Blackwell Publishing

Asian J Androl 2008; 10 (): –
DOI: 10.1111/j.1745-7262.2008.00356.x

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·*Original Article*·

Sperm quality improvement after natural anti-oxidant treatment of asthenoteratospermic men with leukocytospermia

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Abstract

Aim: To study the immune-modulating and anti-oxidant effects of beta-glucan, papaya, lactoferrin, and vitamins C and E on sperm characteristics of patients with asthenoteratozoospermia associated with leucocytosis. **Methods:** Fifty-one patients referred to our Sterility Center for semen analysis were selected. Sperm parameters were assessed before and after patient's treatment with beta-glucan, lactoferrin, papaya, and vitamins C and E. DNA damage was assessed by the acridine orange test and sperm structural characteristics were evaluated by transmission electron microscopy. **Results:** After 90 days of treatment, an increase in the percentage of morphologically normal sperm (17.0 ± 5.2 *vs.* 29.8 ± 6.5) and total progressive motility (19.0 ± 7.8 *vs.* 34.8 ± 6.8) were detected. Structural sperm characteristics as well as chromatin integrity were also improved after treatment. In terms of leukocyte concentration in seminal fluid, a significant reduction was recorded (2.2 ± 0.9 *vs.* 0.9 ± 0.2). **Conclusion:** The treatment of an inflammatory process by the synergic action of immune modulators and anti-oxidants could protect sperm during maturation and migration, leading to improved sperm function. *(Asian J Androl 2008 ***; 10: –)*

Keywords: asthenoteratozoospermia; leukocytospermia; anti-oxidant; beta-glucan; papaya; lactoferrin; vitamin C; vitamin E; chromatin integrity; transmission electron microscopy

1 Introduction

Many factors can impair male reproductive capacity, causing transient or permanent infertility. In these cases, leukocytospermia represents an additional risk factor inducing the production of highly toxic reactive oxygen species (ROS) that impair genital tract accessory glands and sperm cell functions and quality.

High leukocyte counts in seminal fluid are common, even in the absence of inflammatory symptoms, and might be an indirect sign of viral or microbial infections, past testicle trauma, and varicocele. Although white blood cell lines play a physiological role in immune surveillance and in eliminating anomalous sperm, many studies indicate that an increased number of leukocytes in seminal fluid is associated with altered sperm parameters [1–3].

The relationship between inflammation of the genital

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Received 2007-07-27 Accepted 2007-09-17

tract and reduced fertility is unclear. Different stimuli (chemical, biological, and physical) are known to activate cells of the lymphatic system, inducing an inflammatory response accompanied by production of ROS [4]. Scrotal varicocele was found to be associated with elevated ROS levels and decreased levels of anti-oxidants in internal spermatic venous blood compared to peripheral venous circulation [5]. When the body fails to counteract increased production of these radicals, they might cause oxidative stress, affecting sperm. Lipid peroxidation causes structural damage to the acrosome and sperm head and neck, as well as triggering apoptosis and inducing DNA breakage [6]. Altered nuclear chromatin structure or DNA damaged in sperm could be responsible for male infertility. It has been reported that *in vivo* fecundity is decreased when more than 30% of sperm show DNA damage. Moreover, gamete function is impaired by reduced motility and by inhibition of the acrosome reaction and fusigenic capacity [6–9].

Anti-oxidant enzymes in seminal fluid therefore play a fundamental role, replacing the cytoplasmic enzymes lost by sperm during spermiogenesis. Seminal fluid also contains non-enzyme anti-oxidants, such as vitamins C and E, pyruvate, glutathione, and carnitine. Some authors reported that aging, diet, smoking and lifestyle tend to reduce immune defenses and anti-oxidant activity, lowering semen quality and impairing fertilizing capacity [10, 11].

Anti-inflammatory drugs of different types have been found effective in the treatment of asymptomatic and symptomatic leukocytospermia [12, 13], and improved sperm quality has been reported after oral intake of anti-oxidants [14, 15]. The effect of a therapy combining the immune-stimulating properties of beta-glucan and fermented papaya with anti-oxidant vitamins C and E and the bacteriostatic effect of lactoferrin has never been assessed in asthenospermic males. Beta-glucan, a polysaccharide extracted from yeast cell walls, is known for its immune-stimulating properties. It reinforces natural defenses against viral infections, bacteria, fungi, parasites and neoplastic cells, and stimulates tissue repair [16, 17]. Many recent studies have shown the efficacy of papaya against peroxidation and the genotoxicity of free radicals [18, 19]. The anti-oxidant properties of fresh papaya, which contains vitamins and amino acids, are increased by fermentation, improving its immune-modulating activity [18]. Lactoferrin, a protein found in milk and other products of the exocrine glands of the digestive, respiratory, and reproductive systems, is a natural anti-oxidant and a powerful activator of natural killer cells. It modulates the migration, maturation, and function of cells of the immune system [20]. Ascorbic acid and alpha-tocopherol, vitamins C and E, respectively, are normally present in seminal fluid where they counteract peroxidation by virtue of their anti-oxidant properties. Their concentration in seminal fluid is correlated with daily intake.

The aim of the present study was to assess the synergic effect of the immune-modulating and anti-oxidant properties of beta-glucan, papaya, lactoferrin, and vitamins C and E on sperm quality in men with asthenoteratozoospermia and leukocytospermia.

2 Materials and methods

2.1 Patients

We selected 36 patients referred to our Infertility Center for semen analysis after 12–18 months of sexual intercourse without conception. The patients had leukocyte counts in seminal fluid exceeding 1×10^6. Azoospermic men were excluded. The patients enrolled in this study did not have a history of endocrine or anatomical disorders.

The diagnosis of leukocytospermia associated with asthenoteratozoospermia was confirmed by a second spermiogram 1 month after the first. In all patients, microbiological screening of seminal fluid and urine for common microorganisms *Mycoplasma*, *Trichomonas vaginalis* and *Chlamydia trachomatis* gave negative results. The mean age of patients was 32 years (range, 22–47 years).

2.2 Controls

As controls we selected 15 men (mean age 30 years; range, 25–41 years) with the same seminal characteristics, associated leukocytospermia and negative sperm culture, for whom no therapy was prescribed.

2.3 Therapy

The patients were treated for 3 months with two tablets per day of a formula (Fattore M). Each tablet contained 20 mg beta-glucan, 50 mg fermented papaya, 97 mg lactoferrin, 30 mg vitamin C, and 5 mg vitamin E (Progine, Florence, Italy).

2.4 Analysis of seminal fluid

Before the spermiogram, patients observed 4 days of sexual abstinence. Samples were assessed according to World Health Organization (WHO) guidelines [21], using an inverted phase-contrast microscope with Hoffmann lens (Olympus, Milan, Italy) and platform heated to 37°C. Eosin Y was used to detect necrotic sperm. Morphological examination of the specimens was carried out after staining with modified Papanicolaou reagent for sperm. One hundred sperm were counted in each sample and sperm organelle morphological parameters (nucleus, acrosomal and postacrosomal regions, and flagellum) were evaluated by WHO criteria [21]. All patients repeated the spermiogram after 3 months.

2.5 Peroxidase staining

Leucocytes were counted after peroxidase staining. Briefly, 0.0375% H_2O_2 was added to 4 mL benzidine stock solution (0.0125% w/v benzidine [Sigma Aldrich, Milan, Italy, in 50% ethanol). Ten microliters of ejaculate was mixed with 20 mL fresh benzidine–H_2O_2 solution. After 5 min, 160 mL phosphate-buffered saline was added and peroxidase-positive (round brown cells) and peroxidase-negative (unstained) cells counted using a Makler chamber and phase-contrast microscope.

2.6 Acridine orange (AO) staining

Sperm chromatin DNA was assessed by AO (Sigma Chemical, St. Louis, MO, USA) fluorescence method described by Tejada *et al.* [22]. Briefly, after air-drying, the sperm smears were fixed in Carnoy's solution (methanol-glacial acetic acid 3:1) overnight at 4°C. After they were air-dried, the samples were stained with AO solution for 5 min. The AO staining solution was prepared daily by adding 10 mL of 1% AO stock solution in distilled water to a mixture of 40 mL of 0.1 mol/L citric acid and 2.5 mL of 0.3 mol/L $Na_2HPO_47H_2O$, pH 2.5. Then the samples were rinsed and mounted with distilled water. The percentage of sperm with normal double-stranded DNA (green) was determined by randomly scoring 100 sperm under a fluorescence microscope (DMRB; Leica, Lodi, Italy) with × 400 magnification and excitation of 450 nm–490 nm. Red or yellow fluorescent sperm indicated denatured or single-stranded DNA.

2.7 Transmission electron microscopy (TEM)

Aliquots of 19 semen samples, before and after treatment, and 10 samples before and after 3 months without any therapy were examined by TEM. Sperm samples were fixed in Karnovsky's reagent, rinsed overnight in 0.1 mol/L cacodylate buffer (pH 7.2), postfixed in 1% buffered OsO_4, dehydrated, and embedded in Epon-Araldite (Fluka, Milan, Italy). Ultrathin sections were cut with an LKB ultramicrotome (Vienna, Austria) and stained with uranyl acetate and lead citrate. Observations of 100 sperm sections were made with a TEM CM 10 (Philips, Eindhoven, the Netherlands), at magnifications of × 5 000 to × 75 000, by two highly trained evaluators who did not know the identity of the samples.

2.8 Statistical analysis

The data were collected and analyzed using the commercial software GraphPad Prism4 (GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± SD. Standardized skewness and kurtosis values were used to determine the normal distribution of data. The Mann–Whitney *U*-test was used for comparisons. Statistical significance was set at $P < 0.05$.

3 Results

The first spermiogram obtained before therapy showed asthenoteratozoospermia and leukocytospermia; the diagnosis was confirmed by the second spermiogram carried out 1 month later. Mean sperm count, progressive motility, and normal sperm morphology were below the normal range in all patients (Table 1). Staining with peroxidase revealed a high mean leukocyte count. After the end of therapy, there was no significant change in sperm count, but morphology and motility improved, and leukocyte count significantly decreased.

Light microscopy examination of sperm showed diffuse sperm anomalies in patients before therapy. The acrosome was frequently absent or abnormally shaped and the nucleus was frequently malformed. Sperm tails were twisted, disrupted, or altered. All of these tail anomalies could explain the motility reduction. After therapy, a general improvement in sperm features was observed, namely normal-shaped nucleus, acrosome, tail structure and extension.

TEM evaluation of basal samples revealed diffuse structural defects typical of necrosis. The acrosome was absent or reacted, the chromatin was uncondensed or disrupted, and the plasma membrane was broken. The cytoskeletal structures of sperm flagellum were altered and often rolled up, and the mitochondrial helix was frequently disorganized with swollen mitochondria (Figure 1).

Table 1. Sperm parameters evaluated in treated and untreated patients before and after natural anti-oxidant therapy with beta-glucan, lactoferrin, papaya and vitamins C and E. $^{b}P < 0.05$; $^{c}P < 0.01$ (Mann-Whitney *U*-test), compared with ***. WHO, World Health Organization; —, not provided.

Sperm parameters	Treated (n = 36)		Untreated (n = 15)		WHO
	Before	After	Before	After	parameters
Sperm count (1×10^6/mL)	48.5 ± 8.9	46.2 ± 9.3	51.1 ± 6.0	49.0 ± 5.4	> 20
Progressive motility (%)	19.0 ± 7.8	34.8 ± 6.8^{c}	21.3 ± 5.1	23.8 ± 4.2	> 50%
Leukocytes (1×10^6/mL)	2.2 ± 0.9	0.9 ± 0.2^{c}	1.4 ± 0.9	1.8 ± 1.4	< 1×10^6
Normal morphology (%)	17.0 ± 5.2	29.8 ± 6.5^{c}	21.5 ± 6.2	19.1 ± 4.1	> 30%
Eosin test (% necrotic)	49.3 ± 12.1	38.7 ± 6.7^{b}	55.1 ± 9.8	57.2 ± 6.5	< 40%
Acridine orange staining (abnormal sperm %)	16.7 ± 8.0	14.4 ± 6.0	15.8 ± 6.7	16.1 ± 5.4	—

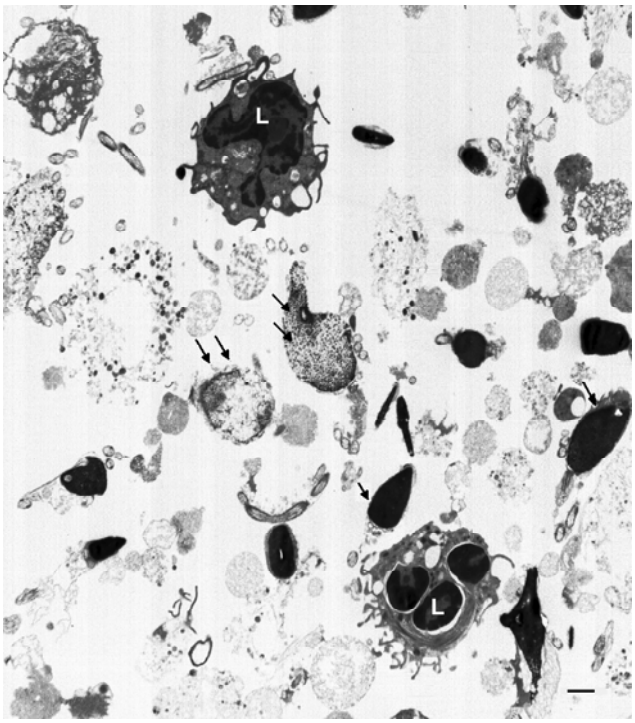

Figure 1. Transmission electron micrograph of sperm sample from a patient before treatment with beta-glucan, lactoferrin, papaya, and vitamins C and E. Leukocytes (L) are present among sperm showing structural defects typical of necrosis: absent or reacted acrosomes (arrows); misshapen nuclei with disrupted chromatin (double arrows); altered axoneme; swollen mitochondria; and broken plasma membrane. Bar = 0.59 μm.

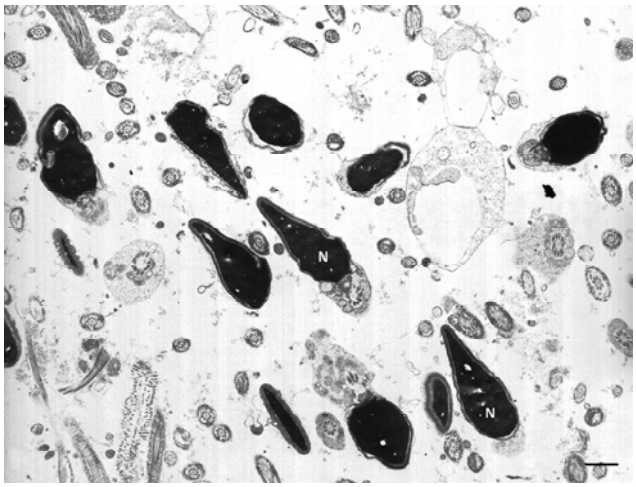

Figure 2. Transmission electron micrograph of sperm sample from a patient treated with beta-glucan, lactoferrin, papaya, and vitamins C and E, showing normal acrosome and nuclei (N), with condensed chromatin. Several tails have normal axonemes and accessory structures. Leukocytes are absent. Bar = 1.28 μm.

After therapy, ultrastructural analysis showed that sperm defects typical of necrosis were reduced compared to the previous examination (Figure 2).

After therapy, eosin Y staining also confirmed a significant reduction in sperm with broken plasma membrane in comparison to basal samples, in which more than half the sperm had broken plasma membranes (Table 1).

The AO staining for detection of sperm with damaged DNA did not reveal any significant change in the mean percentage of orange/red abnormal single-stranded DNA sperm after therapy.

Patients treated with anti-oxidants and beta-glucan did not complain of any side-effects and all reported feeling well. Seven men with a history of recurrent respiratory tract infections contracted fewer infections during treatment.

The spermiogram of 15 untreated patients (controls), carried out 3 months after the first in the absence of any type of therapy, did not show any significant variation in sperm number, motility, or morphology (Table 1). The concentration of leukocytes was almost the same in the two examinations. Eosin Y staining confirmed the presence of a higher percentage of dead sperm compared to WHO parameters. DNA damage detected by AO staining did not show any significant change after 3 months of interval without any therapy (Table 1).

4 Discussion

Asymptomatic inflammatory processes in the testicles associated with leukocytospermia might damage the seminiferous epithelium, the epididymis, or the ejaculatory ducts, altering the process of sperm formation and maturation, as shown by the presence of anomalous sperm [1–3, 23]. A possible mechanism by which leukocytospermia might alter sperm functions could be contact of sperm and ROS produced by leukocytes during co-migration from the seminiferous tubules to the epididymis. In the sperm cell, one of the main sites of lipid peroxidation by ROS is the intermediate tail segment that consists essentially of mitochondria [24]. Therefore, the reduction in sperm motility observed in our patients could also be due to altered membrane permeability with loss of intracellular adenosin triphosphate (ATP) and axonemal damage. ROS also have a negative effect on sperm DNA, leading to fragmentation [25]. It has been suggested that DNA fragmentation could be a possible cause of increased infertility in males [26]. In the present study, natural anti-oxidants and beta-glucan given to patients for 90 days led to improvement in some sperm parameters, namely motility and morphology, and reduction in the number of lymphocytes. The percentage of sperm with DNA damage was also decreased after anti-oxidant treatment, but without reaching statistical significance. These results are presumably due to the double action of the components of the product. Beta-glucan, fermented papaya, and lactoferrin modulate immune and inflammatory responses [16–19, 27], whereas vitamins C and E increase the anti-oxidant defenses of cells.

In men, the combined action of anti-oxidants and beta-glucan seems to protect sperm structure, reducing the effects of free radicals. In fact, not only enzymes, but also anti-oxidant systems, including vitamins C and E, are fundamental for defense of sperm against oxidative damage. The effects of the anti-oxidant therapy on seminal fluid have been the subject of clinical trials that have shown the individual and synergic action of vitamins C and E in protecting sperm against oxidative stress in cases of idiopathic infertility [28, 29]. In some studies, no effects on conventional semen parameters were found after vitamin C and E supplementation [30, 31]. Recently, Ménézo *et al*. [32] reported a decrease in DNA fragmentation but an unexpected increase in sperm decondensation, probably due to the ability of anti-oxidant vitamins to interfere with interchain disulfide bridges of sperm protamines. Moreover, sperm DNA damage has been reported by Greco *et al*. [31] to be efficiently treated by vitamins C and E, given orally. Seminal plasma levels of vitamin C show a positive correlation with the presence of morphologically normal sperm, probably acting at the epididymal level [14]. Fermented papaya, moreover, has strong anti-oxidant properties, eliminating free radicals and increasing superoxide dismutase activity [18, 19].

The results of the present study suggest that the tested formula can be effective in the treatment of certain spermiogenetic alterations, particularly of the nucleus and motility apparatus. Improvement in the main sperm parameters is related to restored cell function sufficient to enable natural fertilization, or at least less invasive assisted reproductive techniques. Treatment with natural supplements containing high concentrations of vitamins C and E can be considered a valid alternative to anti-inflammatory drugs in the treatment of asthenospermia with leukocytosis.

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