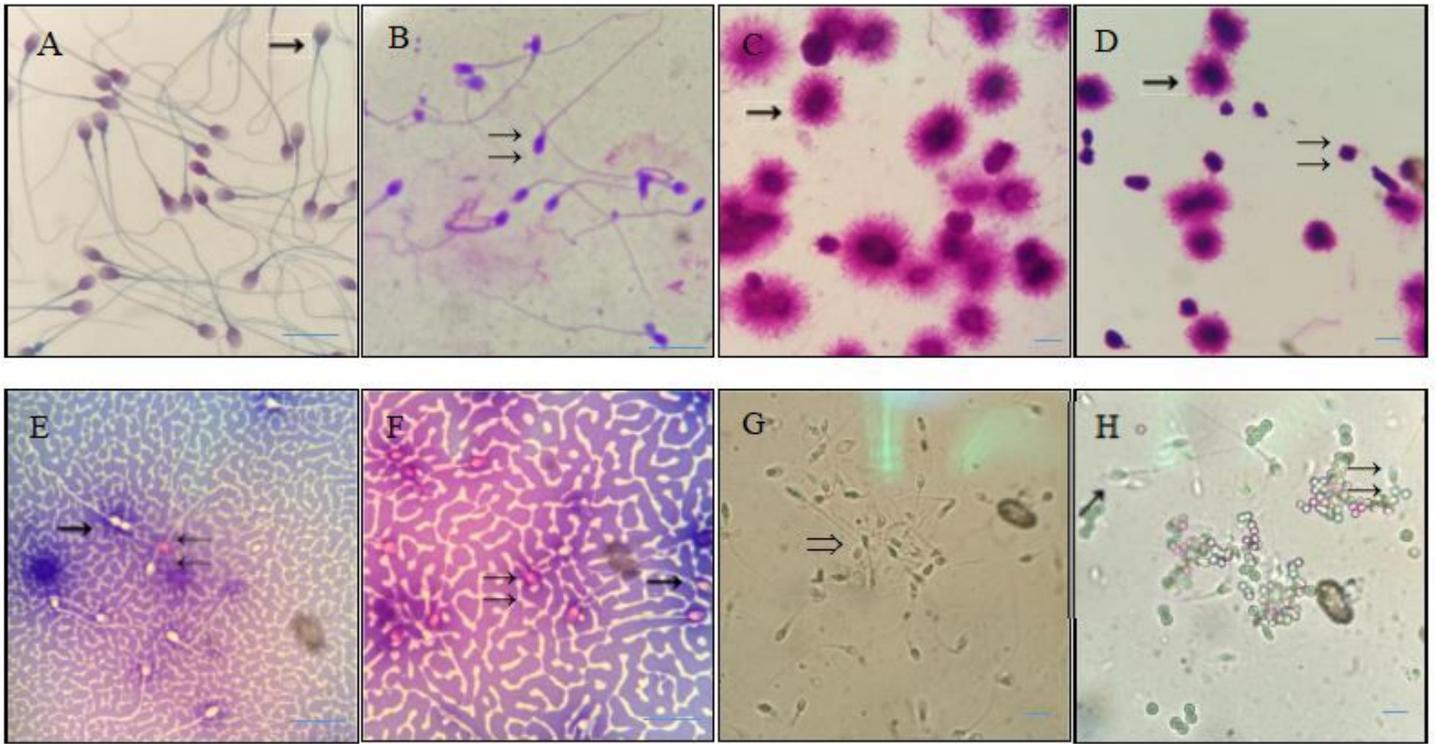


Figure 1

The sperm quality effect chart Sperm morphology (Pap staining). (A) Normal sperm morphology. (B) Abnormal sperm morphology. Sperm DNA fragments (SCD). (C) Sperm with normal DNA spread. (D) Sperm with abnormal DNA spread sperm membrane integrity (aniline staining). (E) Sperm with intact cell membranes. (F) Sperm with incomplete cell membranes. Anti-sperm antibody detection (MAR). (G) Partial sperm agglutination. (H) Sperm being braked (→ indicates normal state, means abnormal state). Magnification: A, B, E, F×1000; C, D, G, H×400). Bar=50μm.

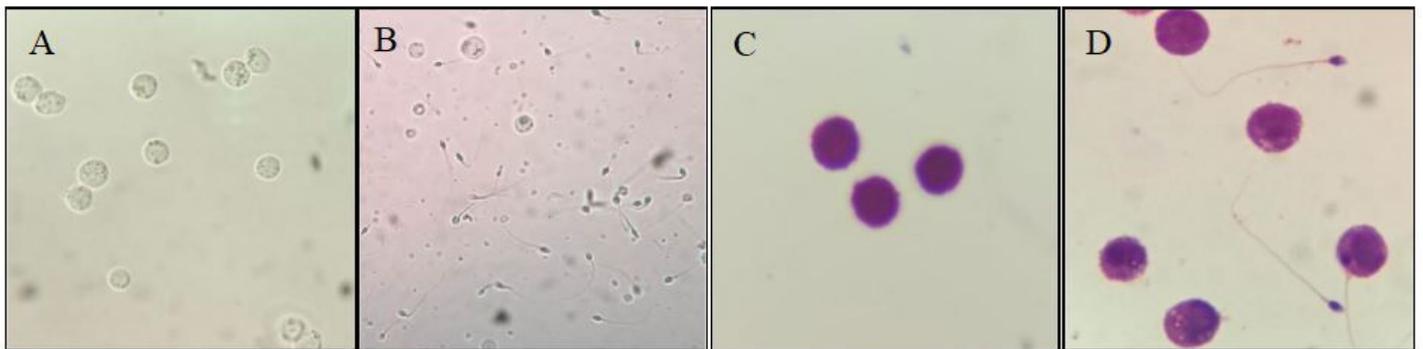


Figure 2

Morphological comparison of macrophages in semen with standard macrophages (A) Morphology of standard macrophages. (B) Morphology of macrophages in semen. (C) Morphology of standard

macrophages after Pap staining. (D) Morphology of macrophages in semen after Pap staining (Magnification: A, B×400; C, D×1000).

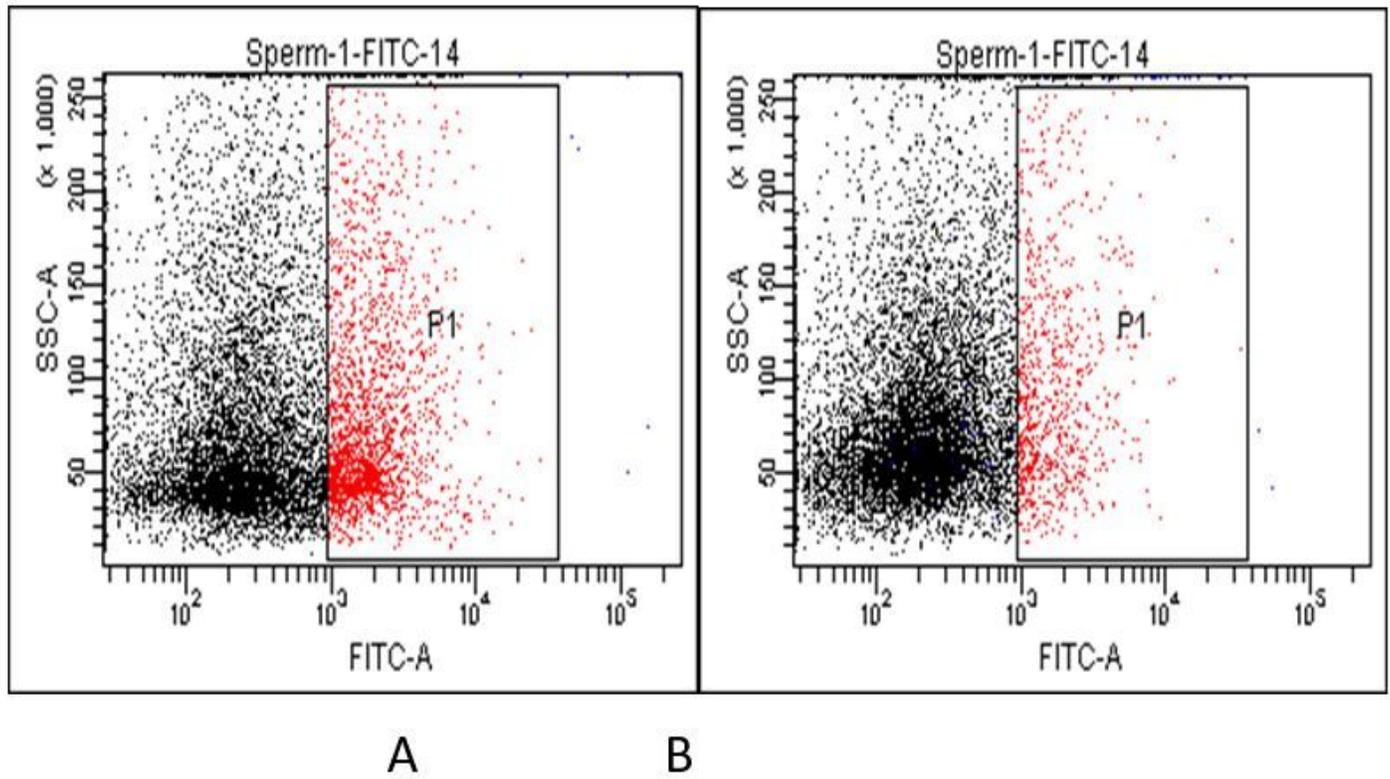


Figure 3

Detection of macrophages in semen after flow cytometry (A) Positive group of macrophages in semen (CD14+). (B) Negative group of macrophages in semen (CD14-).

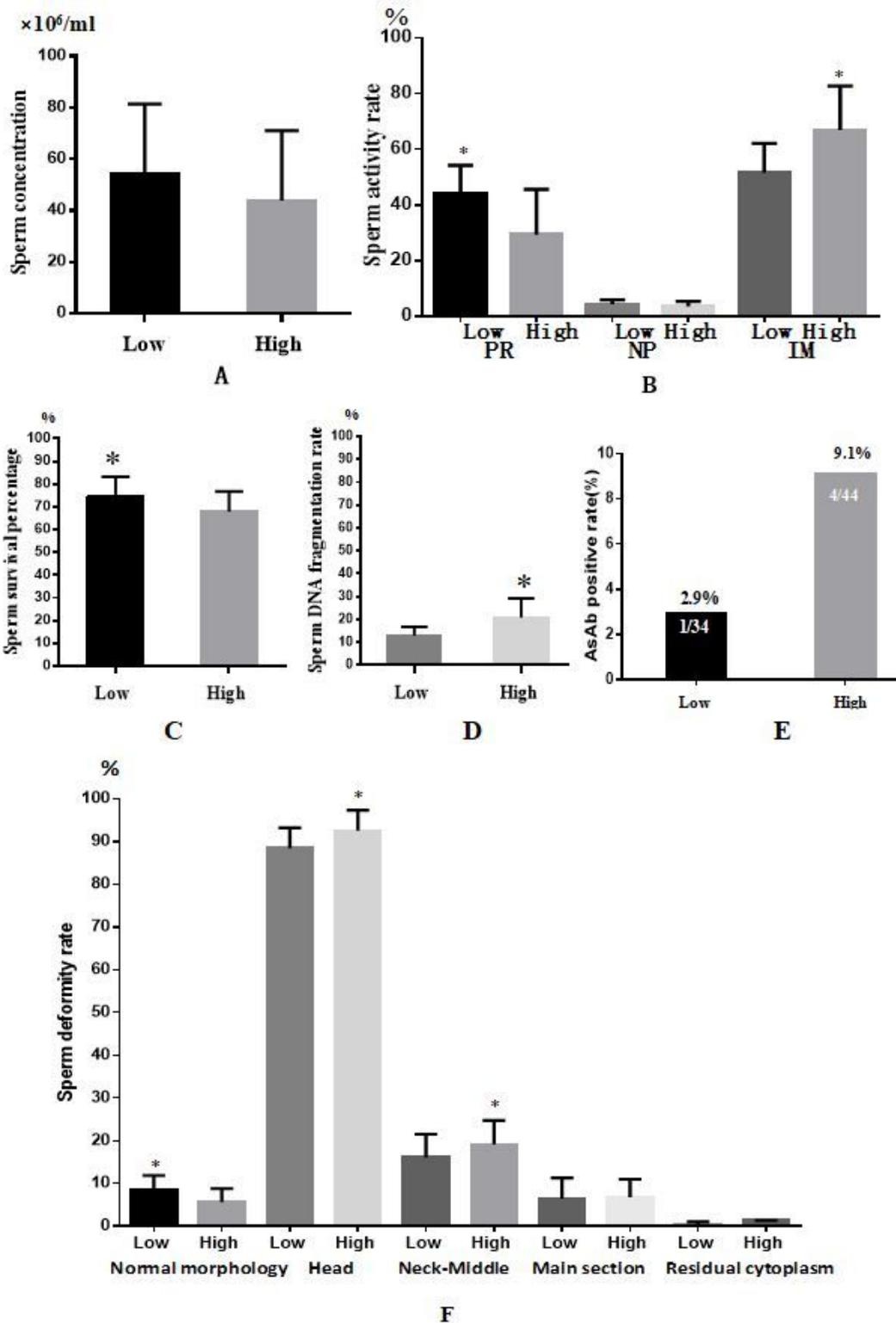


Figure 4

Comparison of sperm quality parameters between the two groups (A) Difference in sperm concentration (B) Comparison of sperm motility (C) Comparison of sperm membrane integrity (D) Comparison of sperm DFI (E) Comparison of AsAb (F) Comparison of sperm morphology. *Indicates statistical difference between the two groups ($P < 0.05$).

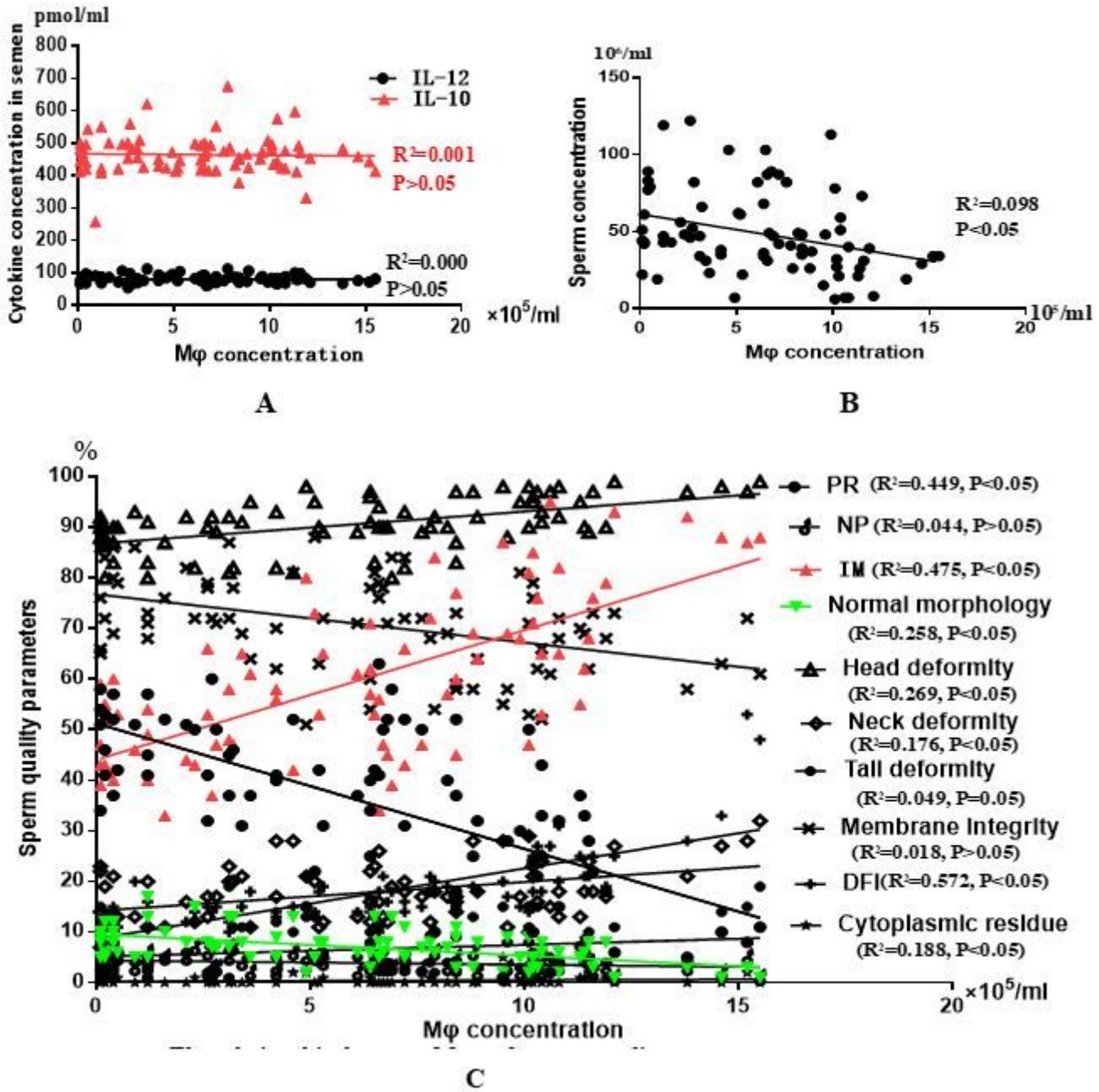


Figure 5

The linear relationship between macrophages and sperm quality (A) The relationship between macrophages and cytokine. (B) The relationship between macrophages and sperm concentration. (C) The relationship between macrophages and sperm quality parameters. R2 represents the degree of correlation; $P<0.05$ indicates there is a linear relationship between them.