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Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of human spermatozoa

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Keywords
DNA oxidation L carnitine motility sperm cryopreservation vitality

Summary

Successful cryopreservation for human spermatozoa markedly influences the reproductive outcomes of assisted reproductive technologies. But in spite of its usefulness, cryopreservation significantly decreases sperm quality. L carnitine has been found to improve the quality of spermatozoa in selected cases with male infertility. Here, we examined the efficacy of L carnitine in improving sperm motility and vitality and reducing sperm DNA oxidation during cryo preservation. Semen samples from infertile patients ($n = 22$) were collected and analysed. Cryopreservation medium supplemented with L carnitine was mixed with the semen at a ratio of 1 : 1 (v/v). The final L carnitine concentra tion in each cryovial was 0.5 mg ml^{-1} per 5×10^6 cell ml^{-1}. Controls were cryopreserved without addition of L carnitine. After 24 h of cryopreservation, thawed sperm samples were analysed for motility, vitality and DNA oxidation. Sperm vitality was assessed by the eosin nigrosin test, while sperm DNA oxida tion was measured by flow cytometry. Addition of L carnitine significantly improved sperm motility and vitality ($P < 0.05$) compared with the control. The flow cytometry experiment showed no statistical difference ($P > 0.05$) in the levels of DNA oxidation between samples and controls. In conclusion, L carnitine improves human sperm motility and vitality, but has no effect on sperm DNA oxidation after cryopreservation.

Introduction

Human sperm cryopreservation was first introduced in the 1960s as a useful procedure to manage male infertility (Sherman, 1973). Its importance is underlined in a variety of circumstances that may negatively affect sperm quality such as chemotherapy treatments and surgical infertility interventions. It is also useful as it allows storage of donor semen until seronegativity for hepatitis and HIV is confirmed (Dillon & Fiester, 2012). Successful sperm cryopreservation therefore greatly influences the repro ductive outcomes of assisted reproductive technologies (ART). But in spite of its usefulness, cryopreservation sig nificantly decreases motility, vitality and chromatin integ rity of spermatozoa (Nijs & Ombelet, 2001; Zribi *et al.*, 2010).

L carnitine (LC), structurally defined as 3 hydroxy 4 (trimethylazaniumyl) butanoate, is a biomolecule derived from the amino acid lysine. In the human body, LC can be synthesised *de novo*. It is described as a conditionally essential nutrient and is largely obtained from meat, fish and dairy products (Tanphaichitr & Leelahagul, 1993). LC facilitates the entry of long chain fatty acids into mitochondria for oxidation and ATP production (Tanp haichitr & Leelahagul, 1993). Besides, LC has been found to exert an antioxidant activity (Gulcin, 2006).

Menchini Fabris *et al.* (1984) observed a positive corre lation between free LC in human semen and sperm motility and sperm count. Others have shown a correla tion between male infertility and lower levels of LC in semen (Matalliotakis *et al.*, 2000; Li *et al.*, 2007). Further, dietary supplementation with LC has been found to improve the quality of spermatozoa (Kozink *et al.*, 2004; Yeste *et al.*, 2009). Studies performed *in vivo*, as well as *in vitro*, have shown that LC reduces oxidative stress induced DNA damage (Haripriya *et al.*, 2005; Qi *et al.*, 2006; Thangasamy *et al.*, 2009). Moreover, LC has been found to decrease hydrogen peroxide induced DNA dam age and improve the *in vitro* blastocyst development rate in mouse embryos (Abdelrazik *et al.*, 2009). Although

various studies have investigated the role of LC in improving sperm quality, its effectiveness on human sperm quality during cryopreservation is still undefined. Thus, the main goal of this study was to examine the effect of free LC in improving sperm motility and vitality and reducing sperm DNA oxidation during cryopreservation.

Materials and methods

Sample collection

This study was approved by the institutional review board of Cleveland Clinic Hospital. Semen samples from infertile patients ($n = 22$) were collected by masturbation after 72 h sexual abstinence and analysed for sperm viscosity, volume, concentration and motility according to the guidelines of the World Health Organization (1999).

Cryopreservation of spermatozoa

After the initial semen analysis, two equal aliquots (each containing 5×10^6 spermatozoa) were prepared from each sample. All aliquots were cryopreserved using TEST Yolk buffer (TYB; Irvine Scientific, Santa Ana, CA, USA). TYB containing LC (Sigma Tau, Pomezia, Italy) was added to the first aliquot at room temperature, while the second aliquot received treatment with LC free TYB (control). The final LC concentration in each cryovial was 0.5 mg ml^{-1} per 5×10^6 cell ml^{-1}. An aliquot of the cryopreservation medium equal to 25% of sperm sample volume was added to the specimen and gently mixed for 5 min using a Hema Tek aliquot mixer (Miles Scientific, Elkhart, Indiana). This was repeated to give a final 1 : 1 (v/v) ratio of cryopreservation medium to the sperm sample. Cryovials (1.5 ml; Corning, Pittsburg, PA, USA) containing the specimens were placed in the freezer at −20 °C for 8 min and in the liquid nitrogen vapours at −80 °C for 2 h. The vials were then transferred to liquid nitrogen at −196 °C. Twenty four hours after, the samples were frozen; the vial was removed and thawed by incubation at 37 °C for 20 min. Spermatozoa were resuspended in human tubal fluid medium immediately after thawing and analysed for motility, vitality and DNA oxidation.

Measurement of sperm motility and vitality

A total amount of 5 µl of each aliquot was used for evaluation of motility using a microcell slide chamber (Conception Technologies, San Diego, CA, USA).

Sperm vitality was measured using the eosin nigrosin test. Briefly, to one drop of the sperm sample 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 (Banihani *et al.*, 2012). A thin smear was prepared by pipetting 10 µl onto a slide and allowed to air dry. Slides were then mounted with a coverslip using Accu mount media (Olympus America Inc., Center Valley, PA, USA). Measurements were performed on 200 spermatozoa counted on each slide in duplicate sets using the ×100 objective. Percentage of unstained (white) and stained (pink) spermatozoa was calculated. The unstained spermatozoa were considered viable, while the stained ones were considered nonviable.

Measurement of sperm DNA oxidation

Sperm DNA oxidation was evaluated using the flow cytometric OxyDNA assay kit (Calbiochem, San Diego, CA, USA). The assay is based on using a direct fluorescent protein binding method targeting oxidised sites (8 oxoguanine) of DNA (Banihani *et al.*, 2012).

Briefly, cryopreserved samples were washed twice in phosphate buffered saline (PBS), resuspended in 2% paraformaldehyde and placed on ice for 15 30 min. Spermatozoa were again washed and resuspended in 70% ice cold ethanol by centrifugation at x300 ***g*** for 5 min. 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 the dark. The staining solution contained fluorescein isothiocyanate (FITC) labelled protein conjugate and distilled water. All spermatozoa 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. Data acquisition was achieved 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). 10 000 spermatozoa 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) (Banihani *et al.*, 2012).

Statistical analysis

All values were documented as mean ± standard deviation. Differences were considered significant at $P < 0.05$ against control. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by paired Student's t test using the SPSS/PC computer software (SPSS 10.0.7; SPSS Inc., Armonk, NY, USA).

Results

Figure 1 shows the effect of LC supplementation at 0.5 mg ml^{-1} on human sperm motility during cryopres ervation. The motility of cryopreserved spermatozoa sig nificantly increased ($P < 0.05$) by about 21.5% after adding LC compared with the control (without LC) (Table 1).

Figure 2 shows the cryoprotective effect of LC supple mentation at 0.5 mg ml^{-1} on human sperm vitality as evaluated by eosin nigrosin test. The vitality of spermato zoa significantly increased ($P < 0.05$) by about 13.6% after adding LC (Table 1).

The cryoprotective effect of LC supplementation at 0.5 mg ml^{-1} on human sperm DNA oxidation as evalu ated by flow cytometry is shown in Fig. 3 as a representa tive experiment. Although there is a decrease in the mean value of FITC fluorescence for spermatozoa cryopreserved with LC compared with those cryopreserved without LC (control), both means were not statistically different (P value > 0.05), indicating insignificant difference in the baseline level of sperm DNA oxidation between the two conditions (Table 1).

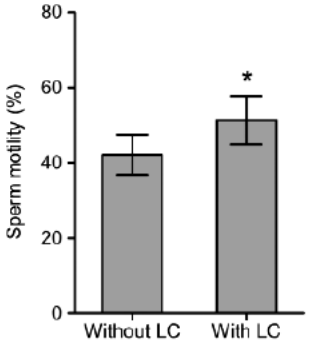

Fig. 1 Cryoprotective effect of L carnitine (LC) supplementation at 0.5 mg ml^{-1} on human sperm motility. Sperm motility significantly increased after adding LC (*P value < 0.05).

Table 1 Mean values and their standard deviations of sperm motility, vitality and intensity of fluorescein isothiocyanate (FITC) fluorescence

Variable	Without L carnitine	With L carnitine
Motility (n 22)	42.2 ± 5.3	51.3 ± 6.4*
Vitality (n 22)	43.3 ± 5.5	49.2 ± 7.8*
Intensity of FITC fluorescence (n 12)	75.2 ± 19.1	69.2 ± 17.3

*$P < 0.05$.

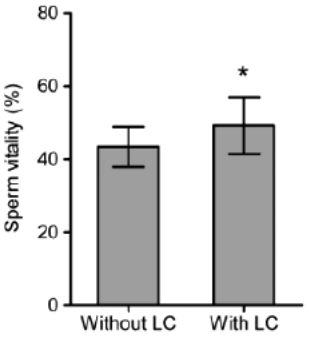

Fig. 2 Cryoprotective effect of L carnitine (LC) supplementation at 0.5 mg ml^{-1} on human sperm viability as evaluated by eosin nigrosin test. Sperm viability significantly increased after adding LC (*P value < 0.05).

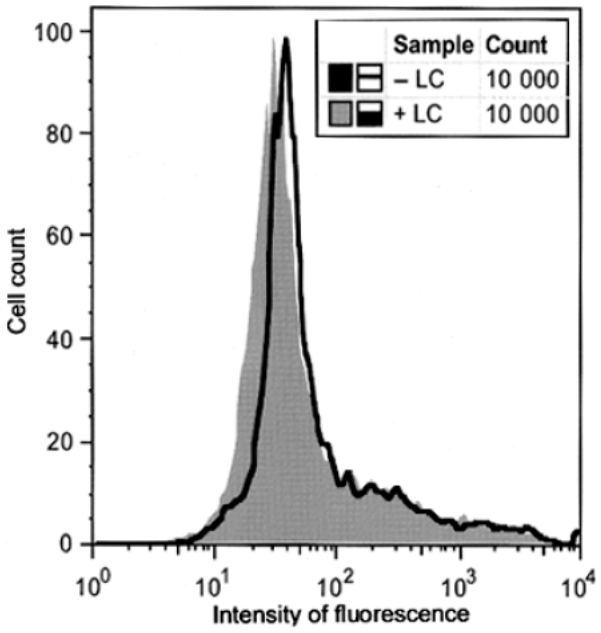

Fig. 3 Cryoprotective effect of L carnitine (LC) supplementation at 0.5 mg ml^{-1} on human sperm DNA oxidation as evaluated by flow cytometry. Adding LC to the cryopreserved spermatozoa did not alter the level of sperm DNA oxidation (P value > 0.05). The x axis represents the intensity of sperm labelled fluorescein isothiocyanate (FITC) fluores cence. Data are representative of 12 independent experiments.

Discussion

In our previous work, we have shown that LC concentra tions (0.5 1.0 mg ml^{-1}) increase human sperm motility and vitality during sperm incubation at 37 °C (Banihani *et al.*, 2012). Similar improvement in sperm motility *in vitro* has been documented earlier after adding acetyl L carnitine (ALC), an acetylated form of LC, to human semen at 37 °C (Tanphaichitr, 1977). This beneficial

effect of LC to human sperm quality is due to its antioxi dant properties as well as its function in sperm metabo lism (Agarwal & Said, 2004; Gulcin, 2006). The antioxidant activity of LC may be due to its ability to chelate free ferrous ions, inhibit generated superoxide ions and detoxify accumulated hydrogen peroxide species (Gulcin, 2006). LC facilitates the transport of activated fatty acids across the inner membrane of mitochondria, allowing their β oxidation to produce ATP (Steiber *et al.*, 2004), hence providing energy for use by spermatozoa. In addition, accumulation of reactive oxygen species (ROS) in spermatozoa leads to a depletion in the ATP reservoir, which, in turn, negatively affects sperm quality (Dokmeci, 2005). The properties of LC as a ROS scavenger and an energy production facilitator could thus be responsible for its beneficial effects on sperm motility and vitality.

Freezing semen at −20 °C for 7 days was found to sig nificantly decrease the intracellular concentrations of free LC in human spermatozoa (Suter & Holland, 1979). Another study found that human sperm cryopreservation decreased the intracellular levels of ALC in 14 of the 15 cases studied (Reyes Moreno *et al.*, 2000). In this work, we have shown that adding LC to human sperm cryopro tectant increases significantly their motility and vitality after thawing. Accordingly, adding LC to the cryoprotec tive medium may decrease the loss of LC from the intra cellular compartment of spermatozoa, which preserves, at least in part, sperm motility and vitality after thawing.

The cryoprotectants used in sperm freezing may alter the intracellular content of LC. Setyawan *et al.* (2009) found that certain cryoprotectants (glycerol (GLY), 1,1,1 tris (hydroxymethyl) propane [2 ethyl 2 hydroxymethyl propane 1,3 diol] (THP), ethylene glycol (EG), 1,1,1 tris (hydroxymethyl) ethane [2 hydroxymethyl 2 methyl pro pane 1,3 diol] (THE), propane 1,2 diol (PD2), propane 1,3 diol (PD3) and dimethylsulphoxide (DMSO)) reduced the intracellular LC content in bovine spermato zoa. Consequently, the addition of LC to the cryopreser vation medium of human spermatozoa (0.5 1.0 mg ml^{-1}) could be advantageous in maintaining its cellular reserves (Banihani *et al.*, 2012).

L carnitine, in cells rather than spermatozoa, has been found to reduce oxidative stress and damage to DNA (Berni *et al.*, 2008; Abdelrazik *et al.*, 2009; Thangasamy *et al.*, 2009). In the present work, we examined whether LC had similar effects on oxidative DNA damage to human spermatozoa *in vitro* after cryopreservation thaw ing. We have monitored DNA oxidation as a primary marker for antioxidant activity of LC because oxidative DNA damage induced by ROS during the cryopreserva tion process is known to precede DNA fragmentation. Therefore, DNA oxidation, typically measured by assess ing the 8 hydroxy guanosine (oh8G), would be a good indicator for any LC induced antioxidant activity of sperm DNA. Contrary to our findings on sperm motility and vitality, our results suggest an insignificant decrease in the level of sperm DNA oxidation after adding LC to the cryoprotectant medium. Similar work showed that the addition of alpha tocopherol (vitamin E), a fat solu ble antioxidant, to cryopreservation medium did not alter the post thaw DNA fragmentation of spermatozoa (Tay lor *et al.*, 2009). These findings are in line with our find ings, which do not show a significant effect of LC in decreasing DNA oxidative damage in spermatozoa.

Compared with DNA in somatic cells, mammalian sperm DNA is highly compacted (6 fold more highly condensed) (Ward & Coffey, 1991). Moreover, the majority of antioxidants in somatic cells are present within their cytoplasm, while in spermatozoa, a signifi cant amount of the antioxidants is present in seminal plasma outside the spermatozoa because spermatozoa lose the majority of their cytoplasm during maturation (Donnelly *et al.*, 1999). Accordingly, the differences in the packaging of DNA and the distribution of antioxi dants between somatic cells and spermatozoa may attri bute to different outcomes when measuring the effect of *in vitro* supplementation of LC to sperm cryopreserva tion medium compared with the baseline sperm DNA damage. In addition, the sample size obtained ($n = 12$) may have been insufficient to establish statistical differ ence in sperm DNA oxidation between the cryopre served samples supplemented with LC and their controls (without LC); therefore, increasing the sample size may show appreciable improvements in the DNA oxidation results.

In conclusion, LC enhances sperm motility and vitality following *in vitro* cryopreservation, without altering the baseline of sperm DNA oxidation. Our findings provide a platform to further investigate and design protocols to test the beneficial effects of LC supplementation on cryopreserved human spermatozoa.

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