Article

Candida albicans experimental infection: effects on human sperm motility, mitochondrial membrane potential and apoptosis

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Abstract

Studies suggest *Candida albicans* infection has a negative effect on sperm function, including fertilizing ability. Assisted reproduction treatment using spermatozoa from a patient with unrecognized *C. albicans* infection did not result in fertilization. Preliminary evidence suggested an effect on sperm motility and apoptosis. This study was undertaken to evaluate the effects of experimentally induced *C. albicans* infection on motility, membrane mitochondrial potential (MMP), chromatin packaging and apoptosis [membrane phosphatidylserine (PS) externalization and DNA fragmentation] of spermatozoa isolated from normozoospermic healthy men. Motile spermatozoa were isolated by swim-up from 13 normal volunteers and exposed to increasing concentrations (0, 1000, 10,000, and 100,000 cfu/ml) of the fungus for 3 and 24 h. *C. albicans* was isolated from vaginal swabs, after identification, freshly prepared for experiments. Following incubation, sperm motility decreased significantly ($P < 0.05$ from 10,000 cfu/ml) and spermatozoa with reduced MMP or PS externalization, an early sign of apoptosis, increased in a time- and concentration-dependent manner. Sperm DNA fragmentation and chromatin integrity increased slightly after exposure to *C. albicans*, but the increase did not reach statistical significance. This study showed that *C. albicans* infection may decrease the functional competence of spermatozoa by reducing motility and MMP and by promoting molecular apoptosis mechanisms.

Keywords: Candida albicans, *chromatin integrity, DNA fragmentation, mitochondrial membrane potential, phosphatidylserine externalization, sperm motility*

Introduction

Male accessory gland infections have been reported to affect approximately 15% of male infertile patients (Weidner *et al.*, 1991; Diemer *et al.*, 2003). Various microbial agents may affect sperm function by infecting the urogenital tract at different sites. Infections have been reported to alter spermatozoon motility, morphology (Menkeveld and Kruger, 1998) and functional biochemistry (Zalata *et al.*, 1998).

Candida albicans is an opportunist pathogen of the human urogenital tract. It is virulent when filamentation is present because it can adhere to many substrates (Mitchelle, 1998). It can negatively affect sperm function. Human spermatozoa from healthy volunteers, exposed to *C. albicans*, show a strong decrease in sperm motility and increased agglutination (Tuttle *et al.*, 1997). More recently, Tian and colleagues (2007) showed a similar in-vitro effect of *C. albicans* on sperm motility from healthy donors. Signs of sperm membrane alteration have also been reported. Light and transmission electron microscopy examination demonstrated that *C. albicans* attach to spermatozoa, mainly to the head. Multiple ultrastructural

lesions have been detected that could explain spermatozoon immobilization (Tian *et al.*, 2007).

The success of assisted reproduction treatment may be compromised in patients with male accessory gland infections, although it is difficult to estimate the effect on fertilization, cleavage and pregnancy rates. A lack of fertilization after assisted reproduction treatment in a patient with initial prostatitis treated with antibiotics who later developed an unrecognized *C. albicans* infection has been previously reported (Burrello *et al.*, 2004). The patient had normal sperm parameters and the couple underwent conventional IVF for female factor infertility. After IVF procedures, no oocyte fertilization resulted and almost all spermatozoa in the incubation medium were found to be immotile. *C. albicans* was detected in the sperm sample, whereas no bacteria grew. A new semen sample was requested from the patient to perform intracytoplasmic sperm injection (ICSI) on the same oocytes and a low fertilization rate resulted. The spermatozoa were studied in an attempt to understand why there was no fertilization following IVF procedures. An increased percentage of motile spermatozoa with chromatin packaging abnormalities, membrane phosphatidylserine (PS) externalization and DNA fragmentation was found. These findings suggested that *C. albicans* infection reduced the capability of spermatozoa to maintain their motility in the incubation medium and damaged sperm chromatin/DNA. Additional data are needed to understand the precise mechanism(s) by which *C. albicans* may affect sperm fertilizing capability. This study was undertaken to evaluate the in-vitro effects of increasing concentrations of *C. albicans* on motility, membrane mitochondrial potential (MMP), chromatin packaging and apoptosis of spermatozoa isolated from normozoospermic healthy men. As far as is known, this is the first study exploring the effects of *C. albicans* on mitochondrial function in human spermatozoa.

Materials and methods

Sperm preparation

Spermatozoa were obtained from 13 normozoospermic healthy men. Semen was collected by masturbation after 3–5 days of abstinence and sperm parameters were evaluated according to the World Health Organization (1999) criteria. After 30 min liquefaction at 37°C, motile spermatozoa were isolated using a swim-up procedure as previously reported (Calogero *et al.*, 1998). The pellet was overlaid with 0.5 ml Biggers, Whitten and Whittingham (BWW) medium and left to incubate for 30 min, at the end of which the supernatant was collected. Sperm density was adjusted with BWW medium to a concentration ranging from 20 to 30 × 10^6 spermatozoa/ml.

An aliquot containing 5 × 10^6 spermatozoa was incubated with increasing concentrations of *C. albicans* (0, 1000, 10,000, and 100,000 cfu/ml) in a water-jacketed incubator at 37°C under 5% CO_2 atmosphere for 3 and 24 h. At the end of the incubation, the following parameters were evaluated: total sperm motility; MMP (assessed by the cationic dye JC-1 and flow cytometry); sperm chromatin packaging quality following DNA staining with propidium iodide (PI); the presence of phosphatidylserine (PS) on the outer leaflet of the cell membrane using the annexin V/PI assay; and DNA fragmentation using the TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay, as previously reported (Perdichizzi *et al.*, 2007).

C. albicans production

C. albicans was isolated from vaginal swabs. It was grown on Sabouraud's dextrose agar (Oxoid, Milan, Italy) at 37°C, overnight, in aerobic conditions, and identified by the API Candida system (bioMerieux SA, Marcy-l'Etoile, France). The *C. albicans* suspension was freshly prepared the day before the experiment by incubation at 37°C, overnight, in brain heart infusion under aerobic conditions. A final suspension, containing 4 × 10^6 cfu/ml, was prepared in physiological saline, starting from an initial concentration of 1 × 10^8 cfu/ml obtained using a 0.5 McFarland standard. The exact concentrations of the suspension were verified by spectrophotometer ThermoSpectronic Genesis UV (Thermo Fisher Scientific, single cell holder, 6 position auto turret, USA).

Flow cytometry

The EPICS XL (Coulter Electronics, IL, Italy) flow cytometer, equipped with a 488 nm argon laser light source, was used for flow cytometry analysis. Three fluorescent detectors were used according to the assay utilized to measure the fluorescence corresponding to green (FL-1 detector 525 nm wavelength band), orange (FL-2 detector 620 nm wavelength band) and red (FL-3 detector 620 nm wavelength band). In all, 20,000 events were measured for each sample at a flow rate of 50–100 events/second and analysed using SISTEM II™ Software, 3.0 Version. The debris was gated out, by drawing a region on forward versus side scatter dot plot enclosing the population of cells of interest, as previously reported (Perdichizzi *et al.*, 2007). The compensations and the settings were adapted according to the assay utilized.

JC-1 staining

To evaluate a decrease in MMP, a marker of early apoptosis, the cell suspension was adjusted to a density of 1 × 10^6 cells/ml and incubated for 10 min at 37°C in the dark with JC-1 at a final concentration of 5 µg/ml (Molecular Probes, Milan, Italy). This molecule, which is able to enter the mitochondria selectively, exists in a monomeric form emitting at 527 nm after excitation at 490 nm. However, depending on the membrane potential, JC-1 is able to form aggregates that are associated with a large shift in emission (590 nm). Thus, the colour of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized. At the end of the incubation period, cells were washed in phosphate-buffered saline (PBS) and analysed. For the analysis of the cells stained with JC-1, the photomultiplier (PMT) value of the detector in FL-1 was set at 529 V, FL-2 PMT at 543 V; FL1-FL2 compensation was 12%, FL2-FL1 compensation was 28%, as previously reported (Aljabari *et al.*, 2007; Perdichizzi *et al.*, 2007).

Annexin V/PI assay

Early macrophage and phagocyte recognition during apoptosis is due to cell membrane structure change. PS, which is confined to the inner leaflet of the plasma membrane, is externalized during the early phases of apoptosis. PS externalization in cells undergoing apoptosis may be detected by fluorescein isothiocyanate (FITC)-

labelled annexin V, a Ca^{++}-binding protein, able to interact with negatively charged phospholipids, such as PS. In addition, during apoptosis, cells externalize PS before they lose the capacity to exclude PI. Consequently, the simultaneous labelling with annexin V and PI makes it possible to distinguish between vital cells and apoptotic or necrotic cells.

Staining with annexin V/PI was performed using a commercially available kit (annexin V–FITC apoptosis detection kit, Sigma Chemicals, Milan, Italy), as previously reported (Perdichizzi *et al*., 2007). An aliquot containing 0.5×10^6 spermatozoa/ml was re-suspended in 0.5 ml of binding buffer, labelled with 10 µl of annexin V–FITC plus 20 µl of PI, incubated for 10 min in the dark, and immediately analysed. Signals were detected through FL-1 (FITC) and FL-3 (PI) detectors. The cell population of interest was gated on the basis of the forward-scatter (FSC) and side-scatter (SSC) properties. The different labelling patterns in the bivariate PI/annexin V analysis identify the different cell populations: FITC negative and PI negative were designated as viable cells; FITC positive and PI negative as apoptotic cells (cells with PS externalization); and FITC positive and PI positive as late apoptotic or necrotic cells.

PI staining

During spermiogenesis, the sperm genome undergoes profound structural remodelling. Histones are substituted with protamines and a number of intra- and inter-molecular disulphide bridges are formed. This results in an approximately six-fold greater chromatin compactness compared with somatic cells. Because of this, sperm PI staining has to be performed following membrane permeabilization to allow cytochrome penetration within the nucleus. Once the nucleus has been reached, PI gives rise to two different peaks of fluorescence: the first peak identifies a cell population with compact chromatin and consequently a low number of free ligation sites; and the second peak identifies a cell population with loosely packed chromatin, which contains a high number of free ligation sites.

PI staining was performed following cell membrane permeabilization, as previously reported (Perdichizzi *et al*., 2007). Briefly, 1×10^6 spermatozoa were incubated in 1 ml PBS containing 50 µg/ml of PI (Sigma Chemicals), 0.1% sodium citrate, and 0.1% Nonidet P40 (Sigma Chemicals), 100 Kunits/ml of RNase type A (Sigma Chemicals) in the dark, at room temperature. After 30 min, flow cytometry analysis was performed. In this assay, only the FL3 detector was used. To gate out and thus exclude from the analysis doublets and cell aggregates, a 'doublet discrimination module' was used. The peak width was estimated using the coefficient of variation (CV) of the signals within each peak.

TUNEL assay

Sperm DNA fragmentation following *C. albicans* exposure was assessed by the TUNEL assay using the commercially available Apoptosis Mebstain kit (Beckman Coulter, IL, Milan, Italy), as previously reported (Perdichizzi *et al*., 2007). To obtain a negative control, TdT was omitted from the reaction mixture. The positive control was obtained by pre-treating spermatozoa with 1 µg/ml deoxyribonuclease I, RNase-free (Sigma Chemicals) at 37°C for 60 min before labelling. The debris was eliminated following the same procedure described above. Light-scattering and fluorescence data were obtained at a fixed gain setting in logarithmic mode. The percentage of FITC-labelled spermatozoa was determined in the FL-1 channel of the flow cytometer.

Statistical analysis

Results are reported as mean ± SEM. The data were analysed by 1-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range test. Correlation between total motility and MMP was carried out using the Pearson test. The software Statistics Package for Social Sciences (SPSS) 9.0 for Windows was used for statistical evaluation (SPSS Inc., Chicago IL, USA). A statistically significant difference was accepted when the *P*-value was lower than 0.05.

Results

Experimentally induced *C. albicans* infection had a concentration-dependent inhibitory effect on total sperm motility (**Figure 1**). After 3 h of incubation, the effect became significant at a concentration of 10,000 cfu/ml ($P < 0.05$ versus *Candida* 0, 1-way ANOVA). The inhibitory effect became significantly stronger at a concentration of 100,000 cfu/ml ($P < 0.05$, versus *Candida* 1000 cfu/ml, 1-way ANOVA). After incubation for 24 h, *C. albicans* infection significantly decreased the percentage of motile spermatozoa in a concentration depending manner from 1000 to 100,000 cfu/ml ($P < 0.05$).

The inhibitory effect of C. albicans on sperm motility was paralleled by a negative impact on sperm MMP (**Figures 2** and **3**). After 3 and 24 h of incubation, *C. albicans* significantly increased the percentage of spermatozoa with decreased MMP from the lowest concentration tested ($P < 0.05$ versus *Candida* 0, 1-way ANOVA). A highly significant direct correlation between decreased sperm total motility and reduced MMP was found ($r = 0.82$, $P < 0.001$).

C. albicans infection had a concentration-dependent effect on PS externalization, a precocious sign of apoptosis (**Figures 4** and **5**). After 3 and 24 h of incubation, *C. albicans* significantly increased

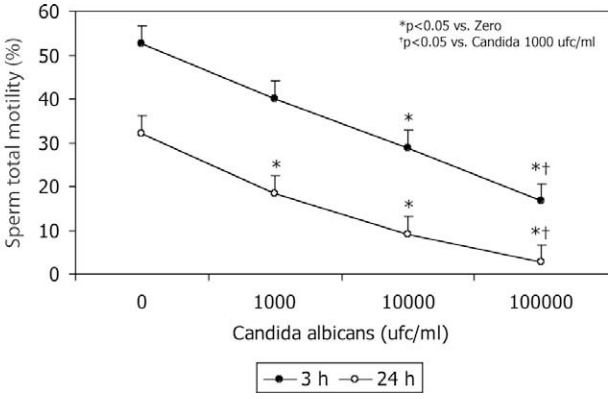

Figure 1. Time-course and dose-response of experimentally induced *Candida albicans* infection on total motility of spermatozoa separated by swim-up from 13 healthy normozoospermic men.

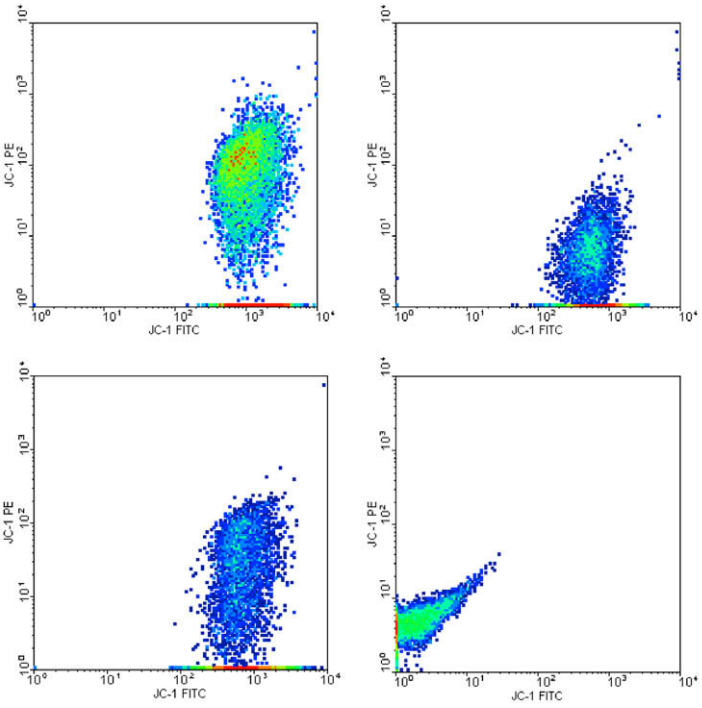

Figure 2. Representative flow cytograms showing the effects of *Candida albicans* infection (10^5 cfu/ml) on sperm mitochondrial membrane potential after (**B**) 3 and (**D**) 24 h of incubation. The effects for controls with no *C. albicans* infection after (**A**) 3 and (**C**) 24 h of incubation are also reported. FITC = fluorescein isothiocyanate.

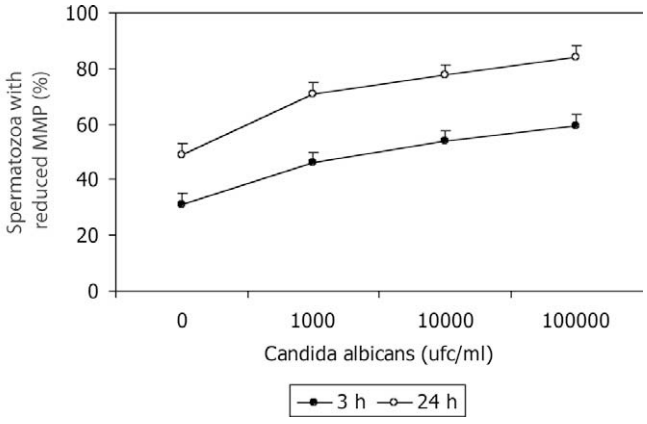

Figure 3. Time-course and dose-response of experimentally induced *Candida albicans* infection on the mitochondrial membrane potential (MMP) of spermatozoa separated by swim-up from seven healthy normozoospermic men.

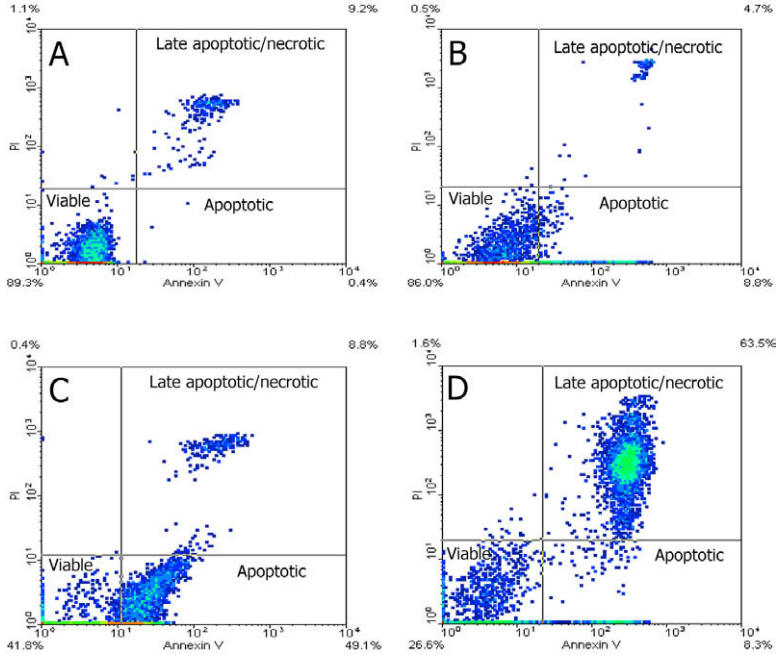

Figure 4. Representative flow cytograms showing the effects of *Candida albicans* infection (10^5 cfu/ml) on phosphatidylserine externalization in spermatozoa after (**B**) 3 and (**D**) 24 h of incubation. The effects for controls with no *C. albicans* infection after (**A**) 3 and (**C**) 24 h of incubation are also reported. PI = propidium iodide.

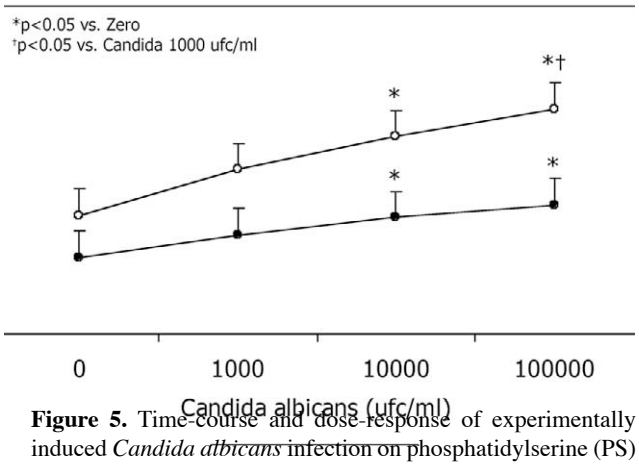

Figure 5. Time-course and dose-response of experimentally induced *Candida albicans* infection on phosphatidylserine (PS) externalization (apoptotic cells) in spermatozoa separated by swim-up from 10 healthy normozoospermic men.

the percentage of spermatozoa with PS externalization from the concentration of 10,000 cfu/ml ($P < 0.05$ versus *Candida* 0, 1-way ANOVA). At a concentration of 100,000 cfu/ml after 24 h of incubation, the effect was significantly stronger ($P < 0.05$ versus *Candida* 1,000, 1-way ANOVA).

C. albicans infection had no statistically significant effect on the percentage of spermatozoa with DNA fragmentation, a late sign of apoptosis (**Table 1**) and it did not significantly alter sperm chromatin condensation (**Table 2**).

Discussion

Experimental infection with *C. albicans* decreases sperm motility. The degree of this effect was associated with the concentration of the fungus and the duration of the contact with spermatozoa. Three hours were sufficient to significantly modify the percentage of motile spermatozoa at a concentration of 10,000 cfu/ml (*Candida*:spermatozoon ratio: 1:500) and the effect was more marked at a concentration of 100,000 cfu/ml (*Candida*:spermatozoon ratio: 1:50). After 24 h of incubation, there was a statistically significant decrease in sperm motility starting from the lowest concentration tested (i.e. 1000 cfu/ml) (*Candida*:spermatozoon ratio: 1:5000) and the effect became significantly stronger at higher concentrations. These experiments were performed with a concentration of *C. albicans* lower than that used in a previous study, which was 1:1 (Tian *et al.*, 2007). This evidence suggests that the damage caused by *C. albicans* on spermatozoa may be of greater magnitude if the ratio *Candida*:spermatozoa increases.

The first to report a negative impact of experimentally induced *C. albicans* infection on sperm motility were Tuttle and colleagues who found a motility decrease soon after spermatozoa came into contact with the fungus (Tuttle *et al.*, 1977). They also reported a significant degree of sperm non-specific agglutination detected after 2 and 4 h of incubation. A significant reduction of sperm motility and signs of membrane alteration were found to correlate with the length of infection (0, 1, 2, 4 h). A clear head to head sperm agglutination with *C. albicans* interposition was reported (Tian *et al.*, 2007). It was postulated that the reduction of sperm motility following incubation with *C. albicans* was due to the formation of a mechanical barrier (Huwe *et al.*, 1998). However, subsequent studies challenged this assumption. In fact, it is only at the beginning that motility reduction can be ascribed to the presence of a mechanical barrier that causes sperm agglutination. Later on, mitochondrial and tail alterations appear, which may contribute to the sperm motility decrease. In addition, spermatozoa in contact with *C. albicans* undergo acrosomal swelling, vesiculation (outer membrane) and rupture (Tian *et al.*, 2007), which may impair sperm fertilization capability. It has been reported that in the presence of *C. albicans* no fertilization occurred after IVF and ICSI (Burrello *et al.*, 2004).

In the presence of *C. albicans*, the percentage of spermatozoa with reduced MMP increased significantly, which correlated strongly with sperm motility decrease. As far as is known, this is the first study reporting the effects of *C. albicans* on mitochondrial function in human spermatozoa. A loss of mitochondrial integrity has already been reported in macrophages incubated with phospholipomannan, a surface component of *C. albicans* (Ibata-Ombetta *et al.*, 2003). The mitochondrial effect of *C. albicans* on human spermatozoa was significantly detectable starting from the lowest concentration used both after 3 and 24 h of incubation.

MMP reduction is a marker of initial apoptotic phenomena and shows a positive correlation with sperm motility (Wang *et al.*, 2003). A correlation between MMP, sperm motility and viability has been reported (Evenson *et al.*, 1982; Auger *et al.*, 1989, 1993). This confirms the great linkage between mitochondrial functional status and sperm quality (Marchetti *et al.*, 2002).

MMP alterations as well as PS externalization are characteristic of the early stages of apoptosis in somatic cells. These events precede other alterations of programmed cell death, such as DNA fragmentation (Kroemer *et al.*, 1997). A significant correlation between translocation of PS and loss of MMP has been demonstrated (Barroso *et al.*, 2006). The present results showed that experimentally induced *C. albicans* infection increased the number of spermatozoa with PS externalization, but not with DNA fragmentation. This apparent discrepancy may be due to a different length of incubation with *C. albicans* to achieve these effects. A greater concentration of the fungus is necessary to achieve sperm DNA fragmentation.

C. albicans had no effect on chromatin condensation under the experimental conditions used in this study. Since an increased number of spermatozoa with chromatin packaging abnormalities in a man with *C. albicans* infection has been previously reported (Burrello *et al.*, 2004), it may be postulated that higher *C. albicans* concentration and/or a longer fungus–spermatozoa interaction may be needed to see such an effect. Alternatively, it may require an in-vivo mediated mechanism.

In conclusion, it was found that *C. albicans* infection reduces sperm motility after a short time of interaction. This effect strongly correlated with reduced MMP. An increased number of spermatozoa showed precocious signs of apoptosis. These findings may explain the reduced functional competence of spermatozoa in the presence of *C. albicans* infection.

Table 1. Effects of experimentally induced *Candida albicans* infection on DNA fragmentation of spermatozoa separated by swim-up from 10 healthy normozoospermic men.

Candida albicans *(cfu/ml)*	*3 h incubation (%)*	*24 h incubation (%)*
0	13.9 ± 2.3	14.5 ± 2.3
1000	13.4 ± 2.5	15.1 ± 2.3
10,000	14.8 ± 2.2	16.8 ± 2.3
100,000	14.9 ± 2.2	17.8 ± 2.4

Table 2. Effects of experimentally induced *Candida albicans* infection on the chromatin integrity of spermatozoa separated by swim-up from 10 healthy normozoospermic men.

Candida albicans *(cfu/ml)*	*3 h incubation (%)*	*24 h incubation (%)*
0	12.7 ± 2.4	13.7 ± 2.4
1000	13.4 ± 2.4	14.1 ± 2.5
10,000	13.8 ± 2.3	14.9 ± 2.4
100,000	14.2 ± 2.4	15.9 ± 2.6

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