

The improvement of Sperm Parameters and Chromatin Quality by Vitamin C

Mangoli E¹, Pouretezari M¹, Anvari M^{1,2}, Talebi AR^{*1,2}, Nahangi H¹

1-Department of Biology & Anatomy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
2-Research & Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

*Corresponding author: Prof_talebi@hotmail.com

Abstract:Antioxidants are the main defense factors against oxidative stress (ROS) induced by free radicals. Vitamin C (vit.C) and vitamin E is believed to be the primary component of the antioxidant system of the spermatozoa and are one of the major membrane protectants against ROS and LPO attack. There is a relationship between activity of these antioxidant and function of sperm. Vitamin C which is belonging to non-enzymatic antioxidant is used as a supplemented drug to improve sperm quality in male infertility. Given the importance of sperm in reproductive and Generation health other hand and harmful effects of oxidative stress the aim of this study was to evaluate the protective effects of vitamin C on sperm parameters and chromatin quality in mice. 14 adult male mice were divided equally into two groups .mice of group 1 served as control fed on basal diet, group 2 received basal diet and vitamin C (10 mg/kg/daily, intraperitoneal) for 35 days. Finally, right tail of epididymis was cut in Ham's F10. Released sperm were used to analyze number, motility, morphology (Pap-staining) and viability (eosin-Y staining) of the sperm and DNA integrity and chromatin condensation assessments were ready by standard cytochemical techniques including: (AOT): Acridine orange, (AB): Aniline Blue, (TB): Toluidine blue and chromomycin A3 (CMA3). In vitamin C mice, a significant Increase was found in sperm number, sperm viability and sperm morphology compared to control group. Also a significant increase was found in sperm TB⁺ (normal) and Ao⁻ (double-stranded DNA) in vitamin c group compared to control group. It was concluded, vitamin C not only is able to improvement the sperm parameters but also increases sperm chromatin condensation and quality in mice.

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Introduction:

Vitamin C (vit.c) (chemical names: ascorbic acid and ascorbate) is a six-carbon lactone which is synthesized from glucose by many animals. Vitamin C is synthesized in the liver in some mammals and in the kidney in birds and reptiles. However, several species—including humans, non-human primates, Indian fruit bats, guinea pigs, and Nepalese red-vented bulbuls—are unable to synthesize vitamin C. When there is insufficient vitamin C in the diet, human suffer from the potentially lethal deficiency disease scurvy [Stewart CP 1953].

Vitamin C (ascorbic acid) has been associated with fertility and it may have evolutionary significance [Millar 1992]. It has also been shown to be important for reproduction in several other mammalian species [Luck 1995]. Lower deficient ascorbate levels in human have been associated with increased abnormal sperm morphology, low sperm counts, reduced fertility, and agglutination [Dawson 1990].

Recently, the generation of oxidants, also described as reactive oxygen species, in the male reproductive tract has become a real concern because of their potential Harmful effects, at high levels, on sperm quality and function [Sharma RK 1996]. In the

context of human reproduction, a balance normally exists between ROS production and antioxidant scavenging activities in the male reproductive tract. As a result of such balance, only minimal amounts of ROS remain, and they are needed for the regulation of normal sperm functions, such as the acrosome reaction, sperm capacitation, and sperm-oocyte fusion [Aitken RJ 1999]. The production of excessive amounts of ROS in semen can overwhelm the antioxidant defense mechanisms of sperm and seminal plasma and causes oxidative stress [Sikka SC 2001]. Production of high levels of ROS in the reproductive tract is detrimental not only to the fluidity of the sperm plasma membrane but also to the integrity of DNA in the sperm nucleus [Aitken RJ 1999]. Strong evidence suggests that DNA fragmentation commonly observed in the spermatozoa of infertile men is mediated by high levels of oxidative stress [Kodama H 1997]. The antioxidant's role of this vitamin has been reported in reducing testicular oxidative stress [Kutlubay R 2007].

Hence male gamete supplies 50 % of the embryonic genome, any anomalies in sperm DNA can affect embryonic development. It is generally accepted that there is a clear relation between sperm DNA damage and reproductive outcomes [Talebi AR 2011]

A]. Furthermore sperm chromatin condensation has an important role in male fertility, early embryonic growth and pregnancy results [Talebi AR 2006]. In the process of spermatogenesis, the extent of sperm chromatin compaction changes deeply when histones are replaced in a stride mode by testis-specific nuclear proteins, transitional proteins and finally by protamines. Each anomalies during expression of sperm-specific nucleoproteins change sperm chromatin structure and may cause male infertility [Talebi 2011 A]. The inter and intra-molecular disulphide bonds of protamine molecules are crucial for sperm nuclear compaction and stabilisation. It is believed that this kind of nuclear compaction protects sperm genome from external damages include oxidative stress, temperature height and acid-induced DNA denaturation [Carrell DT 2007].

Animals and treatments:

During the course of this experiment, we followed the recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and killing of the animals. Detailed information about animals and treatments has been reported previously [Manjanatha 2006]. Totally 14 Adult male mice (10 weeks old, 35g) that they divided to 2 group each containing 7 mice. Mice of group 1 served as control fed on basal diet, group 2 received basal diet and vitamin C (10 mg/kg/daily, intraperitoneal) [shrilatha 2007]. They were held in cages and were housed in a controlled environment with a temperature range of 25±3°C and mean relative humidity of 50±5%. The experimental proposal was agreed by our university ethics committee. After 35 days (one duration of spermatogenesis in mice is about 32 days), the mice were killed.

Materials and methods:

A small part of the cauda epididymis of each mouse was dissected and located in 1 mL of pre-warmed Hams F10 medium (37°C, 5% CO₂). Gentle tearing of the tissue was done to make spermatozoa swim out into the culture medium. The dishes were placed in the incubator for 15 min.

Sperm analysis:

Sperm count:

The dissected epididymis of each animal was transferred into 10 ml Ham's F10 medium and cut into small slices, in order to swim out the sperm into the medium. After 10 min of diffusion, 1 ml of the solution was diluted with 9 ml formaldehyde fixative. The diluted solution was transferred into each chamber of Neubauer hemocytometer and sperm heads was

manually counted under a microscope. Sperm count was performed according to WHO guidelines [WHO 1999] and data were expressed as the number of sperm per ml [Momeni H R 2009].

Sperm motility:

Assessment of sperm motility was done according to WHO protocol [WHO 1999]. In brief, 10 µl of the sperm suspension was placed on a microscopic slide and covered lipped. A minimum of five microscopic fields were assessed to evaluate sperm motility on at least 200 sperm for each animal. The percentage of sperm motility was analyzed for following motion parameters: percentage of progressively motile sperm (PMS), non-progressively motile sperm (NPMS) and non-motile sperm (NMS). [Momeni H R 2009].

Sperm viability:

For the assessment of viability we used from eosin y staining that discriminate live sperm from dead sperm by staining cytoplasm of cell. In brief, 10µl of the sperm sample was placed on a microscopic slide and 10µl of eosin 0.5 % added to it and then covered lipped and saw with light microscope x 40. Then counted at least 100 sperm for each animal and discriminate live sperm that was not stained and dead sperm that was red [cao 2011].

Sperm morphology:

For studying the sperm morphology, a drop of sperm suspension was smeared onto a clean glass slide. The smear was then air dried and fixed in a mixture of equal parts ethanol and ether. The slides were then stained with Papanicolaou stain. Dried stained slides were scanned under oil immersion (100 objectives) for morphological abnormalities. A total of 100 sperms per sample were classified according to their morphology; such as normal, coiled mid piece, hair pin (a kink at the annulus, usually 180°), bent tail (a kink at the annulus, usually 90°), coiled tail, double head, amorphous head, triangular head, pin head and cytoplasmic droplet. Sperm abnormality was expressed as percent. [ZohreZare 2010].

Sperm chromatin/DNA evaluation:

DNA integrity and chromatin condensation assessments were ready by standard cytochemical techniques including acridine orange test (AOT), aniline blue (AB), toluidine blue (TB), chromomycin A3 (CMA3). All dyes and chemicals were purchased from Sigma Aldrich Company (St Louis, MO, USA). [Talebi AR 2011 C]

Aniline blue (AB) staining:

Aniline blue selectively stains lysine-rich histones and has been used for the purpose of those sperm chromatin condensation anomalies that are related to residual histones. To do this staining, air-dried smears were set from washed semen samples and

then fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH=3.5) for 7 min. In light microscopic evaluation, 200 spermatozoa were counted in different areas of each slide using $\times 100$ eyepiece magnification [Talebi AR 2011 A].

Toluidine blue (TB) staining:

Toluidine blue is a metachromatic dye which determines both the quality and the quantity of sperm nuclear chromatin condensation/DNA fragmentation via binding to phosphate groups of DNA strands [Joao Ramalho 2009]. Briefly, air-dried sperm smears were permanent in fresh 96% ethanol-acetone (1: 1) at 4 °C for 30 min and then hydrolysed in 0.1 N HCl at 4 °C for 5 min. after that, the slides were rinsed 3 times in distilled water for 2 min and in the end stained with 0.05% TB in 50% citrate phosphate for 10 min at room temperature. In each sample, at least 200 spermatozoa were counted under light microscopy using $\times 100$ eyepiece magnifications [Talebi AR 2008].

Acridine orange test (AOT):

Acridine orange is a metachromatic fluorescence probe for demonstration of degree of sperm nuclear DNA susceptibility to in-situ acid-induced denaturation by distinction between native double-stranded DNA (green fluorescent) and denatured single-stranded DNA (red fluorescent). Briefly, the air-dried smears were fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4°C for at least 2 hrs. Each sample was stained by freshly prepared AO (0.19 mg/ml in McIlvain phosphate-citrate buffer (pH=4) for 10 min. Smears were assessed on the same day using fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter [Talebi AR 2011 A].

Chromomycin A3 staining:

Chromomycin A3 is fluorochrome specific for guanosine cytosine-rich sequences and is used for estimate of the degree of protamination of sperm chromatin (Carrell DT 2007). For this purpose, the smears were dried first and then fixed in Carnoy's solution at 4°C for 10 min. The slide was treated with 150 μ l of CMA3 (0.25 mg/ml) in McIlvain buffer for 20 min. After staining in darkroom, the slides were washed in buffer and mounted with buffered glycerol. In each sample, at least 200 spermatozoa were counted under fluorescent microscope with a 460-nm filter and $\times 100$ eyepiece magnifications [Lolis D 1996].

It should be noted that in AB staining, the percentages of unstained or pale blue stained (normal spermatozoa) and dark blue stained (abnormal spermatozoa) were reported.

In TB staining, the chromatin quality of sperm was assessed according to metachromatic

staining of sperm heads in following scores: 0, light blue (good chromatin); 1, dark blue (mild abnormal chromatin); 2, violet; and 3, purple (severe chromatin abnormality). So, the sum of spermatozoa with score 1, score 2 and score 3 was considered as TB⁺ or sperm cells with abnormal chromatin, whereas score 0 spermatozoa were considered as TB⁻ or spermatozoa with normal chromatin.

For AOT, the percentages of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated.

In CMA3 staining bright yellow-stained chromomycin-reacted spermatozoa (CMA3+) were considered as abnormal form and yellowish green-stained non-reacted spermatozoa (CMA3-) were considered as normal form.

Statistical analysis:

Statistical analysis was performed by spss 18 for Windows (SPSS Inc., Chicago, IL, USA). Student's t-test was applied to evaluate the data and the term 'statistically significant' was used to signify a two-sided *P value* < 0.05 for sperm parameters and cytochemical tests.

Results and Discussion:

Table 1 shows the means and statistical analysis of the various sperm parameters in two groups. This table reveals that sperm count, rapid and total motilities, morphology and viability with significant (*P*<0.05) between Groups A (vitamin C) and B (control).

Table 2 shows the results of analysis of sperm chromatin and DNA integrity. Regarding to TB, AO, CMA3 and AB tests, with significant (*P*<0.05) between Groups A (vitamin C) and B (control).

Table 1: The results of semen analysis in vitamin C (Group A) and control mice (group B).

Variables	Vitamin C mice (group A)	Control mice (group B)	P-value
	Mean \pm SD	Mean \pm SD	
Count($\times 10^6$)			
Rapid motility(Grade a)	138 \pm 32.393	100 \pm 12.5	
Slow motility(Grade b)	19.857 \pm 4.375	21.142 \pm 3.0237	.013
Non progressive motility(Grade c)	22 \pm 3.829	23.142 \pm 5.241	.534
Immotile sperm(Grade d)	29.428 \pm 4.825	31.714 \pm 4.644	.65
Total motility(Grade a,b,c)	30.142 \pm 3.76	4.644	.018
Normal morphology Viability (%)	71.285 \pm 5.964	76 \pm 4.618	.124
	81 \pm 4.163	76 \pm 3.696	.035
	77 \pm 5.686	4.634	.015

Statistically significant (T-Test), *P value* <0.05.

Table 2: The results of sperm chromatin/ DNA evaluation in vitamin C (group A) and control mice (group B).

Variables	Vitamin C mice (group A)	Control mice (group B)	p-value
	Mean±SD	Mean±SD	
AB	12.714± 1.704	13.142 ±1.345	.611
TB	12.428 ±2.699	20.857± 2.34	0
CMA3	1.428 ±1.133	1.857 ±1.345	.531
AO	4.228± 1.718	7.428± 2.249	.014

Statistically significant (T-Test), P value <0.05.

ROS can cause alterations in sperm plasma membrane and reduction in motility and fertilizing ability of spermatozoa [Raina 2002]. Because ROS and their derivatives are reactive, they can damage various biomolecules including lipids, proteins and DNA [Lee Ho 1996]. Many studies are also being conducted on vitamin C and its roles in spermatozoa quality. The effect of vitamin C in reducing the level of oxo8dG in DNA supports the hypothesis that adequate antioxidant protection is essential to maintain the genetic integrity of sperm cells and to minimize the risks of mutations in germ cells that may lead to birth defects, genetic disease. Sperm adduct analysis is a non invasive means to evaluate the genetic toxicity of endogenous and exogenous compounds, and in this regard the measurement of the oxo8dG level in sperm DNA may help in understanding optimum antioxidant intakes [CESAR G 1991]. The experiments conducted by Thiele and Colleagues indicate that the infertile men had lower levels of ascorbate and elevated levels of ROS with respect to the controls. They also established correlations and showed that low levels of vitamin C were correlated with high percentages of sperm with abnormal motility and morphology. Our results also raise the sperm count and normal morphology sperm in the vitamin group than the control group. Low levels of vitamin C were also correlated with higher levels of ROS [Thiele, J.J 1995]. Treatment with vit.c, Vit. E and their combination increased lipido (reaction time), ejaculate volume, total sperm output, sperm concentration, sperm motility index, total motile sperm, packed sperm volume, initial hydrogen ion concentration (pH), and semen initial fructose concentration. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were decreased, whereas glutathione S-transferase (GST) showed a significant increase in seminal plasma of treated animals compared with the controls. The results from this study indicated that supplementation of drinking water with antioxidant ascorbic acid, Vit.E and their combination reduced the production of free radicals and can improve rabbit semen quality, [Yousef MI 2003]. Reduced sperm

count and poor sperm quality can also be responsible for male factor infertility. In fact most cases of infertility are due to abnormal sperm count or low sperm motility. Our results also raise the sperm count sperm in the vitamin C group than the control group and this is due to the effect of vitamin C on antioxidant enzymes oxidant effects could improve. But, unlike in study Armit and colleagues studied the Antioxidants reduced the LPO, and improved sperm motility and viability in vitro under induced oxidative stress. [Amrit KB 2008], in our study no change was found in movement in the vitamin C group compared to the control group.

Sperm DNA damage is significantly increased in men with idiopathic and male factor infertility and in men who failed to initiate a pregnancy after assisted reproductive techniques. Such an increase may be related to high levels of seminal oxidative stress. [Saleh RA 2003]. Damage to the DNA of germ cells can lead to mutation, which may result in birth defects and genetic diseases. The very high endogenous rate of oxidative DNA damage and the importance of dietary vitamin C in preventing this damage have prompted an edition of these factors in human sperm DNA [CESAR G 1991].

There is evidence to show that infertile men possess substantially more sperm DNA damage than do fertile men and that this chromatin damage may adversely affect reproductive outcomes [Zini A 2008]. Studies of infertile men with high levels of sperm DNA damage have shown that antioxidant therapy is effective in improving sperm DNA integrity or pregnancy rates. In men with unselected infertility, the effect of dietary antioxidants on sperm DNA integrity is equivocal with one of two controlled trials showing a benefit of antioxidants on sperm DNA integrity.

The levels of sperm-derived ROS (measured in sperm preparation shaving minimal leukocyte contamination) have been associated with sperm DNA damage, although no ROS threes hold level above which sperm DNA damage is detected has been established [Saleh RA 2003]. Moreover, the levels of sperm DNA oxidation are higher in infertile compared to fertile men [Shen HM 1999]. Cao showed that increased oxidative stress, enzymatic and non-enzymatic antioxidant is reduced levels in leydig cells and an important factor for impaired spermatogenesis and consequently a significant reduction in epididymal sperm count [Cao L 2004]

Previous studies have shown that antioxidant treatment such as vitamin E improves sperm nuclear DNA integrity in mice with elevated sperm DNA damage [pourentezari M 2012]. However, reports concerning the clinical usefulness of antioxidants in the treatment of male infertility are controversial [Agarwal 2004].

This is especially study in which the possible effect of this treatment by Aniline blue (AB) staining, Toluidine blue (TB) staining, Acridine orange test (AOT) and Chromomycin A3 staining.

We saw the significant difference in percentage of TB-reacted spermatozoa between two groups; this showed that Vitamin C can improve of nuclear chromatin condensation. Hammadeh showed that DNA damage is induced by oxidative assaults but the physiological antioxidant situation in the seminal plasma does not seem to be strongly associated with either a reduction in the DNA damage or an increase in the decondensation (Hammadeh 2006).

In AO test, we saw notable difference between two groups. As the AO test has the potential to differentiate the single-stranded DNA from double-strand ones, it can be concluded that the vitamin C has decreased the denaturation of sperm DNA strands. It should be considered that this finding was obtained by other researcher but using different assessment. According to our results, Ciara M.Hughes and colleagues showed DNA damage was induced by 30 Gy X-irradiation. DNA strand breakage was measured using the comet assay. Sperm DNA was protected from DNA damage by ascorbic acid (600 mM), alpha tocopherol (30 and 60 mM) and urate (400 mM). These antioxidants provided protection from subsequent DNA damage by X-ray irradiation. In contrast, acetyl cysteine or ascorbate and alpha tocopherol together induced further DNA damage. Supplementation in vitro with the antioxidants ascorbate, urate and alpha tocopherol separately has beneficial effects for sperm DNA integrity [Ciara M.Hughes 1998].

In AB staining that shows the sperm cells with excessive histones, we did not found a significant difference between groups. We can say that the vitamin C doesn't have improvement effects on histone-protamines replacement during the testicular phase of sperm chromatin packaging. To compare our data with others, we didn't see any similar study by this test in literature.

In CMA3 staining, although we saw the difference in percentage of CMA3-reacted spermatozoa between two groups, but it was not statistically significant. As we noted before, the CMA3 test shows the protamine deficiency in the process of sperm chromatin condensation

It is indicated that the level of oxidative stress is high in hyperglycemia state [Mahesh T 2004], due to excess production of reactive oxygen species (ROS) and decreased efficiency of anti-oxidant enzyme defences [Steger RW 1997]. Oxidative stress is harmful to sperm function and a significant factor in the etiology of male infertility [Kartikaya Makker

2009]. In addition, oxidative stress impairs male fertility by changing the cell function like sperm motility [Kartikaya Makker 2009, Agarwal 2005], increase in DNA damage by induction of gene mutations, DNA denaturation, base pair oxidation and DNA fragmentation [Agarwal 2005].

In conclusion, our study showed that in the cases of vitamin c, almost Majority of the sperm parameters had a statistically significant Increase in comparison with controls and also we demonstrated that spermatozoa of vitamin C mice had more chromatin condensation and Increase DNA integrity than spermatozoa of control group. this study in compare with recent study around effect of vitamin E on sperm showed that vitamin E was the most effective than vitamin C on sperm parameters but it is controversial and needs to the more study.

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