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Comparison and evaluation of capacitation and acrosomal reaction in freeze-thawed human ejaculated spermatozoa treated with L-carnitine and pentoxifylline

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Summary

Cryopreservation is used to preserve the spermatozoa; however, it leads to a reduction in sperm quality. L-carnitine (LC) influences sperm motility and preserves the sperm membrane and DNA integrity. The objectives of this study were to evaluate the protective effects of LC on the membrane integrity of normal human spermatozoa and compare it with pentoxifylline (PT) during cryopreservation. Thirty normal semen samples, prepared by swim-up procedure, were divided into three aliquots: a control without any treatment and two experimental aliquots that were incubated in PT or LC for 30 min. All aliquots were cryopreserved and thawed after 48 hr. To evaluate the percentages of intact, acrosomal-reacted and capacitated spermatozoa, lectin histochemistry and flow cytometry were performed by wheat germ agglutinin, peanut agglutinin and Con A. Statistical analyses were performed using ANOVA. LC supplementation elevated the percentage of noncapacitated spermatozoa compared with control and PT-treated samples and the percentages of acrosomal intact spermatozoa compared with PT-treated samples. PT pre-treatment improved the motility but not membrane integrity. LC supplementation reduced the percentages of acrosomal-reacted spermatozoa compared with the control and PT-treated samples. Although LC did not improve motility, it protected the plasma membrane and acrosomal integrity. Therefore, LC may be the superior choice compared to PT for maintaining the sperm integrity.

KEYWORDS

acrosomal reaction, capacitation, carnitine, cryopreservation, pentoxifylline

1 | INTRODUCTION

Two of the major concerns in medical fields are infertility and subfertility that have medical and social effects on the patients. Infertility can be defined as the pregnancy failure after at least 1 year without using any contraceptive approaches. Reports show that 10–15 per cent of couples are infertile and male infertility is responsible for about half of them (Poongothai, Gopenath, & Manonayaki, 2009). Male infertility including chemotherapy or radiation-related germ cell loss due to cancer therapy (Mohazzab et al., 2011), some kind of hepatitis, HIV (Dillon & Fiester, 2012) and some surgical treatments may lead to testicular failure (Di Santo et al., 2012).

One of the most effective approaches used to preserve the spermatozoa is semen cryopreservation. However, cryopreservation leads to a reduction in some critical sperm parameters such as motility, viability (Bagchi, Woods, & Critser, 2008), crossing through cervical mucus, acrosome reaction (Nijs & Ombelet, 2001) and activity of some acrosomal enzymes including acrosin (Mack & Zaneveld, 1987). Cryopreservation has been detected to modify the surface sugar distribution on the sperm membrane. These sugars play a critical role in sperm physiology such as capacitation, sperm membrane permeabilisation, acrosomal reaction and fertilisation (Talaei, Esmaeelpour, Aekiyash, & Bahmanpour, 2010). The evidence suggested glycocalyx content modification influences the male fertility and may lead to

infertility (Purohit, Laloraya, & Kumar, 2008). A significant decrease in the antioxidant content of the spermatozoa and an increase in reactive oxygen species (ROS) were shown after cryopreservation of the semen samples (Saraswat et al., 2012). Modifications in mammalian sperm phospholipid asymmetry were reported previously during freeze–thaw procedure due to high ROS production (Duru, Morshedi, Schuffner, & Oehninger, 2001). High ROS content was shown to have a detrimental effect on plasma membrane by increasing the susceptibility to lipid peroxidation and, subsequently, it led to a decrease in sperm qualification such as motility (Bansal & Bilaspuri, 2010). As a result, it reduced fertilising capability of the cryopreserved spermatozoa. Therefore, an increase in ROS content along with a decrease in antioxidant enzymes induced cell death (Wang et al., 2003).

The microenvironment content may exert some effects on the sperm quality (Talaei-khozani et al., 2011). For instance, antioxidant supplementation can prevent the sperm damage by neutralising ROS (Saraswat et al., 2012). L-carnitine (LC), as an unusual amino acid present in male reproductive system, has antioxidant property and plays a pivotal role in increasing the sperm viability and motility. The concentration of LC in mammalian epididymis is 200 times higher than that in the blood (Pekala, Patkowska-Sokola, & Bodkowski, 2011). Some reports showed that infertile men had less amount of LC in their semen (Menchini-Fabris, Canale, Izzo, Olivieri, & Bartelloni, 1984; Zare, Imani Mohammadi, Mofid, & Dashtnavard, 2009).

Another antioxidant used extensively in assisted reproductive technologies is pentoxifylline (PT). PT can act as ROS scavenger and protect the plasma membrane integrity. PT is also used in sperm cryopreservation techniques (Yovich, 1993). There is a controversy about the impacts of PT on cryopreserved spermatozoa. Some reports showed beneficial effects of PT supplementation on the reduction of the acrosomal reaction occurrence after cryopreservation (Esteves, Sharma, Thomas, & Agarwal, 1998); in contrast, others detected that PT supplementation increases the frequency of the acrosomal-reacted spermatozoa in the thawed samples (Mirshokraei, Hassanpour, Mehdizadeh, & Taheri, 2011). However, any supplementation may be toxic and have detrimental effect on the spermatozoa such as cell death (Sato & Ishikawa, 2004).

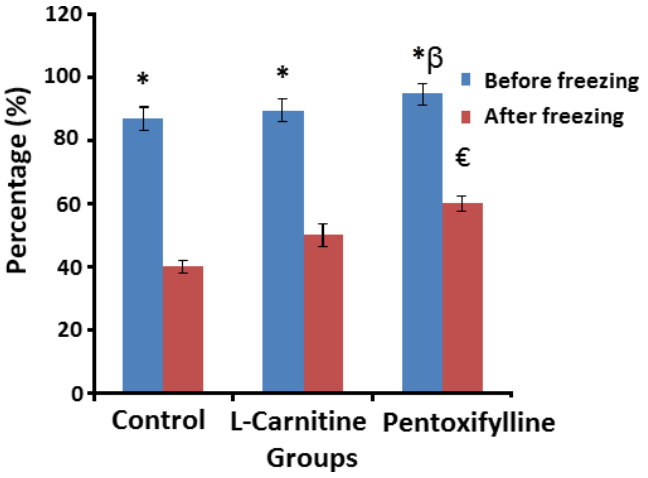

FIGURE 1 Sperm motility in the samples pre-treated with pentoxifylline and L-carnitine before and after freezing compared with the control aliquots. *Siginificant difference with sperm motility of the samples after freezing ($p < .05$). β Siginificant difference with sperm motility in control and L-carnitin-teated samples ($p < .05$). € Siginificant difference with sperm motility of the control and L-carnitin-teated samples ($p < .05$)

The current study attempted to evaluate and compare the protective effects of PT and LC on the plasma membrane integrity of normal human spermatozoa during cryopreservation. We used three lectins: peanut agglutinin (PNA) to detect the acrosomal intact spermatozoa, wheat germ agglutinin (WGA) to detect noncapacitated spermatozoa and concanavalin A (ConA) to detect acrosomal-reacted cells.

2 | MATERIALS AND METHODS

2.1 | Sperm preparation

Semen samples were collected from 30 healthy male volunteers with informed consents from cytogenetic clinic in Shiraz, Iran. The study design was approved by ethic committee of University with reference no IR.SUMS.REC.1394.S139. All the subjects were asked to abstain from ejaculation 2–3 days before sample collection. The inclusion criteria were normal sperm count, motility, morphology, viability according to the WHO criteria (WHO, 2010).

2.2 | Sperm preparation by Swim-up method

One millilitre of liquefied semen was transferred to a sterile round-bottomed centrifuge tube, and mixed with 2 ml of culture medium, Ham's F10, containing %20 human serum albumin (HSA). Then, they were centrifuged for 10 min at 500 g. After centrifugation, the supernatant was discarded, 1 ml of the medium was added again without disturbing the pellet and the sample was left in the incubator at an angle of 45° to swim up the spermatozoa for 1 hr. The motile spermatozoa left the pellet and swam into the supernatant; then, the fluid was transferred in another sterile round-bottomed tube and mixed to the medium up to 1 ml.

2.3 | Experimental design

Each sample was divided into three equal aliquots: control group, including 0.25 ml of the sample and 0.25 ml of Ham's F10 containing HSA; LC-supplemented group, including 0.25 of the sample and 0.25 ml of 3.6 mM LC (1 ml medium containing 7 mg LC); and PT-supplemented group, including 0.25 ml of the sample and 0.25 ml of 3.6 mM PT (1 ml medium containing 1 mg of PT). The final molarity of LC or PT was 1.8 mM. The PT and LC concentrations were chosen according to the study performed previously on the effect of PT and LC on sperm parameters (Aliabadi, Karimi, & Talaei-Khozani, 2013). All aliquots were incubated at 37°C for 30 min. The samples were assessed for motility using light microscopy (Nikon, E200, Japan) according to the WHO criteria for sperm motility (World Health Organization, 2010). According

to WHO criterion, the sperm motility can be scored into three categories: progressive, the spermatozoa move forward linearly or in a large circle; nonprogressive, the spermatozoa move in site or move slowly without any forward progress such as moving in small circles or beating flagellum; and immotile, without any movement (World Health Organization, 2010). The percentage of motile spermatozoa was calculated by counting 10 randomly selected fields and counting the number of progressive and the total number of spermatozoa.

2.4 | Cryopreservation

0.5 ml of each aliquot was transferred to a 2 ml cryotube and mixed with 0.5 ml of cryoprotective medium (Life Global, USA) drop by drop. The cryotubes were placed in the horizontal position at 5 cm above to the surface of liquid nitrogen for 20 min. Then, it was quickly plunged into liquid nitrogen for more storage.

After 48 hr, the cryotubes were taken out and 1 ml of fresh culture medium added to each sample; then, they were left to thaw at 37°C for 10 min. Finally, they were centrifuged at 500 g for 10 min. Spermatozoa were resuspended in Ham's F10 containing 20% HSA. After thawing, the sperm motility was evaluated.

2.5 | Lectin histochemistry

An aliquot of the thawed sample was placed on a slide and a smear was prepared. Then, they were fixed with 2% paraformaldehyde for 20 min and, subsequently, they were washed with phosphate buffer saline (PBS) for 30 min. To stain with lectins, the samples were treated with FITC-conjugated WGA, Con A and PNA (All from Sigma, USA) at a dilution of 10 μg/ml for 2 hr at dark. After washing with PBS, the samples were counterstained with 4′,6-diamidino-2-phenylindole for 5 min. The specimens were observed with a fluorescent microscope (Nickon, Eclipse, E600).

2.6 | Flowcytometry

Control, LC- and PT-supplemented groups were washed with 800 ml of PBS, centrifuged at 290 g for 10 min and fixed with 2% paraformaldehyde for 30 min at 4°C. Thereafter, the aliquots were centrifuged and the palates were resuspended in PBS. The aliquots were exposed with FITC-conjugated WGA, PNA and ConA at a dilution of 10 mg/ml for 2 hr at 37°C in humidified environment in darkness. After washing with PBS, the frequencies of the spermatozoa stained with

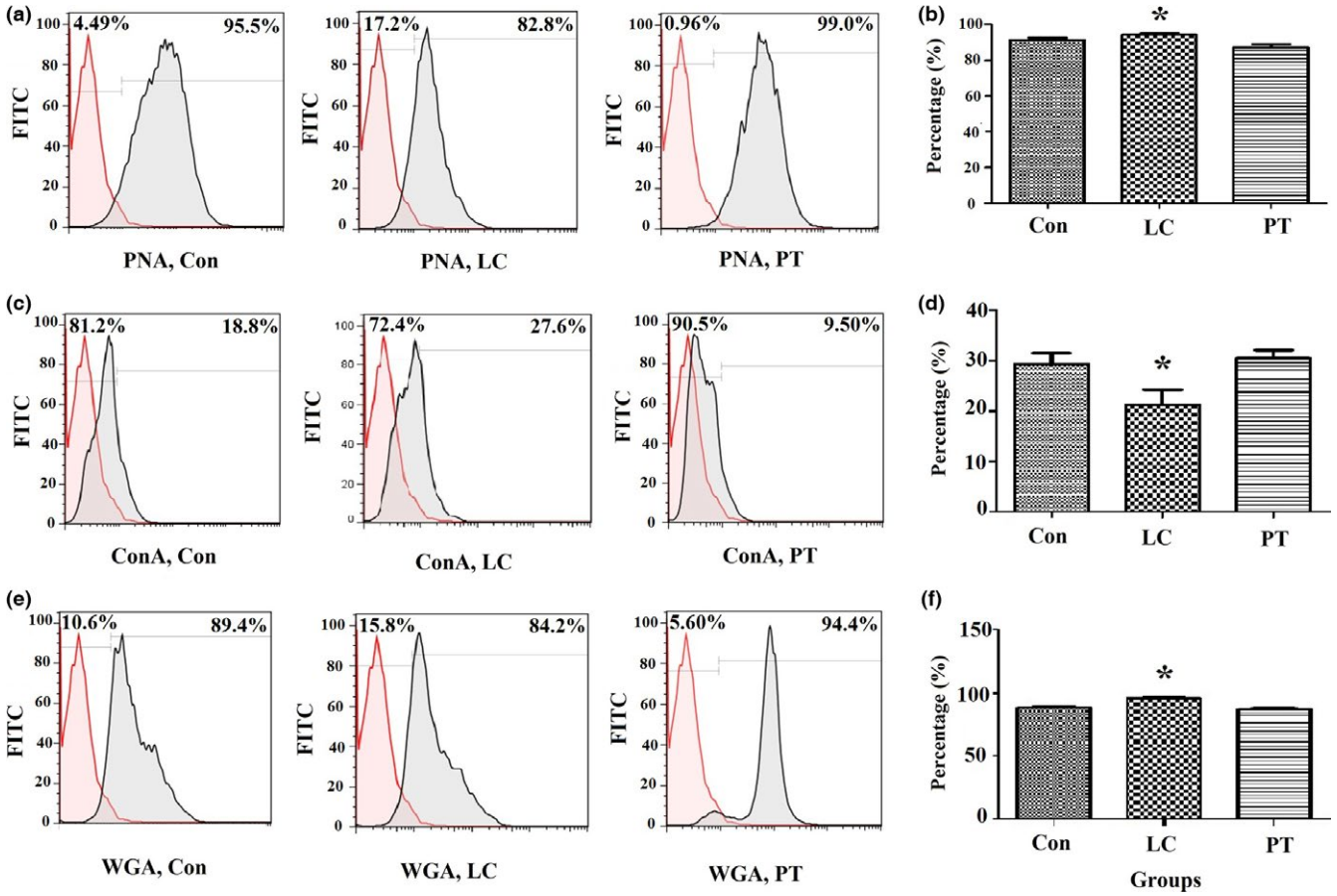

FIGURE 2 The flow cytometry showed that L-carnitine (LC) increases the frequency of the intact spermatozoa compared with pentoxifylline (PT; a and b). LC also decreases the frequency of the acrosomal-reacted spermatozoa (c and d) and increases the noncapacitated spermatozoa (e and f) compared with PT and control £ Significant difference with pentoxifylline ($P < .05$), * Significant difference with pentoxifylline and control ($P < .05$) Con, control; LC, L-carnitine; PT, pentoxifyllin

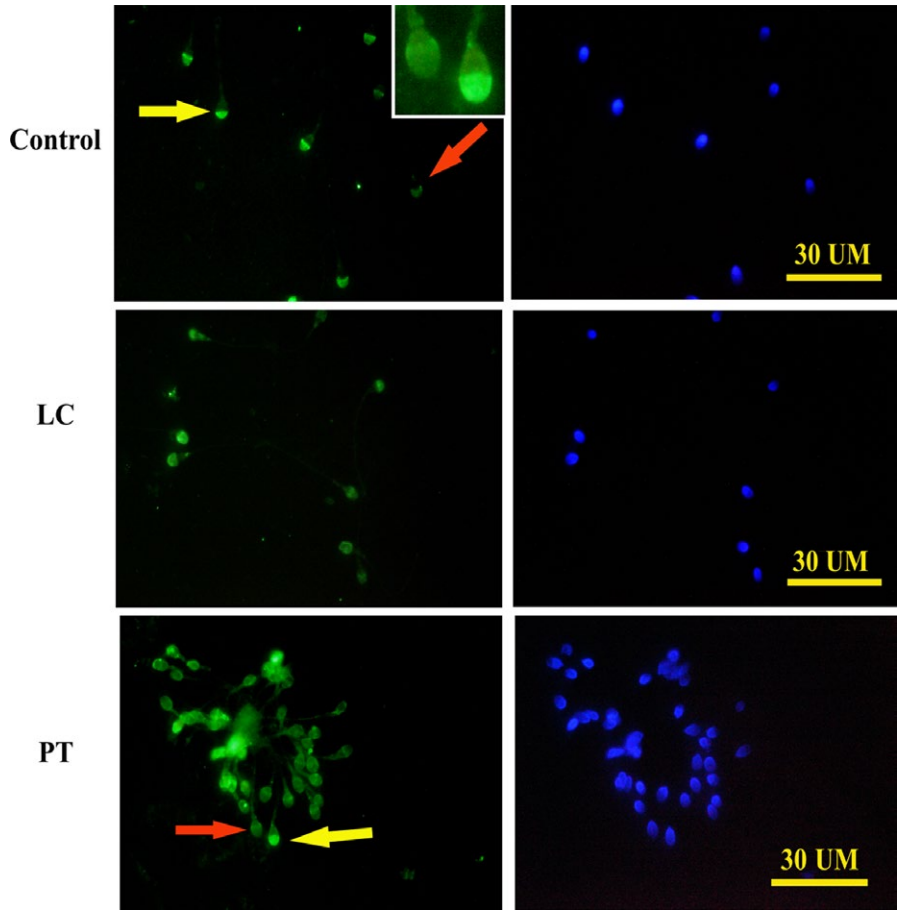

FIGURE 3 The lectin histochemistry with peanut agglutinin (PNA) shows the reactivity properties of the spermatozoa pre-treated with L-carnitine (LC) and pentoxifylline (PT) before freezing procedure. Reacted spermatozoa with PNA show intact spermatozoa and nonreacted one indicate those release the acrosome content. At above right of the figure, the higher aggrandisement of the intact and acrosomal-reacted spermatozoa was depicted. The frequency of the intact spermatozoa (yellow arrow) is higher in LC-treated aliquots and acrosomal-reacted spermatozoa (red arrow) were more frequent in PT-treated samples

FITC-conjugated lectins were assessed by FL1 channel flow cytometry (BD, USA). The data were analysed by flowjo software.

2.7 | Statistical analyses

The data were presented as mean values ± standard deviations (*SD*). The results were analysed statistically by one-way analysis of variance (ANOVA) and least standard deviation (LSD) tests. SPSS version 16 for windows was used to analyse the data. A *p*-value <.05 was considered as the statistically significant difference. The graphs were depicted by prism.

3 | RESULTS

3.1 | Sperm motility assessment

Before freezing and after 30-min incubation of the samples with reagents, the percentages of motile spermatozoa in all aliquots were assessed. The data showed that the frequency of the progressive spermatozoa significantly increased in PT-supplemented aliquots compared with both control and LC-supplemented aliquots ($p = .001$).

The comparison of each samples with their counterpart after freezing and thawing revealed that the freeze–thaw process significantly decreased the sperm motility ($p = .001$). After thawing, the frequency of the progress motile spermatozoa were significantly higher in PT-supplemented aliquots compared with both other aliquots ($p = .001$). LC supplementation did not show any significant impact on the sperm motility after thawing procedure (Figure 1).

3.2 | Effects of LC and PT on sperm reactions

The frequency of the intact spermatozoa was shown by flow cytometry analyses. Incubation for 30 min before freezing led to a significant increase in the percentages of acrosomal intact spermatozoa in LC-supplemented compared with PT-supplemented aliquots ($p = .0001$). LC treatment showed no change in the percentages of PNA-reacted spermatozoa compared with control aliquots (Figure 2a,b). Lectin reactivity of the spermatozoa showed that the acrosome content can be stained with PNA. The anterior part of the head of intact spermatozoa was stained with PNA intensely; in contrast, acrosomal-reacted spermatozoa were stained uniformly with PNA (Figure 3). The partial release of the acrosome led to formation of a PNA-positive bond around the equatorial part of the sperm head.

Con A stained mannose residues in the posterior membrane of the acrosomal-reacted spermatozoa. The incubation for 30 min before freezing with LC led to a significant decrease in the percentages of acrosomal-reacted spermatozoa compared with those incubated with PT and control (both $p = .0001$; Figure 2c,d). Lectin histochemistry revealed that the anterior part of the acrosomal-reacted sperm head was

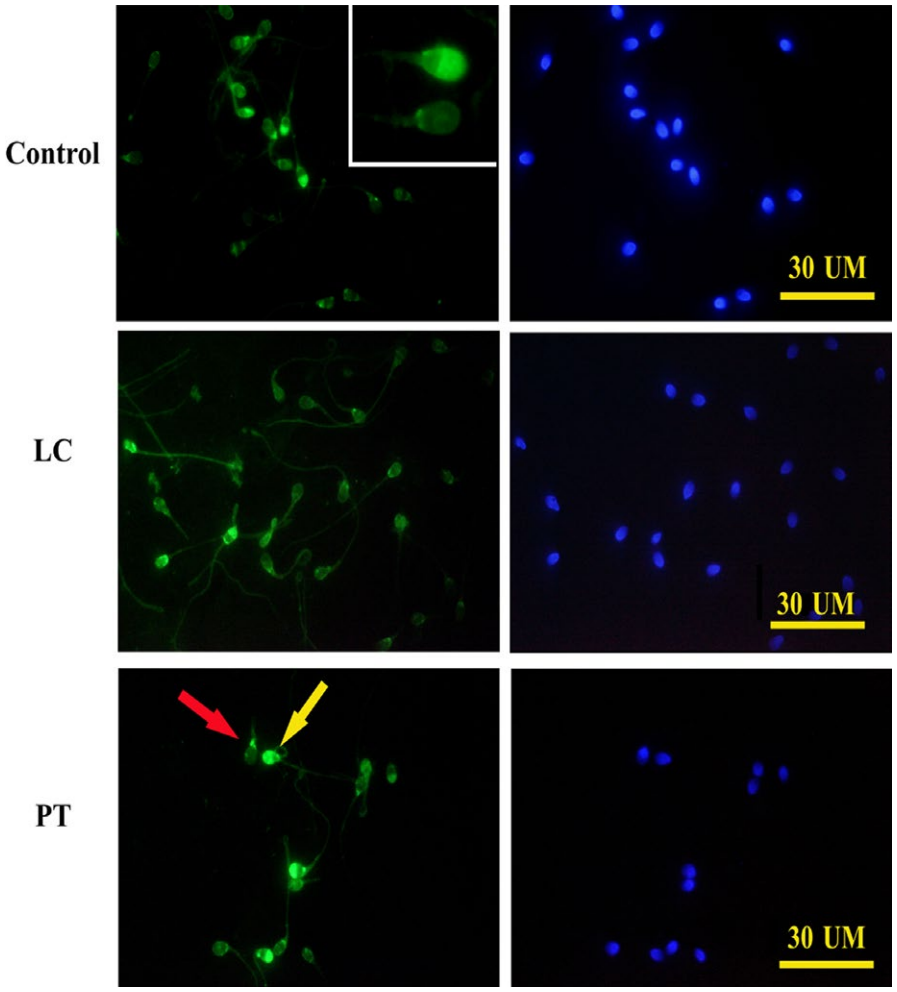

FIGURE 4 The lectin histochemistry with Con A shows the reactivity properties of the spermatozoa pre-treated with L-carnitine (LC) and pentoxifylline (PT) before freezing procedure. Reacted spermatozoa with concanavalin A are the spermatozoa that release acrosome content and those remained non-reacted indicates intact spermatozoa. At above right of the figure, the higher aggrandisement of the intact and acrosomal-reacted spermatozoa was depicted. The frequency of the acrosomal-reacted spermatozoa (yellow arrow) is higher in PT-treated aliquots and intact spermatozoa (red arrow) are more frequent in LC-treated samples

stained strongly with Con A, while the intact spermatozoa showed a uniformly staining pattern (Figure 4).

Flow cytometry analyses with FITC-conjugated WGA showed that the incubation for 30 min before freezing led to a significant increase in the percentages of noncapacitated spermatozoa in LC-supplemented compared with PT-supplemented aliquots (p = .0001) and also in the percentages of noncapacitated ones compared with control spermatozoa (p = .0001; Figure 2e,f). Lectin histochemistry revealed that the sperm populations in the smears reacted to various lectins differentially according to their states (intact versus capacitated). WGA reacted to the anterior part of the sperm heads and formed a cap for each spermatozoan. The noncapacitated sperm heads reacted with WGA uniformly with weak reaction (Figure 5).

4 | DISCUSSION

The fertilisation rate has been demonstrated to reduce significantly by freeze–thaw procedure and it may be attributed to cold shock and sperm membrane dysfunction due to oxidative stress or physical damage (Talaei et al., 2010; Nazm Bojnourdi et al., 2008). In addition, phosphatidylserine flip-flopping, as the initial stage of cell death, has been shown to be significantly higher in frozen-thawed human spermatozoa than that of fresh spermatozoa (Aggarwal, 2000). Also, glycoconjugate distribution pattern has been detected to changes in hamster apoptotic germinal epithelial cells (Seco-Rovira et al., 2013). Antioxidant supplementation has been shown to protect the sperm plasma membrane damage by ROS production during freezing and thawing process. ROS formation reduction or antioxidant supplementation impacts cellular condition and in this way, the sperm quality is preserved (Shahrzad, Zahiri, Ghasemi, & Kargar Jahromi, 2013).

There are many studies focusing on the impact of cryopreservation procedures on the sperm plasma membrane damages due to ROS production and decrease in the antioxidant level (Bansal & Bilaspuri, 2010; Bucak, Atessahin, & Yuce, 2008). Both antioxidant in the seminal plasma, which is separated during sperm ART technique manipulations, and lack of the cytoplasmic components in mature spermatozoa containing antioxidants have detrimental effects on the sperm resistance to ROS (Bucak et al., 2008; Seco-Rovira et al., 2013). Therefore, the spermatozoan needs antioxidant supplementations. In the current study, LC supplementation, as an antioxidant before freezing, showed an increase in the number of intact acrosome and noncapacitated spermatozoa. However, PT was superior in improvement of motility.

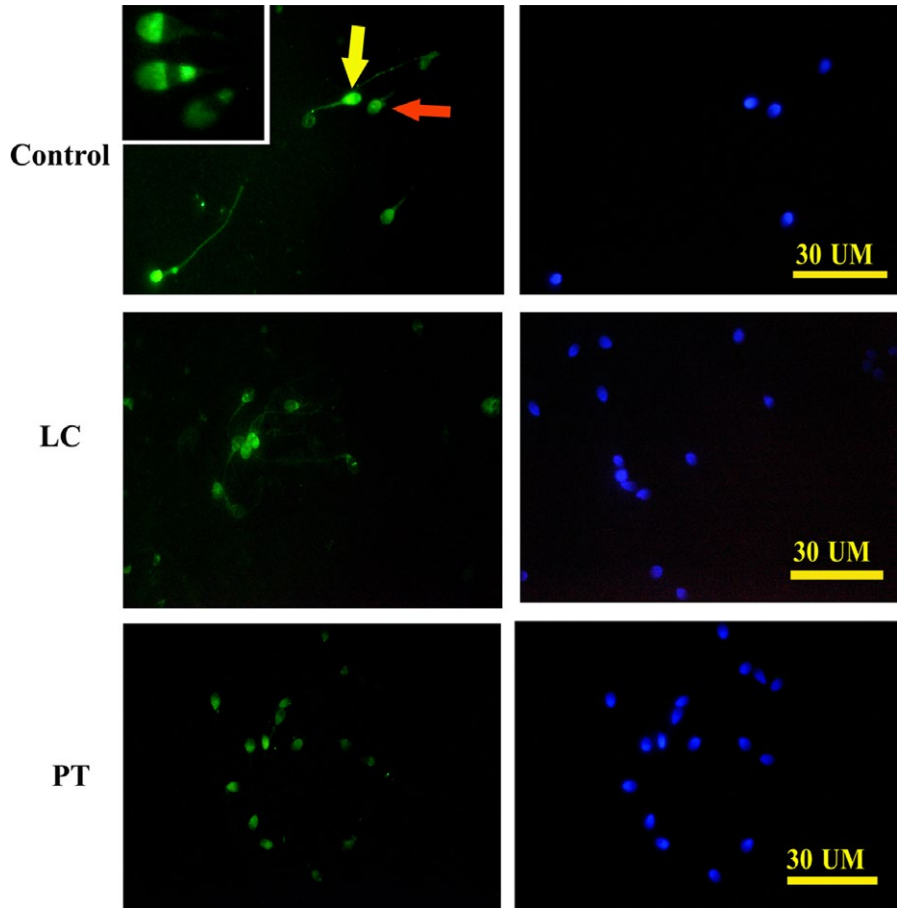

FIGURE 5 The lectin histochemistry with wheat germ agglutinin (WGA) shows the reactivity properties of the spermatozoa pre-treated with L-carnitine (LC) and pentoxifylline (PT) before freezing procedure. Reacted spermatozoa with WGA are noncapacitated spermatozoa and those remained nonreacted indicate capacitated spermatozoa. At above left of the figure, the higher aggrandisement of the intact and acrosomal-reacted spermatozoa was depicted. The frequency of the noncapacitated spermatozoa (yellow arrow) is higher in LC aliquots and capacitate one (red arrow) are more frequent in PT-treated samples

L-carnitine has been reported to protect the spermatozoa from ROS. High concentration of LC in the epididymal fluid has been suggested to stabilise the sperm plasma membrane, increase the sperm survival and reduce acrosome-reacted sperm frequency (Banihani, Agawal, Sharma, & Bayachou, 2014; Manee-in et al., 2014). Our results showed that LC administration led to a significant increase in the number of spermatozoa with intact acrosome as demonstrated by PNA reaction. LC caused a significant increase in the number of noncapacitated spermatozoa as indicated by WGA reaction. These showed the administration of the LC inhibited early capacitation of the spermatozoa and, at the same time, it caused a significant decrease in the number of acrosomal-reacted spermatozoa. The presence of LC in the culture medium mimics in vivo condition in epididymis.

Oral administration of LC induced the maturation of the spermatozoa, improved the sperm motility and maintained sperm cell membrane integrity (Dokmeci, 2005). In vitro administration of the spermatozoa with LC led to a decrease in the sperm acrosomal reaction, and in this way, it could increase the number of the intact spermatozoa as our results indicated (Deana, Indino, Rigoni, & Foresta, 1988). Using LC, as a substance with antioxidant properties, may prevent ROS production and reinforce the cell antioxidant defence (Shahrzad et al., 2013).

Review articles revealed a controversy in the effects of PT on the induction of the sperm capacitation and acrosomal reaction. Some authors reported that PT treatment induced capacitation in fresh human and hamster spermatozoa (Kay, Coutts, & Robertson, 1994; Seshagiri, Thomas, Sreekumar, Ray, & Mariappa, 2003). The results obtained by Mirshokraei et al. (2011) in fresh samples indicated that PT exerted a beneficial effect on the sperm motility but a detrimental effect of PT on sperm capacitation. Esteves et al. pre-treated the spermatozoa with PT before freezing and showed no improvement in motility and viability. They also reported the acrosomal reaction loss during the freeze–thaw process (Esteves, Spaine, & Cedenho, 2007). Our results also showed that PT improved the sperm motility but had no significant impact on the number of capacitate and acrosomal-reacted spermatozoa after thawing. In contrast, the other articles reported a beneficial effect of pre-exposed spermatozoa with PT in viable intact spermatozoa (Esteves et al., 1998). PT is a toxic agent and reduces the sperm survival if it is exposed for a longer period of time (Sato & Ishikawa, 2004). As our data showed, PT enhanced the sperm motility but could not protect the sperm plasma membrane integrity during freeze–thaw process. In contrast, although LC had no impact on the motility, it could protect the plasma membrane.

5 | CONCLUSION

Despite the superiority of PT in enhancement of sperm motility, LC, as a natural agent in the spermatozoa in vivo microenvironment,

acted more efficiently in inhibition of sperm capacitation and acrosomal reaction than PT as indicated by enhancement in frequency of WGA- and PNA-positive cells. Therefore, LC pre-exposure may improve the sperm cryopreservation quality, reduce damages caused by cryopreservation and as a result, may improve the sperm membrane integrity.

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