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Original Article

Effects of L-Carnitine and Pentoxifylline on Carbohydrate Distribution of Mouse Testicular Sperm Membrane

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Abstract

Background: The glycoconjugate content of sperms indicates their physiological and fertility properties. Lectin reactivity is indicative of intact, capacitated, and acrosome-reacted sperms. In the epididymis, sperms experience maturation, glycoconjugate modification, and simultaneously, higher L-carnitine (LC) concentrations. The aim of this project was to evaluate the effects of LC and Pentoxifylline (PF) on the integrity, capacitation, and acrosomal reaction of sperms by studying their lectin reactivity.

Methods: Mouse testicular sperm samples were divided into three parts. Each sample was added Ham's F10 (control) or media containing 1.76 mM LC or PF. At 30 and 90 minutes after incubation, sperm motility was assessed. Peanut agglutinin (PNA), wheat germ agglutinin (WGA), and Concanavalin A (Con A) were used to detect non-acrosome-reacted, non-capacitated, and acrosome-reacted sperms, respectively and the frequency was evaluated by flow cytometry. Statistical analysis was performed using the ANOVA.

Results: Sperm motility increased after 30 and 90 minutes of incubation in the LC- and PF-treated cultures (P=0.001). LC administration created a significant increase in the percentage of the non-acrosome-reacted sperms compared to the control sperms after 30 and 90 minutes (P=0.02 and P=0.03, respectively). The frequency of the non-capacitated sperms in the LC-treated group increased compared to the control sperms after 30 minutes significantly (P=0.01).

Conclusion: Although the administration of LC and PF enhanced sperm motility, LC also impacted glycoconjugates on the sperm surface. Glycoconjugates are involved in the interaction between the sperm and the zona pellucida and subsequently fertilization, thereby probably influencing the male fertility state.

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Introduction

Glycocalyx is composed of glycoconjugates such as glycoproteins, glycolipids, and proteoglycans.[1] In mammals, the glycoproteins of testicular germ cells are important in the sperm differentiation and interactions with Sertoli cells during spermatogenesis.[2] Glycocalyx also

plays an important role in sperm protection against the female immune system, acrosome reaction, and ability of the sperm to fertilize oocytes.[3] Some cases of male infertility may be in consequence of changes in the sperm surface glycoconjugates.[4] In mammals such as humans, the sperms that leave the testis are infertile and some biochemical changes are needed to fertilize the sperms in the reproductive system of males and females. Purohit et al.[4] in 2008 showed that the reaction to wheat germ agglutinin (WGA) of the sperms extracted from oligozoospermic cases was diminished. WGA detects GlcNac residues on the glycocalyx located on the acrosomal membrane surface. GlcNac, as a permanent part of the sperm membrane, is an N-glycan and contributes to the physiochemical properties of the membrane.[4] N-glycans confer extraordinary flexibility to the membrane, which is vitally important for motility.[5]

Lectins are employed *as* an excellent tool for examining glycoconjugate changes in mammalian cells during development, cell differentiation, and maturation. Lectin histochemistry studies have shown that all cell types of spermatogenesis lineage contain mannose, N-acetylglucosamine, sialic acid, galactose, and N-acetylgalactosamine in human,[2] and mouse.[6] The presence of glycoconjugates, including those recognized by lectins such as Concanavalin A (Con A), WGA, and peanut agglutinin (PNA), from the testicular to the ejaculated sperm membrane, has been previously studied.[7] These glycoconjugates are involved in spermatogenesis,[2] capacitation, and acrosomal reaction.[8] The modification of the glycoproteins of the sperm surface is one of the aspects of sperm maturation, which occurs in the epididymis and is correlated with many sperm functions.[9] Acrosome reaction is one of the most important events that occur prior to fertilization.[10] It has been shown that whereas some glycoconjugate-reacted lectins such as Con A and PNA induce acrosome reaction, WGA does not.[11]

In Assisted Reproductive Technology (ART), one of the most important considerations is the production of excessive free radicals, including reactive oxygen species (ROS), due to laboratory manipulations, cellular waste products, presence of large numbers of leukocytes, and immature germ cells. Therefore, sperm samples from males undergoing ART programs have been subjected to oxidative stress and are susceptible to oxidative damage, especially when the samples are exposed to low levels of protective antioxidant.[12] Research has shown that ROS impacts on cellular components such as lipid and protein and the carbohydrate components,[13] of the cell membrane. Immature sperms such as sperms extracted from the testis (TESE) produce much more ROS than ejaculated mature sperms.[14] Sperm stimulators such as Pentoxifylline (PF) and L-carnitine (LC) are ROS scavengers.[12] The epididymis produces many chemicals such as LC, the concentration of which in the epididymis is twice that present in the testis in rats.[15] LC plays an important role in the onset of sperm motility and sperm maturation and it subsequently increases sperm fertility.[16] It has been suggested that high concentrations of carnitine in the epididymal fluid serve to stabilize the sperm plasma membrane,[17] confer a beneficial effect on cell survival, and reduce the number of acrosome-reacted sperms.[18]

The current literature suggests that a large number of agents such as PF improve sperm quality; however, any supplementation may exert both positive and negative effects on the various aspects of sperm functions. For instance, antioxidants such as PF act as a stimulator of the sperm,[19] and can protect the sperm plasma membrane integrity.[20] PF also has a protective effect on the reduction of the occurrence of acrosome reaction during the freeze-thaw process.[21] In patients with oligoasthenozoospermia, treatment of pre-frozen sperms with PF is thought to improve induced acrosome reaction.[22] However, PF is toxic and leads to a reduction in sperm survival if administered for longer than 90 minutes.[23]

With regard to the detrimental effects of free radicals on the structural integrity of membrane glycoconjugates and sperm function, we sought to use a non-toxic antioxidant to reduce oxidative stress. PF is a toxic antioxidant, while LC is a non-toxic antioxidant supposed to preserve the glycoconjugate content of the sperm. Therefore, it is postulated that adding LC could yield an intact sperm with a normal glycoconjugate pattern. The present study was designed to investigate the effects of LC and PF on the glycoconjugate content in the testicular sperm membrane in vitro. We made use of three lectins: PNA to detect acrosome intact sperms; WGA to detect non-capacitated sperms; and Con A to detect acrosome-reacted cells.

Materials and Methods

Animals

Forty-eight male BALB/c mice, weighing 25-30 grams, were acclimatized to the laboratory condition (12 hours of light and 12 hours of darkness at a temperature of 22-24ºC). The mice were kept ad libitum. The animal experiments were approved by the Ethics Committee of Shiraz

University of Medical Sciences.

Lectins

Fluorescein isothiocynate (FITC)-conjugated lectins (Sigma, USA), including PNA, Con A, and WGA, were used to detect N-acetylgalactosamine/galactose, mannose, and sialic acid, respectively. WGA also detects N-acetylglucosamine.

Sperm Preparation

Forty-eight healthy male mice were chosen for the experiments. Testes of 6 mice were removed from the animal under deep anesthesia. The testes were washed with saline and Ham's F10 (Sigma, USA). Under a stereo microscope, the tunica albuginea was separated from the testes, and seminiferous tubules were scattered by two syringes gently. In order to separate red blood cells, Ham's F10 was added to the samples and centrifuged at 500 rpm for 10 minutes. The palette was transferred to a Petri dish, containing Ham's F10, and cut into pieces. The sample was, thereafter, vortexed for one minute to extract the sperms from the tubules.[24] The sample was allowed to remain for one hour at room temperature [23] before it was centrifuged at 500 rpm for 10 minutes. The Leydig and Sertoli cells were placed on the bottom, and the supernatant contained sperms. The supernatant was centrifuged at 1200 rpm for 10 minutes. The palettes that contained sperms were resuspended in 1 mL of Ham's F10.[24] All the experiments were performed 8 times.

Experimental Design

One mL of the sperm sample was aliquoted into three parts. Equal volumes of Ham's F10 and Ham's F10 containing 3.6 mM of LC (Sigma, USA) or PF (Sigma, USA) were added to the aliquoted sperm samples. Therefore, the final concentration of 1.76 mM of LC or PF was obtained.[19]

Sperm Motility Assay

Sperm motility was assessed 30 and 90 minutes after incubation at room temperature. All the motility assessments were performed according to the World Health Organization (WHO) guidelines.[25] To evaluate sperm motility, the sperm cells were classified as immotile (IM, no movement), non-progressive motile (NP, all other patterns of motility with an absence of forward progression, e.g. swimming in small circles, the flagella force hardly displacing the head, or when only a flagella beat can be observed), and progressively motile (PR, spermatozoa moves actively, either linearly or in a large circle, regardless of the speed).[25] The percentage of the motile sperms was calculated according to a previous work conducted by Moreira et al.[26]

Lectin Histochemistry

The smears were prepared from the aliquoted sperms. The smears were fixed with paraformaldehyde for 20 minutes and washed in phosphate buffered saline (PBS) for 30 minutes. Then, the sperms were incubated in FITC-conjugated lectins (10 µL/mL) for 2 hours in the dark. The smears were, subsequently, washed in PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. The specimens were observed under the fluorescent microscope (Nickon, Eclipse, E600).

Flow Cytometry

All the samples were incubated in the media, LC or PF, and were washed with 800 µL of PBS and centrifuged at 1200 rpm for 10 minutes before they were fixed with 2% paraformaldehyde for 30 minutes at 4°C. The fixed samples were centrifuged and resuspended in PBS. Afterward, the samples were incubated in FITC-conjugated lectins (10 µL/mL) for 2 hours at 37°C in a humidified atmosphere in the dark. They were then washed twice in PBS, and the percentage of the lectin-reactive sperms was measured via FL1 channel flow cytometry. The percentage of the spermatozoa that reacted with FITC-conjugated lectins was analyzed by histogram using WINmdi 2.8 software. The mean of fluorescence intensity was also analyzed using FlowJo software.

Statistical Analyses

All the results are presented as mean±SE (standard error of mean). The statistical analyses were performed using the One Way Analysis of Variance (ANOVA) and the Least Significant Difference test (LSD) using SPSS version 15 for Windows. A p value less than 0.05 was considered a statistically significant difference.

Results

Sperm Motility Assay

The data showed a significant increase in the percentage of the progressive sperms exposed to PF compared to the control sperms at 30 minutes (P=0.001) and 90 minutes (P=0.007) after incubation. There was a significant decrease in the percentage of the immotile sperms and a significant increase in the percentage of the non-progressive sperms in the presence of LC and PF compared with the control samples at 30 minutes (P=0.000 and P=0.000) and 90 minutes after incubation (P<0.001 and P<0.001), respectively. Table 1 summarizes the data for the sperm motility assay.

Table 1: The comparison of the testicular sperm motility percentages (mean±S.E; n=8) at 30 and 90 minutes after incubation in the media (control), L-carnitine- and Pentoxifylline

Time (min)	Motility percentages Group	Immotile (mean±S.E)	Non progressive (mean±S.E)	Progressive (mean±S.E)
30	Control	71.43±1	28.19±0.9	0.26±0.13
	L-carnitine	54.71±1.5*	44.58±1.4*	0.68±0.17
	Pentoxifylline	45.51±0.9*¥	53.08±0.8*¥	1.38±0.3*†
90	Control	76.14±1.5	23.77±1.5	0.07±0.07
	L-carnitine	65.80±1.5*	34.10±1.5*	0.08±0.08
	Pentoxifylline	61.30±1.5*†	38.13±1.4*†	0.54±0.16*¥

*Significant difference with control group (P<0.008); †Significant difference with L-carnitine-treated group (P<0.05); ¥Significant difference with L-carnitine-treated group (P<0.009)

Lectin Histochemistry Distribution Pattern

The data demonstrated no changes in the distribution pattern of the glycoconjugates in the testicular sperms in the presence of the additives compared with the media. All the lectins reacted with acrosome: PNA reacted to the middle piece and WGA and Con A reacted to all the parts of the testicular sperms. However, the reaction to Con A was less intense in the acrosome region of the LC- and PF-treated sperms (figure 1).

Effects of LC and PF on Sperm Reaction to WGA

At 30 minutes after incubation, the LC-treated samples exhibited a significant increase in the percentage of the WGA-reactive sperms compared with the control sperms (P=0.01).

At 90 minutes after incubation, there was no significant difference in the percentage of the WGA-reactive sperms between the aliquoted samples; however, a non-significant increase was observed between the percentages of the WGA-reactive sperms in the two treated samples and control samples (figure 2 and table 2).

At 30 and 90 minutes after incubation, the mean of fluorescence intensity for WGA did not show a significant difference between the experimental samples and the control sperms (table 3).

Effects of LC and PF on Sperm Reaction to PNA

At 30 minutes after incubation, the LC-treated sperms showed a significant increase in

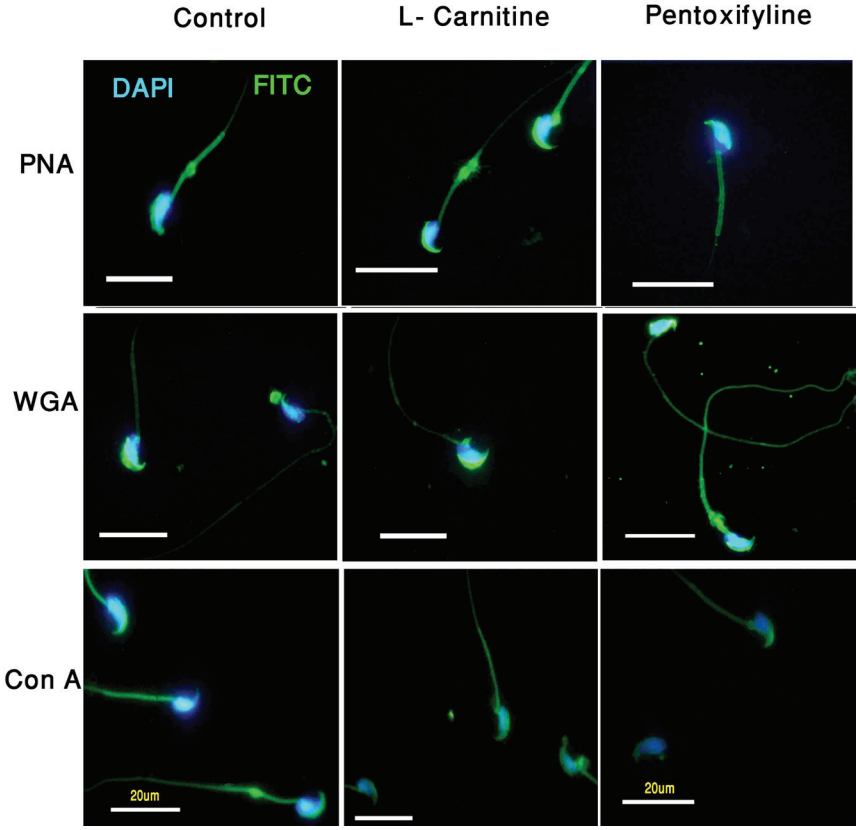

Figure 1: Lectin histochemistery of the testicular sperms exposed to the media (control), L-carnitine and Pentoxifylline at 30 minutes. The acrosomal region reacted to all lectins. PNA reacted with the middle piece, and WGA and Con A reacted to all the parts of the testicular sperm. Scale bar is 20 µm.

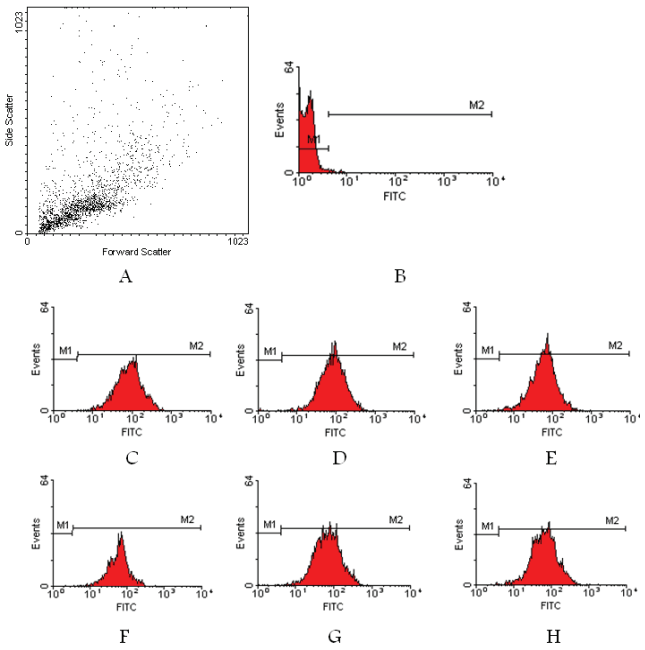

Figure 2: Comparison of WGA-positive sperms after exposure to the media (control), L-carnitine and Pentoxifylline. A: dot plot; B: unstained; C: L-carnitine-exposed sample at 30 minutes (99.72±0.08) (P=0.01); D: Pentoxifylline-exposed sample at 30 minutes (99.54±0.14); E: control sample at 30 minutes (99/08±0.23); F: L-carnitine-exposed sample at 90 minutes (99/80±0.05); G: Pentoxifylline-exposed sample at 90 minutes (99/75±0.04); H: control sample at 90 minutes (99/47±0.21) after incubation

Table 2: The percentages of lectin-reactive sperms (mean±S.E; n=8) at 30 and 90 minutes after incubation in the media (control), L-carnitine and Pentoxifylline

Time (min)	Reacted sperms (%) Group	WGA (mean±S.E)	ConA (mean±S.E)	PNA (mean±S.E)
30	Control	99.08±0.23	83.49±3	89.27±0.5
	L-carnitine	99.72±0.08*	74.38±6	94.09±1.4*
	Pentoxifylline	99.54±0.14	74.53±5	90.24±1.1
90	Control	99.47±0.21	89±3	90.95±1.1
	L-carnitine	99.80±0.05	83.21±5	94.57±1.3*
	Pentoxifylline	99.75±0.04	88.64±3	91.88±0.8

*Significant difference from control (P<0.05)

the percentage of the PNA-reactive sperms compared with the control sperms (P=0.02). PF exhibited no change in the percentage of the PNA-reactive sperms compared with the control and LC aliquots.

At 90 minutes after incubation, the LC-treated samples displayed a significant rise in the percentage of the PNA-reactive sperms compared with the control sperms (P=0.03). The percentage of the PNA-reactive sperms did not show a significant change in the PF-treated samples compared with the control and LC-treated sperms (figure 3 and table 2).

At 30 and 90 minutes after incubation, the mean of fluorescence intensity for PNA did not show a significant difference between the experimental samples and the control sperms (table 3).

Effects of LC and PF on Sperm Reaction to Con A

At 30 and 90 minutes after incubation, the data showed that the percentage of the Con A-reactive sperms had a drop in the LC- and PF-treated samples compared with the control samples; nevertheless, the difference did not constitute statistical significance (figure 4 and table 2). In the LC-treated samples, the mean of fluorescence intensity for Con A displayed a significant decrease compared with the control samples at 90 minutes after incubation (P=0.02) (table 3).

Discussion

Augmentation of the ability of the sperm to fertilize the oocyte is the aim of many ART studies. In the testicular sperm extraction (TESE) technique, it is important to have good quality, matured sperms for the successful application of ART. Our data showed that while the incubation of the samples with LC and PF conferred an increase both in testicular sperm motility and percentages of the PNA- and

Table 3: The mean of fluorescence intensity (mean±S.E; n=8) of lectin-reactive sperms at 30 and 90 minutes after incubation in the media (control), L-carnitine and Pentoxifylline

Time (min)	MFI Group	WGA (mean±S.E)	ConA (mean±S.E)	PNA (mean±S.E)
30	Control	44.53±6	7.10±0.7	56.76±7.4
	L-carnitine	60±10	5±0.6	65.45±7.3
	Pentoxifylline	52.19±4	5.73±0.8	64.18±7.5
90	Control	45.49±5	8.58±0.6	54.87±7.9
	L-carnitine	54.66±9	6±0.6*	62.05±9.7
	Pentoxifylline	48.98±4	6.75±0.9	58.73±5.7

*Significant difference from control (P<0.05)

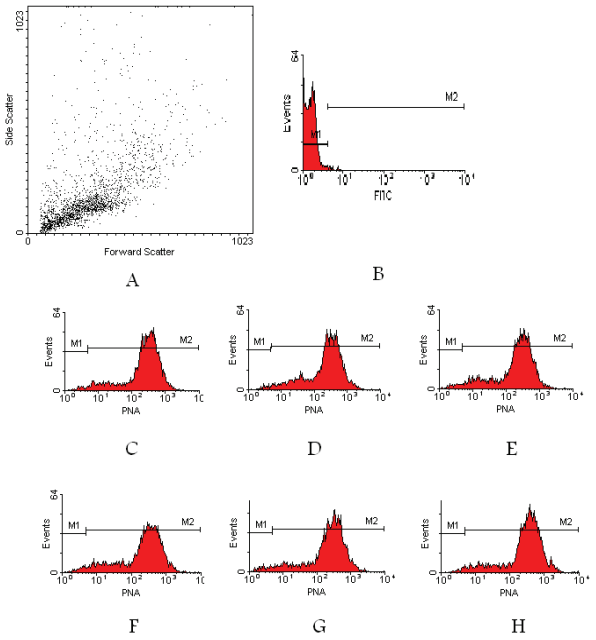

Figure 3: Comparison of PNA-positive sperms after exposure to the media, L-carnitine and Pentoxifylline. A: dot plot; B: unstained; C: L-carnitine-exposed sample at 30 minutes (94.09±1.4) (P=0.02); D: Pentoxifylline-exposed sample at 30 minutes (90.24±1.1); E: control sample at 30 minutes (89/27±0.5); F: L-carnitine-exposed sample at 90 minutes (94/57±1.3) (P=0.03); G: Pentoxifylline-exposed sample at 90 minutes (91/88±0.8); H: control sample at 90 minutes (90/95±1.1) after incubation

WGA-reactive sperms, it led to a decrease in the number of the Con A-reactive sperms by comparison with the control group. Our findings also revealed that the mean of fluorescence intensity was also decreased in the acrosome-reacted sperms in the LC-treated group compared with the control samples as indicated by the flow cytometry of the Con A-reactive sperms. Poor reaction to PNA has been demonstrated by lectin histochemistry in human and mouse spermatogonia; however, the reaction is known to increase in other spermatogenesis lineages as the acrosome development gradually progresses. WGA and Con A also react to mouse and human spermatogenesis lineages. The presence of galactose/N-acetylgalactosamine, mannose, N-acetylglucosamine, and sialic acid in testicular germ cells can also be detected by lectin histochemistry.[2,6] In the present study, lectin histochemistry displayed that the administration of LC and PF did not change the distribution pattern of glycoconjugates in the sperms after 30 or 90 minutes of incubation.

PNA can be deemed an intact sperm acrosome marker in human,[27]and mouse.[28] The ultrastructure of sperms is reported to show that the PNA-binding site is situated on the outer acrosomal membrane.[29] Acrosome-reacted sperms are believed to react with Con A strongly, and the ultrastructure of sperms is reported to demonstrate that the Con A-binding site is situated on the inner acrosomal membrane.[30] WGA reacts more intensely to intact and non-capacitated sperms, so WGA can be considered as a non-capacitated mouse sperm marker.[31]

ROS is produced by ART manipulations.[12] Gil-Guzman,[14] showed that the ROS production occurred much more frequently in immature sperms (TESE) than in mature ones. Oxidative stress can influence cell membrane components such as carbohydrates.[13] Glycoconjugates have

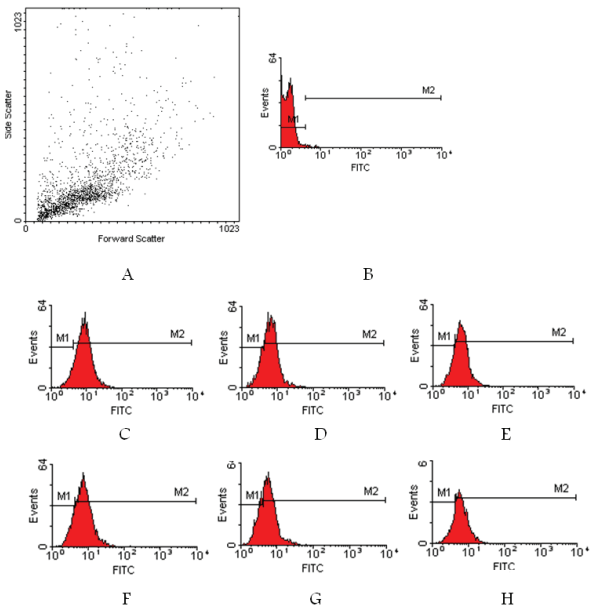

Figure 4: Comparison of Con A positive sperms at exposure to the media, L-carnitine and Pentoxifylline. A: dot plot; B: unstained; C: control sample at 30 minutes (83.49± 3); D: Pentoxifylline-exposed sample at 30 minutes (74.53±5); E: L-carnitine-exposed sample at 30 minutes (74.38±6); F: control sample at 90 minutes (89±3); G: Pentoxifylline-exposed sample at 90 minutes (88.64±3); H: L-carnitine-exposed sample at 90 minutes (83.21±5) after incubation

important roles in the interaction between sperm and zona pellucida and, subsequently, in fertilization;[32] therefore, any change in the membrane glycoconjugates may affect fertility.[4] ROS scavengers such as LC and PF may affect sperm metabolism,[12] motility,[33,34] and membrane.[17,20] Our results showed that the LC administration led to a significant rise in the number of the sperms with intact acrosomes as demonstrated by PNA reaction. LC caused a significant increase in the number of the non-capacitated sperms after 30 minutes of incubation as indicated by WGA reaction. As the time progressed, LC effected an increase in the number of the sperms with intact acrosomes, but no change in the number of the non-capacitated sperms was observed; this showed that the administration of LC inhibited the early capacitation of the sperms as indicated by WGA reaction. The in vivo administration of LC, as an antioxidant, can affect sperm maturation and motility,[35] and stabilize the sperm cell membrane.[17] The in vitro administration of LC diminishes acrosomal reaction,[18] and thus increases the number of intact sperms, which chimes in with our results. LC is also thought to protect the sperm plasma membrane and affect carbohydrate metabolism.[18]

PF acts as an anti-oxidant,[19] and can protect the fresh sperm plasma membrane,[20] normal frozen-thawed sperm,[21] and oligoasthenozoospermic patients.[22] The protective effect of PF on the acrosome loss was also reported by Esteves et al.[21] Our results also showed that PF did not impact on the incidence of capacitated or acrosome-reacted sperms. Review articles have revealed a controversy vis-à-vis the effects of PF on the induction of sperm capacitation and acrosomal reaction. Some authors have reported that PF treatment can induce capacitation in human,[36] and hamster.[37] Mirshekari et al.[38] showed no changes in the number of the capacitated canine sperm by a low dose of PF administration;[38] our data confirm this finding.

Although both LC and PF increase sperm motility, it seems that LC prevents early capacitation and acrosome loss more effectively than PF. Our data showed that LC prevented early capacitation (as indicated by WGA reaction) and subsequently inhibited acrosome reaction (as indicated by PNA and Con A reaction). Our previous work demonstrated that LC administration also improved nuclear maturation criteria more effectively than PF.[39]

Conclusion

PNA- and WGA-positive sperms indicated the intact and non-capacitated sperm populations, respectively, in the present study. Whereas supplementation of the testicular sperm samples with both LC and PF enhanced sperm motility, LC was more efficient in the inhibition of sperm capacitation and acrosomal reaction as indicated by the enhancement in the percentages of WGA- and PNA-positive cells. Our results showed that LC was more suitable than PF for utilization in the ART

protocol for increasing sperm integrity.

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Conflict of Interest: None declared.

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