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Vitamin C attenuates detrimental effects of diabetes mellitus on sperm parameters, chromatin quality and rate of apoptosis in mice

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ABSTRACT

Objectives: The main goal was to examine the protective effects of vitamin C on sperm parameters, sperm chromatin condensation and apoptosis in experimentally-induced diabetic mice.
Study design: 28 adult Syrian mice were divided into 4 groups. In Group1, the mice were diabetic that received a single dose of Streptozocin (STZ) (200 mg/kg) intra-peritoneally (ip). Group 2 was included diabetic mice that received vitamin C (10 mg/kg/daily, ip). Mice in group 3 were received vitamin C and group 4 was considered as control. After 35 days, sperm analysis was done accordingly. To asses sperm chromatin and DNA quality, we used aniline blue (AB), toluidine blue (TB), chromomycin A3 (CMA3), acridine orange (AO) and terminal transferase mediated deoxyuridine triphosphate biotin end labeling (TUNEL) tests.
Results: All of the sperm parameters (count, motility, morphology and viability) had significant reduction in diabetic mice but, the data showed a significant increase in all of the sperm parameters in diabetic + vitamin C when compared with diabetic and control animals ($P < 0.05$). There were significant differences ($P < 0.001$) between groups regarding to TB staining (48.8 ± 5.92 vs 34.3 ± 4.13), AO test (35.9 ± 6.11 vs 20.8 ± 2.89) and TUNEL test (39.42 ± 7.18 vs 22.00 ± 3.65) in diabetic and diabetic + vitamin C groups, respectively. Nevertheless, in CMA3 and AB staining assays, there were not any significant differences between different groups.
Conclusion: Vitamin C, as a potent antioxidant, can attenuate detrimental effects of diabetes mellitus on the sperm parameters, chromatin quality and apoptosis in an experimental model.

Introduction

Diabetes mellitus (DM) is one of the most important diseases that threatens health of more than 171 million people in the world [1]. DM may affect male reproductive function at multiple levels including endocrine control of spermatogenesis [2]. Recently, it has been reported that spermatozoa of diabetic mice had less chromatin condensation and low DNA integrity compared to control group [3]. Shalaby and Hamowieh, showed that DMcan disrupt spermatogenesis and may have detrimental impacts on sperm parameters [4].

Vitamin C or ascorbic acid, is one of the main antioxidant factors in biological systems [5]. It was shown that intake of antioxidants such as vitamin C and E can reinforce the stability of testicular blood barrier and protect sperm DNA from oxidative stress [6]. In the male reproductive system, vitamin C is a well-known antioxidant for protection of spermatogenesis and it has a critical role in improvement of semen quality and sperm fertility potential both in human [7] and animals [8]. Shrilatha and Muralidhara reported the protective effect of vitamin C on testicular oxidative stress and genotoxic effects using a diabetic mice model [8]. There is a clear negative relationship between sperm chromatin/DNA damage and reproductive outcomes [9]. Furthermore, sperm chromatin condensation has a key role in male fertility, early embryonic growth and pregnancy outcomes [9].

There are many kinds of tests for sperm chromatin/DNA evaluation which show different forms of damages. Chromatin structural probes by nuclear dyes with cytochemical bases are

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sensitive, easy and inexpensive which do not require unique device like flow cytometry [9].

Our previous work showed that DM has detrimental effects on sperm parameters and chromatin quality in mice [3]. This study was designed to evaluate the effects of vitamin C administration on sperm chromatin condensation and apoptosis following DM in male mice.

Materials and methods

Animals and care

28 Adult male Syrian mice (10 weeks old, 35 g) were divided into 4 groups. Group 1 was included diabetic mice that received a single dose Streptozocin (STZ) (200 mg/kg) intra-peritoneally (ip). Group 2 consisted diabetic mice that received vitamin C (10 mg/kg/daily, ip). Mice in group 3 received vitamin C (10 mg/kg/daily, ip) and group 4 serving as the control group without any treatment. The mice were held in separate cages about 35 days (about one duration of spermatogenesis) and were housed in a controlled environment with a temperature ranged 25 ± 2 °C and mean relative humidity of 50 ± 5%. They were fed "mice chow" and had access to water ad libitum. The experimental proposal was approved by our university ethics committee.

Streptozotocin treatment and induction of diabetes

Diabetes was induced via a single i.p. injection of buffered solution (0.1 mol/l of citrate, pH 4.5) of STZ (200 mg/kg). Diabetes criterion was hyperglycemia (blood glucose >250 mg/dl) (range 250–700) measured 72 h post injection [3,8].

Epididymal sperm preparation

Animals were anesthetized by ketamine and xylazine (150 mg/kg and 10 mg/kg, respectively) and their dissected cauda epididymis were placed in 1 ml of pre-warmed Ham's F10 medium for 30 min. Gentle tearing was done to swim-out spermatozoa into culture medium [3].

Sperm analysis

Sperm parameters including count (10^6/ml), motility, viability and normal morphology (%) were evaluated for 200 spermatozoa of each animal. Sperm count and motility was assessed using Makler chamber. Motility was expressed as percentage of progressive (rapid and slow) and non-progressive. Sperm viability and morphology were evaluated by Eosin and Papanicolaou staining tests, respectively [3,10].

Sperm chromatin/DNA integrity assessments

All of the dyes and chemicals were purchased from Sigma Aldrich Company (St Louis, MO, USA). The effectiveness of dyes was tested with and without acid denaturation of some sperm specimens and they were considered as positive and negative controls, respectively [10].

Aniline blue (AB) staining

AB selectively stains lysine-rich histones and has been used for anomalies that are related to residual histones [9,11]. Briefly, air-dried smears from washed semen samples were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature (RT). 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 each slide using 100× eyepiece magnification. Unstained or pale blue stained (normal spermatozoa or AB^-) and dark blue stained (abnormal spermatozoa, AB^+) cells were reported as percentages [3].

Toluidine blue (TB) staining

TB is a metachromatic dye which determines both quality and quantity of sperm nuclear chromatin condensation via binding to phosphate groups of DNA strands. Air-dried sperm smears were fixed in fresh 96% ethanol and acetone (1:1) at 4 °C for 30 min and then incubated in 0.1 N hydrochloric acid (4 °C, 5 min). Then, the slides were washed 3 times with distilled water for 2 min and finally stained with 0.05% TB in 50% citrate phosphate for 10 min [3]. In each sample, at least 200 spermatozoa were counted under light microscopy using 100× eyepiece magnification according to metachromatic staining of sperm heads in following scores: 0: light blue (good chromatin); (1) dark blue (mild abnormal chromatin); (2) violet and purple (sever chromatin abnormality) [9]. The spermatozoa with score 0 were considered as normal cells (TB^-) and spermatozoa with violet and purple heads (scores 1 and 2) were considered as abnormal cells (TB^+).

Acridine orange test (AOT)

AO is a metachromatic fluorescence staining that is used to determine the rate of DNA denaturation [12]. Briefly, the air-dried smears were fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4 °C for at least 2 h. 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 [3,13]. At least 200 spermatozoa were evaluated and the rate of normal (green fluorescent) and abnormal cells (red fluorescent) were reported as percentage.

Chromomycin A3 staining

CMA3 is fluorochrome specific for guanosine cytosine-rich sequences and is used to estimate of the degree of protamination of sperm chromatin [10]. Briefly, the smears were dried and fixed in Carnoy's solution at 4 °C for 10 min. The slides were treated with 150 μl 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 [3]. In each sample, at least 200 spermatozoa were counted under fluorescent microscope with a 460-nm filter and 100× eyepiece magnification and the percentage of $CMA3^+$ spermatozoa was reported. Bright yellow-stained chromomycin-reacted spermatozoa (CMA3+) were considered as abnormal and yellowish green-stained no reacted spermatozoa (CMA3−) were considered as sperm with normal protamination [3,11].

Evaluation of sperm apoptosis by TUNEL assay

In terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) test, the ends of fragmented DNA, either single or double-stranded, are tagged to labeled nucleotides (dUTP) in a reaction catalyzed by the enzyme TdT [14]. The smears were fixed in methanol (RT, 30 min) and then the slides were washed in phosphate-buffered saline (PBS, pH 7.4). The permeabilization was done by 0.1% Triton X-100 (Merck, Germany) and 0.1% sodium citrate for 2 min on ice. After washing with PBS, 30 μl of TUNEL mixture (Roche, USA) was added to each sample and incubated for 60 min at 37 °C in a humid chamber in the darkness. Then, the slides were washed three times with PBS and analyzed with fluorescence microscope under 100× magnification [15]. The nuclei of sperm cells with fragmented DNA ($TUNEL^+$) showed bright green color, whereas

the nuclei of the normal cells (TUNEL$^-$) were seen pale green. At least 200 spermatozoa were assessed and the rates of TUNEL$^+$ spermatozoa were reported as percentage.

Statistical analysis

The data were shown as mean ± SD. One way ANOVA test was used to evaluate the significant differences between 4 groups and the Tukey post-test was operated for determination of differences between each two groups. The term 'statistically significant' was used to signify a two-sided *P*-value <0.05 for sperm parameters and *P*-value <0.001 for cytochemical tests.

Results

Table 1 shows the sperm parameters in four groups. This table reveals that all of the sperm parameters except slow and non-progressive motility (grades b and c) had significant differences ($P < 0.05$) between four groups. The data showed that DM can impair sperm parameters and Vitamin C can reverse detrimental effects of DM on sperm parameters.

Table 2 shows the results of analysis of sperm chromatin condensation and DNA integrity status using different assays. There were statistically significant differences ($P < 0.001$) between groups with regard to TB, AO and TUNEL tests. TB staining showed that the rates of TB+ were 48.8 ± 5.92 and 34.3 ± 4.13 in diabetic and diabetic + vitamin C groups, respectively. AO and TUNEL staining methods showed that the rates of abnormal spermatozoa were 35.9 ± 6.11 vs 20.8 ± 2.89 and 39.42 ± 7.18 vs22.00 ± 3.65 in diabetic and diabetic + vitamin C groups, respectively. But, in CMA3 and AB staining there were not any significant differences between different groups.

Comments

The findings of present study showed that almost all of the sperm parameters had significant decrease following DM and vitamin C can improve them. Although, there is no similar study on

Table 1
The results of semen analysis in different groups.

Variables	Diabetic (1) $n=7$ Mean ± SD	Diabetic + vitamin C (2) $n=7$ Mean ± SD	Vitamin C (3) $n=7$ Mean ± SD	Control (4) $n=7$ Mean ± SD	*P*-value
Sperm count ($\times 10^6$)	22.6 ± 7.33	73.5 ± 22.5	143.7 ± 32.12	115.2 ± 33.03	0.037[a] 0.000[b,c,d,e,f,g]
Rapid motility (%) (grade a)	4.10 ± 1.79	12.6 ± 3.92	19.5 ± 3.92	21.9 ± 4.43	0.001[a,d,e] 0.000[b,c,f] 0.910[g]
Slow motility(%)(grade b)	16.00 ± 5.75	22.90 ± 2.55	22.20 ± 3.93	23.5 ± 4.32	0.113[a,c] 0.269[b,f] 0.960[d,e,g]
Non progressive motility (%) (grade c)	24.20 ± 3.64	26.8 ± 4.21	29.1 ± 4.14	30.2 ± 5.90	0.651[a,d,e,g] 0.081[b,f] 0.009[c]
Immotile sperm (%) (grade d)	55.7 ± 4.42	37.7 ± 3.43	30.2 ± 5.22	24.3 ± 3.94	0.000[a,b,c,e,f] 0.038[d,g]
Total motility (%) (grade a–c)	44.3 ± 4.42	62.3 ± 3.43	70.8 ± 6.49	75.7 ± 3.94	0.000[a,b,c,d,e,f] 0.293[g]
Normal morphology (%)	53.00 ± 5.85	69.3 ± 3.88	89.9 ± 2.92	83.2 ± 6.10	0.962[a,g] 0.000[b,c,d,e,f]
Viability (%)	47.8 ± 5.92	64.9 ± 4.17	72.5 ± 6.34	77.6 ± 4.67	0.000[a,b,c,d,e,f,g]

[a] Difference between diabetic and diabetic + vitamin C (DV) group.
[b] Difference between diabetic and vitamin C group.
[c] Difference between diabetic and control group.
[d] Difference between DV and vitamin C group.
[e] Difference between DV and control group.
[f] Difference between vitamin C and diabetes group.
[g] Difference between vitamin C and control group.

Table 2
The results of sperm chromatin/DNA evaluation in different groups.

Variables	Diabetic $n=7$ Mean ± SD	Diabetic + vitamin C (2) $n=7$ Mean ± SD	Vitamin C (3) $n=7$ Mean ± SD	Control (4) $n=7$ Mean ± SD	*P*-value
Aniline blue (AB) (%)	13.71 ± 2.05	12.85 ± 1.67	12.71 ± 1.7	13.14 ± 1.34	0.765[a,b,f] 0.969[c,d,e,g]
Toluidine blue (TB) (%)	48.8 ± 5.92	34.3 ± 4.13	12.8 ± 2.48	21.1 ± 2.72	0.000[a,b,c,d,e,f,g]
Acridine orange (AO) (%)	35.9 ± 6.11	20.8 ± 2.89	4.8 ± 1.81	7.00 ± 2.66	0.000[a,b,c,d,e,f,g]
Chromomycin A3 (CM A3) (%)	3.14 ± 1.77	2.85 ± 1.67	1.42 ± 1.13	1.85 ± 1.34	0.964[a,g] 0.318[c,d,e] 0.172[b,f]
TUNEL (%)	39.42 ± 7.18	22.00 ± 3.65	13.71 ± 2.13	12.85 ± 2.79	0.000[a,b,c,d,e,f]

[a] Difference between diabetic and diabetic + vitamin C (DV) group.
[b] Difference between diabetic and vitamin C group.
[c] Difference between diabetic and control group.
[d] Difference between DV and vitamin C group.
[e] Difference between DV and control group.
[f] Difference between vitamin C and diabetes group.
[g] Difference between vitamin C and control group.

using vitamin C in diabetic mice, but, Shalaby and Hamowieh [4] showed that oral administration of *Zingiber officinale* as an antioxidant to diabetic rats can induce a significant increase in sperm progressive motility, sperm count and viability as well as decrease in percentage of sperm morphological abnormalities. La Vignera et al. in a brief review stated that clinical and experimental DM changes sperm parameters in both humans and animals. They suggested that involved mechanisms in the beginning of these alterations are hormonal changes and enhanced oxidative stresses in them [16]. In line with our results, Arikawe et al. also showed that in Alloxan-induced diabetic rats, all of the sperm parameters were significantly lower than control values [17].

Our data showed that vitamin C can improve sperm chromatin condensation in epididymal phase of sperm chromatin compaction and also reduce DNA fragmentations and apoptosis in diabetic mice. But regarding to AB and CMA3 tests, we found that vitamin C did not have any beneficial effects on histone–protamine replacement in testicular phase of sperm chromatin compaction.

To the best of our knowledge, this is the first investigation on the relationship between sperm chromatin condensation and administration of the antioxidants in DM. Another unique trait of present study is use of cytochemically-based dyes that distinguish almost all of the problems that may be produced in process of testicular and epididymal phases of sperm chromatin remodeling.

Regarding to TB staining, we found a significant difference among groups. This showed that DM may cause changes in both the quality and the quantity of nuclear chromatin condensation and can increase sperm DNA fragmentation and vitamin C can have protective effects on it. To compare our data with others, we did not find any similar study in the literature. In AO test, the results showed that the rate of abnormal sperm cells would be increased following DM. As the AO test can differentiate the single-stranded DNA from double-strand ones, it can be concluded that the DM may increase the denaturation of sperm DNA strands and vitamin C, as a potent antioxidant, may decrease sperm DNA damage induced by DM via reducing production of ROS.

In CMA3 and AB staining methods, we did not find significant differences between groups. So, it seems that DM does not have any detrimental effects on histone–protamines replacement during the testicular phase of sperm chromatin packaging as it was previously shown [3].

Regard to AOT, it should be noted that the flow cytometry as a good instrument can detect the stained spermatozoa with more accuracy; but, this method in form of sperm chromatin structural assay (SCSA) is so expensive and it is unusual in clinical examinations. On the other hand, the chromatin structural probes using nuclear dyes with cytochemical bases are simple, sensitive and inexpensive. However, to decrease the false positive sperm in AOT, we used flat glassy slides to reduce the effect of dye's microlake and also, in counting method, all of the sperm samples were checked twice by two expert technician in a blind manner and inter observer variations were not significant. There are some controversies in usage of vitamins in order to decrease sperm chromatin abnormalities. Silver et al. showed that high intake of vitamin E, and C did not have any beneficial effect on sperm chromatin condensation in men with fertility problem [18]; that was against to our results. This difference may be due to different mechanisms of infertility in patients of two studies. In verification of our results, Greco et al., demonstrated that sperm DNA damage assessed by TUNEL can be well treated with oral antioxidants during a relatively short time period [7].

Oxidative stress impairs male fertility by changing the cell function like sperm motility and increase in DNA damage [19–21]. As it was mentioned earlier, DM as a metabolic disease may lead to induce oxidative stress and ROS production could be detrimental for male reproduction system, sperm function and DNA integrity status [22,23].

Hence, in this study, we did not measure the amount of ROS production following DM, the results may be changed with different amount of ROS in different species as well as human; so, we suggest additional study on ROS and DM.

In conclusion, Our study, for the first time, showed that although the DM may have detrimental effects on sperm fertility potential and DNA integrity, but, consumption of vitamin C, as a potent antioxidant, has beneficial effects on sperm parameters, sperm function and also prevents sperm chromatin abnormalities and apoptosis in experimentally-induced diabetic mice.

Condensation

At this study we want to find the protective effect of vitamin C on sperm parameters, chromatin quality and apoptosis in diabetic mice.

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