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# Relationships between bacteriospermia, DNA integrity, nuclear protamine alteration, sperm quality and ICSI outcome

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ABSTRACT

The Aim of this study was to evaluate the effects of bacteriospermia on human sperm parameters, nuclear protamines, DNA integrity and ICSI outcome in patients enrolled for ICSI treatment. 84 unselected couples consulting in infertility and obstetrics clinic and enrolled for ICSI treatment were included in this study. The semen specimens were screened bacteriologically; semen and sperm parameters were also evaluated according to WHO guidelines. DNA integrity, protamines concentration and protamine deficiency were estimated by TUNEL assay, AU-PAGE and Chromomycin (CMA3) respectively. The results of this study revealed that 34.52% of studied semen samples were infected with bacteria. The isolated bacteria were identified as *Staphylococcus aureus*, *Staph. epidermidis*, *Staph. haemolyticus*, *Escherichia coli*, *Enterococcus faecalis* and *Streptococcus agalactiae.* Bacteriospermia had a significant ($p < .010$) negative effect on sperm parameters; concentration, motility, progressive motility and chromatin condensation. Moreover, high DNA fragmentation with low P1 and P2 concentrations were noticed in infected patients in comparison to non-infected patients but non-significant. Also, the fertilization rate decreased significantly ($p < .05$) with infected patients. In conclusion: bacteriospermia has significant negative effect on sperm quality and fertilization rate in patients who underwent ICSI treatment.

## 1. Introduction

Invasion of bacteria into the male genital system has been shown to be associated with poor sperm function, leading to infertility [1].

Fraczek and Kurpisz reported that, 60% of patients treated with assisted reproductive technology (ART) had suffered inflammation or infection. And the bacteriospermia was directly related to 15% of male infertility which creates a serious problem in andrology [2].

It was found that the presence of pathogenic organisms in semen samples negatively affects sperm parameters [2,3], which inversely effects artificial reproduction techniques [4].

The quality of sperm and oocyte is very important for achievement of the fertilization process. The bad quality of sperm and/or oocytes reduces the fertilization rate [5]. It was reported that the bacteria presence in semen samples have a significant relationship with poor sperm motility [6] and recurrent pregnancy miscarriages [7].

In addition many bacterial species have negative effects on sperm conventional parameters, chromatin condensation and DNA integrity [8].

Protamines (P1,P2) are highly essential nuclear proteins in sperm, which provide tight packaging to sperm DNA, resulting in condensed chromatin and the cessation of gene expression [9]. In human sperm, the ratio of protamine 1 and protamine 2 (P1/P2 ratio) is approximately 1 in fertile men [10,11].

Abnormal P1/P2 ratios have been shown to be associated with increased sperm DNA fragmentation, lower fertilization rates, poor embryo quality and reduced pregnancy rates [12,13]. Other studies confirmed that the abnormal m-RNA ratio of Protamine-1 and protamine-2 have a negative impact on sperm parameters and fertilization capability [5,14]. A positive correlation between sperm protamine deficiency and increased sperm DNA fragmentation [15] as well as reduced semen and sperm quality were also detected [16].

The purpose of the present study was to detect the effects of bacteriospermia on human sperm parameters, nuclear protamine, DNA integrity and ICSI treatment outcomes.

## 2. Materials and methods

### 2.1. Subjects

The study was conducted on 84 couples enrolled for intra-cytoplasmic sperm injection (ICSI) treatment at the clinic of infertility and

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obstetrics at the University of Saarland, Homburg-Germany. This study was approved by the institutional review board, University of Saarland, Germany, and signed consent was obtained from each participant in the present study. All studies involving human subjects were conducted in accordance with Declaration of Helsinki guidelines.

### 2.2. Semen specimen collection and analysis

Semen analysis was performed within 60 min of collection to detect sperm concentration, motility, progression, morphology, and WBCs concentration. Sperm parameters were evaluated according to the recent World Health Organization guidelines [17,18].

#### 2.2.1. Sperm count and motility evaluation

Sperm count and motility were estimated by using sperm counting Makler Chamber (Sefi Medical Instruments Ltd) and bright-field microscope (magnification, 15 × 20). After 60 min of collection, 5 µl of the liquefied semen sample was transferred to the Makler Chamber. Sperm concentration was calculated by dividing the total number of sperms counted in 100 squares by 10 and the results were recorded as $10^6$/ml (total sperm number in 100 squares/10 × $10^6$/ml). Sperm motility and progression were evaluated as follows:

Total motility% = total number of motile sperms/sperm concentration × 100%.

Sperm progression motility% = total number of progressive sperms/sperm concentration × 100%.

#### 2.2.2. Sperm morphology analysis

For sperm morphology reading, 10 µl of sperm suspension was used to prepare sperm smear on a glass slide and left at room temperature to dry. Then the smears were stained by Papanicolaou stain according to WHO guidelines [17].

A total of 200 sperms per slide were analyzed under 100× oil-immersion bright-field objective and a 15× eyepiece to differentiate between normal and abnormal sperms according to WHO criteria [17].

#### 2.2.3. Leukocytes quantification in seminal plasma

The Endtz test was used for quantification of leucocytes in the seminal plasma according to Shekarriz et al. [19].

Briefly, a 20 µl liquefied semen specimen was transferred to a small tube containing 20 µl phosphate-buffered saline (pH 7.0) and 40 µl benzidine solution. Then the content was mixed well and left at room temperature for 5 min. The leukocytes were stained dark brown.

Makler chamber (Sefi Medical Instruments Ltd) under the bright-field objective (magnification, 20×) was used for leukocytes counting and evaluation, with the same method we performed our sperm count evaluation. Significant leukocytospermia were defined at ≥$10^6$ WBC/ml.

### 2.3. Bacteriological study of semen samples; isolation and identification of bacteria

All specimens were collected under aseptic condition and rapidly transferred to a microbiology laboratory within 1 h, this is in accordance to the World Health Organization guidelines [17]. The samples were cultured in two kind of bacteriological media (blood agar and MacConkey agar) within 3 h of collection and then incubated for 48 h at 37 °C. Three replicates were done for each specimen.

Semen samples were considered positive for bacteriospermia when the number of bacterial colonies was > $10^3$ colony forming units (CFU)/ml [20].

Bacterial identification was carried out biochemically by using VITEK 2 system and colorimetric reagent cards (gram-positive and gram-negative cards) according to the manufacturer's instructions with aid of Bergey's manual of determinative bacteriology [21].

### 2.4. Sperm protamine deficiency assay (Chromomycin A3)

Chromomycin $A_3$ (CMA3) assay was used to evaluate the sperm protamine deficiency which is a good indicator for sperm chromatin condensation. This test was performed according to Manicardi et al. [22]. Sperm slides were prepared by transfering 10 µl of sperm suspension to microscope slides. After the slides dried, smears were fixed by methanol–glacial acetic acid 3:1 at 4 °C for 20 min and then allowed to air dry at room temperature. 20 µl of $CMA_3$ stain solution was added to each slide and covered by cover slips before being incubated in the dark for 20 min at room temperature. The slides were washed in PBS buffer and mounted with 1:1 (v/v) PBS/glycerol then kept overnight at 4 °C in the dark. For estimation, a total of 200 spermatozoa were analyzed on each slide by using fluorescence Microscope BH2-RFCA (Olympus, Japan) with yellow fluorescence filter, to distinguish spermatozoa that stain bright yellow (CMA3 positive) from those that stain a dull yellow (CMA3 negative).

### 2.5. Sperm DNA fragmentation assay

DNA fragmentation was assessed using TUNEL assay as previously described by Borini et al. [23]. The assay was performed using the *in-situ* cell death detection kit: fluorescein following the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany). In this test, we used the terminal deoxynucleotidyl transferase (TdT) to detect The DNA broken regions. TdT enzyme will catalyze the addition of dUTPs, and this will be labelled with a marker.

TUNEL assay: semen samples were washed in semen wash buffer (COOK Medical) and centrifuged at 250 × *g* for 10 min to remove the seminal plasma. 10 µl of sperm suspension was used for sperm smear preparation on glass slide then allowed to air dry.

For fixation, the slides were incubated in 4% paraformaldehyde phosphate–buffered saline, pH 7.4 at room temperature for two hours, and then washed with PBS. Thereafter, the slides were incubated with 0.1% triton X-100 in 0.1% sodium citrate, pH 6.0, for 15 min at RT in purpose to do sperm permeabilisation.

Fifty µl of the TdT-labelled nucleotide mixture (50 µl of enzyme solution and 450 µl of label solution) was added to each slide and incubated in a humidified chamber at 37 °C overnight in the dark. Negative controls without TdT enzyme were run in each replicate. Then the slides were washed twice in PBS buffer (Fig. 1).

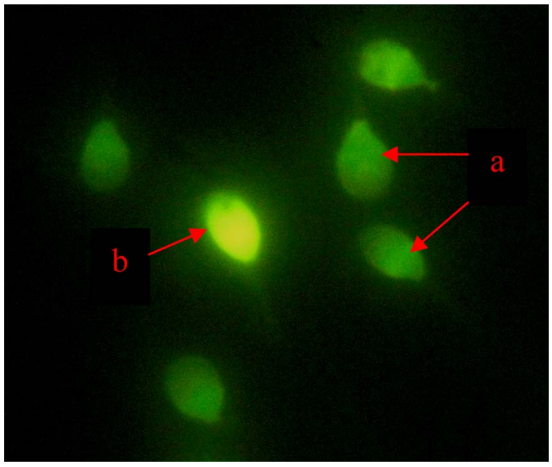


**Fig. 1.** Chromomycin A3 (CMA3) stain for analysis of sperm protamines deficiency. It shows two types of sperms: Sperms with normal protamines components (CMA3 negative fluorescent dull yellow) (a). Sperm with protamine deficiency (CMA3 positive fluorescent bright yellow) (b). Magnification = 15 × 100. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)