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Human sperm DNA oxidation, motility and viability in the presence of L-carnitine during *in vitro* incubation and centrifugation

S. Banihani , R. Sharma , M. Bayachou , E. Sabanegh & A. Agarwal

Keywords
Antioxidants DNA oxidation human sperm L carnitine motility viability

Summary

In vitro incubation and centrifugation is known to decrease human sperm quality. In the human body, besides its antioxidant effects, L carnitine (LC) facilitates the transport of activated fatty acids from the cytosol to the mitochondrial matrix. In this study, we investigated the effect of LC on human sperm motility, viability and DNA oxidation after incubation and centrifugation, following the sperm preparation protocols of assisted reproduction. Normozoospermic semen samples ($n = 55$) were analysed according to the World Health Organization (WHO) guidelines. LC concentrations that are not toxic to spermatozoa as determined by sperm motility and viability were standardised after 2 and 4 h of incubation at 37 °C. Semen samples to which the optimal LC concentrations were added were also centrifuged for 20 min at 300 ***g*** and analysed for sperm motility, viability and DNA oxidation. Sperm motility was improved at 0.5 mg ml ^{1} LC after incubation and centrifugation with 5×10^{6} sperm ml ^{1}. Higher concentration of LC (50 mg ml ^{1}) significantly decreased sperm motility and viability. LC did not alter the baseline of sperm DNA oxidation during both incubation and centrifugation. In conclusion, LC may enhance sperm motility following incubation and centrifugation, while it might not affect sperm viability and DNA oxidation.

Introduction

Infertility affects one in seven couples who are trying to conceive (Lamb & Lipshultz, 2000; Brugh & Lipshultz, 2004). Decreased semen quality, a measure of the ability of semen to achieve fertilisation, is a primary cause of male infertility. Usually, poor semen quality is characterised by low sperm motility and viability. Assisted reproductive techniques (ART) such as intra uterine insemination and *in vitro* fertilisation with or without intracytoplasmic sperm injection are the most successful therapeutic means for male factor infertility.

Sperm preparation protocols used in ART involve sperm incubation and centrifugation. It was reported that sperm motility and viability decrease over time after ejaculation (Singer *et al.*, 1980). Further, a rapid decline in motility was noted after incubating periods lasting more than 4 h at 37 °C (Jeulin *et al.*, 1982). A recent report suggested that different centrifugation protocols adversely affect sperm recovery (Matas *et al.*, 2007). Increased centrifugation speeds (>500 ***g***) significantly increases the number of dead spermatozoa (Makler & Jakobi, 1981). Centrifugation has also been shown to increase the reactive oxygen species (ROS) formation in semen, which may affect sperm survival (Shekarriz *et al.*, 1995).

L Carnitine (LC) is a naturally occurring molecule, derived from the amino acid lysine. In cellular systems, LC serves as a facilitator for the transport of activated fatty acids into the mitochondrial matrix, so that they can be broken down through β oxidation to produce ATP (Steiber *et al.*, 2004). Very low concentrations of LC have been reported in azoospermic men compared with

normospermic control (Lewin *et al.*, 1981). Further, lower concentrations of LC were noted in oligoasthenozoospermic men, and LC supplementation was shown to improve semen quality in these people (Ng *et al.*, 2004). A number of studies have examined the role of LC in reducing the cellular oxidative stress (Vanella *et al.*, 2000; Apak *et al.*, 2004; Silva Adaya *et al.*, 2008). Studies reported an association between lower levels of LC in semen and male infertility (Matalliotakis *et al.*, 2000; Li *et al.*, 2007). Dietary supplementation with LC has been reported to improve sperm quality (Kozink *et al.*, 2004; Yeste *et al.*, 2009). Further, a positive correlation between free LC in human semen and sperm count and motility has been reported (Menchini Fabris *et al.*, 1984). In addition, it was suggested that LC might be regarded as an index of androgenisation (Abbaticchio *et al.*, 1985). LC showed a protective effect against DNA damage in lymphocytes and brain cerebral cortex of aged rats (Haripriya *et al.*, 2005; Thangasamy *et al.*, 2009). Furthermore, LC was reported to decrease actinomycin D, hydrogen peroxide and TNF alpha induced DNA damage and improve the *in vitro* blastocyst development rate in mouse embryos (Abdelrazik *et al.*, 2009).

The main goal of this study was first to standardise the LC doses that are not toxic to spermatozoa and examine LC effectiveness in improving sperm motility and viability, and decreasing sperm DNA oxidation induced by *in vitro* incubation and centrifugation.

Materials and methods

Sample collection

This study was approved by the institutional review board of Cleveland Clinic Hospital. Fifty five normozoospermic men were selected and analysed on the basis of normal semen analysis according to the World Health Organization (1999) guidelines and used to study the effectiveness of LC on sperm motility, viability and sperm DNA oxidation during incubation and centrifugation. Samples were collected by masturbation following of 48 72 h of sexual abstinence. Following liquefaction at 37 °C for 20 min, semen specimens were evaluated for volume, sperm concentration, total cell count, motility and morphology. A 5 μl aliquot of the sample was used for evaluation of concentration and motility using a microcell slide chamber (Conception Technologies, San Diego, CA, USA).

Effect of various LC concentrations on sperm motility and viability

Sixteen semen samples were examined. Each sample was divided into six equal aliquots; each contained 5×10^6 sperm mL ^{1}. Samples were supplemented with various concentrations of LC dissolved in phosphate buffered saline (PBS), pH 7.4. LC concentrations tested were 0.1, 0.5, 1.0, 10 and 50 mg ml ^{1}. The control contained PBS in place of LC. Sperm motility and viability were assessed after 2 and 4 h incubation at 37 °C.

Measurement of sperm viability

Sperm viability was measured by eosin nigrosin staining. To one drop of liquefied semen placed on a Boerner slide, two drops of 1% aqueous eosin were added and mixed with a wooden stirrer for 15 s. Next, three drops of 10% aqueous nigrosin were added and mixed well. A thin smear was made after pipetting 10 μl onto a slide and allowed to air dry. Slides were mounted with a coverslip using Accu mount media. Two hundred spermatozoa on each slide were counted in duplicate using an ×100 objective and the percentage of viable (unstained) spermatozoa and nonviable (stained) were calculated.

Effect of LC on sperm motility and viability during centrifugation

Semen samples ($n = 19$) were divided into two equal aliquots; each aliquot (5×10^6 sperm) was supplemented with 0.5 mg ml ^{1} of LC dissolved in human tubal fluid (HTF); control was supplemented with HTF in the place of LC. Sperm motility and viability were assessed directly after centrifugation at 300 ***g*** for 20 min.

Effect of LC on sperm motility and viability for various sperm concentrations during sperm incubation

Semen samples from 12 donors were used to examine the optimal sperm concentration for studying the effects of LC. Sperm concentrations used were 5, 10, 20 and 40×10^6 ml ^{1}. Each aliquot was supplemented with 0.5 mg ml ^{1} LC dissolved in PBS. Control contained PBS in place of LC. Sperm motility and viability were measured immediately (time 0) and after 2 h incubation at 37 °C.

Effect of LC on sperm DNA oxidation

Oxidative damage to DNA was evaluated using the flow cytometric OxiDNA assay kit (Calbiochem, San Diego, CA, USA). The assay is based on utilising a direct fluorescent protein binding method targeting oxidised sites in DNA. Briefly, sperm samples were treated with LC concentration at 0.5 and 50 mg ml ^{1}, incubated for 2 h at 37 °C, washed twice in PBS, resuspended in 1% paraformaldehyde at a concentration of 2

4×10^6 cell ml ^{1} and placed on ice for 15 30 min. Spermatozoa were again washed and resuspended in 70% ice cold ethanol by centrifugation at 300 *g* for 5 min as per the kit instructions. The ethanol supernatant was removed, and the sperm pellets were washed twice in wash buffer and resuspended in 100 μl of the staining solution for 1 h at room temperature in dark. The staining solution contained fluorescein isothiocynate (FITC) labelled protein conjugate and distilled water. All cells were further washed using rinse buffer, resuspended in 250 μl and incubated for 30 min in the dark on ice for flow cytometry measurements. Control samples without LC supplementation were subjected to the same assay. Negative controls were prepared without FITC staining, while the positive controls were prepared in the presence of 0.5 mM H_2O_2 and 0.25 mM $FeSO_4$. To study the effect of LC on sperm DNA oxidation during centrifugation, sperm samples (5×10^6 cell ml ^{1}) were treated with one volume HTF supplemented with LC concentration at 0.5 mg ml ^{1}, centrifuged for 20 min at 1600 rpm, then submitted to the same treatment as described earlier.

Data acquisition was performed within 30 min on a flow cytometer equipped with a 515 nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA, USA). Ten thousand cells were examined for each assay at a flow rate of 100 cells s ^{1}. The FITC (log green fluorescence) was measured on FL1 channel. Data analysis was performed using FLOWJO v4.4.4 software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

All values were reported as mean ± SD. Differences were considered significant at $P < 0.05$ against control group. Statistical analyses were operated using one way analysis of variance (ANOVA) followed by an unpaired Student's *t* test.

Results

Effect of LC on sperm motility and viability on incubation

The effect of various LC concentrations (0.1, 0.5, 1.0, 10 and 50 mg ml ^{1}) on sperm motility and viability was examined after 2 and 4 h incubation periods at 37 °C (Fig. 1). Sperm motility was significantly increased after LC supplementation at 0.1, 0.5 and 1.0 mg ml ^{1} after 2 h incubation (55.0 ± 16.9%, 58.4 ± 13.9% and 52.4 ± 19% respectively) compared with the control (39.4± 15%; $P < 0.05$) (Fig. 1a). Semen samples supplemented with LC and evaluated after the 4 h incubation period did not show any significant increase in sperm motility compared with the control (Fig. 1a). Semen supplementation with LC did not show a statistically significant increase in sperm viability at any concentrations tested compared with the control at both incubation periods (Fig. 1b). LC concentration of 50 mg ml ^{1} significantly reduced sperm motility and viability compared with the control at the two incubation periods ($P < 0.05$; Fig. 1a,b).

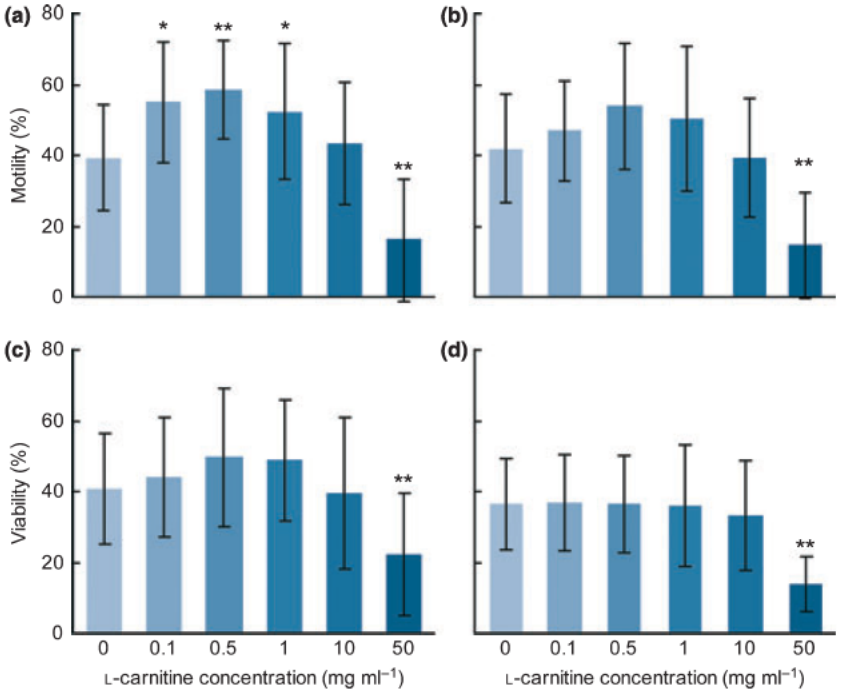

Fig. 1 Effect of various *in vitro* incubation times with L carnitine (LC) concentrations at 37 °C on sperm motility for (a) 2 h and (b) 4 h; viability (c) 2 h and (d) 4 h. Data are mean values ± standard deviations of sperm motility and viability obtained from analysis of sixteen different semen samples. *P* values for samples and their controls were measured for all LC concentrations (*P value < 0.05; **P value < 0.01); $P < 0.05$ was considered statistically significant.

Effect of LC on sperm motility and viability during sperm centrifugation

Figure 2 shows the effect of LC on sperm motility and viability after sperm centrifugation for 20 min at 1600 rpm. Figure 2a shows the mean values ± standard deviations of sperm motility for semen samples centrifuged in the presence of LC (0.5 mg ml ^{1}) and samples centrifuged in absence of LC (control). A significant difference ($P < 0.05$) in average sperm motility (37.5 ± 16.6 versus 24.0 ± 12.8%) was observed between spermatozoa centrifuged with LC compared with the control. The viability was comparable between sperm samples treated with LC compared with those without LC treatment (38.0 ± 12.0 versus 34.2 ± 12.6% respectively) (Fig. 2b).

Effect of LC on sperm motility and viability for various sperm concentrations

The effect of LC at 0.5 mg ml ^{1} concentration on sperm motility and viability was evaluated for various sperm concentrations (5, 10, 20 and 40 million ml ^{1}) (Fig. 3). Control samples without LC supplementation were subjected to the same assay conditions. Motility and viability of spermatozoa were assessed immediately after 2 h incubation at 37 °C. A statistically significant increase in sperm motility was observed in semen samples supplemented with LC compared with the control only for a sperm concentration of 5×10^{6} ml ^{1} ($P < 0.05$; Fig. 3a). No increase in sperm viability was seen after addition of LC at all sperm concentrations tested (Fig. 3b).

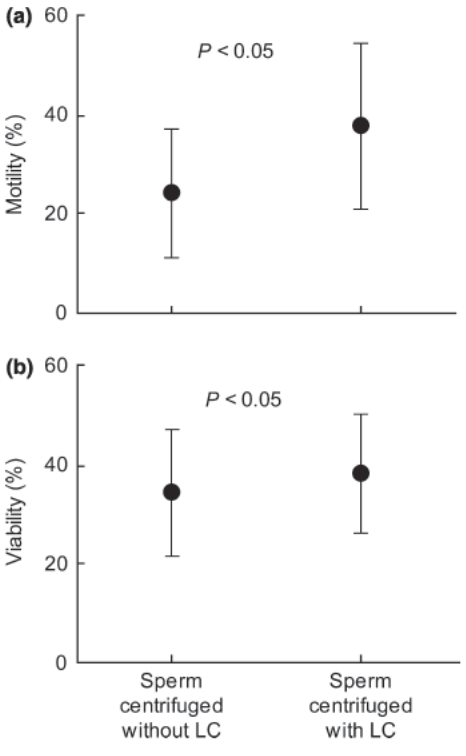

Fig. 2 Effect of centrifugation on (a) sperm motility with and without (L carnitine) LC; (b) sperm viability with and without LC. Sperm aliquots ($n = 19$) were centrifuged for 20 min at 300 *g*. All data are mean values ± standard deviations. $P < 0.05$ are considered statistically significant.

Effect of LC on sperm DNA oxidation

The effect of LC on sperm DNA oxidation during incubation and centrifugation is shown in Fig. 4. Flow cytome

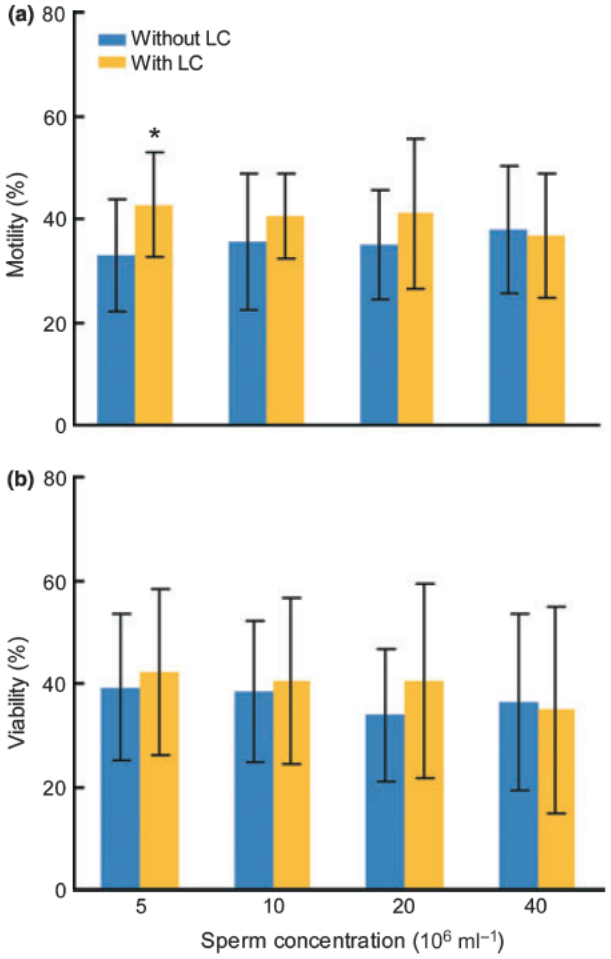

Fig. 3 Effect of L carnitine (LC) (0.5 mg ml^{-1}) on (a) sperm motility and (b) viability following incubation with varying sperm concentrations (5, 10, 20 and 40 × 10^{6} ml^{-1} motility and viability of spermatozoa were assessed immediately after 2 h *in vitro* incubation at 37 °C. The histograms are mean values ± standard deviations obtained from analysis of twelve different semen samples (yellow). Controls (blue) were assayed without addition of LC. *P* values for samples and their controls were measured for all sperm concentrations (*P value < 0.05); $P < 0.05$ was considered statistically significant.

try analysis of FITC labelled spermatozoa showed that spermatozoa treated with the LC at 0.5 and 50 mg ml ^{1} for 2 h at 37 °C did not increase the intensity of the FITC fluorescence, indicating an insignificant effect on the baseline DNA oxidation (Fig. 4a1 a2).

Figure 4b shows the effect of LC on the baseline of sperm DNA oxidation during centrifugation as evaluated by flow cytometry. Sperm samples centrifuged with LC at 0.5 mg ml ^{1} concentration did not show a significant dif ference in the intensity of FITC fluorescence, and thus in sperm DNA oxidation, compared with those without LC supplementation.

Discussion

Studies examining effects of LC on sperm quality in gen eral have focused on the effects of LC *in vivo*. Recent findings in this context show that administration of LC might antagonise the oxidative as well as the pro inflam matory pathways that attenuate sperm motility (Garolla *et al.*, 2005; Abd Allah *et al.*, 2009; Yeste *et al.*, 2009). In the present study, we have focused on the role of LC in improving sperm quality *in vitro*. In the first series of experiments, we standardised the LC concentrations, util ising semen from normozoospermic donors and estab lished that 0.1, 0.5 and 1.0 mg ml ^{1} concentrations were not toxic.

To study the effect of LC on sperm motility and viabil ity during sperm centrifugation, we used LC at a concen tration of 0.5 mg ml ^{1}. Sperm aliquots with and without LC (control) were centrifuged for 20 min at 300 ***g***, sperm motility and viability were assessed. Our results suggest that LC at the standardised concentrations was beneficial and improved sperm motility, in particular, after the 2 h incubation period. A significant increase in sperm motil ity was observed in the centrifuged spermatozoa supple mented with LC compared with the control. Similar enhancement in sperm motility *in vitro* has been reported after adding acetylcarnitine, an acetylated form of carni tine, to semen at 37 °C (Tanphaichitr, 1977). This posi tive effect of LC on sperm motility is due to its role in sperm metabolism as well as its antioxidant properties (Agarwal & Said, 2004; Gulcin, 2006). LC facilitates the transport of long chain fatty acids across the inner mem brane of mitochondria, so that they can be broken down through β oxidation to produce ATP (Steiber *et al.*, 2004); therefore, providing readily available energy for use by spermatozoa. Accumulation of the ROS in sperma tozoa leads to ATP depletion, lipid peroxidation and insufficient axonemal phosphorylation (Dokmeci, 2005). The property of LC as a scavenger for ROS could thus be responsible for its positive effect on sperm motility. This is in agreement with a study by Balercia *et al.* (2005),

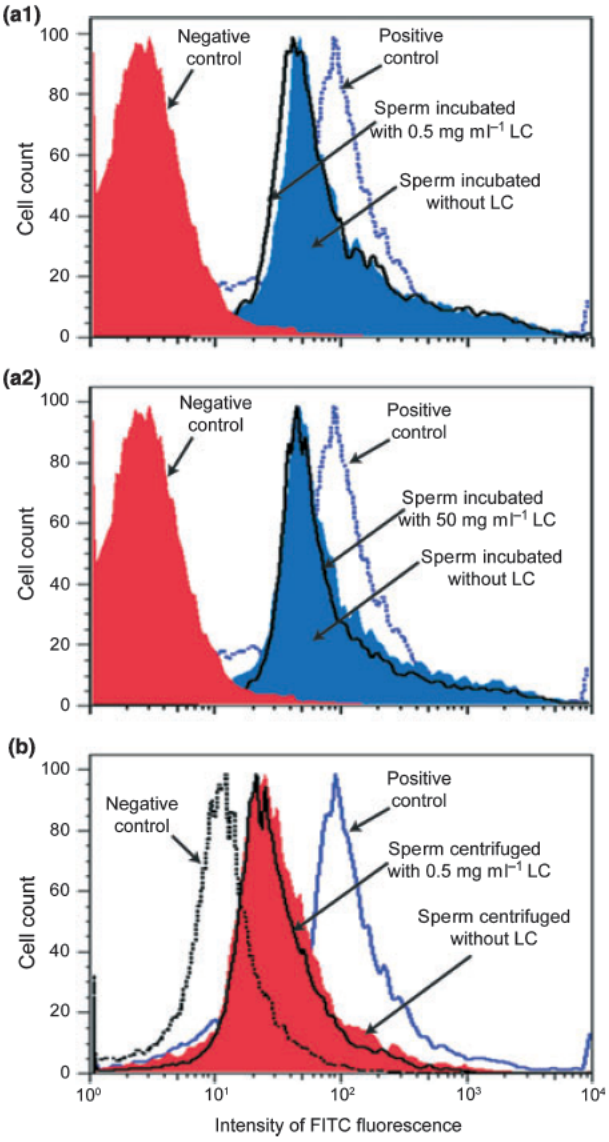

Fig. 4 Effect of L carnitine (LC) on sperm DNA oxidation during sperm incubation and centrifugation and evaluated by flow cytometry. (a1) Spermatozoa incubated for 2 h with 0.5 mg ml^{-1} and (a2) 50 mg ml^{-1} LC concentrations at 37 °C. (b) Spermatozoa centrifuged in the presence of 0.5 mg ml^{-1} LC for 20 min at 300 ***g***. Sample con trols assayed in the absence of LC in both incubation and centrifuga tion. Negative controls were analysed without FITC staining; positive controls contained H_2O_2 induced sperm DNA oxidative damage. The *X* axis represents the intensity of sperm labelled FITC fluorescence. Data are representative of four independent experiments.

who showed that LC and acetylcarnitine administration *in vivo* increased total seminal antioxidant activity as well as sperm motility in idiopathic asthenozoospermic patients.

L Carnitine contributes in shielding sperm membranes against harmful ROS because of its antioxidant activity

(Gulcin, 2006). It also decreases the availability of lipids for peroxidation *via* easing the transport of fatty acids to the mitochondrial matrix for β oxidation (Thangavel & Panneerselvama, 1998). Neuman *et al.* (2002) reported that dietary supplementation with LC reduces sperm lipid peroxidation in roosters; this may preserve sperm membranes, thereby increasing their viability (Neuman *et al.*, 2002). We therefore anticipated that *in vitro* incubation of semen samples with LC would improve sperm viability. Contrary to our expectation, we observed that semen supplementation with LC showed only a slight but statistically insignificant effect in improving sperm viability during incubation. Similar insignificant effects were reported earlier by Duru *et al.* (2000), who studied the influence of acetyl L carnitine on sperm membrane damage after cryopreservation thawing cycles. Similarly, using *in vivo* systems, it was found that dietary supplementation with LC did not improve the membrane integrity of pony stallion spermatozoa (Deichsel *et al.*, 2008). A more recent study by Yeste *et al.* (2009) showed that the addition of LC to the boar's diet had no effect on their sperm viability. In contrast, LC administration to infertile human men with poor sperm motility (<50%) was effective in improving membrane integrity of the ejaculated spermatozoa (De Rosa *et al.* (2005). In our *in vitro* study, we only added LC to semen samples with normal motility (>50%). A similar *in vitro* study utilising semen samples from infertile subjects is needed to assess LC effect on sperm viability during incubation and substantiate our findings.

Furthermore, Duru *et al.* (2000) used two assays; eosin Y staining and annexin V Cy3 in their study to measure sperm viability after cryopreservation thawing. These investigators observed significant discrepancy between the percentages of spermatozoa marked as 'live' between eosin Y staining and annexin V Cy3 assay. The eosin staining showed a lower percentage of viable spermatozoa compared with the annexin V Cy3 assay. These investigators attributed this discrepancy to the different mechanisms through which these assays operate. In our study, we measured sperm viability using the eosin staining assay, which is based on the membrane permeability for the eosin stain. Therefore, the mechanism by which this assay recognises live spermatozoa could be a contributing factor in this insignificant effect of LC on sperm viability.

The influence of LC on sperm motility and viability of various sperm concentrations was also investigated (Fig. 3). We supplemented different concentrations of spermatozoa with LC at 0.5 mg ml ^{1}. Our results from this experiment suggest that LC was effective in increasing sperm motility only when utilising sperm concentration of 5×10^{6} cell ml ^{1}. At higher sperm concentrations, the decrease in the ratio of the sperm number against available LC molecules may explain the lack of effect in improving the sperm motility. On the other hand, sperm viability did not increase utilising any of the sperm concentrations. These results are in agreement with our experiment earlier. This information may be exploited in exploring and designing protocols that aim to improve sperm motility in patients with low sperm count, especially oligozoospermic men.

L Carnitine has been shown to improve resistance to oxidative stress by decreasing DNA damage in *Ataxia telangiectasia* cells (Berni *et al.*, 2008). Moreover, Thangasamy *et al.* (2009) reported that LC significantly reduces DNA damage in lymphocytes of aged rats. We also recently demonstrated the effectiveness of LC in reducing H_2O_2 induced DNA damage and improvement of *in vitro* blastocyst development rate in mouse embryos (Abdelrazik *et al.*, 2009). In the present study, we examined whether LC had similar effects on human sperm DNA *in vitro*. We incubated the sperm samples with the standardised LC concentration 0.5 mg ml ^{1}, at 37 °C for 2 h. We did not see the decrease in the extent of the sperm DNA oxidation (Fig. 4a1). Similarly, we did not see a significant effect of LC on the baseline of sperm DNA oxidation after centrifugation compared with control samples (i.e. centrifugation without LC) (Fig. 4b).

Studies *in vitro* show that semen supplementation with an antioxidant does not affect sperm DNA integrity. In fact, Donnelly *et al.* (1999a) showed that neither addition of ascorbate nor addition of α tocopherol to sperm preparation medium affected baseline DNA integrity of spermatozoa. However, both antioxidants provided protection against the H_2O_2 induced DNA damage in spermatozoa (Donnelly *et al.*, 1999b). Similar reports showed that addition of vitamin E to cryopreservation medium did not alter the post thaw DNA fragmentation of spermatozoa (Taylor *et al.*, 2009). These results are in line with our observations, which do not show a significant effect of LC as an antioxidant in reducing DNA oxidative damage in spermtozoa.

One explanation for the undetectable effect of LC on sperm DNA oxidation may be the fact that we used only semen samples from normozoospermic subjects in our study. In the present study, we measured the effect of LC on DNA oxidation in spermatozoa induced only by *in vitro* incubation and centrifugation, but not by exogenous induction of oxidative damage. This might be another likely reason for the lack of a significant effect of LC on sperm DNA oxidation as reported by others (Donnelly *et al.*, 1999b; Berni *et al.*, 2008; Abd Allah *et al.*, 2009; Abdelrazik *et al.*, 2009).

Another reason for lack of improvement in DNA oxidation may be the fact that mammalian sperm DNA is

highly compacted (6 fold more highly condensed) com pared with DNA in somatic cells (Ward & Coffey, 1991). Also, the majority of antioxidants in somatic cells are present within their cytoplasm, while in spermato zoa, a significant amount of the antioxidants are present in seminal plasma outside the spermatozoa, because spermatozoa lose most of its cytoplasm during matura tion (Donnelly *et al.*, 1999a). Therefore, the differences in the DNA packaging and the distribution of antioxi dants between spermatozoa and somatic cells might introduce variations in outcomes when measuring the effect of *in vitro* supplementation of antioxidants on DNA oxidation.

It has been shown that cell treatment with high con centrations of antioxidants *in vitro* increases the levels of 8 oxoguanine moieties on DNA, a biomarker for DNA oxidative damage (Gutteridge, 1994). Increasing DNA oxidative damage may lead to apoptosis and cell death (Wang *et al.*, 2003; Hurh *et al.*, 2004. We therefore exam ined whether high LC concentration decreased *in vitro* sperm quality by inducing sperm DNA oxidation. We measured the 8 oxoguanine moieties in the sperm DNA after incubating the spermatozoa with 50 mg ml ^{1} LC using flow cytometry. Our results suggest that this might not be the likely mechanism by which the high LC level negatively affects sperm quality *in vitro* (Fig. 4a2). Again the fact that all samples from normozoospermic subjects may also explain the lack of any significant effects of LC on DNA damage.

In conclusion, LC enhances sperm motility following *in vitro* incubation and centrifugation, while it might not significantly affect sperm viability and baseline DNA oxi dation of spermatozoa. These results provide a solid foundation to explore and design protocols to test the potentially beneficial effect of LC supplementation for the spermatozoa prepared for ART.

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