4.1. Experiment No. I

Cyproterone acetate (CPA) induced testicular dysfunction in Wistar strain male albino rat: a study for infertile model animal

4.1.1 Significance of the study

This experiment was performed to confirm the development of an infertile rat model by sensor analysis covering spermatogenic, androgenic and histological domains in cyproterone acetate (CPA) treated condition. Depending on previous work, the dose and the duration of cyproterone acetate treatment were determined in connection with development of infertile model animal.

4.1.2 Experimental Design

To fulfil the aim of the experiment, twelve male Wistar strain albino rats were used. The average body weight of rats was 120 ± 10 g and they were of 80-90 days old. Experimental animals were kept in clean, dry and sterilized polypropylene cages with 12 hour light: 12 hour dark cycle. The room temperature and relative humidity of the room were 25 ± 2 ºC and 45-60% respectively. During pre-experimental period, all the animals were handled in regular basis to acclimatise them with the human contact and the environment of the room to minimise the physical stress imposition on them which may occur during experimentation. Animals were allowed to have a free access to standard rat chew and water ad libitum.

Prior approval was taken from the Institutional Ethic Committee (IEC) [IEC/3/C-4/14, dated 03/11/2014] to conduct the experiment as per CPCSEA guideline.

Animals were categorised broadly in two groups:

Group I: ***Vehicle treated control group-*** Six, healthy, fertile, normal male rats were subjected to oral administration of distilled water by force feeding through gavage at the volume of 0.5 ml/ 100 g body weight per day for 30 days.

Group II: ***CPA treated group-*** Another six male albino rats were allowed for the oral intubation of cyproterone acetate at 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days through gavage.

The duration of the experiment was 30 days. Animals were treated with CPA in 12 hours fasting condition at 8 A.M. Then at 12 .30 P.M. and 8 P.M. animals were provided with food to avoid drug nutrient interaction.

On 31st day of the experimental duration, all the animals were sacrificed by using euthanasia followed by decapitation. Blood was collected from dorsal aorta followed by serum separation by centrifugation at 3000 g for 5 min for analysing toxicity marker. Body weight and organo-somatic indices including testicles, cauda epididymis and pair of prostate were recorded after dissection from the body. Finally all the separate organs and serum were preserved at -20 º C for the assessment of relevant parameters except cauda epididymis which was used for spermiological sensors.

4.1.3 Parameters and methods

Sperm count was assessed by dissecting the cauda of epididymis following the standard protocol **(Pant and Srivastava, 2003)**. Sperm viability **(WHO, 1999)** and sperm motility **(Zemjanis, 1977)** were determined to cover spermatogenic domain. Androgenic profile was assessed by determining the activities of Δ^5, 3β-HSD **(Talalay, 1962)** and 17β-HSD **(Jarabak et al., 1962)**. Testicular cholesterol level was also measured to confirm the status about androgen synthesis **(Plummer, 1995).** Oxidative stress domain was assessed by measuring the activity of catalase **(Beer, 1952)** and superoxide dismutase **(Marklund and Marklund, 1974)** in sperm pellet, prostate and testis. Toxic effect of CPA was determined by

analysing the activities of serum GOT and GPT by kit method **(Henry et al., 1960)**. Histology of testis was performed by haematoxylin-eosine staining following standard protocol **(Ghosh et al., 2014)** and seminiferous tubular diameter was measured by using the software "Dewinter calliper pro 3.0" along with qualitative assessment of seminiferous tubular diameter **(Ghosh et al., 2014).**

'Analysis of varience' (ANOVA) followed by multiple comparison two-tail *t*-test was adopted **(Sokal and Rohle, 1997)** for analysis the collected data statistically and to find out whether the result were significant or not in respect to independent variable.

4.1.4 Results

4.1.4.1 Body weight and organo-somatic indices

Final body weight of the CPA treated albino rat showed a significant ($p < 0.05$) downward deviation in comparison to the vehicle treated control. Similarly, testicular organo-somatic indices such as the weight of testicles, seminal vesicle, epididymis, prostate in CPA treated were decreased significantly ($p < 0.05$) in respect to the vehicle treated control **(Table 4.1.1)**.

4.1.4.2 Sperm count, sperm motility and sperm viability

A significant ($p < 0.05$) diminution was noted in sperm count after the treatment with CPA at 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day compared to the vehicle treated control. Number of viable sperm were also significantly ($p < 0.05$) decreased in CPA treated group in respect to the vehicle treated control after CPA treatment for 30 days **(Table 4.1.2)**.

4.1.4.3 Activity of androgenic key enzymes

Activities of androgenic key enzymes such as testicular Δ^5, 3β-HSD and 17β-HSD were significantly ($p < 0.05$) decreased after CPA treatment at 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days in comparison to the vehicle treated control **(Fig. 4.1.1)**.

4.1.4.4 Testicular cholesterol

Significant ($p < 0.05$) elevation in the testicular cholesterol level was noted after oral administration of CPA for 30 days in CPA treated group in comparison to the vehicle treated control **(Fig. 4.1.2)**.

4.1.4.5 Catalase activity

Activity of catalase in sperm pellet, testis and prostate showed a significant ($p < 0.05$) downward deviation in CPA treated group when comparison was made with the vehicle treated control **(Fig. 4.1.3)**.

4.1.4.6 Activity of SOD

Superoxide dismutase activity was significantly ($p < 0.05$) decreased in sperm pellet, testis and prostate compared to the vehicle treated control after 30 days treatment with CPA at 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day **(Fig. 4.1.4)**.

4.1.4.7 Activities of SGOT and SGPT

Serum GOT and GPT activities were significantly ($p < 0.05$) increased after treatment with CPA at 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days in CPA treated group compared with the vehicle treated control group **(Fig. 4.1.5)**.

4.1.4.8 Seminiferous tubular diameter

A significant ($p < 0.05$) diminution in the seminiferous tubular diameter was noted in CPA treated group after treatment with CPA for 30 days when comparison was made by the vehicle treated control **(Fig. 4.1.6)**.

4.1.5 Discussion

The experiment was conducted to develop an infertile model by applying proper dose of CPA i.e. 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days.

Cyproterone acetate is an anti-androgenic drug **(de Voogt, 1992)**. It's anti-androgenic property was noted in the activities of testicular Δ^5, 3β-HSD and 17β-HSD which are the androgenic key enzymes. The enzyme activities were decreased after CPA treatment. This results were again repeated by the elevated level of testicular cholesterol which is the mother molecule of testosterone synthesis **(Joshi et al., 2005)**. This occurs due to low utilization of cholesterol in testicular androgenesis as CPA plays **(Haider and Rai, 1986)**. This anti-androgenic efficacy of CPA has been further supported by diminution in the sex organs such as testis, epididymis, seminal vesicle and prostate **(Haider and Rai, 1986)** as the growth and development of such organs were under the influence of androgen **(Morohashi et al., 2013)**.

Remarkably diminution after CPA treatment was noted in the seminiferous tubular diameter due to interruption in the spermatogenesis by CPA **(Saidapur et al., 1981)**. Cyproterone acetate treated group also showed a low population density of mature germ cell in the seminiferous tubular cavity. Sperm motility, sperm count, sperm viability are the main indicators of semen quality analysis (**WHO, 1999**). Cyproterone acetate directly affects the mid piece and the tail of the sperm **(Fredricsson and Carlström, 1981)**. Thus it affects the sperm motility which was focused in our experiment where sperm motility and viability were significantly decreased after CPA administration. On the other hand, CPA has been reported to reduce spermatogenesis **(Meriggiola et al., 1998)**. This fact was also reflected in this experiment where sperm count was significantly reduced in CPA treated group.

Oxidative stress plays a major role in the development of several pathological conditions including degradation in the reproductive health **(Burton, 2011)**. Free radical generation in the body can always be a cause of low activity of antioxidant enzymes like catalase, superoxide dismutase, peroxidase, glutathione-s-transferase etc. Oxidative stress development in the testis, sperm pellet and prostate was noticed by analysing the activities of SOD and catalase which was significantly decreased in CPA treated group in our experiment as CPA

may synthesise high amount of uncoupler protein in the said reproductive organ (**Stabler et al., 2010**).

Serum GOT and GPT activities were increased in CPA treated group. Cyproterone acetate has already been reported to trigger the risk of hepatotoxicity **(Savidou et al., 2006)**. Beside that free radical generation in high level due to CPA administration also responsible to increase the activities of serum GOT and GPT.

4.1.6 Conclusion

From the experiment, it can be concluded that CPA can be able to develop an infertile animal model by hindering testosterone synthesis, damaging the morphological structure of the sperm and by generation of the oxidative stress in reproductive organs.

Table 4.1.1 Effect of cyproterone acetate on body weight and reproductive organo-somatic indices

Group	Initial body Weight (g)	Final body weight (g)	Testiculo-somatitic indices (g%)	Epididymal somatic indices (g%)	Seminal vesiculo somatic indices (g%)
Vehicle treated control	128 ± 3.1^{a}	145 ± 5.2^{a}	2.51 ± 0.04^{a}	1.16 ± 0.02^{a}	0.27 ± 0.03^{a}
CPA treated	126 ± 2.6^{a}	121 ± 2.1^{b}	1.50 ± 0.04^{b}	0.20 ± 0.01^{b}	0.11 ± 0.03^{b}

Data were expressed as mean ±SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Values with different superscripts (a, b) in each vertical column differ from each other significantly at $p < 0.05$.

Table 4.1.2 Spermiological sensors of the rat after treatment with CPA

Group	Sperm count (Millions/ ml)	Motile sperm (%)	Viabile Sperm	
			Alive spermatozoa (%)	Dead Spermatozoa (%)
Vehicle treated control	26.20 ± 0.99^{a}	76.83 ± 1.66^{a}	87.00 ± 2.32^{a}	13.00 ± 0.18^{a}
CPA treated	14.20 ± 1.05^{b}	46.14 ± 1.05^{b}	40.60 ± 0.85^{b}	59.40 ± 1.23^{b}

Data were expressed as mean ±SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Values with different superscripts (a, b) in each vertical column differ from each other significantly at $p < 0.05$.

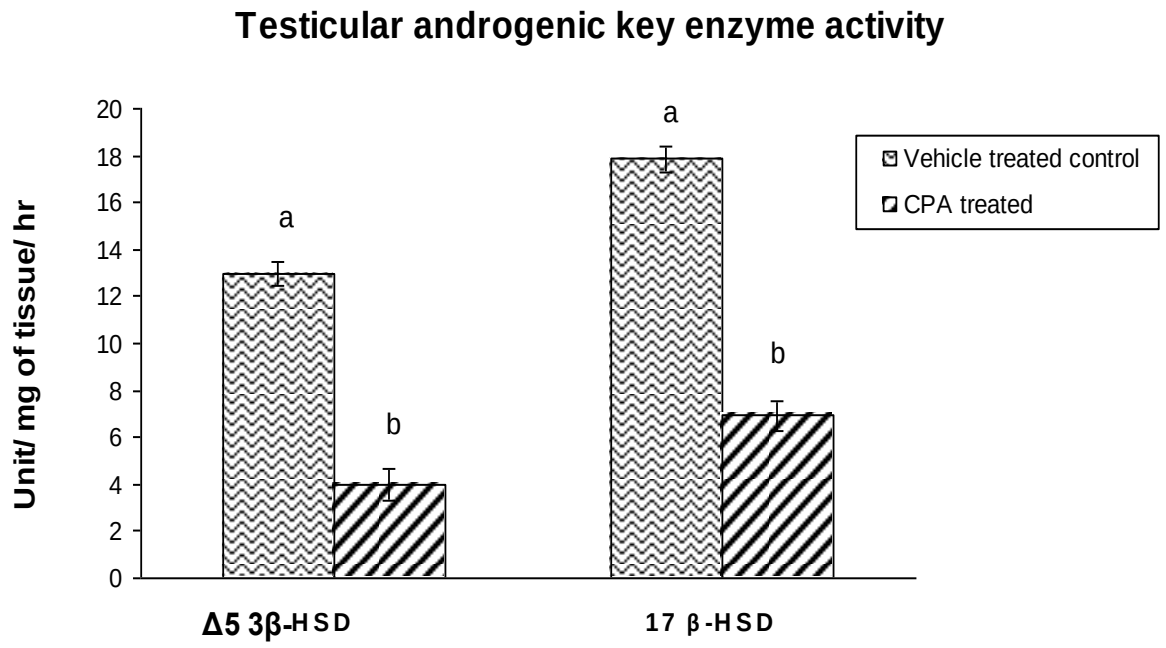


Figure 4.1.1 Activity of testicular Δ^5, 3β-HSD and 17β-HSD after cyproterone acetate treatment. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b) differ from each other significantly, $p < 0.05$

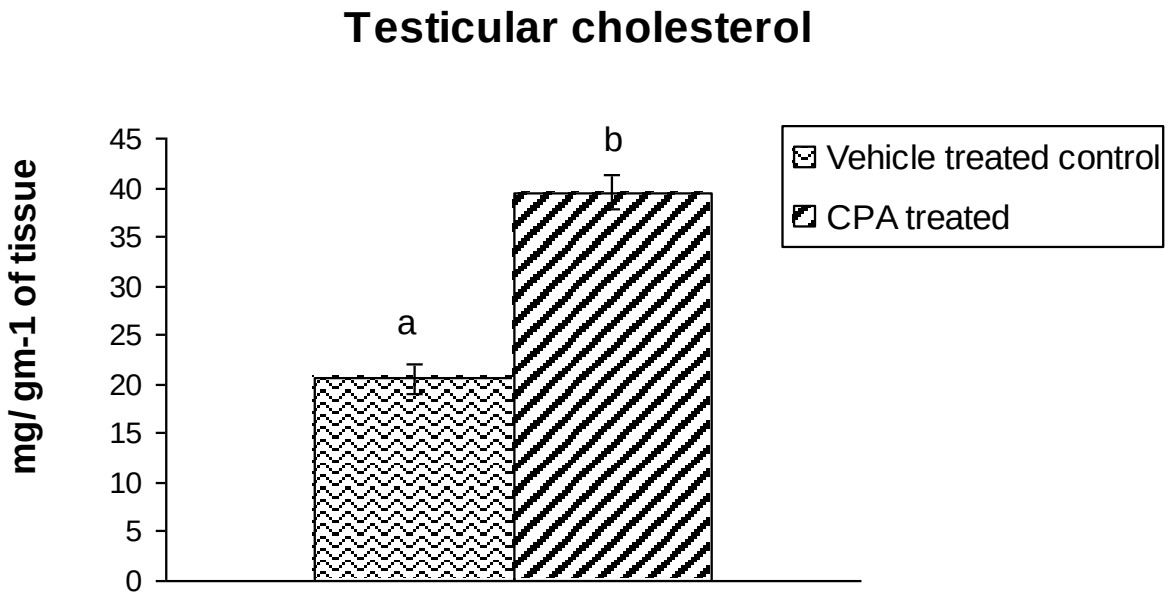


Figure 4.1.2 Effect of cyproterone acetate on testicular cholesterol level in male albino rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b) differ from each other significantly, $p< 0.05$

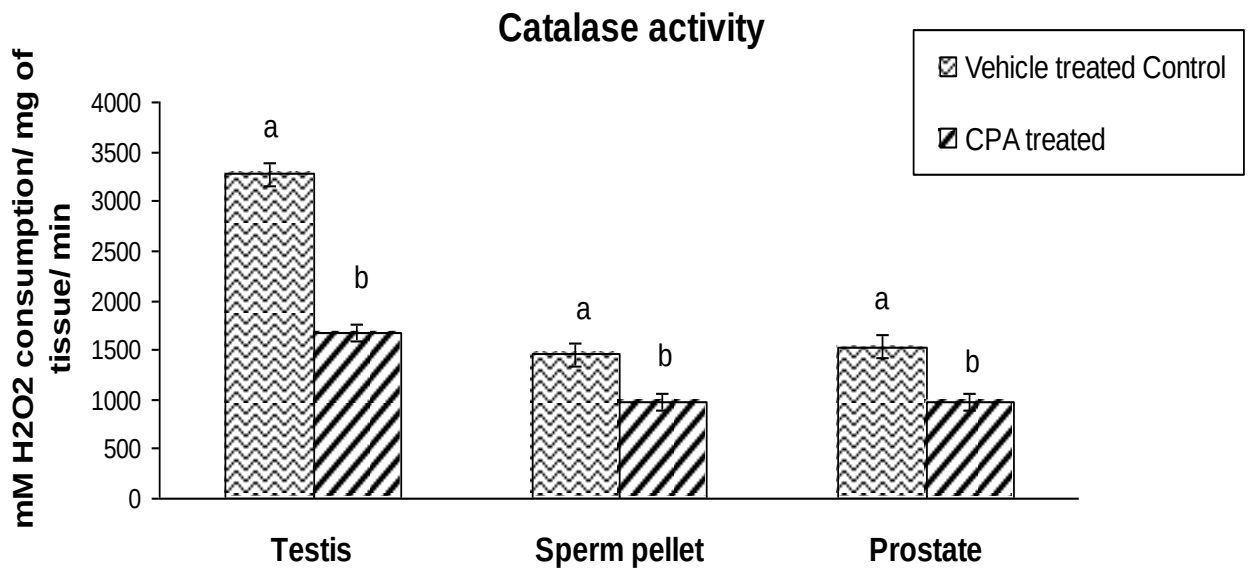


Figure 4.1.3 Catalase activity in testis, sperm pellet and prostate in CPA treated albino rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b) differ from each other significantly, $p < 0.05$

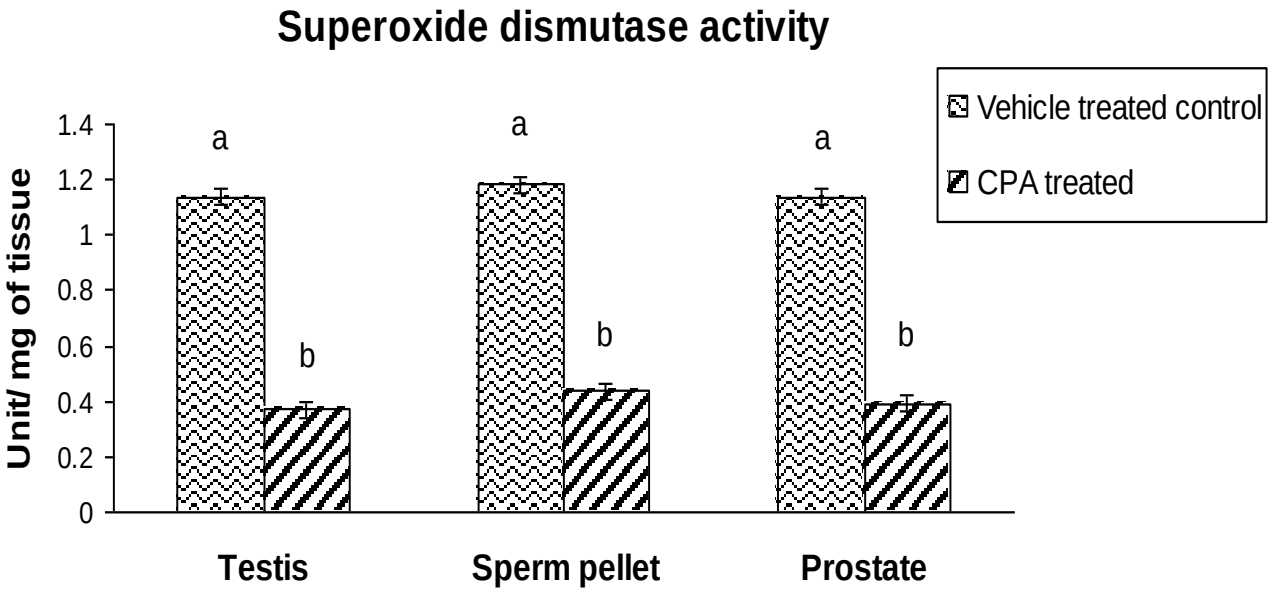


Figure 4.1.4 Adverse effect of cyproterone acetate on superoxide dismutase activity in testis, sperm pellet and prostate in albino rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-tes"t. Bars with different superscripts (a, b) differ from each other significantly, $p < 0.05$

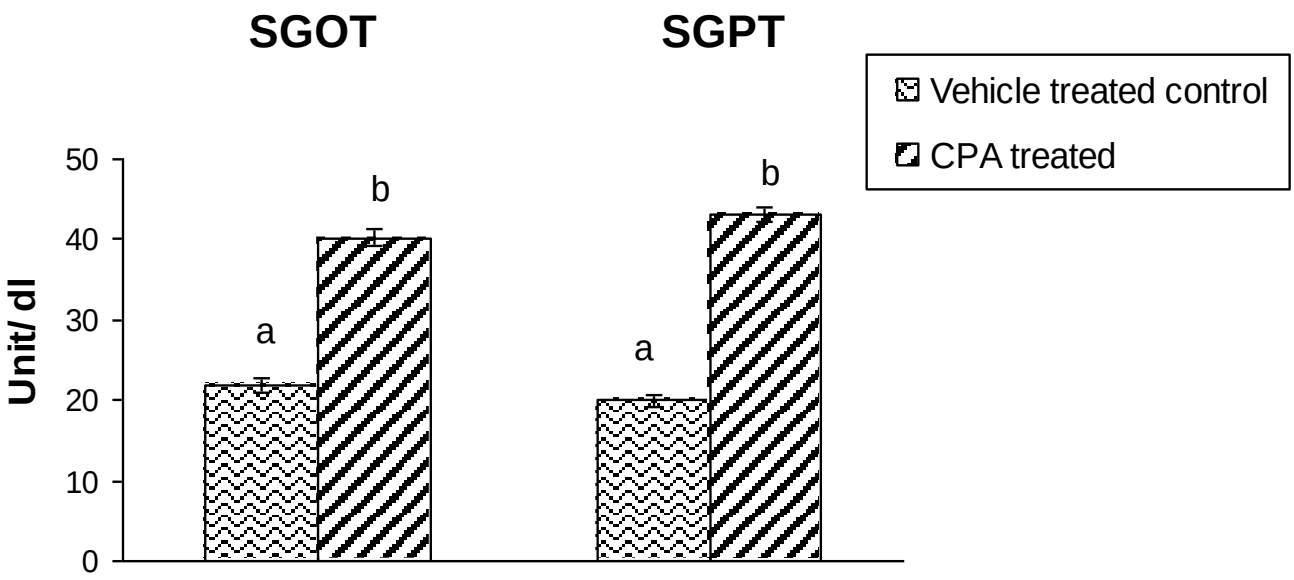


Figure 4.1.5 Toxicity assessment of CPA in general in male albino rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b) differ from each other significantly, $p < 0.05$

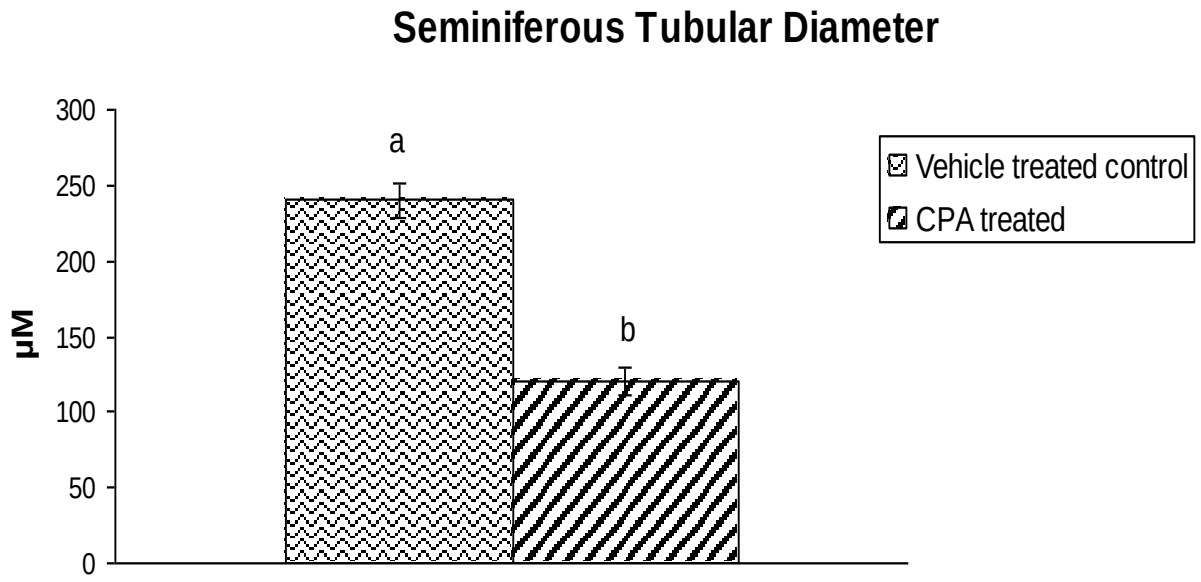


Figure 4.1.6 Negative deviation in the seminiferous tubular diameter after cyproterone acetate administration to the male albino rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b) differ from each other significantly, $p < 0.05$

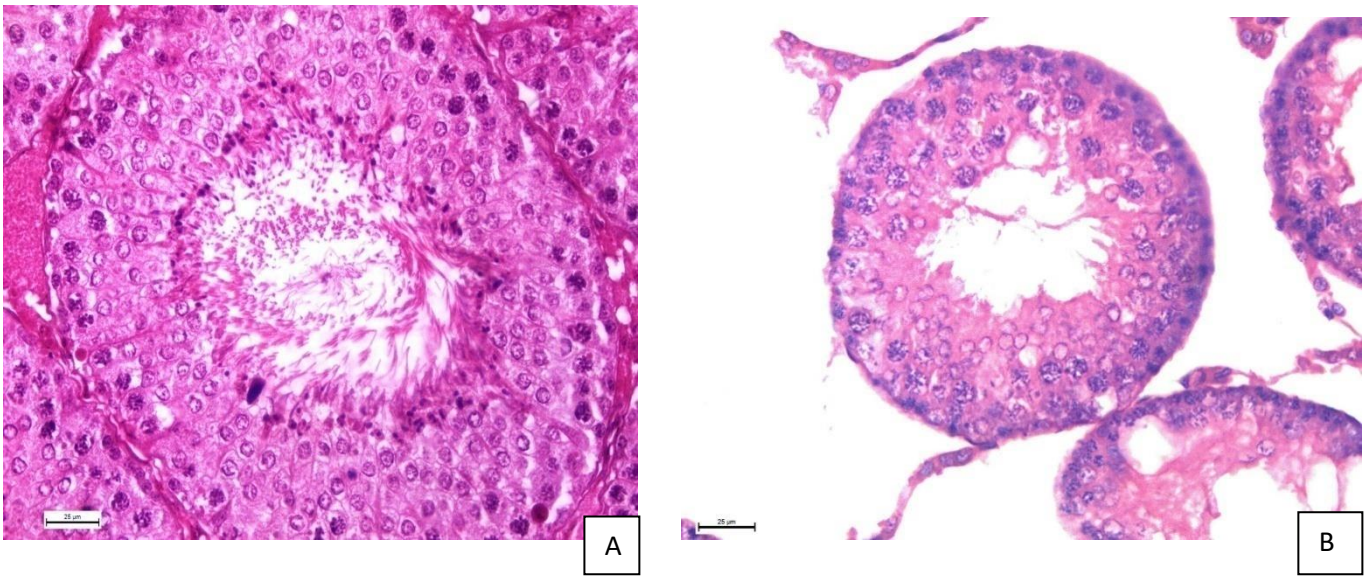


Plate 4.1.1: Histology of testis 400X (Hematoxyline-Eosin Stain). Representative microphotographs of A) Vehicle treated control and B) CPA treated rat where the size of seminiferous tubule in CPA treated rat is remarkably smaller than the vehicle treated control.

4.2. Experiment No. II

Protective efficacy of a potent nutraceutical, lycopene on CPA induced hypo-testicular activities in Wistar strain male albino rat: A dose dependent study for infertility management

4.2.1 Significance of the study

The objective of the present experiment was to find out the most effective dose or potent dose of the lycopene having maximum revival efficacy to the cyproterone acetate (CPA) treated androgenic and spermatogenic interruptions, low anti-oxidative enzyme activity and other related male reproductive domains in Wistar strain male albino rat.

4.2.2 Experimental Design

Thirty-six animals were considered for the fulfilment of the aim of the present experiment. All the experimental animals, weighing 120 ± 10 g were kept in dry and clean polypropylene cage at 25 ± 2 ºC temperature, 12 h light: 12 h dark cycle along with 45-60% related humidity. Fifteen days prior the experiment, all the animals were handled to acclimatize them with human contact in regular basis to minimise the physiological stress due to handling. The animal house was maintained in well ventilated, clean and dry conditions. All the animals were provided with standard feed and water *ad libitum.* Institutional Ethical Committee (IEC) [IEC/3/C-4/14, dated 03/11/2014] has approved the experiment and the whole experiment was conducted as per the guideline of CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) regulation, Govt. of India.

All the animals except the vehicle treated control group were orally administered with CPA for first 30 days of the experiment to achieve infertile state at the concerned dose mentioned below. After first 30 days of CPA administration, six animals were separated and allowed for CPA administration for another 30 days to sustain the infertile state. These animals were

grouped as cyproterone acetate treated group. Other twenty-four animals were divided into four doses of lycopene treated group. The animals of the lycopene treated group were allowed for oral intubation of lycopene at different does along with CPA for last 30 days of the experiment.

The duration of the experiment was 60 days. Animals were divided into six groups and each group contains six animals as follows:

Group I: ***Vehicle treated control group:*** Normal, healthy, fertile animals were subjected to 0.5 ml Tween-80/ 100 g body weight/ day for last 30 days during lycopene treatment.

Group II: ***CPA treated group:*** Animals in this group were allowed for oral administration of cyproterone acetate at 3 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 60 days.

Group III: ***CPA+ lycopene (0.75 mg dose treated group):*** Cyproterone acetate pre-treated proven infertile treated albino rats were administered with lycopene at the dose of 0.75 mg/ 0.5 ml Tween-80/ 100 g body weight/ day for last 30 days of the experiment along with CPA.

Group IV: ***CPA+ lycopene (1.5 mg dose treated group):*** Cyproterone acetate treated infertile rats of this group were orally treated with lycopene at 1.5 mg/ 0.5 ml Tween-80/ 100 g body weight/ day for 30 days along with CPA.

Group V: ***CPA+ lycopene (3.0 mg dose treated group):*** Animals previously treated with CPA with proven infertility, were subjected to oral administration of lycopene at 3.0 mg/ 0.5 ml Tween-80/ 100 g body weight/ day orally for 30 days with CPA.

Group VI: ***CPA+ lycopene (4.5 mg dose treated group):*** Cyproterone acetate induced infertile rats which were previously treated with CPA, were allowed for oral intubation of lycopene at 4.5 mg/ 0.5 ml Tween-80/ 100 g body weight/ day orally for last 30 days of the experiment along with CPA.

Cyproterone acetate was provided at 7 A.M. at 12 hours of fasting condition to the CPA treated or lycopene doses treated groups for the development of infertile condition. After

three hours of the CPA treatment i.e., 10 A.M. food was provided to the above mentioned groups and this protocol was followed for first 30 days. But for last 30 days of the experiment, all lycopene dose treated groups were allowed for CPA administration at 7 A.M. and after 3 hours of the drug administration i.e., 10 A.M., food was provided to these groups. Feed box was cleaned from all the lycopene dose treated groups at 1 P.M. Then at 5 P.M. lycopene was provided at its four different doses to the concerned dose treated groups and just after two hours i.e. at 7 A.M. of lycopene treatment, food was supplied to the animals to avoid nutrient-drug interaction if any.

All the experimental animals were sacrificed by using euthanasia on the 61^{st} day of the experimental duration. Serum was separated after collection of the blood from dorsal aorta and centrifugation at 3000 x g for 5 min for toxicity assessment. Sperms were collected from cauda epididymis. Sperm pellet was prepared by centrifugation of the sperm suspension at 3000 x g for 10 min. Then dissected testis, cauda epididymis and prepared sperm pellet and serum were preserved at -20ºC for analysis of relevant parameters.

4.2.3 Parameters and methods

Sperm motility **(Zemjanis, 1977)**, sperm viability **(WHO, 1999)** and sperm count **(Pant & Srivastava, 2003)** were assessed by standard protocol. These three sensors covered the spermiological domain. Plasma membrane integrity was determined by hypo-osmotic swelling test **(Jayendran et al., 1984)** and acrosomal status **(Gopalkrishnan et al., 1995)** by following standard protocol. Activities of testicular androgenic key enzymes such as Δ^5, 3β-HSD **(Talalay, 1962)** and 17β-HSD **(Jarabak et al., 1962)** were measured as per standard protocol. Confirmation of steroid synthesis was achieved by measuring the level of testicular cholesterol **(Plummer, 1995)**. Serum testosterone **(Srivastava, 2001)** was also measured to determine alteration in androgenenesis. Oxidative stress was measured by analysing the

activities of the anti-oxidant enzymes such as catalase **(Beer, 1952)** and peroxidase **(Sadasivam and Manickam, 2008)** in testis and sperm pellet by standard methods. Level of anti-oxidant end products such as CD **(Slater, 1984)** and TBARS **(Okhawa et al., 1979)** were also measured in testis to confirm the oxidative stress generation. Toxicity was measured by assessing serum albumin **(Rodkey, 1965)**, serum globulin **(Reinhold, 1980)** and serum total protein **(Rodkey, 1965)**. Testicular tissue was allowed for Heamatoxylin-eosin staining and the seminiferous tubular diameter was measured by using a software 'Dewinter calliper pro 3.0' as per the standard protocol **(Ghosh et al., 2014)**. Statistical analysis was conducted as per standard method **(Sokal and Rohle, 1997)**.

4.2.4 Results

4.2.4.1 Sperm motility, Sperm viability, Sperm count

Sperm motility was significantly ($p < 0.05$) decreased in CPA treated group in comparison to the vehicle treated control. Oral administration of lycopene at 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg dose along with CPA resulted significant ($p < 0.05$) recovery in sperm motility towards the vehicle treated control compared to the CPA treated group. Recovery in sperm motility 0.75 mg dose was statistically significant ($p < 0.05$) in comparison to all the other doses but 1.5 mg, 3.0 mg and 4.5 mg did not show any significant *(*$p > 0.05$) difference with each other. In this concern, 1.5 mg dose maximum efficacy in connection with the rectification from infertile condition **(Table 4.2.1)**.

A significant ($p < 0.05$) diminution was noted in sperm viability in CPA treated group in comparison to the vehicle treated control. Lycopene administration at different doses along with CPA showed a significant ($p < 0.05$) recovery in sperm viability towards the vehicle treated control. Among the four doses of lycopene, the lower dose i.e. 0.75 mg dose showed significant ($p < 0.05$) difference in connection with rectification in sperm viability with all the

other doses such as 1.5 mg, 3.0 mg and 4.5 mg. But these higher doses did not significantly ($p > 0.05$) differ with each other. Lycopene at the dose of 1.5 mg/ 0.5 ml tween 80/ 100 g body weight/ day showed maximum recovery out of doses used here in sperm viability **(Table 4.2.1)**.

Sperm count exhibited a significant ($p < 0.05$) downward deviation in CPA treated group compared to the vehicle treated control after treatment with CPA at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day. Lycopene administration at the dose of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg showed a significant ($p < 0.05$) rectification in sperm count towards the vehicle treated control. Lower dose i.e., 0.75 mg focused a significant ($p < 0.05$) difference compared to the higher doses. But 1.5 mg, 3.0 mg and 4.5 mg dose did not exhibit any significant ($p > 0.05$) variation in upward direction with each other **(Table 4.2.1)**. So, 1.5 mg dose was considered as potent dose in this concern.

4.2.4.2 Hypo-osmotic swelling test

Cyproterone acetate treatment showed a significant ($p < 0.05$) diminution in the number of hypo-osmotically swelled coiled-tail of sperms in the CPA treated group compared to the vehicle treated control group. Lycopene administration at the dose of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg exhibited a significant ($p < 0.05$) rectification in the number of coiled tail of sperm. Among all the doses, 0.75 mg showed a significant ($p < 0.05$) difference with all the other doses such as 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg. On the other hand, all the three higher doses did not show any significant ($p > 0.05$) alteration among each other towards the positive direction. In this concern, 1.5 mg dose showed maximum recovery **(Table 4.2.1)**.

4.2.4.3 Acrosomal status

A significant ($p < 0.05$) downward deviation was noted in sperm acrosomal status in CPA treated group after CPA administration at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days in respect to the vehicle treated control. After treatment with

lycopene at the doses of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg exhibited a significant ($p < 0.05$) recovery in the acrosomal status towards the vehicle treated control in respect to the CPA treated group. Remarkable correction in the said parameter was noted in 1.5 mg, 3.0 mg and 4.5 mg doses compared to the 0.75 mg dose. But the higher doses did not show any significant ($p > 0.05$) difference with each other. Maximum recovery was observed at the dose of 1.5 mg dose **(Figure 4.2.1)**.

4.2.4.4 Testicular Δ5, 3β-HSD and 17β-HSD activity

Activities of testicular Δ5, 3β-HSD and 17β-HSD were significantly ($p < 0.05$) deviated in down ward manner in CPA treated group compared to the vehicle treated control. Lycopene administration at the doses of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg exhibited a significant ($p < 0.05$) recovery in the activities of the said androgenic key enzymes compared to the CPA treated group. 0.75 mg dose showed a significant ($p < 0.05$) rectification in the enzyme activitiescompared to the other three higher doses. On the other hand, 1.5 mg, 3.0 mg and 4.5 mg doses did not focus any significant ($p > 0.05$) recovery further. So, 1.5 mg dose showed maximum recovery in enzyme activities **(Figure 4.2.2)**.

4.2.4.5 Testicular cholesterol

Cyproterone acetate administration at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight for 30 days focused a significant ($p < 0.05$) elevation in the cholesterol level compared to the vehicle treated control. Oral administration of lycopene at different doses such as 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg showed a significant ($p < 0.05$) recovery in the cholesterol level towards the vehicle treated control. Result focused that 1.5 mg dose exhibited maximum recovery in the cholesterol level as 0.75 mg dose showed significant ($p < 0.05$) difference with 1.5 mg, 3.0 mg and 4.5 mg dose where as three higher doses did not differ significantly ($p > 0.05$) with each other **(Figure 4.2.3)**.

4.2.4.6 Serum testosterone

Level of serum testosterone was significantly ($p < 0.05$) diminished in CPA treated group compared to the vehicle treated control. Treatment with lycopene at 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg to the CPA pre-treated infertile rat for 30 days resulted a significant ($p < 0.05$) rectification in the serum testosterone level when comparison was made with the vehicle treated control. From the comparative analysis, it was focused that among all the doses, 1.5 mg, 3.0 mg and 4.5 mg dose showed a significant ($p < 0.05$) recovery compared to the 0.75 mg dose of lycopene. There was a non-significant difference ($p > 0.05$) among the higher doses with each other. So, 1.5 mg dose showed maximum recovery in serum testosterone level compared to the other doses **(Figure 4.2.4)**.

4.2.4.7 Assessment of oxidative stress related parameters

Activities of catalase and peroxidase in testis and sperm pellet were significantly ($p < 0.05$) decreased and level of the antioxidative end products such as CD and TBARS levels in testis were significantly ($p < 0.05$) elevated after the administration of cyproterone acetate at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight for 30 days compared to the vehicle treated control. Activities of these antioxidant enzymes and the end product levels in the concern tissues recovered significantly ($p < 0.05$) towards the vehicle treated control after the treatment with lycopene at different doses such as 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days to the CPA pre-treated rat. Result focused that 0.75 mg dose exhibited a significant ($p < 0.05$) rectification of the concern antioxidant markers compared to the 1.5 mg, 3.0 mg and 4.5 mg does. On the other hand, 1.5 mg dose focused maximum recovery capacity than the other does as the rate of recovery among 1.5 mg, 3.0 mg and 4.5 mg is non-significant ($p > 0.05$) with each other **(Figure 4.2.5 to 4.2.7)**.

2.4.8 Serum total protein, albumin and globulin levels

The levels of serum total protein, albumin and globulin were significantly ($p < 0.05$) declined in CPA treated group after administration of CPA at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days when comparison was made with the vehicle treated control. A significant ($p < 0.05$) rectification in their levels was observed after oral administration of lycopene at 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg doses to the CPA pre-treated rat for next 30 days. A significant ($p < 0.05$) correction in the levels of the concern parameters was observed after 1.5 mg, 3.0 mg and 4.5 mg compared to the 0.75 mg dose of lycopene. But these three higher doses were statistically non-significant ($p > 0.05$) with each other. In this concern, 1.5 mg dose exhibited maximum recovery in the levels of the concern markers **(Figure 4.2.8)**.

4.2.4.9 Histometric assessment of testicular tissue

Histological assessment of testicular tissue exhibited a significant ($p < 0.05$) diminution in seminiferous tubular diameter in CPA treated animals compared to the vehicle treated control. Lycopene treatment at the doses of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg/ 0.5 ml tween 80/ 100 g body weight/ day for 30 days resulted a significant ($p < 0.05$) correction in the size of the seminiferous tubular diameter towards the vehicle treated control. From the statistical analysis, it was observed that 1.5 mg dose showed maximum recovery in comparison to the 0.75 mg dose. No further improvement was noted after the treatment with the higher doses i.e. s3.0 mg and 4.5 mg. Non significant ($p > 0.05$) variations was noted among 1.5 mg, 3.0 mg and 4.5 mg doses with each other in connection to the rectification in STD towards the vehicle treated control **(Plate 4.2.1)**.

4.2.5 Discussion

As per the previous experiment, animals were subjected to develop infertile condition at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days.

Experiment II has been performed to focus the potent and minimum dose of lycopene among 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days having maximum efficacy in connection with the rectification of CPA induced male infertility in albino rat.

Level of testicular cholesterol which is the mother molecule of steroid synthesis (**Miller & Auchus, 2011)** was elevated after CPA treatment due to the lack of utilization of cholesterol in steroid synthesis pathway as androgenesis is interrupted due to CPA **(Gao and Mahto, 2016)**. This finding was further strengthening by the analysis of serum testosterone which is the final outcome of steroid synthesis pathway **(Zubeldia-Brenner et al., 2017)** and the main hormonal regulator for male reproductive health. Serum testosterone level was also decreased after 30 days treatment to CPA treated rat. This is due to the anti-androgenic nature of CPA **(Gao and Mahto, 2016)**. Similar outcome was also focused in testicular Δ5, 3β-HSD and 17β-HSD activities which were deteriorated after CPA administration. Result was again supported by the decreased seminiferous tubular diameter may due to inhibition in androgenic binding protein synthesis which interfere spermatogenesis **(Heinlein & Chang, 2002).** Inhibition in the spermatogenesis is associated with diminution in seminiferous tubular diameter as seminiferous tubule is the site for onset of spermatogenesis **(Tripathy et al., 2015).** Lycopene administration at 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg dose rectified all the above mentioned parameter due to the withdrawal of androgenic inhibition by lycopene **(Graeff and Junior, 2010)**.

Other spermatogenic parameters such as sperm motility, viability and count were also declined after CPA treatment may be due to lack of secretion and synthesis of LH hormone because of inhibition in the hypothalamus-pituitary-testicular axis created by CPA **(Migally, 2009)**. Low level of LH may cause oligospermic and or azospermic condition. Simultaneously, acrosomal status also focused the similar pattern of result due to CPA

administration. On the other hand, HOS test reflected a low number of hypo-osmotically swelled tail of sperm which focused the degradation in the sperm membrane potential after CPA administration. This condition is due to the effect of CPA on hypophyseo-hypothalamic level beside its anti-androgenic efficacy **(Fredricsson and Carlsteom, 1981).** Oral administration of lycopene at different doses rectified the condition of above mentioned testicular impairment. Among all the doses 1.5 mg dose showed maximum rate of recovery. The higher doses such as 3.0 mg and 4.5 mg did not exhibit further efficacy in connection to the rectification of the above mentioned parameters towards the vehicle treated control.

Antioxidant enzyme activity with special reference to the catalase and peroxidase in sperm pellet and testis was decreased after CPA administration due to the generation of oxidative stress by CPA **(Tam et al., 2003)**. Lycopene administration at 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg dose rectified the state but among all the doses 1.5 mg dose resulted most promising efficacy. Lycopene administration rectified the antioxidant enzyme activity may be due to its free radical quenching ability which is ten times more potent than α-tocopherol and twice more efficient than β-carotene **(El-Raey and Ibrahim, 2013)** . This result was further strengthen by the maximum revival effect of lycopene at 1.5 mg by analysing the levels of antioxidant end product such as CD and TBARS in testicular tissue. Reduction in serum albumin, globulin is indicator of loss of the functional integrity of cell membranes in renal tissue due to reno-toxicity (**Preethi & Kuttan, 2009**) after CPA administration. The condition was rectified after lycopene administration at different doses probably due to its non-toxic nature. But most prominent result was achieved after the treatment of 1.5 mg dose compared to the other doses. Lycopene at the dose of 1.5 mg showed maximum recovery as up to this dose the concern nutraceutical receptor can able to exert its binding capacity to achieve revival status either by rectifying the androgen synthesis that may enhance the

spermaogenesis process or by minimising the oxidative stress related free radical induced sperm damage that leads to infertility.

4.2.6 Conclusion

From the experiment, it can be concluded that 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days may have the capability to restore CPA induced testicular complication towards control as the lower dose was unable to exert its action properly and the higher doses saturate the concern receptor as a result further rectification can not be achieved

Table 4.2.1: Dose dependent corrective efficacy of lycopene on spermatogenic profile in CPA induced infertile rats

Group	Sperm count (Millions/ ml)	Motile sperm (%)	Viable sperm		Hypo-osmotic swelled sperm count (%)
			Alive spermatozoa (%)	Dead Spermatozoa (%)	
Vehicle treated control	27.20 ± 0.79[a]	74.83 ± 1.56[a]	86.20 ± 2.30[a]	13.80 ± 0.16[a]	60.16 ± 1.0[a]
CPA treated	13.24 ± 1.02[b]	47.12 ± 1.07[b]	38.20 ± 0.65[b]	61.8 ± 1.21[b]	40.5 ± 1.25[b]
Lycopene (0.75 mg) + CPA	17.23 ± 1.07[c]	50.00 ± 1.04[c]	48.23 ± 0.75[c]	51.77 ± 1.21[c]	46.32 ± 1.14[c]
Lycopene (1.5 mg)+ CPA treated	**19.23 ± 1.76[d]**	**64.56 ± 1.87 [d]**	**65.25 ± 1.37 [d]**	**33.75 ± 0.54 [d]**	**51.16 ± 1.32 [d]**
Lycopene (3.0 mg) + CPA treated	20.45 ± 1.76[d]	63.34 ± 1.76[d]	66.45 ± 1.53[d]	32.55± 0.47[d]	52.16± 1.43[d]

Data were expressed as mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p < 0.05$.

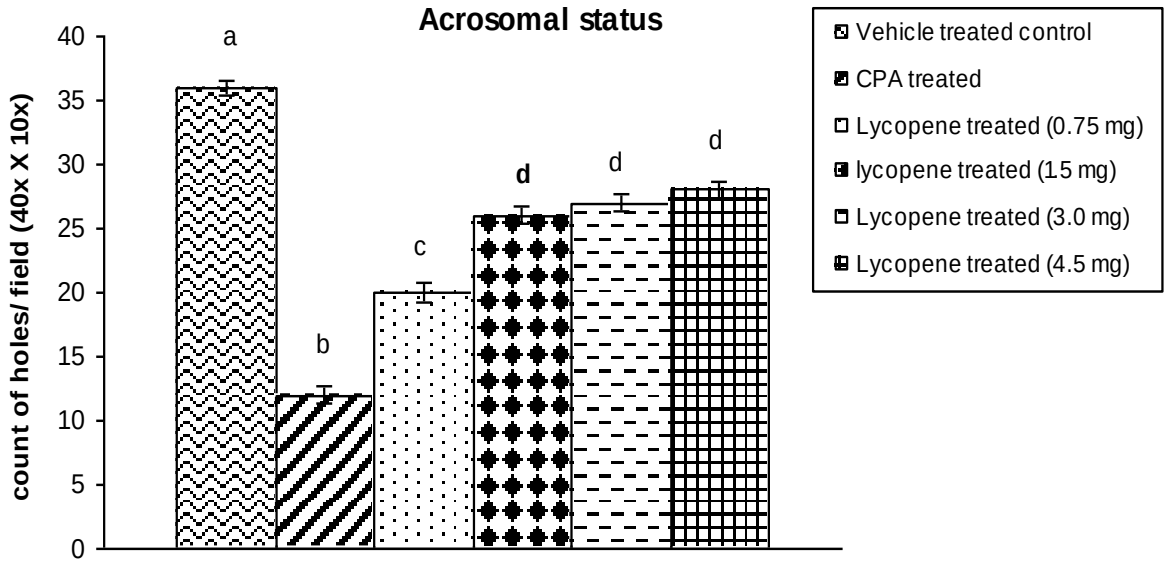


Figure 4.2.1 Rectification in the sperm acrosomal status after lycopene administration at different doses in CPA treated infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

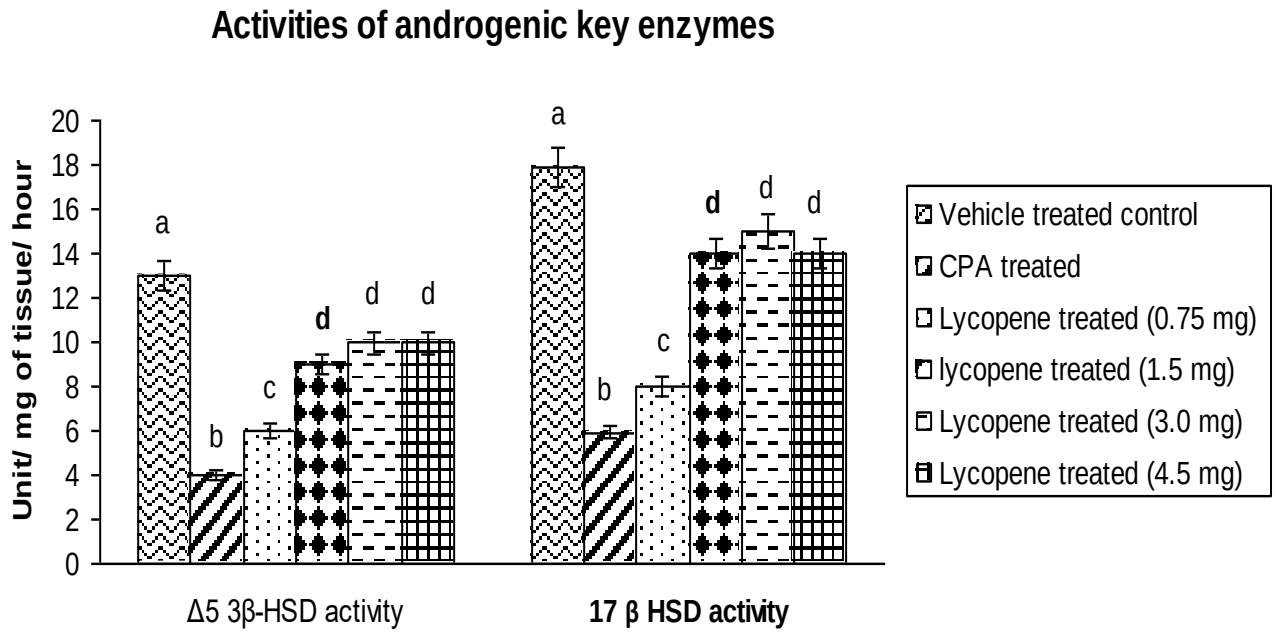


Figure 4.2.2 Effect of different doses of lycopene on androgenic key enzyme activities to the CPA treated rat with andrological abnormalities. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

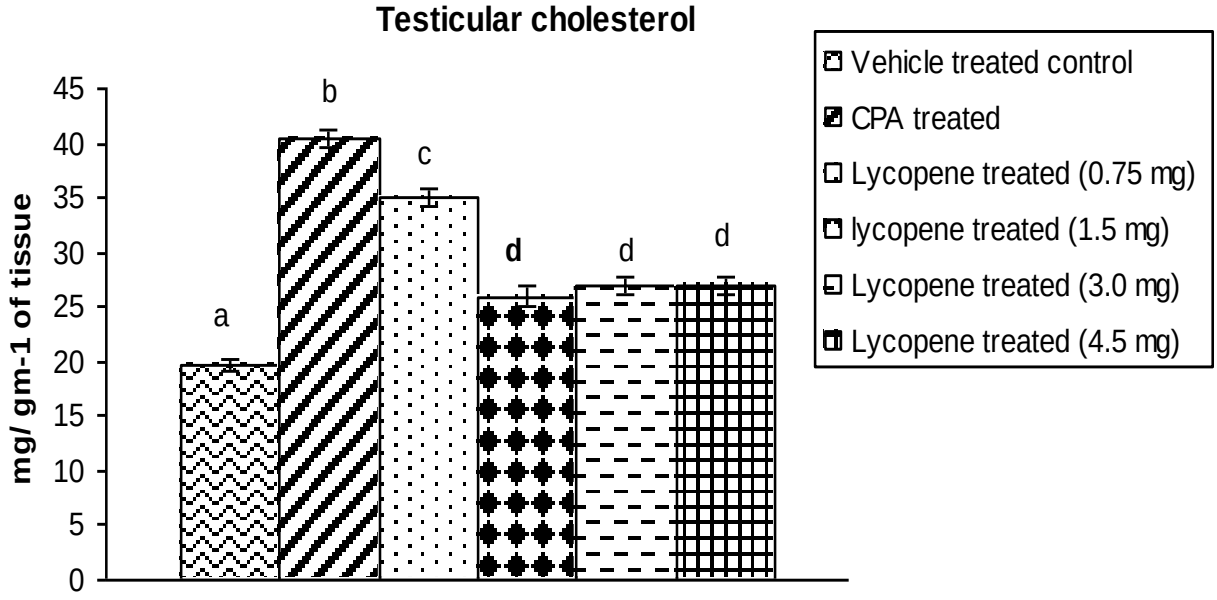


Figure 4.2.3 Corrective efficacy of different doses of lycopene on the testicular cholesterol level on CPA administered infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

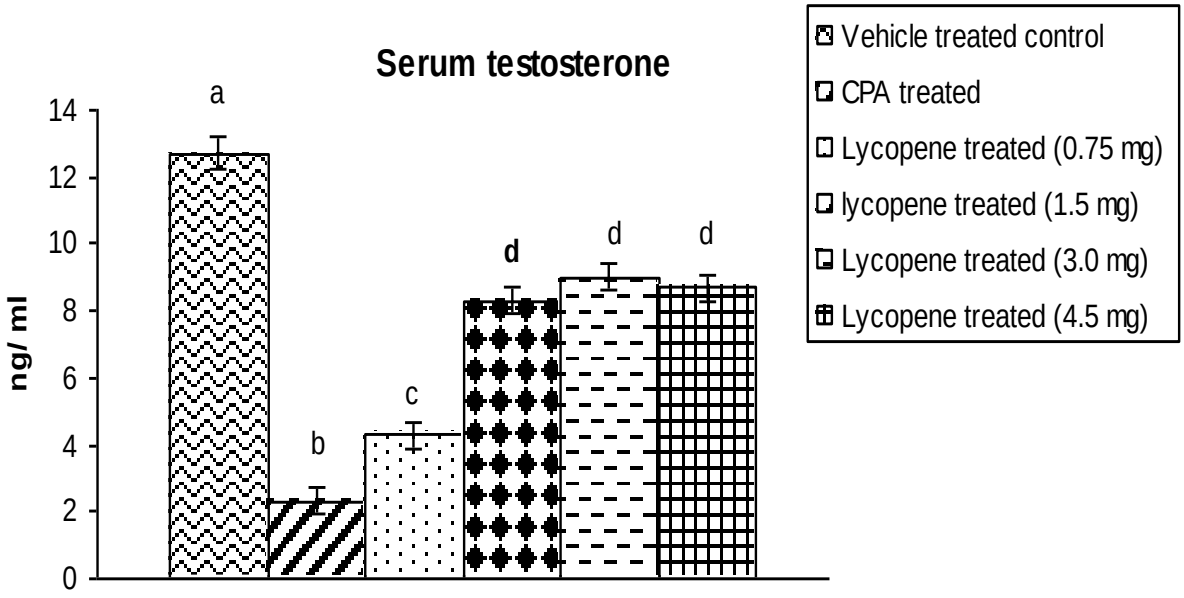


Figure 4.2.4 Attenuation in the serum testosterone level after the treatment with lycopene at different doses to the CPA treated infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

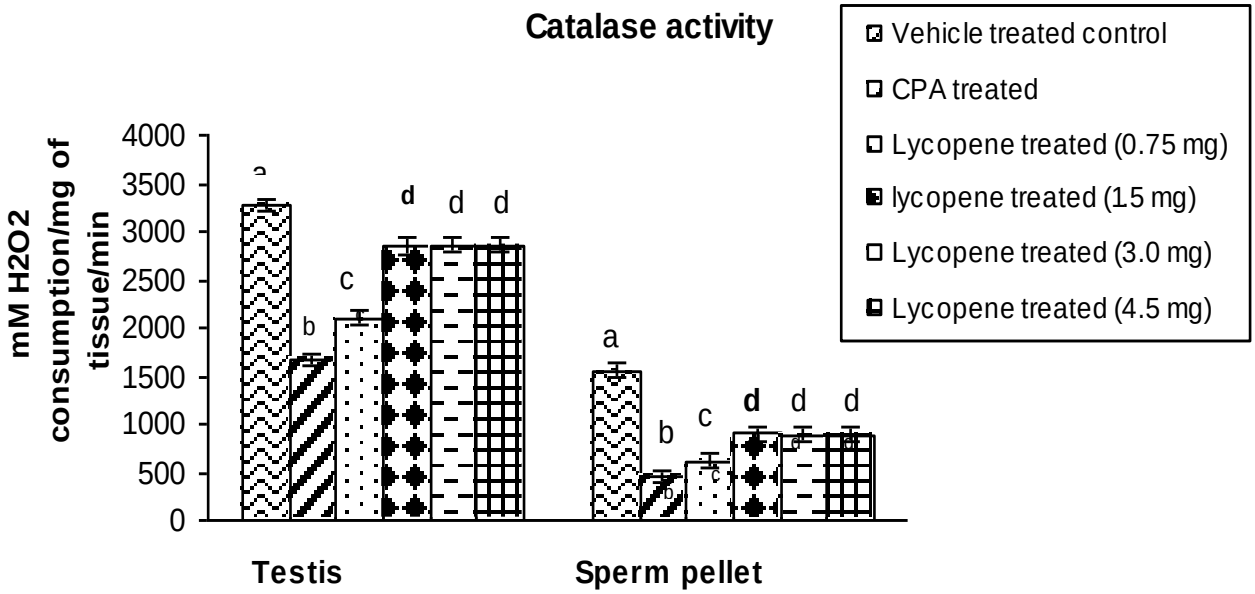


Figure 4.2.5 Protective efficacy of lycopene at the doses of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg in connection to the rectification in catalase activities in testis and sperm pellet to the CPA treated rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

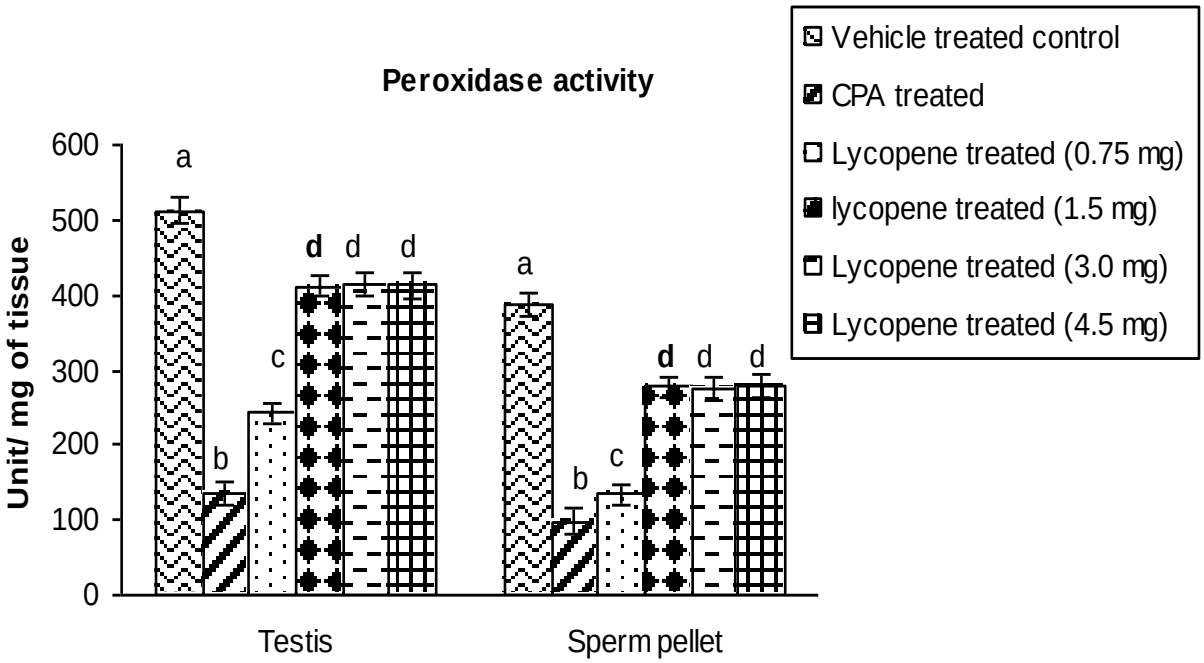


Figure 4.2.6 Dose dependent remedial effect of lycopene on the peroxidase activities in sperm pellet and testis in CPA treated infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

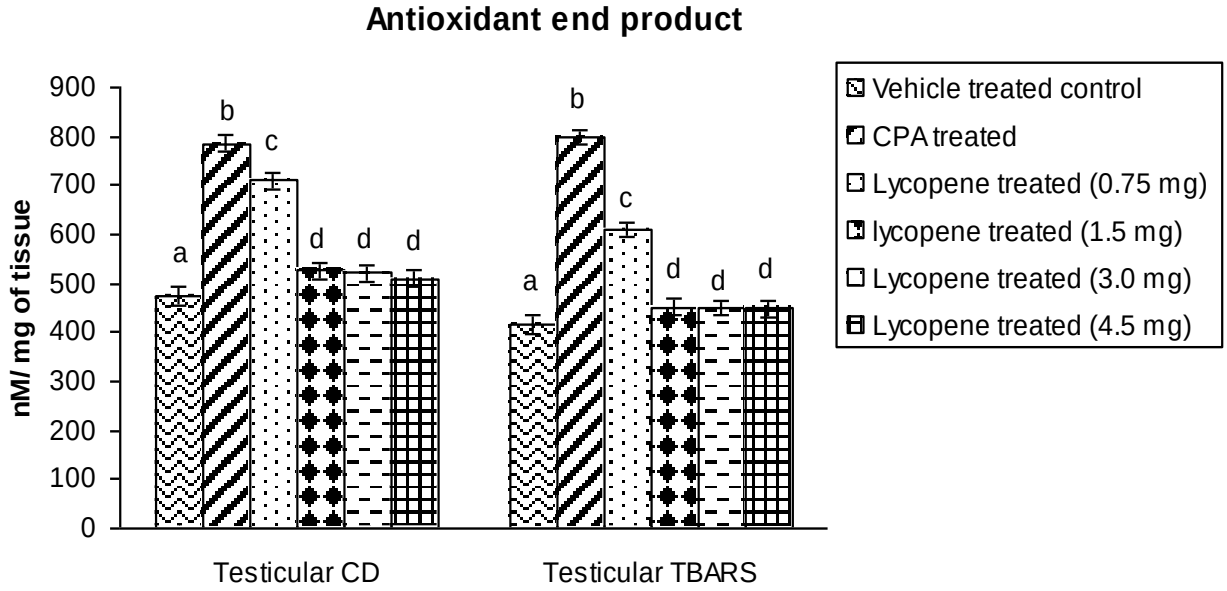


Figure 4.2.7 Protective efficacy of lycopene at the doses of 0.75 mg, 1.5 mg, 3.0 mg and 4.5 mg in connection to the rectification in catalase activity in testis and sperm pellet to the CPA treated rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

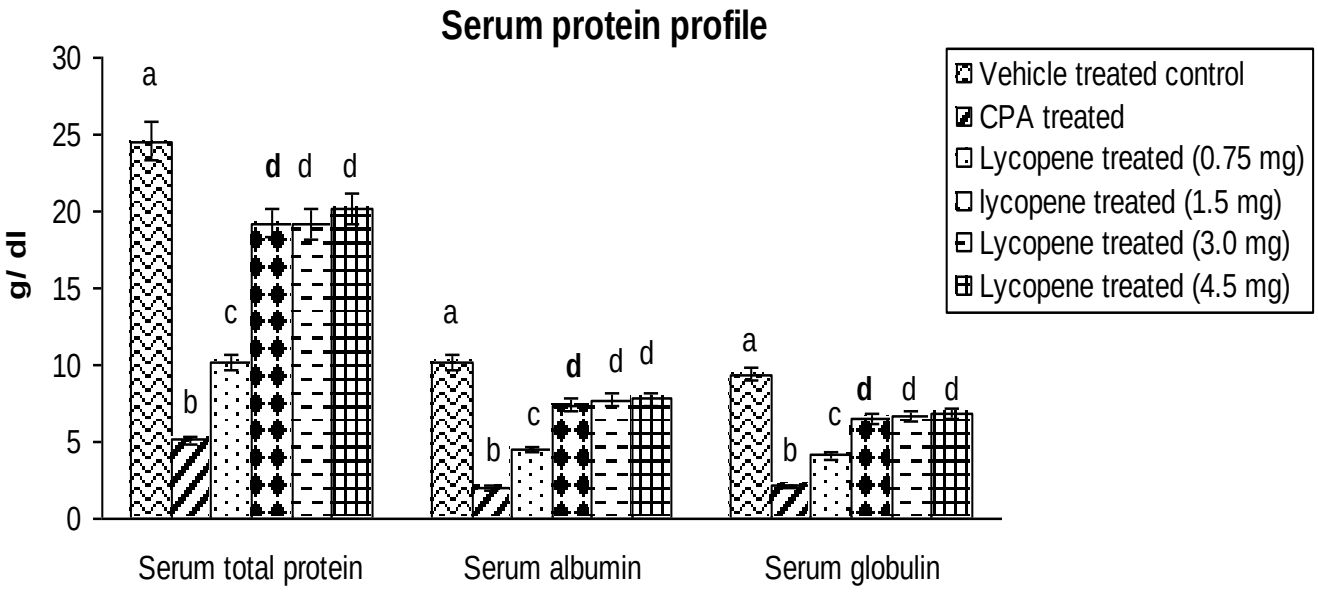
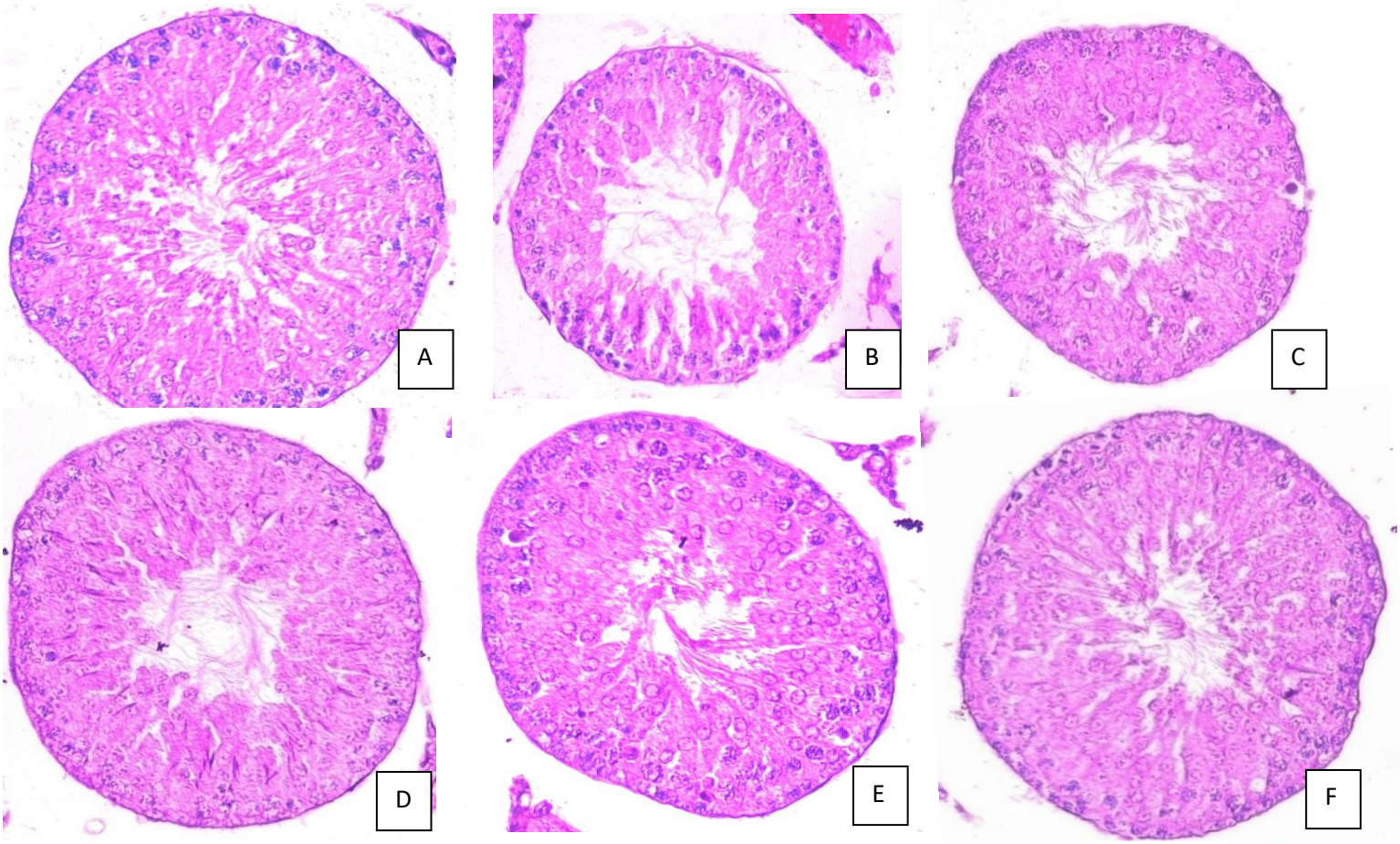


Figure 4.2.8 Recovery in the serum protein profile after the treatment of lycopene at 0.75 mg, 1.5 mg , 3.0 mg and 4.5 mg doses to the CPA induced hypo-testicular activities in rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.



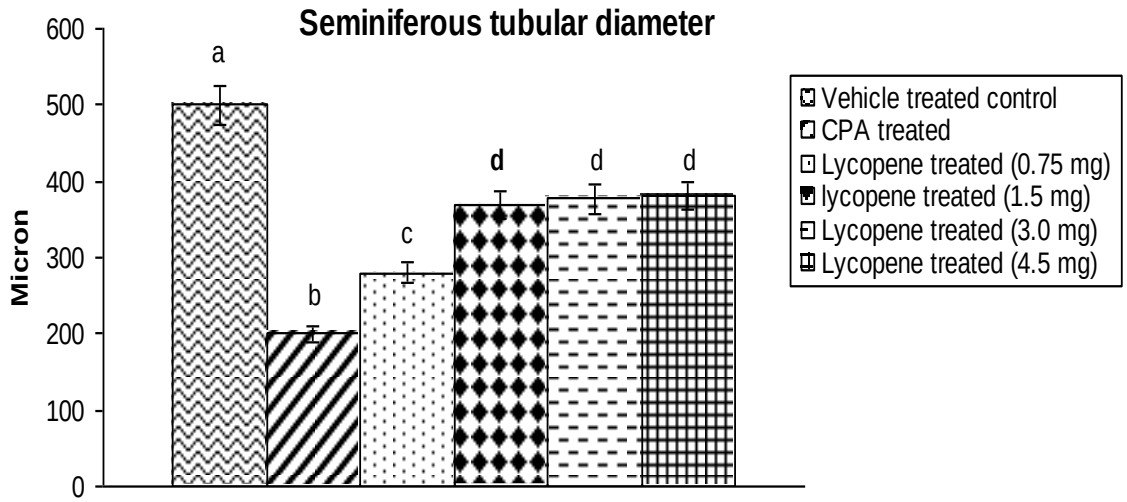


Plate 4.2.1: Histology of testis 400X (Hematoxyline-Eosin Stain). Representative microphotographs of A) vehicle treated control, B) CPA treated rat, C) 0.75 mg lycopene treated, D) 1.5 mg lycopene treated, E) 3.0 mg lycopene treated, F) 4.5 mg lycopene treated rat. Recovery in the seminiferous tubular diameter after treatment with different doses of lycopene to the CPA treated rat which focus the rectification in the sperm density after lycopene treatment. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

4.3. Experiment No. III

Duration dependent efficacy of lycopene on cyproterone acetate induced male andrological hypo-function in male Wistar strain albino rat in connection with male infertility management

4.3.1 Significance of the study

This study was designed to highlight the minimum duration or threshold duration of the treatment protocol by lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day to achieve the maximum remedial effect in a minimum duration on CPA induced infertile rat on the specific duration.

4.3.2 Experimental Design

For the conduction of the experiment, fifty-four Wistar strain albino rat (80-90 days old) weighing about 120 ± 10 g having normal fertile condition were procured from our institutional authorised vendor (Saha Enterprise, Kolkata). All the animals were pre-conditioned for 15 days prior the onset of the experiment in connection to acclimatize them with concern environment and human content. During this acclimatization phase, animals were kept in an ambient temperature of about 25 ± 2º C having humidity 44-45% along with light-dark cycle at the ratio of 12: 12. Animals were supplied with standard rat chew and water *ad libitum*. Animal handling, treatment protocol and sacrifice process were done as per the guideline provided by IAEC after getting approval by IEC (IEC/ 3/ C-4/ 14, dated- 03. 11.2014).

Animals were categorised as follows:

15 days treatment schedule

Total experimental duration was 45 days (30 days CPA treatment+ 15 days lycopene treatment)

Group I: Vehicle treated control: Healthy, normal, fertile rats were provided with distilled water for first 30 days of the experiment. Then 0.5 ml tween -80/ 100 g body weight / day for 15 days were provided to the rat of this group.

Group II: CPA treated group: Animals in this group were provided with cyproterone acetate at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day dose for first 30 days then again for another 15 days of treatment regimen.

Group III: CPA+ lycopene treated group: Cyproterone acetate pre-treated animal were treated with lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 15 days along with CPA at the same dose.

30 days treatment schedule

Total experimental duration was 60 days (30 days CPA treatment+ 30 days lycopene treatment)

Group I: Vehicle treated control: Experimental animals were given with distilled water for 30 days then 0.5 ml tween-80/ 100 g body weight/ day was incorporated for another 30 days of experiment.

Group II: CPA treated group: Albino rats were provided with cyproterone acetate at of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day dose for 60 days.

Group III: CPA+ lycopene: Animals were treated with CPA for 30 days for induction infertility. Cyproterone acetate pre-treated rats were administered with lycopene at the previously mentioned concern dose for 30 days along with CPA.

45 days treatment schedule

Total experimental duration was 75 days (30 days CPA treatment+ 45 days lycopene treatment)

Group I: Vehicle treated control: Experimental rats were allowed for oral administration of distilled water for first 30 days of the experiment. After that 0.5 ml tween-80/ 100 g body weight/ day was provided for another 45 days.

Group II: CPA treated group: Rats were treated with cyproterone acetate at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for first 30 days and then again for another 45 days during the total treatment regimen.

Group III: CPA+ lycopene treated group: Animals of this group were provided with cyproterone acetate for 30 days. Then infertile animals were orally administered with lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 45 days along with CPA.

Vehicle treated control and CPA treated group and CPA+ lycopene treated group for 15 days, 30 days and 45 days duration contains six rats in each for the fulfilment of the aim of this experiment.

For the first 30 days of experiment, vehicle treated control group was provided with distilled water and the rest of the animals (animals of CPA treated group and concern duration dependent group) were administered with CPA at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day. From 31st day of experiment, lycopene was provided to the 15 days, 30 days and 45 days duration treated group at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day along with CPA. All the CPA treated animals were allowed for oral administration of CPA for concern duration to maintain the infertile condition.

Before sacrifice, the final body weight was measured then animals were sacrificed by using euthanasia. After sacrifice, blood was collected from dorsal aorta followed by serum separation after centrifugation for 5 min at 3000 x g. The testis, epididymis and seminal vesicle were collected from the body to measure the organo-somatic indices. Sperm was collected from cauda epididymis. Testis, seminal vesicle was kept in -20ºC for conduction of concern parameters.

4.3.3 Parameters and methods

In this experiment, sperm motility **(Zemjanis, 1977)**, sperm count **(Pant and Srivastava, 2003)** was examined. For morphological study of sperm, acrosomal status **(Gopalkrishnan et al., 1991**) and nuclear chromatin decondensation (NCD) test **(Rodriguez et al., 1985)** were performed. Effect of nutraceutical on CPA induced degradation in reproductive organ was detected by measuring organo-somatic indices including body weight, testiculo-somatic index, epididymal-somatic index and seminal vasiculo-somatic index. Effect of lycopene on androgenic key enzyme activity such as Δ^5, 3β-HSD **(Talalay, 1962)** and 17β-HSD **(Jarabak et al., 1962)** was assessed by standard protocol. Alteration in the androgen synthesis pathway was further analysed by measuring the level of serum testosterone **(Srivastava, 2001)** and testicular cholesterol **(Plummer, 1995)**. Seminal vascular fructose level was also measured by considering the standard method **(Lu et al., 1974)**. Effect of lycopene on spermacokinetics was focused by assessing stage VII spermatogenesis **(Leblond and Clermont, 1952)**. Free radical quenching ability of lycopene was measured by analysing the activity of the anti-oxidant enzymes such as catalase **(Beer, 1952)** and superoxide dismutase (SOD) **(Marklund and Marklund, 1974).** For the assessment of toxicity generation during the duration dependent treatment protocol, activity of serum GOT and GPT **(Henry et al., 1960),** serum

levels of urea, uric acid, blood urea nitrogen and creatinine was measured by standard protocol **(Tiffany et al., 1972; Kabasakilian et al., 1973; Junge et al., 2001).** Statistical analysis of the collected data was performed by standard protocol **(Sokal and Rohle, 1997).**

4.3.4 Results

4.3.4.1 Body weight

Significant ($p < 0.05$) diminution was noted in the body weight of the CPA treated group of all corresponding duration dependent group compared to the vehicle treated control. After administration of lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day, a significant ($p < 0.05$) rectification was observed after treatment for 15 days, 30 days and 45 days treatment schedule. Treatment with lycopene at the concern dose for 30 days and 45 days showed more significant ($p < 0.05$) recovery compared to the 15 days lycopene treated group. But non-significant ($p > 0.05$) rectification was noted between 30 days and 45 days treatment with lycopene **(Table 4.3.1)**.

4.3.4.2 Weight of reproductive organs

The weight of the seminal vesicle testis and epididymis and was significantly ($p < 0.05$) diminished in CPA treated group of all the duration dependent group compared to the corresponding vehicle treated control of 15 days, 30 days and 45 days duration dependent groups. Lycopene administration at the concern dose to all the CPA pre-treated corresponding groups of 15 days, 30 days and 45 days lycopene treated groups, showed a significant ($p < 0.05$) recovery in the weight of the reproductive organs towards the vehicle treated control compared to the CPA treated group. Treatment with lycopene for 30 and 45 days duration, showed more promising and significant ($p < 0.05$) recovery in the body weight compared to the 15 days duration treated group. When the comparison was made between 30

days and 45 days duration dependent groups, then a non-significant ($p > 0.05$) variation was noted **(Table 4.3.1)**.

4.3.4.3 Sperm motility

Sperm motility focused a significant ($p < 0.05$) diminution in the CPA treated groups of all corresponding duration dependent groups when compared with the vehicle treated control of all the concern groups. Lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day along with CPA to the CPA pre-treated followed by lycopene treated animals of 15 days, 30 days and 45 days duration dependent groups exhibited a significant ($p < 0.05$) recovery in the concern parameter towards the vehicle treated control. When the result was compared regarding the rate of recovery among 15 days, 30 days and 45 days duration dependent groups, it was observed that 30 days and 45 days duration of lycopene treatment showed better result in connection to the recovery in percentage of motilsperm compared to the sperm motility. But a non-significant ($p > 0.05$) difference was noted between the 30 days and 45 days duration dependent groups **(Table 4.3.3)**.

4.3.4.4 Sperm count

Sperm count showed a significant ($p < 0.05$) reduction in its number in the animals treated with CPA to the 15 days, 30 days and 45 days duration dependent groups in comparison to the vehicle treated control of the corresponding duration dependent groups. After treatment with lycopene by the potent dose for 15 days, 30 days and 45 days to the CPA pre-treated lycopene treated rats of all the concern duration dependent groups, exhibited a significant ($p < 0.05$) recovery in the sperm count. As per statistical analysis, the recovery rate is more significant ($p < 0.05$) in 30 days and 45 days treatment schedule compared to the 15 days treatment duration of lycopene. On the other hand, non-significant difference ($p > 0.05$) was

noted between 30 days and 45 days duration dependent lycopene treated groups **(Table 4.3.3).**

4.3.4.5 Acrosomal status

In all the duration dependent groups, sperm acrosomal status showed a significant ($p < 0.05$) alteration in downward manner in CPA treated animals of all corresponding groups. All the CPA treated infertile rats when orally administered with lycopene at 1.5 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 15 days, 30 days and 45 days respectively, a significant ($p < 0.05$) correction was observed in sperm acrosomal status in all the corresponding groups towards the vehicle treated control compared to the CPA treated group. Comparative analysis of the data revealed that, in comparison to the 15 days lycopene treated group, 30 days and 45 days lycopene treatment along with CPA resulted more significant ($p < 0.05$) recovery in the said parameter compared to the 15 days duration dependent group. On the other hand, 15 days and 30 days lycopene treated groups did not differ significantly ($p > 0.05$) with each other **(Table 4.3.3).**

4.3.4.6 Nuclear chromatin decondensation (NCD)

A significant ($p < 0.05$) reduction in sperm NCD was observed in CPA treated groups of all the corresponding duration dependent groups compared to the vehicle treated control. Oral administration of the lycopene at the potent dose along with the CPA for 15 days, 30 days and 45 days showed a significant ($p < 0.05$) rectification in the concern parameter towards then vehicle treated control compared to the CPA treated group. Statistical analysis showed that among 15 days, 30 days and 45 days duration dependent groups, 30 days duration and 45 days duration exhibited a significant ($p < 0.05$) and promising result in positive direction compared to the vehicle treated control. But 30 days duration and 45 days duration showed a non-significant ($p > 0.05$) difference with each other (**Figure 4.3.1).**

4.3.4.7 Testicular Δ5, 3β-HSD and 17β-HSD activities

Activities of testicular Δ5, 3β-HSD and 17β-HSD were significantly ($p < 0.05$) decreased in CPA treated group of all the corresponding duration dependent groups compared to the vehicle treated control. Oral administration of lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 15 days, 30 days and 45 days respectively to the lycopene treated group resulted a significant ($p < 0.05$) alteration in positive direction towards the vehicle treated control compared to the CPA treated group. Lycopene treatment for 30 days and 45 days duration showed significant ($p < 0.05$) and effective result in rate of recovery when comparison was made with 15 days duration of lycopene treatment. There was non-significant ($p > 0.05$) difference between the 30 days and 45 days duration dependent group (**Figure 4.3.2, 4.3.3)**.

4.3.4.8 Testicular cholesterol

Significant ($p < 0.05$) elevation in the testicular cholesterol level was noted after CPA treatment at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day to the CPA treated animals of all the corresponding groups. Result focused a significant ($p < 0.05$) rectification in the cholesterol level towards the vehicle treated control after the oral administration of lycopene at 1.5 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 15 days, 30 days and 45 days. From the statistical analysis, it has been observed that among the entire duration dependent lycopene treated groups, 30 days and 45 days duration of lycopene treatment showed promising efficacy in connection to the rectification in cholesterol level compared to the 15 days treated group. A non-significant ($p > 0.05$) difference was noted between 30 days and 45 days duration of lycopene treated groups as further recovery was not noted in 45 days duration dependent group (**Figure 4.3.4).**

4.3.4.9 Serum testosterone

Level of serum testosterone was significantly ($p < 0.05$) deviated downward in CPA treated group compared to the corresponding vehicle treated control group. Lycopene administration at the potent dose for 15 days, 30 days and 45 days respectively resulted a significant ($p < 0.05$) correction in the serum testosterone level towards the vehicle treated control in all the corresponding groups. Result also focused that 30 days and 45 days duration of treatment schedule showed more potency in connection to the recovery in the serum testosterone level compare to the 15 days treatment schedule. On the other hand, 30 days and 45 days duration of lycopene treatment exhibited a non-significant ($p > 0.05$) difference with each other (**Figure 4.3.5**).

4.3.4.10 Seminal vascular fructose

Seminal vascular fructose level showed a significant ($p < 0.05$) elevation in the CPA treated group at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days when comparison was made with the vehicle treated control. Duration dependent efficacy of lycopene with special reference to 15 days 30 days and 45 days duration dependent efficacy of lycopene in seminal vascular fructose level showed a significant ($p < 0.05$) towards the vehicle treated control compared to the corresponding CPA treated groups. When the result of 15 days duration of lycopene treatment compared with 30 days and 45 days duration dependent group, then a significantly ($p < 0.05$) effective recovery was noticed in the concern parameter compared to the vehicle treated control group. Non-significant ($p > 0.05$) difference was noted between 30 days and 45 days duration dependent group as no further recovery was observed (**Figure 4.3.6).**

4.3.4.11 Testicular catalase and SOD activities

Testicular catalase and SOD activities reflected a significant ($p < 0.05$) alteration in negative direction after treatment with CPA compared to the vehicle treated control of corresponding groups. Lycopene administration at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day long with CPA for 15 days, 30 days and 45 days to all the duration dependent corresponding groups, a significant ($p < 0.05$) rectification was observed in the testicular catalase and SOD activities towards the vehicle treated control compared to the CPA treated group. Among three duration schedule of treatment with lycopene such as 25 days, 30 days and 45 days, a significant ($p < 0.05$) recovery in the said enzyme activities was noted in 30 days and 45 days treatment schedule compared to the 15 days duration of lycopene treatment. Simultaneously, a non-significant ($p > 0.05$) difference was noted between 30 days and 45 days of treatment schedule by lycopene as no further rectification was noted in 45 days duration **(Figure 4.3.7, 4.3.8).**

4.3.4.12 Serum GOT and GPT activities

Activities in serum GOT and GPT showed a significant ($p < 0.05$) increase after treatment with CPA at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days in CPA treated animals of all the corresponding groups compared to the vehicle treated control. Lycopene administration at the 1.5 mg/ 0.5 ml tween-80/ 100 g body weight for/ day along with CPA for 15 days, 30 days and 45 days exhibited a significant ($p < 0.05$) rectification in the concern enzyme activities towards the vehicle treated control compared to the CPA treated group. When the result was compared among 15 days duration, 30 days and 45 days duration in connection to the rectification in the enzyme activities, it was observed that 30 days and 45 days duration on lycopene treatment exhibited significant ($p < 0.05$) efficacy compared to the 15 days duration of treatment schedule. But 30 days and 45 days duration of

lycopene treatment showed non-significant ($p > 0.05$) difference with each other (**Figure 4.3.9).**

4.3.4.13 Serum Uric acid, serum creatinine, serum urea, Blood urea nitrogen (BUN)

Significant ($p < 0.05$) elevation in the levels of serum uric acid, serum creatinine, serum urea and blood urea nitrogen was observed in CPA treated group compared to the corresponding vehicle treated groups. After oral administration of lycopene, at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 15 days, 30 days and 45 days exhibited a significant ($p < 0.05$) recovery in the levels of these parameters toward the vehicle treated control compared to the CPA treated group. When the result was compared to the rate of recovery among 15 days, 30 days and 45 days duration of lycopene treatment, it was observed that 30 days and 45 days duration of lycopene treatment showed more potency in recovery of the concern parameters compared to the 15 days duration. On the other side, 30 day and 45 days duration treated group did not show any significant ($p > 0.05$) difference with each other (**Table 4.3.2**).

4.3.4.14 Stage VII spermatogenesis

Quantification of germ cell at stage VII revealed a significant ($p < 0.05$) diminution in the count of ASg, pLSc, mPSc and 7Sd in CPA treated group compared to the vehicle treated control of corresponding groups. Number of all said types of germ cells were significantly ($p < 0.05$) recovered towards the vehicle treated control after the treatment with lycopene at the said dose for 15 days, 30 days and 45 days. The number of germ cell count was significantly ($p < 0.05$) recovered after treatment for 30 days and 45 days when the comparison was made with 15 days. But non-significant ($p > 0.05$) difference was noted between 30 days and 45

days as no further recovery was noted in 45 days compared to 45 days treatment schedule (**Table 4.3.4).**

4.3.5 Discussion

Experiment II has been conduted to search out the potent dose of lycopene in connection to the rectification of CPA induced male infertility. Experiment III has been conducted to focus the threshold duration of lycopene treatment to achieve maximum result within a short period of time.

I this oncern, spermatogenic sensors such as sperm motility and sperm count were analysed and result showed a significant diminution after CPA which lowers the overall sperm concentration that effect the sperm motility and count **(Fogh et al., 1979)**. Acrosome, the cap like structure located in the anterior half of the head in sperm helps in the fertilisation process was also decreased in its states after CPA treatment as CPA deteriorate the morphological structure of the sperm **(Fredricson and Caristrom, 1981**). The result was further strengthening by the nuclear chromatin decondensation test. Lycopene administration the potent dose for 15 days, 30 days and 45 days exhibited a gradual and significant recovery in 30 days and 45 days. Therefore 30 days duration can be considered as threshold duration for lycopene treatment.

Result also focused a significant elevation in testicular cholesterol level which is the precursor for steroid synthesis in the body **(Moon et al.,2016)** due to lack of utilisation in testosterone synthesis after CPA treatment as CPA is an anti-steroidogenic drug **(Wooltorton, 2003)**. Similar result was also focused in the serum testosterone level which was decreased after CPA administration as CPA blocks the androgen receptor **(Wooltorton, 2003)**. Anti-androgenic efficacy of CPA was also highlighted in the deteriorated activities of

Δ5, 3β-HSD and 17β-HSD which are the androgenic key enzymes and intermediate enzymes in steroidogenic pathway (). Lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 15 days, 30 days and 45 days rectified the condition towards the vehicle treated control may be due to the activity of lycopene on androgen receptor that stimulates the steroidogenesis **(Zhang et al., 2010).** Among all the duration dependent groups, 30 days duration of lycopene treated showed most promising recovery in this concern. Another rectification in male reproductive hypo-function was observed by seminal vasicular fructose level which was elevated after CPA treatment and that is may be due to the inhibition in the testosterone synthesis by CPA as the quantity of fructose is regulated by testosterone **(Matsuoka et al., 2006)**. Lycopene administration for 30 days showed remarkable activity in connection to the rectification in seminal vascular fructose level.

Oxidative stress generation is foremost cause for male infertility by damaging the entire cell and leads towards cell death. Cyproterone acetate has been reported to generate high amount of free radical **(Ali, 2008)**. In this experiment, significant diminution in the testicular SOD and catalase was noted CPA treatment. Effect of the free radical production in the body by CPA was also interfered the body weight and the reproductive organ weight which were deterioration after CPA administration **(Choi et al., 2016)**. Oxidative stress can directly effect the spermatogenic cycle. This phenomenon was proved by stage VII spermatogenic cell cycle analysis. Different generations of germ cells focused downward deviation in respect to the vehicle treated control. Lycopene administration for 15 days, 30 days and 45 days showed a significant recovery in the activities of the concern antioxidant enzyme activities in testicular tissue. This is may be due to the antioxidant potentiality of lycopene **(Tripathy et al., 2016)**.

The nitrogenous constituents in blood such as urea, uric acid, creatinine, blood urea nitrogen generally filtered out from the body with the help of kidney. The level of these toxicity

markers increased after CPA treatment which indicates the probability of renal injury muscle dystrophy **(Khedkar et al., 2011).** Similar result was noted in case of GOT and GPT activities in serum. Lycopene administration at potent dose for 15 days, 30 days and 45 days exhibited a ssignificant recovery in the above parameters which focused the non-toxic property of lycopene **(Tripathy et al., 2016).**

4.3.6 Conclusion

From the result it can be concluded that lycopene can improve the CPA induced male androgenic hypo-function by 30 days. As the nutraceuticals receptors were saturated by 30 days, hence the revival effect of lycopene beyond 30 days was not noted. So, 30 days duration of lycopene treatment can be considered as threshold duration of lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day in connection to rectify the CPA induced male infertile condition.

Table 4.3.1: Effect of lycopene in 15 days, 30 days and 45 days duration dependent group on body weight and reproductive organosomatic indices in CPA induced male rat

Groups	**Body Weight (g)**		**Testiculo-somatic index (g)**	**Epididymis-somatic index (g)**	**Seminal vesiculo-somatic index (g)**
	Initial	**Final**			
Vehicle treated control	120 ±1.3 [a]	128 ±1.2[a]	2.46 ± 0.19[a]	0.713 ± 0.016[a]	0.464 ± 0.04 [a]
CPA treated	122 ± 1.1 [a]	115 ± 1.4[b]	0.69 ± 0.008[b]	0.353 ± 0.013[b]	0.178 ± 0.02[b]
15 days lycopene treated	121 ± 2.1 [a]	121 ± 1.0 [c]	1.97 ± 0.03[c]	0.466 ± 0.017[c]	0.215 ± 0.03[c]
Vehicle treated control	119 ± 1.2 [a]	130 ±1.1 [a]	2.43 ± 1.16[a]	0.714 ± 0.003 [a]	0.461 ± 0.03 [a]
CPA treated	118 ± 1.2 [a]	116 ± 1.3 [b]	0.71 ± 0.04[b]	0.352 ± 0.002 [b]	0.180 ± 0.04 [b]
30 days lycopene treated	**120 ± 2.3 [a]**	**125 ± 1.9 [d]**	**2.23 ± 0.03[d]**	**0.585 ± 0.035[d]**	**0.388 ± 0.02[d]**
Vehicle treated control	121 ± 2.1 [a]	130 ± 1.1 [a]	2.42 ± 1.14 [a]	0.712 ± 0.03 [a]	0.462 ± 0.05 [a]
CPA treated	120 ± 2.3 [a]	114 ± 1.0 [b]	0.75 ± 0.05 [b]	0.354 ± 0.04 [b]	0.178 ± 0.04 [b]
45 days lycopene treated	122±2.4 [a]	124 ± 1.5[d]	2.25 ± 0.02[d]	0.587 ± 0.026[d]	0.385 ± 0.01[d]

Data were expressed as mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail t-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p < 0.05$.

Table 4.3.2: Toxicity profile after the administration of lycopene for 15 days, 30 days and 45 days to the CPA treated infertile male rats

Groups	Serum uric acid (mg/dL)	Serum creatinine (mg/dL)	Serum urea (mg/dL)	BUN (mg/dL)
Vehicle treated control	1.42 ± 0.04[a]	0.57 ± 0.004[a]	31.2 ±1.35[a]	13.1 ± 0.35[a]
CPA treated	2.42 ± 0.04[b]	1.11 ± 0.006[b]	55.4 ± 1.66[b]	22.4 ± 0.45[b]
15 days lycopene treated	1.92 ± 0.02[c]	0.76 ± 0.003[c]	48.2 ± 1.69[c]	20.1 ± 0.39[c]
Vehicle treated control	1.43 ± 0.04[a]	0.60 ± 0.03[a]	54.20 ± 1.41[a]	11.32 ± 0.31[a]
CPA treated	2.41 ± 0.05[b]	1.15 ± 0.04 [b]	53.02 ± 1.5 [b]	21.3 ± 0.34 [b]
30 days lycopene treated	**1.72 ±0.04[d]**	**0.67 ± 0.005[d]**	**38.6± 1.42[d]**	**16.8 ± 0.23[d]**
Vehicle treated control	1.41 ± 0.03 [a]	0.58 ± 0.02 [a]	53.1 ± 1.12[a]	12.2 ± 0.25[a]
CPA treated	2.45 ± 0.06 [b]	1.16 ± 0.05[b]	54.2 ± 1.54[b]	**20.43 ± 0.34** [b]
45 days lycopene treated	1.70 ±0.03[d]	0.65 ± 0.005[d]	39.1± 1.46[d]	16.9 ± 0.45[d]

Data were expressed as mean ± SEM (n =6). ANOVA followed by 'Multiple comparison two-tail *t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p < 0.05$.

Table 4.3.3: Spematogenic profile after the administration of lycopene for 15 days, 30 days and 45 days to the CPA treated infertile male rats

Groups	Sperm motility (%)	Sperm count (million/ ml of epididymal fluid)	Acrosomal status (count of holes/ slide)
Vehicle treated control	75.23 ± 1.49[a]	25.52 ± 0.93[a]	34 ± 1.32[a]
CPA treated	46.84 ± 1.06[b]	15.15 ± 1.06[b]	12 ±1.09[b]
15 days lycopene treated	42.48 ± 2.84[c]	15.01 ± 0.85[c]	21 ± 1.10[c]
Vehicle treated control	74.12 ± 1.23[a]	24.12 ± 0.67 [a]	33 ± 1.23 [a]
CPA treated	45.12 ± 1.01[b]	14 12 ± 0.54 [b]	11 ± 1.12 [b]
30 days lycopene treated	**65.98 ± 0.86[d]**	**20.94 ± 0.73[d]**	**25 ± 2.14[d]**
Vehicle treated control	73. 21 ± 1.3 [a]	23.12 ± 0.51 [a]	32 ± 1.23 [a]
CPA treated	**44.12 ± 1.0** [b]	**13.23 ± 1.03** [b]	**10.32 ± 1.09** [b]
45 days lycopene treated	66.11 ± 0.87[d]	21.62 ± 0.75[d]	26 ± 2.09[d]

Data were expressed as mean ± SEM (n =6). ANOVA followed by 'Multiple comparison two-tail *t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p < 0.05$.

Table 4.3.4: Recovery in the spermatogenic cycle after administration of lycopene for 15 days, 30 days and 45 days

Group	Number of germ cells at stage VII of spermatogenic cycle			
	ASg	**pLSc**	**mPSc**	**7Sd**
Vehicle treated control	1.22 ± 0.07[a]	17.25 ± 0.41[a]	20.51 ± 0.24[a]	61.21 ± 2.1[a]
CPA treated	0.42 ± 0.04[b]	9.24 ± 0.43[b]	11.18 ± 0.24[b]	31.92 ± 1.9[b]
15 days lycopene treated	0.75 ± 0.05[c]	12.17 ± 0.39[c]	15.56 ± 0.42[c]	45.50 ± 1.3[c]
Vehicle treated control	1.23 ± 0.04[a]	16.21 ± 0.21[a]	21.32 ± 0.21 [a]	63.11 ± 1.3 [a]
CPA treated group	0.43 ± 0.03 [b]	8.23 ± 0.32 [b]	12 ± 0.13 [b]	33.12 ± 1.5 [b]
30 days lycopene treated	**0.95±0.03[d]**	**15.12±0.45[d]**	**17.21±0.23[d]**	**50.25±1.21[d]**
Vehicle treated control	1.20 ± 0.05[a]	15.43 ± 0.12 [a]	19.21 ± 0.12 [a]	62.14 ± 1.23 [a]
CPA treated	0.41 ± 0.05 [b]	8.12 ± 0.43 [b]	10.54 ± 0.43 [b]	32. 12 ± 1.43 [b]
45 days lycopene treated	0.96±0.05[d]	14.28±0.48[d]	17.54±0.26[d]	51.22±1.27[d]

Data were expressed as mean ± SEM (n =6). ANOVA followed by 'Multiple comparison two-tail *t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p < 0.05$.

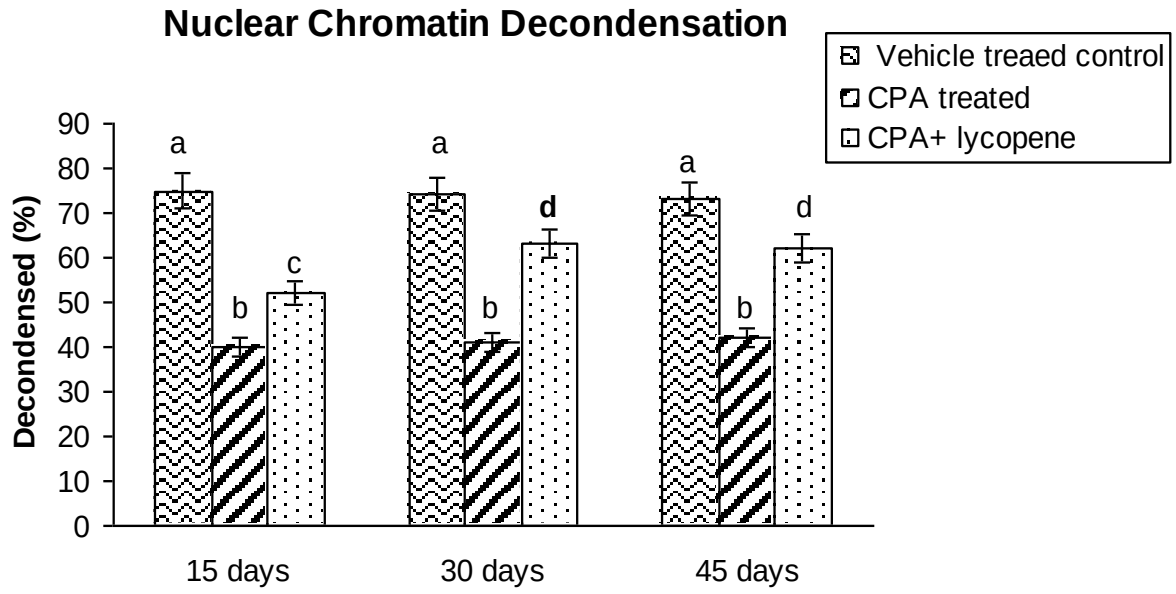


Figure 4.3.1 Corrective efficacy of lycopene on nuclear chromatin decondensation after treatment with 15 days, 30 days and 45 days on CPA induced hypo-testicular dysfunction in male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

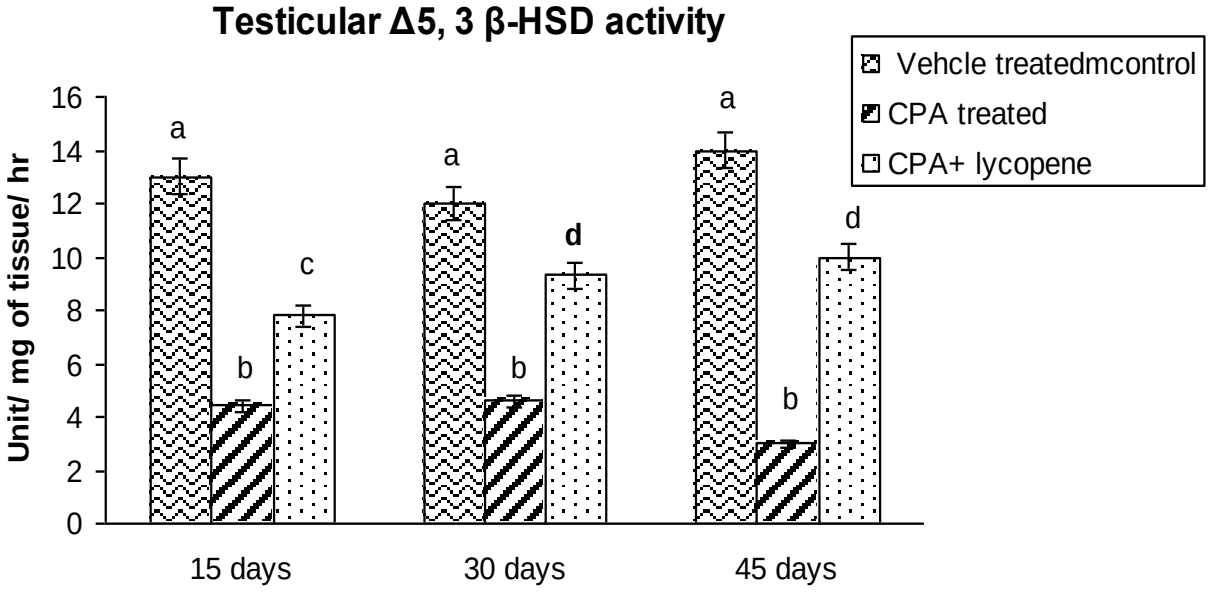


Figure 4.3.2 Rectification in the testicular Δ5, 3β-HSD activity after lycopene administration in different durations in CPA treated infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

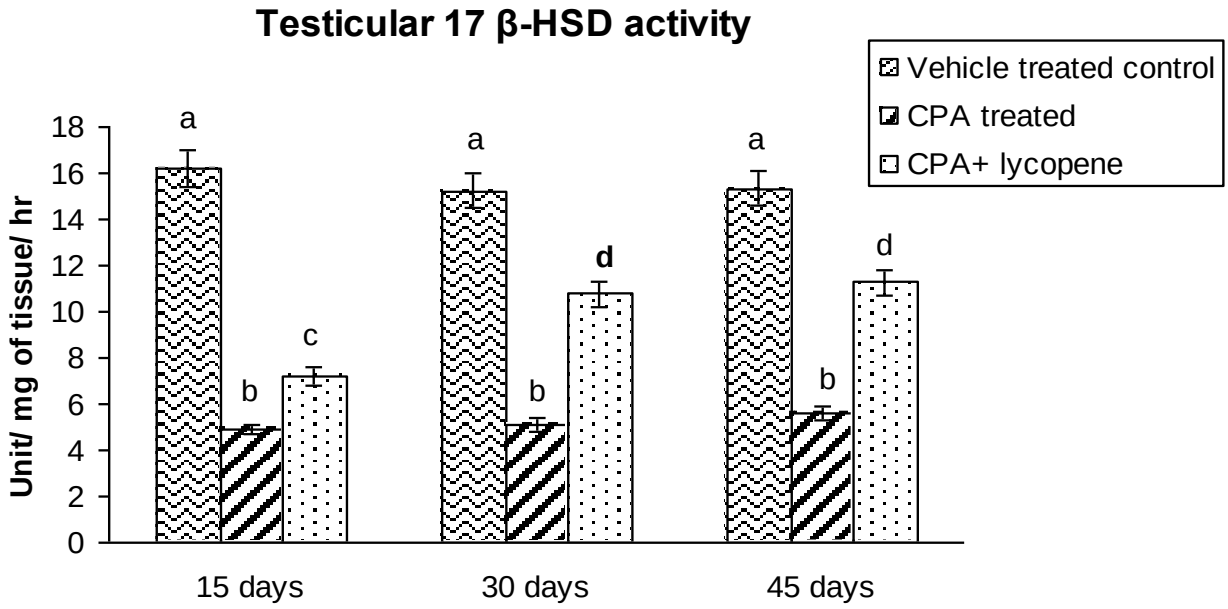


Figure 4.3.3 Correction in the testicular 17, 3β-HSD activity after lycopene administration in different durations in CPA treated infertile rat. Bars were expressed as Mean ± SEM (n= 6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

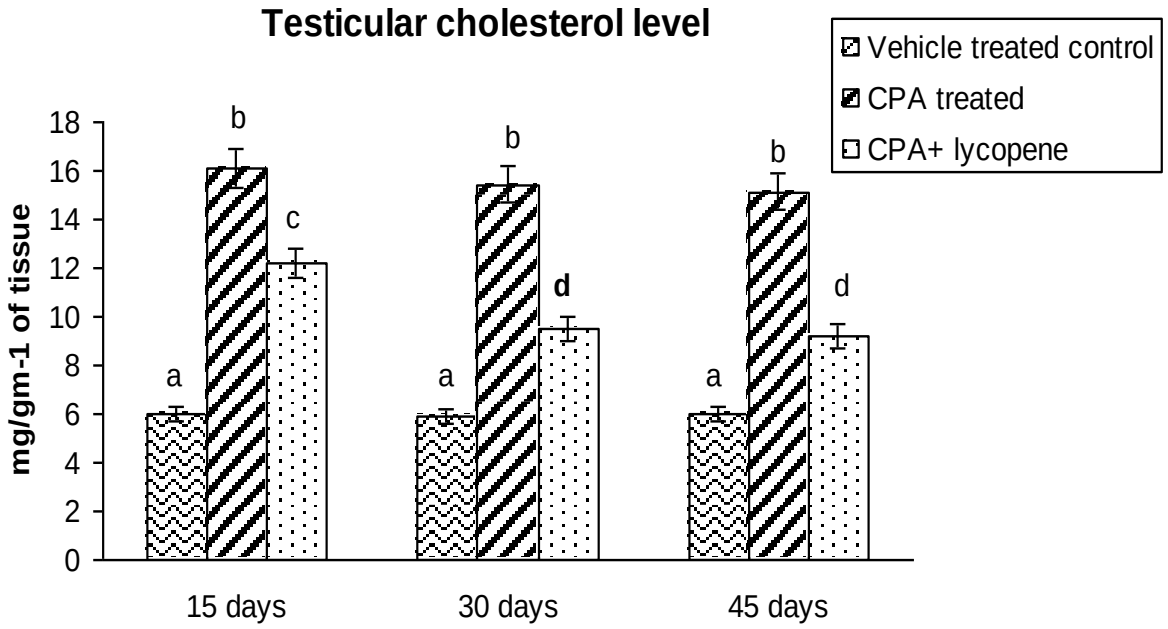


Figure 4.3.4 Rectification in the testicular cholesterol level by duration dependent lycopene treatment on CPA induced male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

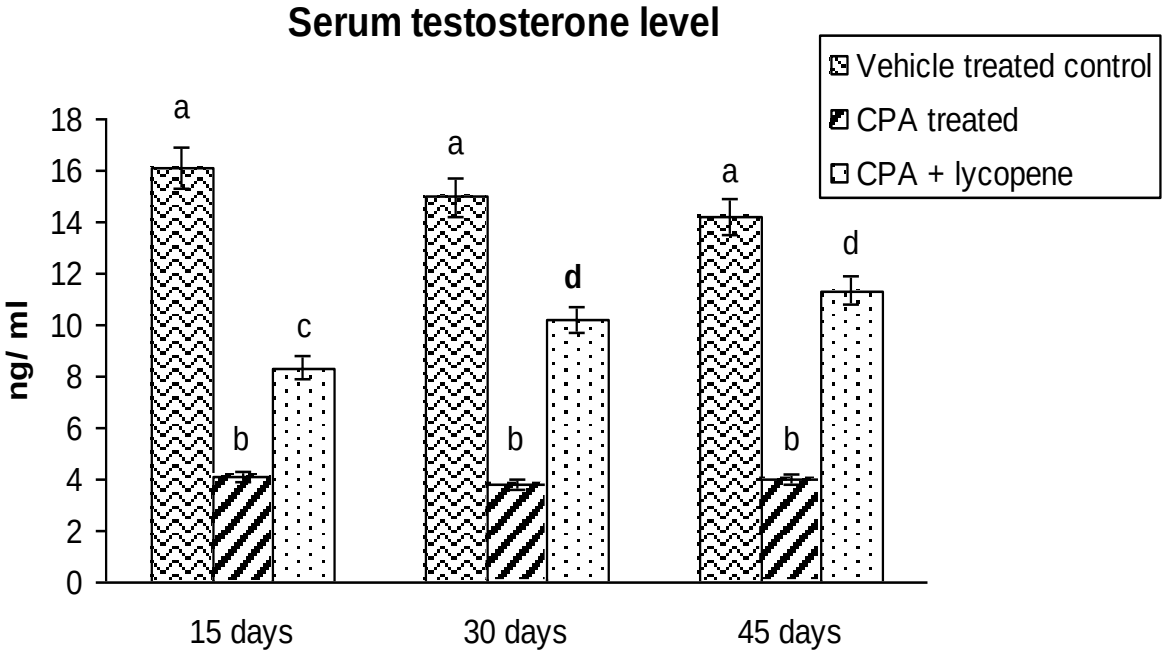


Figure 4.3.5 Ameliorative efficacy of lycopene on testosterone level at different duration depend treatment protocol on CPA induced male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p< 0.05$.

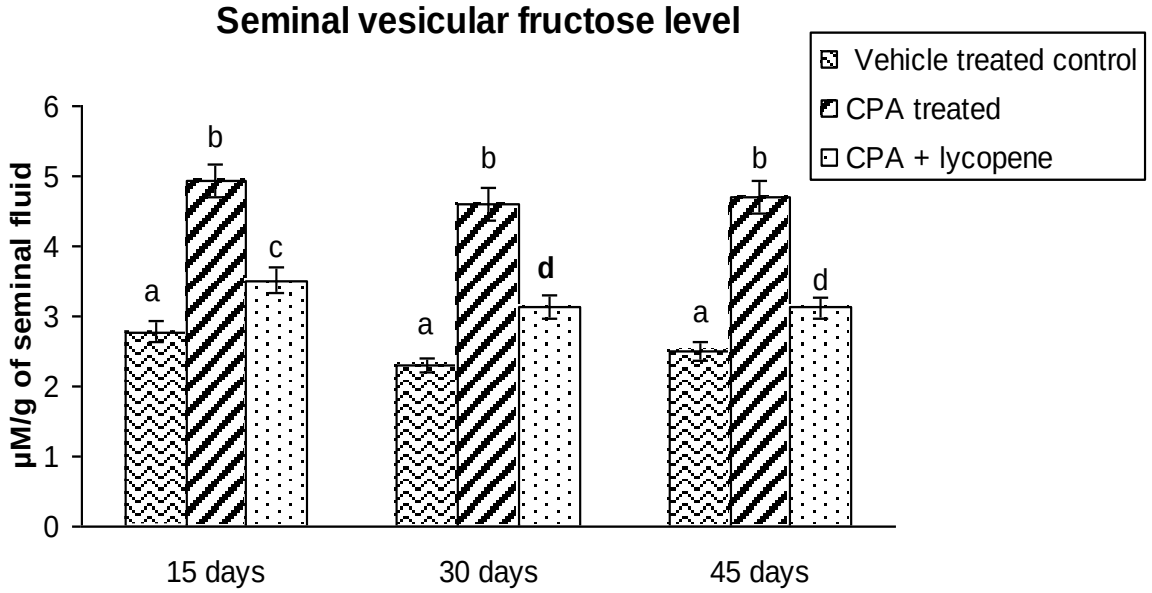


Figure 4.3.6 Duration dependent revival efficacy of lycopene on seminal vascular fructose level on CPA induced male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

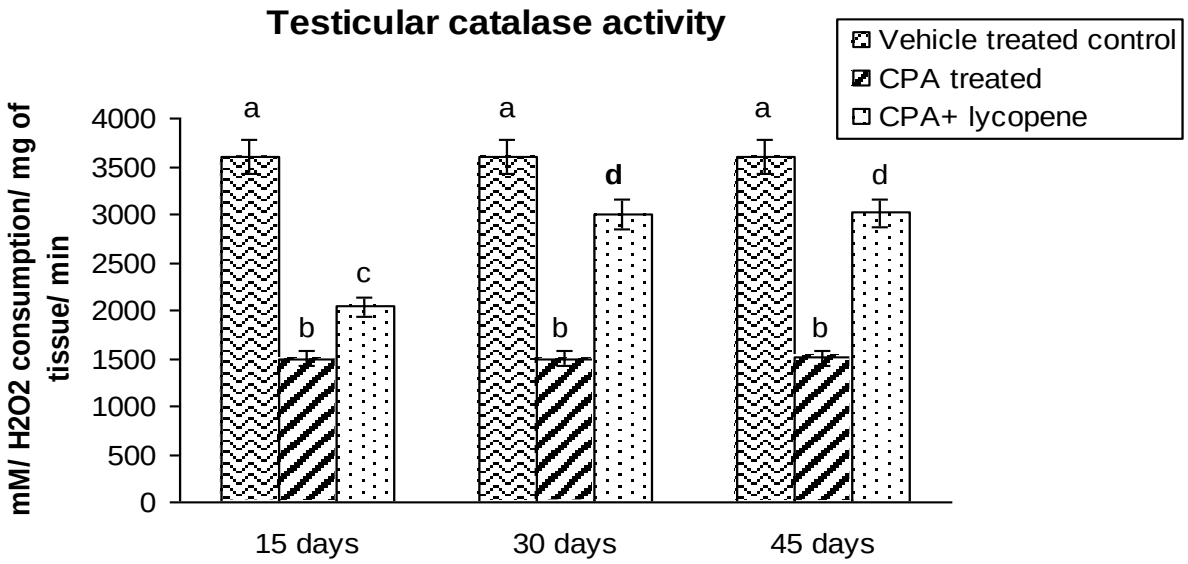


Figure 4.3.7 Duration dependent ameliorative efficacy of lycopene on testicular catalase activity on CPA induced male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

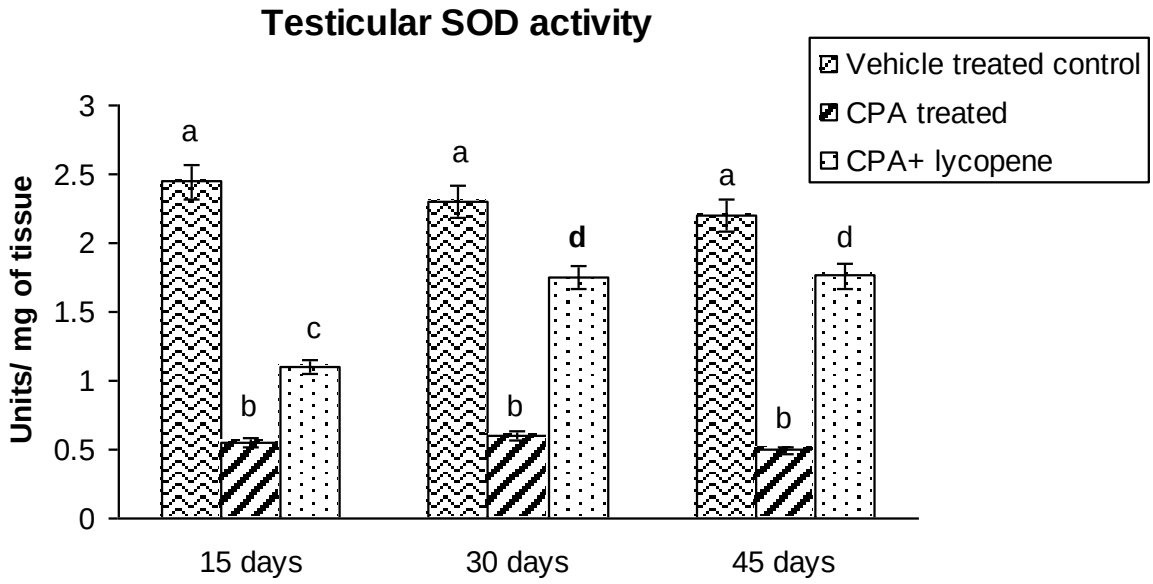


Figure 4.3.8 Correction of testicular SOD activity by duration dependent treatment regimen with lycopene on CPA induced male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

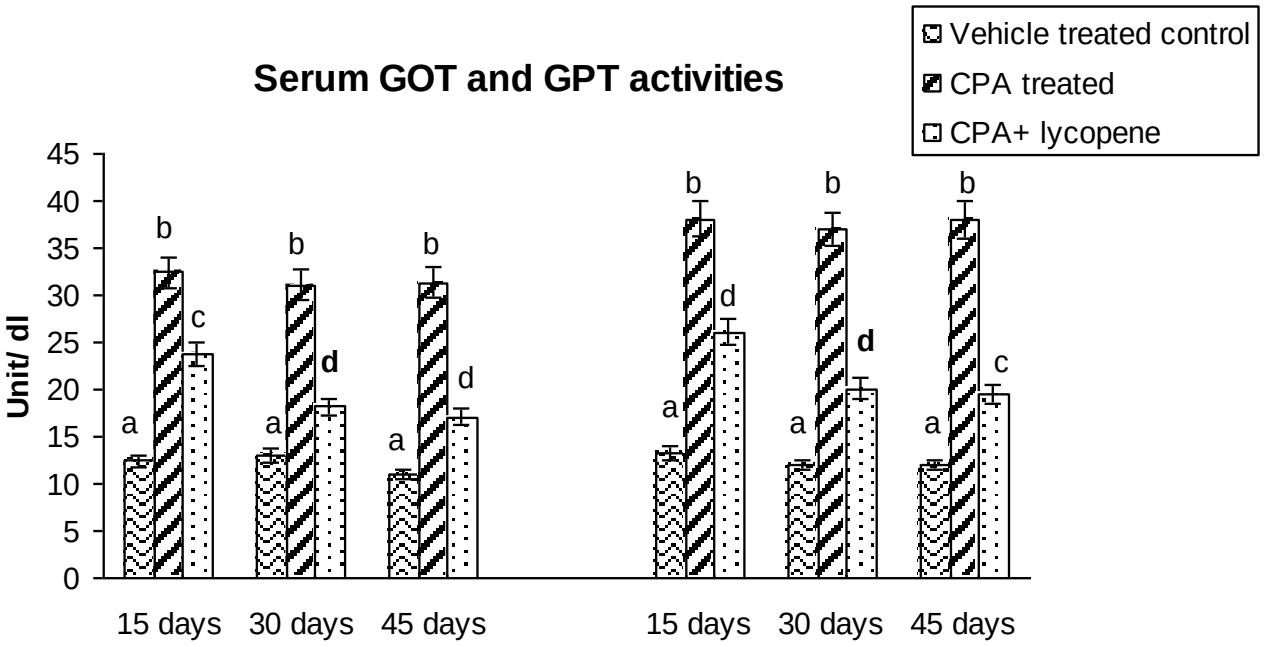


Figure 4.3.9 Rectification in the serum GOT and GPT activities after lycopene treatment in different duration dependent regimen in CPA induced male infertile rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by "Multiple Comparison Two-tail *t*-test". Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

4.4. Experiment No. IV

Reversible or irreversible nature of revival efficacy of lycopene at the potent dose for the management of CPA-induced male reproductive abnormalities: A duration dependent withdrawal study

4.4.1 Significance of the study

The study has been performed to focus the maximum duration of sustainability in recovery of the testicular complication after lycopene treatment at the potent dose and for threshold duration followed by withdrawal of lycopene treatment for 15 day, 30 day and 45 days to the CPA treated male infertile albino rats.

4.4.2 Experimental Design

To fulfil the aim of the experiment, fifty-four Wistar strain male albino rats were obtained from institutional authorized vendor, Shah Enterprise, Kolkata. Animals were of about 80 -90 days old and having body weight 120 ± 10 g average. Before the initiation of the experiment, animals were allowed for acclimatized themselves at laboratory environment and human contact in order to avoid any sudden stress to the animals. The room temperature was 25 ± 2 ºC and humidity was about 44-45% of the room where animals were kept. To maintain the normal circadian cycle of 24 hours, the light-dark cycle at the ratio of 12: 12 was maintained. During this phase, the animals were supplied with sufficient food (rat chew) and water *ad libitum*. Number of animals, process of animals handling, treatment protocol, procedure of sacrifice all were maintained by following the guideline of CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) as approved by IEC (Institutional Ethic Committee) (IEC/ 3/ C-4/ 14, dated- 03. 11.2014).

Animals were classified in three main groups of withdrawal schedule i.e. 15 days, 30 days and 45 days lycopene withdrawal schedule groups. Each schedule group contains three

subgroups such as vehicle treated control group, CPA treated group and CPA and lycopene combine treated groups. Number of animal distribution per group was performed as previous experiments. For the first two months CPA treated groups of all corresponding withdrawal schedule groups were administered with CPA at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day to develop and maintain the infertile status. Cyproterone acetate and lycopene combined treated animals of all corresponding groups were provided with CPA for first one month then lycopene was administered along with CPA for another 30 days followed by withdrawal of lycopene and CPA treatment for 15 days, 30 days and 45 days were considered as duration dependent withdrawal group.

To confirm revival sustainability of lycopene, withdrawal study of CPA with a very small number of animals was conducted to free the experiment from any biasness. The main goal of this study was to focus duration of CPA activity in connection to sustain the infertile condition. In this study CPA was administered for 60 days at the same above mentioned dose followed by withdrawal of CPA treatment for 30 days, 60 days and 90 days. Basic Spermatological parameters such as sperm count, sperm viability, testicular Δ^5, 3β-HSD and 17β-HSD activities, level of serum testosterone, catalase activity was performed. Result showed that CPA can exert its activity to maintain the infertile condition up to 60 days successfully after its withdrawal

Considering that fact, CPA has been withdrawn along with lycopene to the corresponding withdrawal groups focusing the activity of lycopene whereas CPA can able to maintain the infertile condition up to the maximum duration of lycopene withdrawal schedule i.e. 45 days.

Classification of experimental groups was as follows:

15 days withdrawal schedule

Total duration of the experiment of this withdrawal group was 75 days (30 days CPA treatment+ 30 days lycopene+ 15 days lycopene withdrawal)

Group I: Vehicle treated control: Normal, fertile and healthy albino rats of this particular group were supplied with distilled water for first 30 days and then 0.5 ml tween-80/ 100 g body weight/ day for another 30 days. Then again distilled water was provided for another 15 days till their sacrifice.

Group II: CPA treated group: Animals were treated with CPA at 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 60 days and then CPA was withdraw for 15 days till they were sacrificed.

Group III: CPA+ lycopene treated group: Animals previously subjected with CPA for 30 days to induce infertility, were orally administered with lycopene at the dose of 1.5 mg/ 0.5 ml tween 80/ 100 g body weight/ day along with CPA for another 30 days followed by 15 days withdrawal of lycopene treatment

30 days withdrawal schedule

Total duration of the experiment of this withdrawal group was 90 days (30 days CPA treatment+ 30 days lycopene treatment+ 30 days lycopene withdrawal)

Group I: Vehicle treated control: Vehicle treated control animals of this group were allowed to have with distilled water for 30 days of the experiment then during the last 30 days where lycopene was provided to the concern treated group, 0.5 ml tween-80/ 100 g body weight was provided for another 30 days of the experiment. After that, again 0.5 ml distilled water incorporated for another 30 days of withdrawal of lycopene treatment to the concerned group.

Group II: CPA treated group: Animals of this group were incorporated with 3.0 mg CPA/ 0.5 ml distilled water/ 100 g body weight/ day for 60 days. CPA was withdrawal for another 30 days during the withdrawal of lycopene to the withdrawal concerned group.

Group III: CPA+ lycopene treated group: Infertility was induced after cyproterone acetate administration for 30 days. These infertile rats were orally administered with lycopene at the potent dose for threshold duration i.e. 30 days followed by cessation of the lycopene treatment for 30 days along with CPA treatment.

45 days withdrawal schedule

Total duration of the experiment of this withdrawal group was 105 days (30 days CPA treatment+ 30 days lycopene treatment+ 45 days lycopene withdrawal)

Group I: Vehicle treated control: For the first 30 days of experimental schedule, animals were subjected to distilled water for 30 days then 0.5 ml tween-80/ 100 g body weight/ day was administered for last 30 days of the experiment. Then again distilled water was administered for another 45 days during the withdrawal of lycopene to the concerned withdrawal group.

Group II: CPA treated group: Cyproterone acetate was provided for 60 days to develop and maintain infertile model at the concern dose mentioned before. Then CPA was withdrawan for last 45 days of the experiment and distilled water was provided to the rat from that period until their sacrifice.

Group III: CPA+ lycopene treated group: Cyproterone acetate treatment was performed for the first 30 days of the experiment to develop infertile model animal. Then these infertile rats were allowed for administration of lycopene at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 day orally along with CPA and then withdrawal of lycopene treatment for 45 days along with CPA.

Time of drug delivery, lycopene administration and food delivery was followed as per the protocol mentioned in the earlier experiment.

Animals of all the groups were sacrificed at 16^{th} day, 31^{st} day and 46^{th} day of 15 days, 30 days and 45 days withdrawal schedule groups respectively. Blood was collected from dorsal aorta in order to separate the serum by centrifuge the blood at 3000 x g for 5 min. After that sperm was collected from cauda epididymis. Then testes were kept in -20ºC for analysing the concerned reproductive parameters to confirm the reversible or irreversible nature of revival efficacy of lycopene.

4.4.3 Parameters and methods

Spermiological domain was covered by analysing sperm count **(Pant and Srivastava, 2003)** and sperm motility **(Zemjanis, 1977).** Sperm acrosomal status **(Gopalkrishnan et al., 1991)** was performed to confirm the morphological changes of the sperm after withdrawal of lycopene treatment. For the assessment of andrological enzymatic profile of the rat, androgenic key enzymes activity such as Δ^5, 3β-HSD **(Talalay, 1962)** and 17β-HSD **(Jarabak et al., 1962)** were analysed. Level of serum testosterone was assessed by following the standard protocol **(Srivastava, 2001)** as it is the end product of steroidogenic pathway. Antioxidant profile was assessed by measuring the activities of catalase **(Beer, 1952),** SOD **(Marklund and Marklund, 1974),** glutathione-s-transferase **(Hebig et al., 1974)** and peroxidase **(Sadasivam and Manickam, 2008)** in testicular tissue to assess the rectification of oxidative stress after lycopene treatment. Oxidative stress end product was analysed by measuring the levels of CD **(Slater, 1984)** and TBARS **(Okhawa et al., 1979).** For assessment of the levels of some parameters of serum lipid profile such as triglyceride, LDL, VLDL and HDL, standard protocols were followed (**Friedwald et al., 1972).** All the data was analysed by statistical method as per standard protocol **(Sokal and Rohle, 1997)**.

4.4.4 Results

4.4.4.1 Sperm motility and sperm count

Sperm motility and sperm count were significantly ($p < 0.05$) decreased in CPA treated group of all withdrawal schedule groups compared to the corresponding vehicle treated control groups. Treatment with lycopene at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days followed by cessation of lycopene treatment for 15 days, 30 days and 45 days, exhibited a significant ($p < 0.05$) stability in revival status of sperm motility and sperm count compared to CPA treated groups. Statistical analysis revealed a non significant ($p > 0.05$) variation between 15 days and 30 days withdrawal where 45 days withdrawal schedule showed a significant ($p < 0.05$) diminution in sperm motility and sperm count when comparison was made between 15 days and 30 days withdrawal groups. In this concern, 30 days withdrawal is the threshold duration where lycopene can maintain the stable condition of these parameters **(Table 4.4.1)**.

4.4.4.2 Acrosomal status

Acrosomal status showed a significant ($p < 0.05$) diminution in CPA treated group compared to the vehicle treated control of all the corresponding groups. Oral administration of lycopene at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days followed by withdrawal of treatment for 15 days, 30 days and 45 days resulted a significant ($p < 0.05$) stability in maintaining the acrosomal status comparison to the CPA treated group. Statistical analysis revealed that stability of the acrosomal status was maintained up to 30 days as there is a non-significant ($p > 0.05$) difference between 15 days withdrawal and 30 days withdrawal groups. But the condition of acrosomal status was deteriorated significantly ($p < 0.05$) after 45 days withdrawal when the comparison was made with 15 days or 30 days withdrawal group.

Lycopene can able to maintain its revival efficacy up to 30 days withdrawal maximally **(Table 4.4.1)**.

4.4.4.3 Testicular Δ5, 3β-HSD and 17β-HSD activities

Activities of the testicular androgenic key enzymes were diminished significantly ($p < 0.05$) in CPA treated animals of all the withdrawal schedule groups in comparison to the vehicle treated control of all the corresponding groups. Enzyme activities were significantly ($p < 0.05$) recovered after treatment with lycopene at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days and the activities of the enzymes were maintained significantly ($p < 0.05$) after 15 days, 30 days and 45 days withdrawal of lycopene treatment compared to the CPA treated animals of all corresponding groups. It was observed that in 15 days withdrawal and 30 days withdrawal groups, the activities of these parameters were not significantly ($p > 0.05$) differ with each other but on the other hand, level of rectification was not maintained at positive direction after 45 days withdrawal of lycopene treatment and the level of rectification was significantly ($p < 0.05$) deviated compared with 15 days and 30 days withdrawal schedule groups. So, it can be considered that lycopene can able to maintain the enzyme activity up to 30 days withdrawal of lycopeen treatment in reversible manner **(Figure 4.4.1, 4.4.2).**

4.4.4.4 Serum testosterone level

Serum testosterone level was significantly ($p < 0.05$) decreased after CPA treatment at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day in all the withdrawal schedule groups compared to the corresponding vehicle treated control groups. Administration of lycopene at the dose of 1.5 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days followed by cessation of lycopene treatment for 15 days, 30 days and 45 days exhibited a significant ($p < 0.05$) rectification and stability in the level of the concerned parameter

compared to the CPA treated animals of all the corresponding withdrawal schedule groups. Non-significant ($p > 0.05$) difference was observed between 15 days and 30 days withdrawal groups as further diminution in serum testosterone level was not observed at 30 days withdrawal group. On the contrary, a significant ($p < 0.05$) difference was noted in 45 days withdrawal schedule group compared to the 15 days or 30 days withdrawal group that focused that up to 30 days withdrawal of lycopene treatment can stabilise the serum testosterone level optimally **(Figure 4.4.3)**.

4.4.4.5 Seminal vascular fructose

Level of seminal vascular fructose was significantly ($p < 0.05$) increased in CPA treated group after oral administration of CPA at the concerned dose in respect to the vehicle treated control of all corresponding groups. Level was resettled after treatment with lycopene at the potent dose and the rectified level of seminal vascular fructose was maintained for 15 days, 30 days and 45 days withdrawal of lycopene treatment compared to the CPA treated group. Among 15 days, 30 days or 45 days withdrawal groups, a non-significant ($p > 0.05$) variation was observed between 15 days and 30 days as no further diminution was noted in 30 days but 45 days withdrawal of lycopene treatment exhibited a signification ($p < 0.05$) variation in downward manner when comparison was made between 15 days or 30 days withdrawal groups which focused that 30 days withdrawal schedule of lycopene treatment can able to maintain the stability of the concerned parameter optimally **(Fig. 4.4.4).**

4.4.4.6 Antioxidant profile

A significant ($p < 0.05$) diminution was noticed in testicular SOD, catalase, GST and peroxidase activities in CPA treated animals of all the withdrawal schedule groups compared to the vehicle treated control of all the corresponding groups. The enzyme activities were significantly ($p < 0.05$) recovered after administration of lycopene at the potent dose for 30

days which was the threshold duration of the treatment. After that the lycopene treatment was withdrawn for 15 days, 30 days and 45 days and it was observed that the sustainability of the revival efficacy of lycopene was maintained significantly ($p < 0.05$) up to 30 days as 15 days or 30 days withdrawal schedule did not show any significant ($p > 0.05$) difference with each other where as 45 days withdrawal schedule showed a significant ($p < 0.05$) alteration in downward manner compared between 15 days withdrawal and 30 days withdrawal. This result focused that 30 days withdrawal duration is maximally can able to maintain the revival efficacy of lycopene in positive manner **(Figure 4 4.5, 4.4.6, 4.4.7 and 4.4.8)**.

Oxidative stress end product with special reference to CD and TBARS level were significantly ($p < 0.05$) elevated after CPA administration at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ days to the CPA treated animals compared to the vehicle treated control of all the corresponding groups. Administration of lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day orally for 30 days followed by cessation of lycopene treatment for 15 days, 30 days or 45 days exhibited a significant ($p < 0.05$) stability in the rectification of CD and TBARS levels compared to the CPA treated animals. Non-significant ($p > 0.05$) alteration was noted between 15 days and 30 days withdrawal groups but after 45 days withdrawal of lycopene treatment a significant ($p < 0.05$) elevation was observed in the levels of the end products compared to the 15 days and 30 days withdrawal treated group **(Figure 4.4.9).**

4.4.4.7 Triglyceride, LDL, VLDL and HDL levels

Significant ($p < 0.05$) elevation was observed in triglyceride, LDL and VLDL levels where as significant ($p < 0.05$) deterioration was observed in HDL level after the administration of CPA at the dose of 3.0 mg/ 0.5 ml distiller water/ 100 g body weight/ day in all the withdrawal schedule groups compared to the corresponding vehicle treated control

groups.Lycopene treatment at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days resettled the condition towards the vehicle treated control. After withdrawal of lycopene treatment for 15 days, 30 days or 45 days exhibited a significantly ($p < 0.05$) stabled condition in the concern parameters in connection with maintaining the level of the above parameters compared to the CPA treated group. But statistics showed a non-significant ($p > 0.05$) difference between 15 days and 30 days withdrawal groups. 45 days withdrawal group showed a significant ($p < 0.05$) difference in these parameters when comparison was made with 15 days and 30 day withdrawal. So, 30 days withdrawal considered as the optimal duration in maintaining the level of the said parameters **(Table 4.4.2)**.

4.4.5 Discussion

Experiment IV has been performed to find out the reversible or irreversible nature of lycopene after its withdrawal of treatment for 15 days, 30 days and 45 days. To evaluate the nature of recovery and stability after its withdrawal, spermatogenic profile, structural changes of the sperm, anti-oxidant profile, toxicity profile, androgenic hormone profile, seminal vascular fructose were assessed for the confirmation of the study.

Oxidative stress is one of the major causes of infertility. Cyproterone acetate is reported to produce oxidative stress by generating huge amount of free radical **(Ali, 2008)**. Results focused the similar conditions where activities of the antioxidant enzymes such as SOD, peroxidase, catalase and GST in testicular tissue were significantly deteriorated after CPA administration. Result was strengthening by analysing the levels of CD and TBARS that showed significant elevation. Lycopene administration recovered the antioxidant profile by rectifying the antioxidative enzyme activities and end product level as lycopene has the highest free radical quenching ability and it can trap paroxyl radical **(Islamian and Mehrali, 2015)**. Duration dependent withdrawal study was carried out to focus the recovery of

oxidative stress after cessation of lycopene treatment for 15 days, 30 days or 45 days. It has been observed that the remedial effect of lycopene was cessed after 46 days of withdraw of lycopene treatment and that is may be due to the recovery of oxidative stress because of insufficient supply of exogenous antioxidant **(Tan et al., 2018)**. Stress also can damage the sperm motility and sperm count directly **(Agarwal et al., 2014).** On the other hand, effect regarding the destruction in sperm count and sperm motility was noted due to the effect of CPA on androgenesis. Results focused a significant diminution in Δ5, 3β-HSD and 17β-HSD activities which are responsible for the conversion of androstenedione to testosterone and dehydroepiandrostenedione to androstenediol in testosterone synthesis pathway **(Burger, 2002).** It has been observed that 30 days lycopene treatment followed by 30 days cessation of treatment stabilise the enzyme activities towards the vehicle treated control. For confirmation of the scenario, serum testosterone was assessed which focused the same result where stable pattern of recovery was sustained up to 30 days and further deterioration was observed at 46 days. This is may be due to the withdrawal of stimulatory activity of lycopene in steroidogenic pathway. This was further strengthening after quantification of level of seminal vascular fructose which was elevated after CPA administration. This is probably due to the impairment in the activities of fructose metabolic enzyme **(Heinz et al., 1968)** as a result fructose may unavailable to the sperm as it is main source of fuel **(Mukai and Travis, 2012**). This results low level of sperm count and sperm motility. Experiment also enlighten the same result where the sperm motility and sperm count was recovered after lycopene treatment and the irreversible nature of lycopene was maintained up to 30 days afterwards the parameters were deteriorated which focused the reversible nature of the lycopene activity. It is may be due to the cessation of stimulatory activity on testicular pituitary axis after withdrawn of lycopene treatment (**Robberecht and Denef, 1988**). Acrosomal status also focused the

similar type of results where stable condition after lycopene administration and withdrawal of treatment was maintained up to 30 days and afterwards the acrosomal status was decreased.

In this experiment, important parameters of serum lipid profile such as triglyceride, LDL, VLDL and HDL levels were analysed. Increased levels of triglyceride, LDL and VLDL and low level of HDL were linked with the risk of hyperlipidemia that may leads towards the infertility as research has shown the direct association with hyperlipidemia and infertility **(Liu et al., 2017)**. Withdrawal of lycopene treatment for 30 days maintained the stable condition but after that the reversible nature of lycopene was observed as degradation in the level of the concerned parameters was noticed after 45 day withdrawal of lycopene treatment.

4.4.6 Conclusion

From the experiment, it may be concluded that lycopene may exerts its activity by minimising the CPA induced oxidative stress related spermatogenic problems in irreversible manner up to 30 days by lowering the free radical generation and stress induced reproductive degradation. After cessation of treatment for 45 days for chronic period the said remedial state was not maintained which focused the reversible manner of lycopene treatment.

Table 4.4.1: Spematogenic profile after cessation of lycopene treatment for 15 days, 30 days, 45 days to the CPA treatment male albino rat.

Groups	Sperm motility (%)	Sperm count (million/ ml of epididymal fluid)	Acrosomal Status (count of holes/ slide) (%)
Vehicle treated control	84.23 ± 3.49^{a}	22.52 ± 1.03^{a}	31 ± 1.32^{a}
CPA treated	36.84 ± 2.21^{b}	6.15 ± 0.72^{b}	11 ± 1.03^{b}
CPA+ lycopene treated (15 days withdrawal)	51.48 ± 2.84^{c}	12.94 ± 0.94^{c}	21 ± 1.10^{c}
Vehicle treated control	81.87 ± 2.34^{a}	20.54 ± 1.23^{a}	34 ± 1.23^{a}
CPA treated	34 ± 1.21^{b}	5.23 ± 0.43^{b}	10 ± 1.03^{b}
CPA+ lycopene treated (30 days withdrawal)	**51.98 ± 2.76^{c}**	**11.01 ± 0.85^{c}**	**23.93 ± 2.14^{c}**
Vehicle treated control	83 ± 2.12^{a}	23 ± 1.05^{a}	32 ± 1.65^{a}
CPA treated	35 ± 2.34^{b}	4.21 ± 0.54^{b}	12 ± 1.01^{b}
CPA+ lycopene treated (45 days withdrawal)	45.11 ± 2.09^{d}	5.75 ± 0.005^{d}	45.11 ± 2.09^{d}

Data were expressed as mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p < 0.05$.

Table 4.4.2: Levels of serum tryglyceride, VLDL, LDL, HDL after cessation of lycopene treatment for 15 days, 30 days, 45 days to the CPA treated male albino rat.

Groups	Tryglyceride (mg/ dl)	VLDL (mg/ dl)	LDL (mg/ dl)	HDL (mg/ dl)
Vehicle treated control	90 ± 2.49[a]	16.45 ± 1.03[a]	20.43 ± 1.32[a]	40.12 ± 1.56[a]
CPA treated	140 ± 3.21[b]	28.54 ± 2.12[b]	35.54 ±1.03[b]	24.32 ± 1.34[b]
CPA+ lycopene treated (15 days withdrawal)	115 ± 2.84[c]	20.00 ± 1.64[c]	26.21 ± 1.10[c]	32.23 ± 1.34[c]
Vehicle treated control	89.32 ± 2.34[a]	18.54 ± 1.83[a]	21.34 ± 1.23[a]	42.12 ± 1.45[a]
CPA treated	138.3 ± 3.21[b]	30.3 ± 2.43[b]	36.65 ± 1.03[b]	25.21 ± 1.23[b]
CPA+ lycopene treated (30 days withdrawal)	**117 ± 2.76[c]**	**21.34 ± 1.85[c]**	**27.65 ± 2.14[c]**	**31.23** ± 1.21[c]
Vehicle treated control	92 ± 2.12[a]	17.56 ± 1.05[a]	19.54 ± 1.65[a]	39.21 ± 1.32[a]
CPA treated	141.3 ± 2.34[b]	29.43 ± 2.54[b]	34.32 ± 1.01[b]	23.12 ± 1.12[b]
CPA+ lycopene treated (45 days withdrawal)	129.21 ± 2.09[d]	25.67 ± 1.9[d]	38.43 ± 2.09[d]	27.21 ± 1.54[d]

Data were expressed as mean ±SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Values with different superscripts (a, b, c, d) in each vertical column differ from each other significantly $p<0.05$.

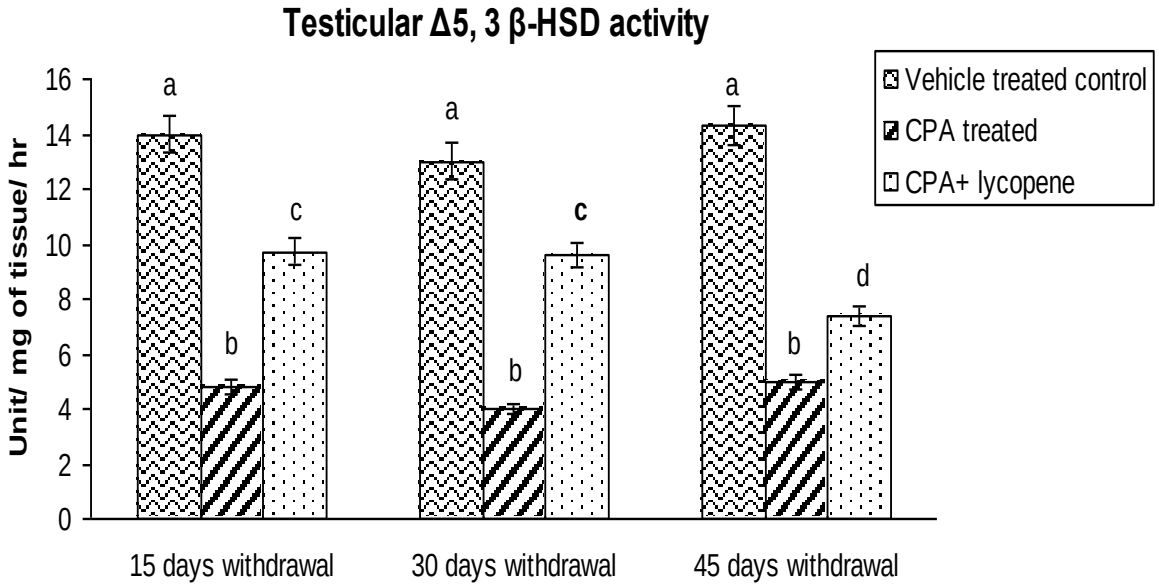


Figure 4.4.1 Effect of 30 days treatment followed by cessation of lycopene treatment for 15, 30 or 45 days on testicular Δ5, 3β-HSD activity in CPA treated rat. Bars were expressed as Mean ± SEM (n= 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, p <

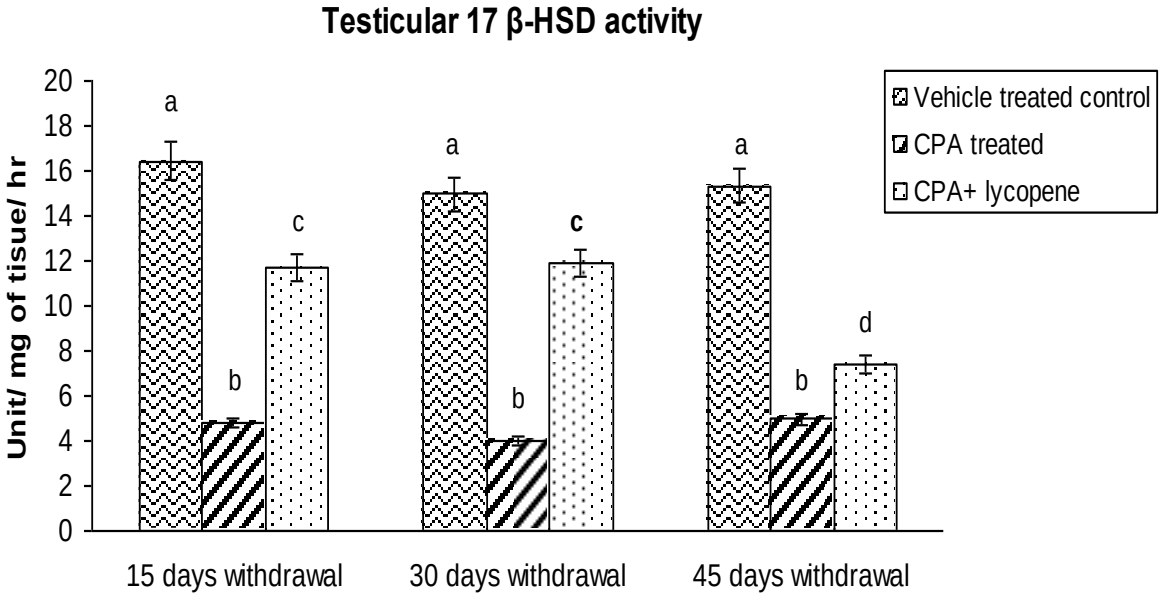


Figure 4.4.2 Activity of 17β-HSD in testicular tissue after the treatment with lycopene for 30 days followed by withdrawal of treatment for 15, 30 or 45 days. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

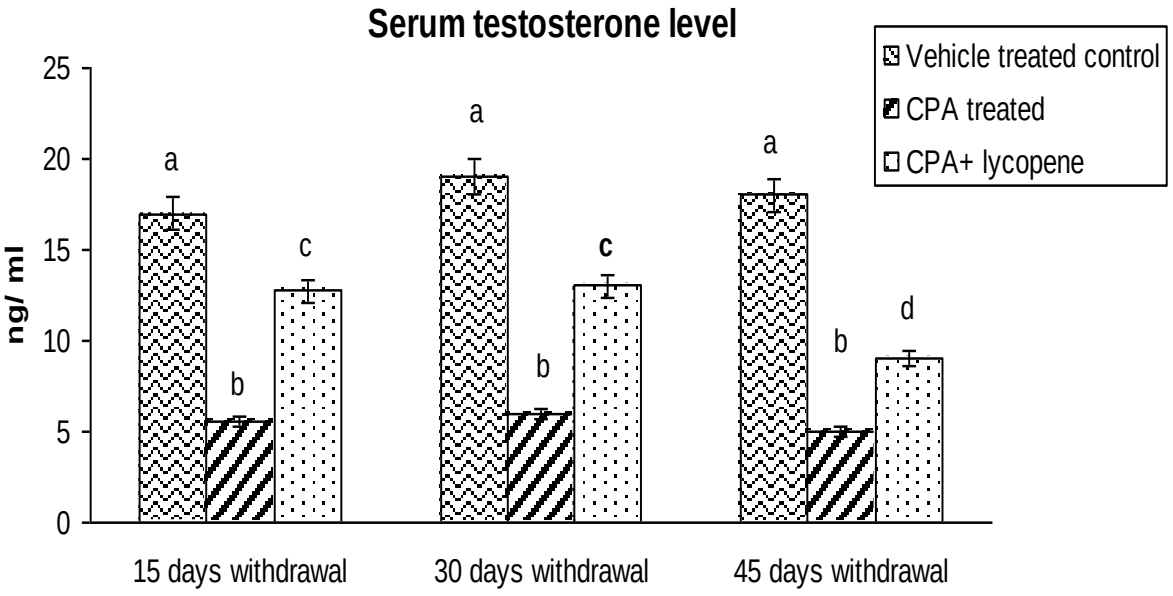


Figure 4.4.3 Rectification in serum testosterone level after treatment with lycopene for 30 days followed by cessation of treatment for 15, 30 or 45 days in CPA treated albino rats. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, p <

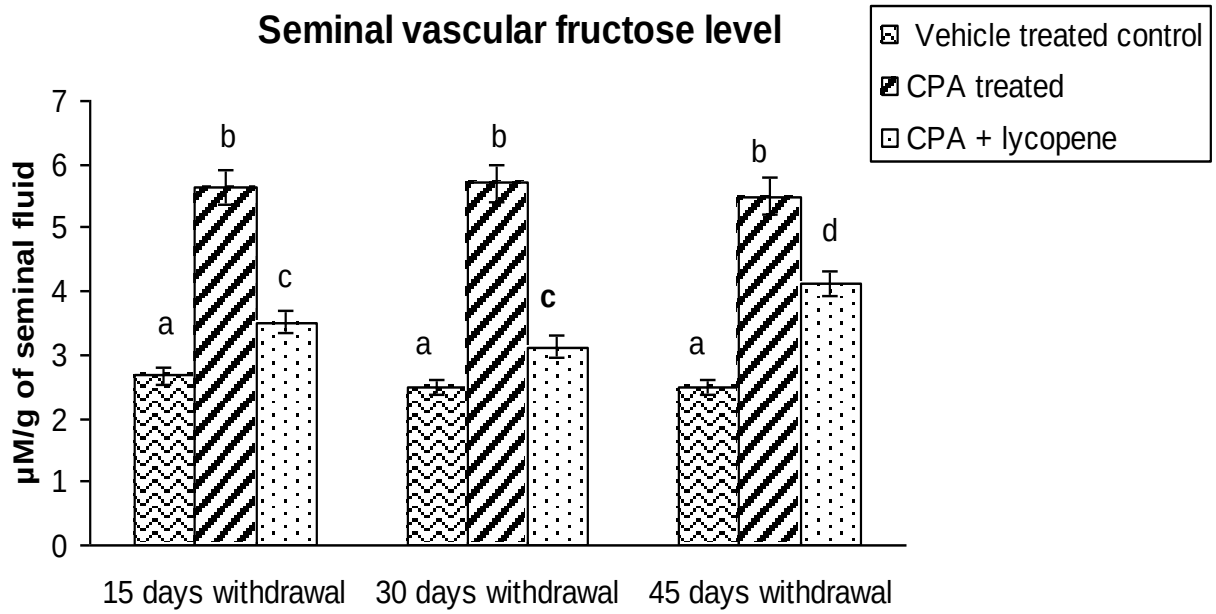


Figure 4.4.4 Rectification in seminal vascular fructose level after treatment with lycopene for 30 days followed by cessation of treatment for 15, 30 or 45 days in CPA treated albino rats. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, p <

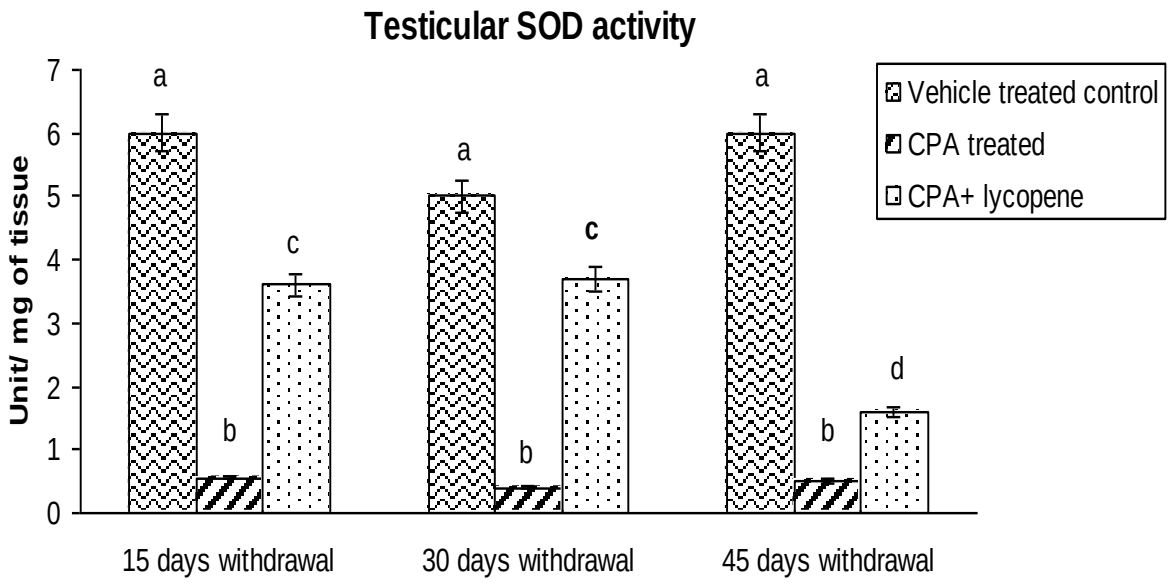


Figure 4.4.5 Testicular SOD activity after the treatment with lycopene for 30 days followed by withdrawal of treatment for 15, 30 or 45 days in CPA treated albino rats. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

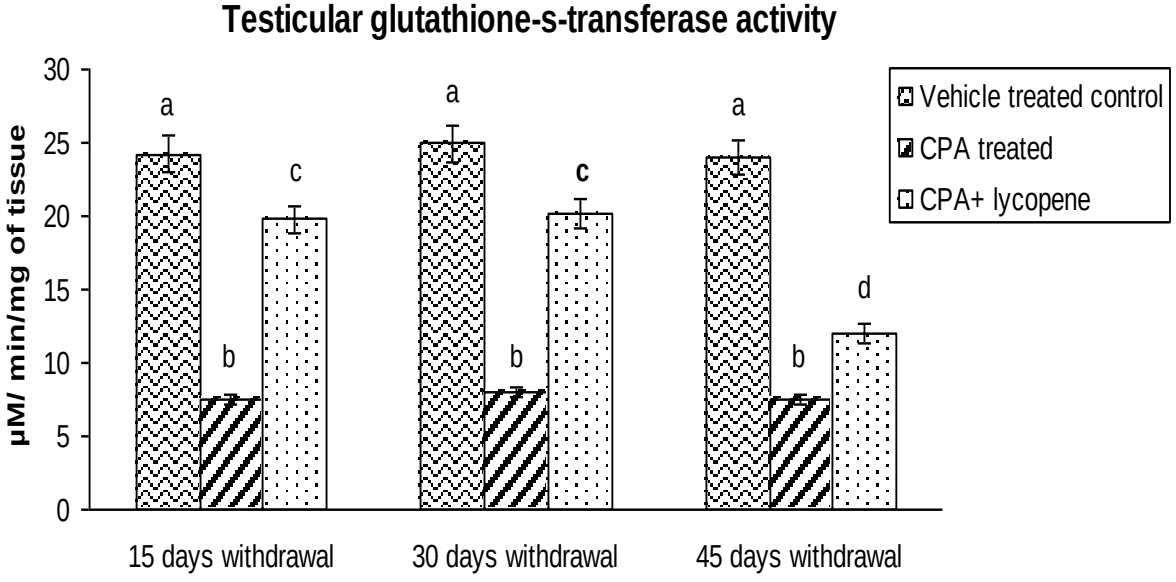


Figure 4.4.6 Activity of testicular GST after 30 days of lycopene treatment followed by cessation of treatment for 15, 30 or 45 days in CPA treated infertile albino rats. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

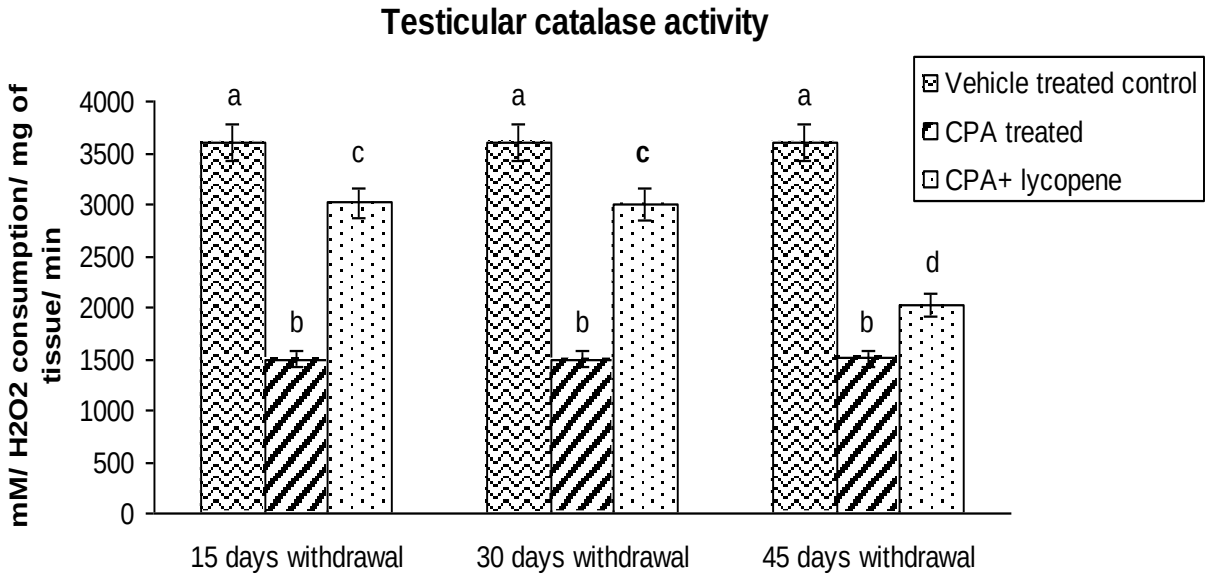


Figure 4.4.7 Testicular catalase activity after oral administration of lycopene for 30 days followed by withdrawal of treatment for 15, 30 or 45 days in CPA treated infertile albino rats. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly,

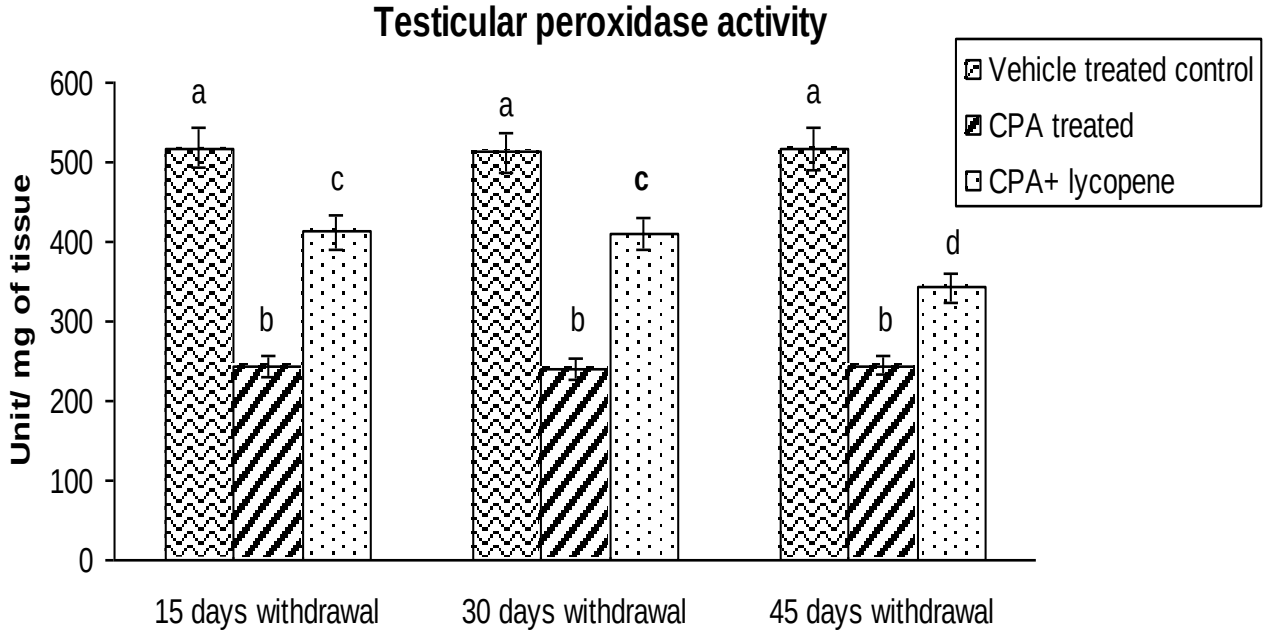


Figure 4.4.8 Effect of withdrawal of lycopene treatment for 15, 30 or 45 days on testicular peroxidase activity on CPA induced hypo-testicular dysfunction in male albino rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

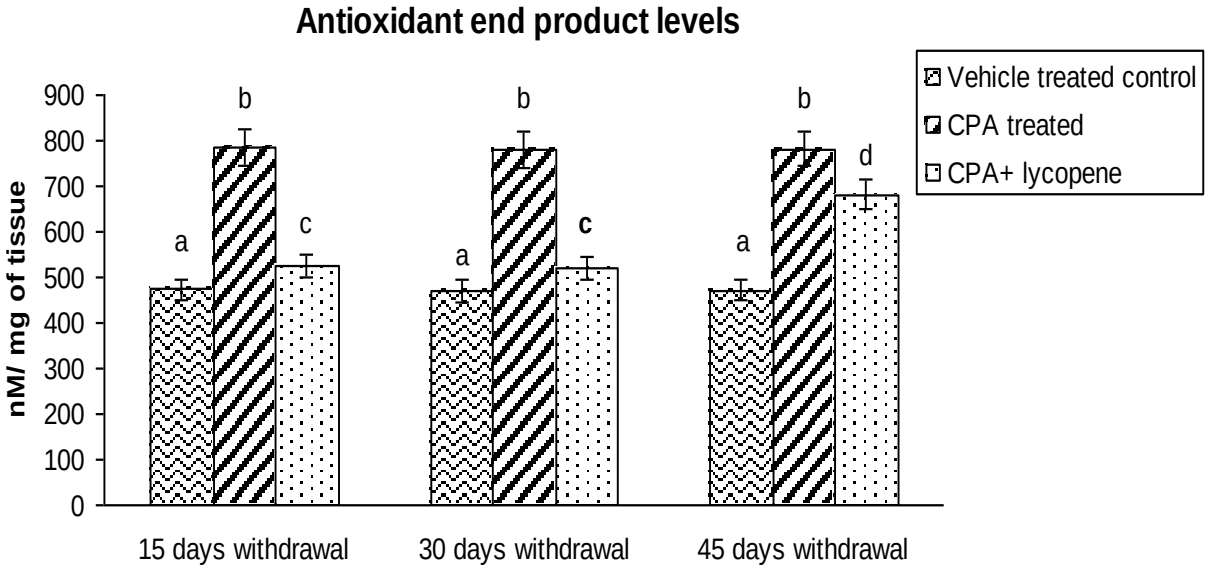


Figure 4.4.9 Effect of withdrawal of lycopene treatment for 15, 30 or 45 days on testicular antioxidant end products levels on CPA induced hypo-testicular dysfunction in male albino rat. Bars were expressed as Mean ± SEM (n =6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c, d) differ from each other significantly, $p < 0.05$.

4.5. Experiment No. V

Defensive mechanism of lycopene on testicular impairment in CPA-induced infertility in male Wistar Strain albino rat: A molecular transection study

4.5.1 Significance of the study

From the previous experiments, the potent dose and the threshold duration of lycopene treatment in connection with the rectification of CPA-induced infertility was achieved. With that information the present experiment has been framed to search out the molecular mechanism behind the rectification of CPA-induced testicular impairment after lycopene administration and to unfold the nutrient-gene interaction. Several domains have been covered such as gene and protein expression of androgenic key enzymes, antioxidant enzymes, apoptotic markers and immuno-histochemistry of testicles to confirm the actual mechanism of action behind the revival efficacy of lycopene through genomic pathway.

4.5.1 Significance of the study

The present experiment was performed by considering eighteen albino rats. The average weight of the animals was 120 ± 10 g and they have kept in sterilized and dry polypropylene cages at about 25 ± 2ºC.To maintain all the normal physiological process as per circadian cycle, the animals were kept in 12 h light: 12 h dark cycle and the humidity of the room was maintained at 45-60%. About 15 days prior the experimentation, animals were allowed to get familiar with the human contact in daily basis in order to minimise the physical stress due to animal handling at the time of forceful drug administration. All the experimental animals were kept in airy, dry scientifically equipped animal house. Experiment was conducted after getting the animal ethical clearance from IEC having number [IEC/3/C-4/14, dated 03/11/2014]. The whole protocol of experiment and animal handling was maintained as per

the guideline provided by CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) regulation, Govt. of India.

Animals were classified in three groups such as vehicle treated control, CPA treated and CPA+ lycopene treated groups. Cyproterone acetate treated groups and CPA+ lycopene treated groups were treated with oral administration of CPA at the dose of 3.0 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days to develop infertile condition. After 30 days, the CPA administration was continued in CPA treated group for another one month to maintain the infertile condition through out the experiment. CPA+ lycopene treated group was allowed to incorporate with lycopene at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for last 30 days of the experiment along with CPA.

The detail of the grouping is discussed below:

Group I: ***Vehicle treated control***: Proven fertile albino rats of this group were administered with distilled water at the dose of 0.5ml/ 100 g body weight/ day orally for first 30 days then tween-80 was administered at the same dose for last 30 days of the experiment.

Group II: ***CPA treated group:*** Animals were subjected to CPA at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 60 days in order to develop infertile condition.

Group III: ***CPA+ lycopene treated*****:** CPA pre-treated infertile rats were allowed for oral administration of lycopene at the dose of 1.5 mg/ 0.5ml tween-80/ 100 g body weight/ day for last 30 days. CPA was also administered at the same time, at the same dose to maintain the infertile condition at the same time.

Time of CPA, lycopene administration and feeding time were maintained as per the interval mentioned in the previous experiment. Total duration of the experiment was for 60 days. At 61st day of the experiment, all the animals were sacrificed by using euthanasia. Sperm was collected from cauda epididymis for viability test and sperm mitochondrial integrity test. Comet assay was also performed after sacrifice. Testes were dissected. One testis of each

animal was stored in -20ºC for gene and protein expression study. On the other hand, another testis has been kept in Bouin's solution for analysis of immuno-histochemistry.

4.5.3 Parameters and methods

Just after sacrifice, sperm viability **(Graham et al., 1990)** and sperm mitochondrial integrity **(Garner et al., 1997)** were assessed through flow cytometry. Sperm DNA damage was assessed by comet assay **(Sing et al., 1988)**. Gene expression study of testicular androgenic key enzyme (Δ5, 3β-HSD and 17β-HSD), anti-oxidant enzymes (SOD, catalase and peroxidase) and apoptotic markers (Bax, Bcl-2, caspase 3, 8, 9, cytochrome-c) was performed by qRT-PCR study **(Meena et al., 2012; Ghosh et al., 2014)**. Western Blot analysis of testicular Δ5, 3β-HSD and 17β-HSD, SOD, catalase, Bax, Bcl-2 and caspase 3 was performed for protein expression study **(Maheshwari et al., 2001)**.The immuno-histochemistry of the testicular tissue was also conducted by using TACS TdT-DAB In-situ Apoptosis Detection Kit (Travigen Inc., Gaithersburg, MD, USA) **(Shikone et al., 1994)**. Statistical analysis of the data was performed by following standard protocol **(Sokal and Rohle, 1997).**

4.5.4 Results

4.5.4.1 Gene expression of androgenic key enzymes

Significant ($p < 0.05$) down regulation was noted in the gene expression of testicular Δ5, 3β-HSD and 17β-HSD in CPA treated group in comparison to the vehicle treated control group. Lycopene administration to the CPA pre-treated infertile rat (CPA + lycopene treated group) at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days along with CPA exhibited a significant ($p < 0.05$) recovery in the expression pattern of the concerned androgenic markers towards the vehicle treated control **(Figure 4.5.1 and 4.5.2)**.

4.5.4.2 Gene expression of antioxidant enzyme markers

Expression pattern of testicular catalase was significantly ($p < 0.05$) deviated in negative direction after administration of CPA at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day in comparison to the vehicle treated control. After administration of lycopene to the CPA + lycopene treated group at the concern dose for 30 days along with CPA, significantly ($p < 0.05$) recovered the gene expression pattern of the enzymatic marker in positive direction towards the vehicle treated control **(Figure 4.5.3)**.

Significant ($p < 0.05$) diminution in the expression pattern of testicular SOD was observed in CPA treated infertile group compared to the vehicle treated control. After lycopene administration at the dose of 1.5 mg/ 0.5ml tween-80/ 100 g body weight/ day for 30 days to the CPA+ lycopene treated group along with CPA showed a significant ($p < 0.05$) rectification in the expression pattern towards the vehicle treated control **(Figure 4.5.4)**.

Similar result was observed in testicular peroxidase gene expression where a significant ($p < 0.05$) down regulation was noted in CPA treated group compared to the vehicle treated control. Oral administration of lycopene to the CPA pre-treated rat (CPA + lycopene treated group) at the potent dose for 30 days exhibited a significant ($p < 0.05$) rectification in the gene expression of the concerned marker towards the vehicle treated control **(Figure 4.5.5).**

4.5.4.3 qRT-PCR study of testicular apoptotic markers

Gene expression of testicular Bax was significantly ($p < 0.05$) increased in CPA treated group compared to the vehicle treated control. Lycopene administration to the CPA + lycopene treated group at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days to the CPA+ lycopene treated group showed a significant ($p < 0.05$) recovery in the testicular Bax gene expression towards the vehicle treated control **(Figure 4.5.6)**.

On the other hand, anti-apoptotic gene i.e. testicular Bcl-2 gene exhibited a significant ($p < 0.05$) down regulation after administration of CPA at the dose of 3.0 mg/ 0.5 ml distilled

water/ 100 g body weight/day for 30 days compared to the vehicle treated control. Lycopene treatment at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/day to the CPA + lycopene treated group for last 30 days showed a significant ($p < 0.05$) rectification in the concerned gene expression towards the vehicle treated control **(Figure 4.5.7)**.

4.5.4.4 Gene expression study of testicular cytochrome –c, caspase 3, 8 and 9

qRT-PCR study of testicular cytochrome- c, caspase 3, 8 and 9 gene expression showed a significant ($p < 0.05$) up regulation in CPA treated group when comparison was made with the vehicle treated control. Lycopene administration to the CPA + lycopene treated group at the potent dose for threshold duration exhibited a significant ($p < 0.05$) correction in the gene expression of concerned markers towards the vehicle treated control **(Figure 4.5.8 and 4.5.9)**.

4.5.4.5 Protein expression of Δ5, 3β-HSD and 17β-HSD

Significant ($p < 0.05$) down regulation was observed in protein expression of testicular Δ5, 3β-HSD and 17β-HSD in CPA treated rat at the concern dose compared to the vehicle treated control. Lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days to the CPA + lycopene treated group, showed a significant ($p < 0.05$) rectification in the protein expression of the androgenic key enzyme marker towards the vehicle treated control **(Figure 4.5.10 and 4.5.11)**.

4.5.4.6 Western blot analysis of testicular antioxidant marker

Protein expression of testicular catalase and SOD was significantly ($p < 0.05$) deviated in down ward manner in CPA treated group compared to the vehicle treated control group. After oral administration of lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day to the CPA + lycopene treated group for 30 days exhibited a significant ($p < 0.05$) rectification in the expression pattern of protein of the concerned markers towards the vehicle treated control **(Figure 4.5.12 and 4.5.13)**.

4.5.4.7 Protein expression study of testicular Bax, Bcl-2 and caspase 3

Western blot study of testicular Bax and caspase 3 showed a significant ($p < 0.05$) up regulation in the transcript level in CPA treated group after treatment with CPA at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days in comparison to the vehicle treated control. Lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day to the CPA+ lycopene treated group exhibited a significant ($p < 0.05$) rectification in the expression pattern of the concern genes towards the vehicle treated control **(Figure 4.5.14 and 4.5.16)**.

Similarly Bcl-2 gene expression of testicular tissue was significantly ($p < 0.05$) diminished in CPA treated group when compared to the vehicle treated control. After lycopene administration at the potent dose to the CPA pre-treated infertile rats (CPA + lycopene treated group) resulted a significant ($p < 0.05$) correction in the Bcl-2 gene expression pattern towards the vehicle treated control **(Figure 4.5.15)**.

4.5.4.8 Sperm viability and sperm mitochondrial integrity by flow cytometry

Sperm viability was assessed through flow cytometry analysis. Results focused a significant ($p < 0.05$) diminution in the percentage of viable sperm compared to the vehicle treated control. The percentage of viable sperm was significantly ($p < 0.05$) rectified towards the vehicle treated control after oral administration of lycopene to the CPA + lycopene treated group at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days **(Figure 4.5.17)**.

Flow cytometry analysis of sperm mitochondrial integrity focused a significant ($p < 0.05$) reduction in the percentage of healthy or polarized mitochondrial membrane in CPA treated infertile rat when comparison was made with the vehicle treated control. Oral administration of lycopene at the concerned dose to the CPA pre-treated infertile rat (CPA + lycopene

treated group), exhibited a significant ($p < 0.05$) correction in the polarized mitochondrial membrane which is towards the vehicle treated control **(Figure 4.5.18).**

4.5.4.8 Quantification of ISEL positive cell

Vehicle treated control group contains very minimum number of ISEL positive cells which was denoted by brown spots. In CPA treated group, there is a significant ($p < 0.05$) elevation in the ISEL positive cells compared to that of vehicle treated control. Treatment with lycopene to the CPA + lycopene treated group at the dose of 1.5 mg/ 0.5 ml tween 80/ 100 g body weight/ day for 30 days resulted a significant reduction ($p < 0.05$) in ISEL positive cells as per the qualitative analysis and the rectification is towards the vehicle treated control **(Figure 4.5.19).**

4.5.4.9 Comet assay

Genotoxicity which is denoted by successful comet is relatively higher in CPA treated infertile albino rats when comparison was made with the vehicle treated control. Lycopene administration at the dose of 1.5 mg/ 0.5 ml tween 80/ 100 g body weight/ day for 30 days to the CPA + lycopene treated group, exhibited a remarkable rectification in the number of comet and the rate of recovery was towards the vehicle treated control **(Figure 4.5.20)**.

4.5.5 Discussion

Experiment V has been conducted to find out the molecular aspect of in connection with the management of CPA induced testicular hypo-function with special reference to oxidative stress mediated apoptosis of germ cell that result sperm DNA damage and 'switching on' of the relevant genes which are responsible for apoptosis in germ cell by lycopene-gene interaction.

Oxidative stress plays a prime role for male infertility induction. Sperm membrane is made of polyunsaturated fatty acid which is damaged by the huge amount of reactive oxygen spices that was produced due to oxidative stress **(Gharagozloo and Aitken, 2011)**. In this concern,

administration of CPA was responsible for the significant down expression of the anti-oxidant enzymes such as catalase, SOD and peroxidase in testicular tissue. Oral administration of lycopene significantly resettled the antioxidant gene expression pattern towards the vehicle treated control due to its free radical quenching property which is twice potent than β-carotene and ten time more potent than α-tocopherol **(El-Raey et al. 2013)** or due to its direct effect on anti-oxidant enzyme gene expression. This result was also reflected in protein expression study by western blot where the protein expression of sperm catalase and SOD was also resettled compared to the CPA treated group. This mechanism may be dependent on nutraceutical-gene interaction signalling pathway **(Ara et al., 2013)** of the concerned gene.

Apoptosis is induced by oxidative stress mediated free radical generation which is regulated by a series of signal cascade under some specific situation. It is initiated firstly by altering mitochondrial membrane potential or depolarization of the mitochondria **(Wagner et al., 2018).** In this study, sperm mitochondria integrity assessment by flow cytometric analysis revealed the high percentage of depolarised mitochondria in CPA treated group compared to the vehicle treated control. This is may be due to the role of cyproterone acetate that damaged the mitochondrial membrane integrity and directly helps in apoptosis by caspase activation **(Khanna and Jain, 2000)**. Up regulation in Bax gene expression and down regulation of Bcl-2 gene further strengthen stress mediated apoptosis induction **(Singh et al., 1988)** which takes place after CPA administration. Significant elevation in cytochrome-c expression further focused the role of CPA to initiate caspase cascade pathway to commit cell death **(Wang and Youle, 2009).** Simultaneously, significant elevation in the gene expression of caspase 3, caspase 8 and caspase 9 is highlighted the activation of both the intrinsic and extrinsic pathways of apoptosis. Lycopene administration resettled the caspase cascade system as a whole towards the vehicle treated control by inhibiting ROS mediated apoptosis

(Mathew et al., 1999). On the other hand, quantification of immuno-histochemistry supported the onset of testicular apoptosis after CPA administration and rectification of apoptosis was also observed here after lycopene administration due to its anti-apoptotic nature **(Gao et al., 2001).**

Experiment exhibited a significant diminution in the gene expression of testicular Δ^5, 3β-HSD and 17β-HSD which are the main androgenic key enzymes. Enzyme activity was recovered after lycopene treatment. Similar result was observed in the protein expression study of the same androgenic markers and the outcome was as same as previous. The anti-androgenic nature of the CPA was further proved by significant diminution in sperm viability, revealed by flow cytometric analysis. This phenomenon was probably due to inhibition in the synthesis of one of the main components of spermatogenesis i.e., androgenic binding protein which interfere spermatogenesis **(Heinlein and Chang, 2002)**.

4.5.6 Conclusion

From the present experiment it may be concluded that lycopene can able to manage the CPA induced hypo-testicular dysfunction cum infertility by altering ROS induced apoptosis. In this context, two hypothesis can be drawn. In hypothesis I, lycopene may correct ROS indued sperm degradation by destroying free radicals. Hypothesis II focused that lycopene may improve the anti-apoptotic and antioxidant gene expression by lycopene-gene interaction.

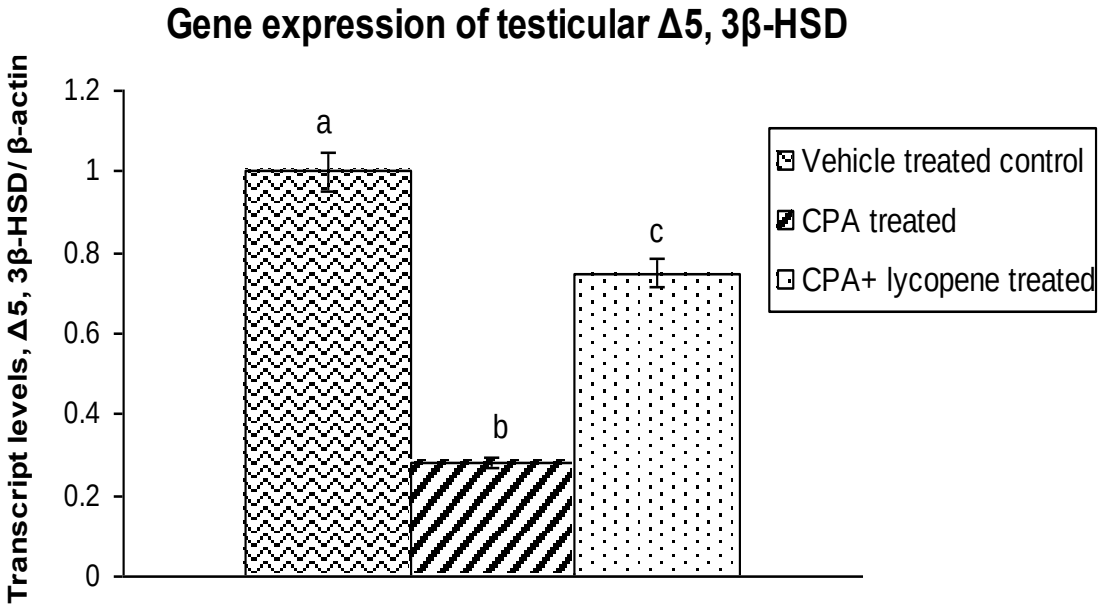


Figure 4.5.1 Correction in the gene expression by qRT-PCR of testicular Δ5, 3β-HSD after lycopene administration in CPA treated infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

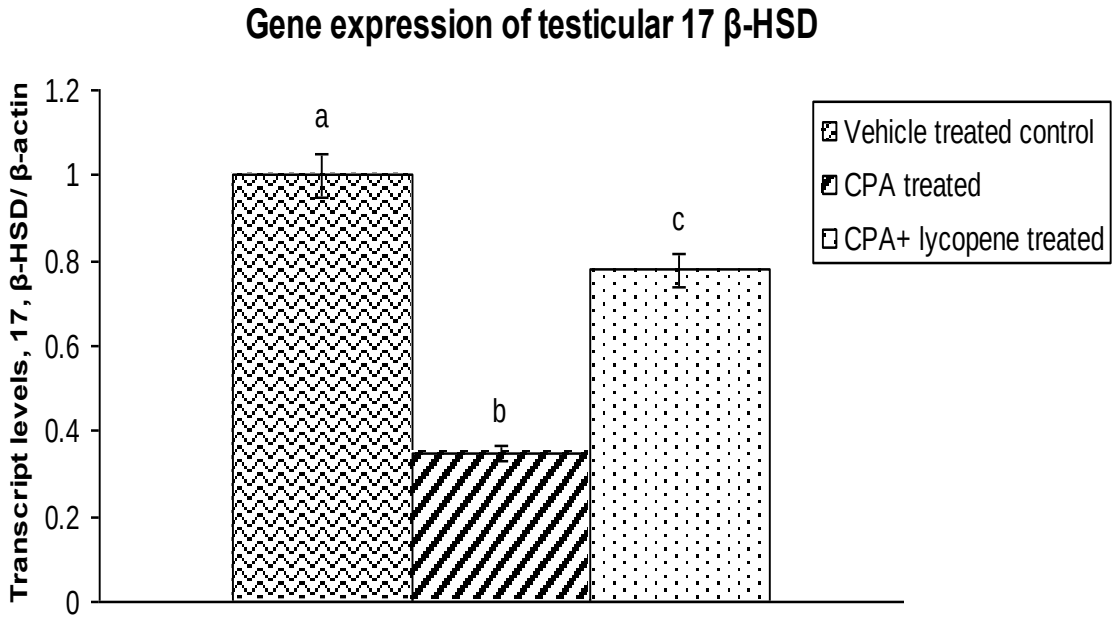


Figure 4.5.2 qRT-PCR study of testicular 17β-HSD gene expression after oral administration of lycopene in CPA pre-treated infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

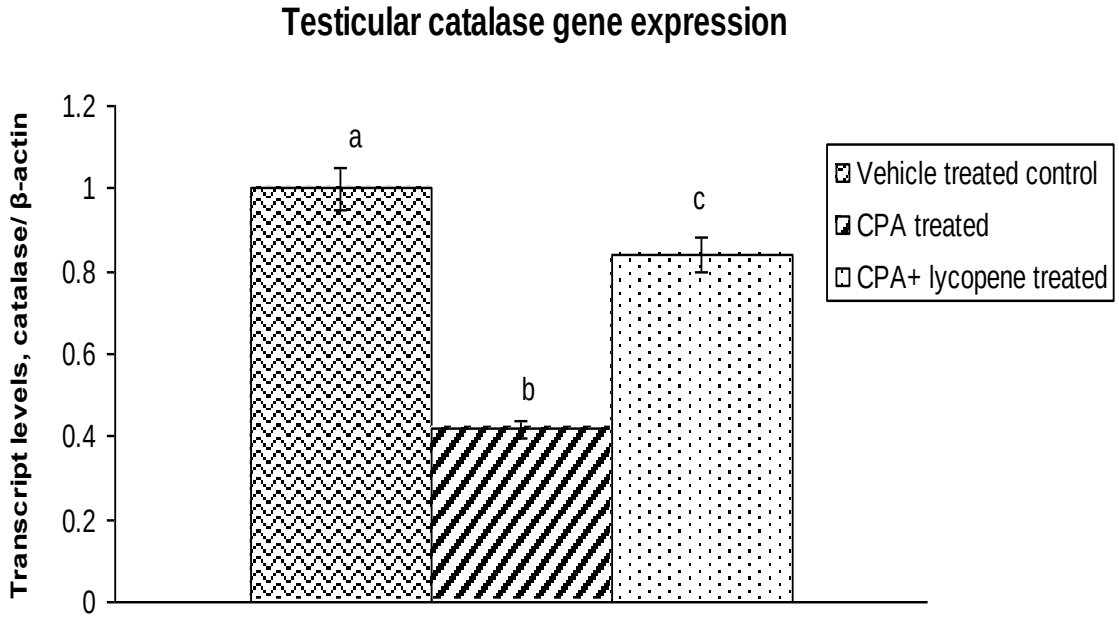


Figure 4.5.3 Up-regulation in testicular catalase gene expression after lycopene treatment in CPA pre-treated infertile albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

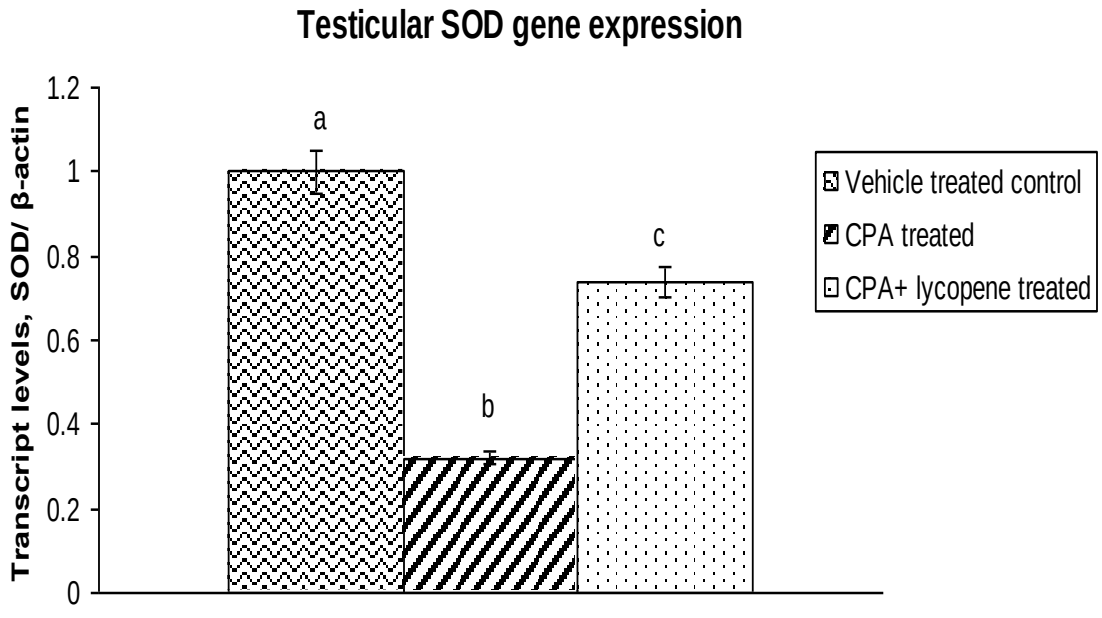


Figure 4.5.4 Correction in the testicular SOD gene expression after lycopene treatment in CPA pre-treated infertile albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

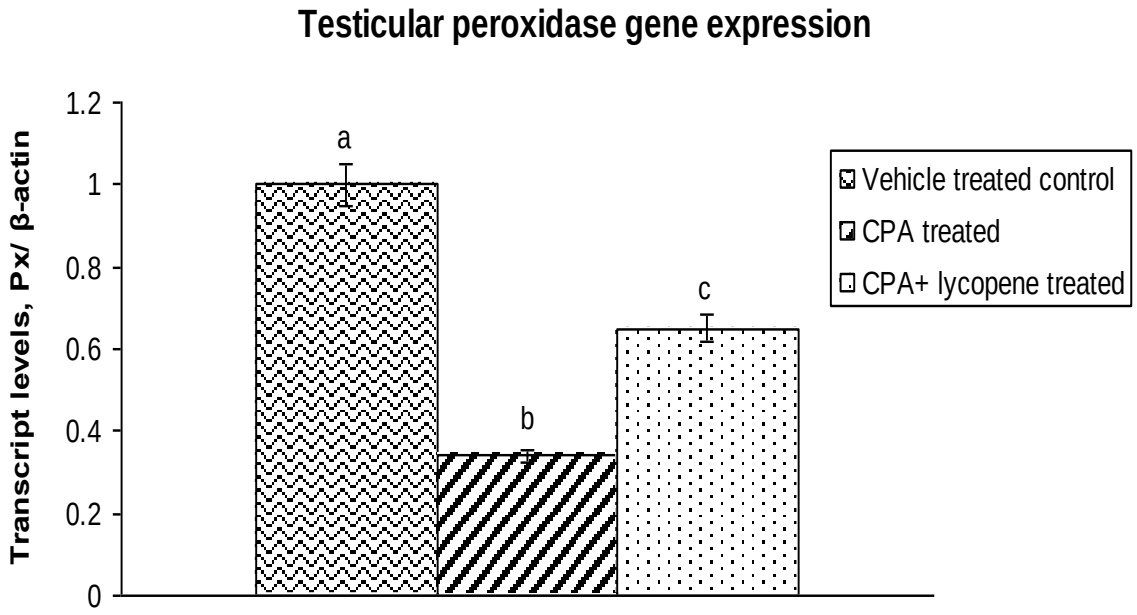


Figure 4.5.5 qRT-PCR study of testicular peroxidase gene expression after lycopene treatment in CPA pre-treated infertile albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

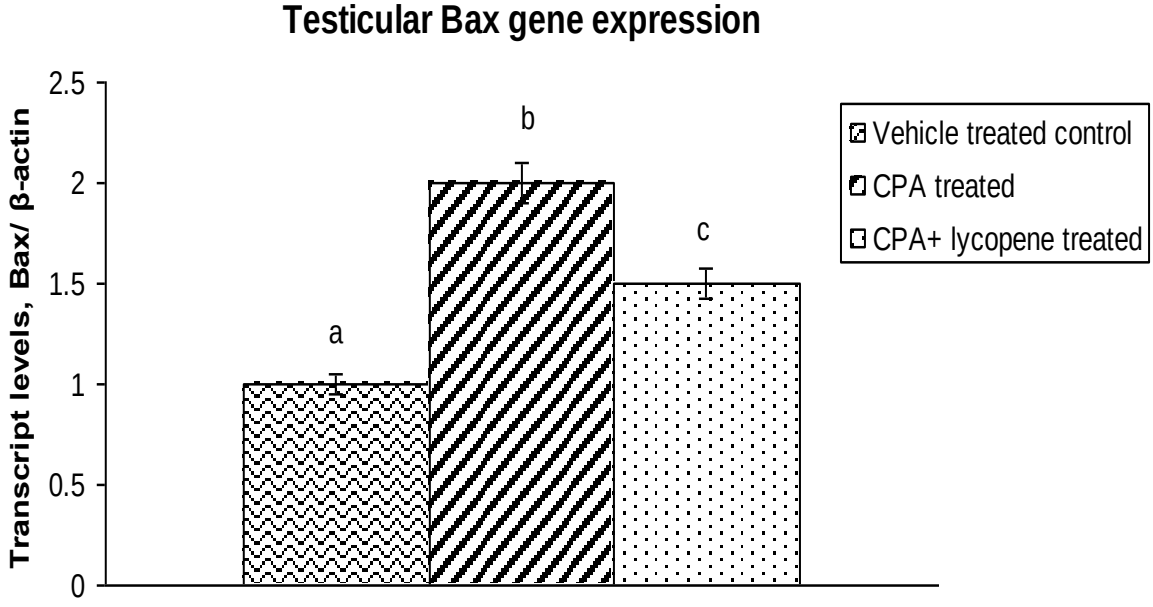


Figure 4.5.6 Rectification in the gene expression of testicular Bax after lycopene treatment in CPA pre-treated infertile albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

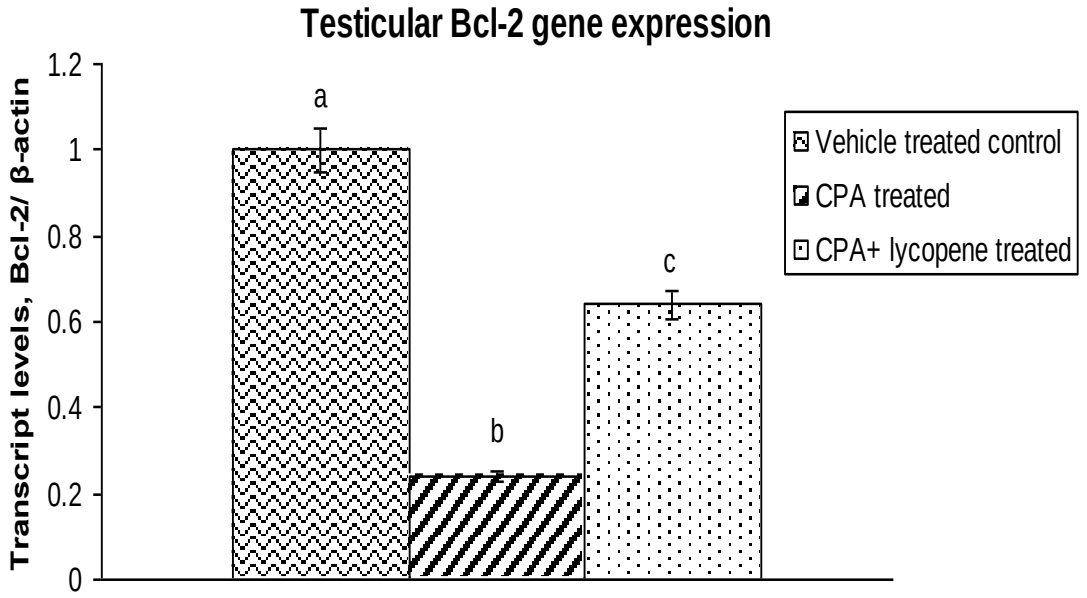


Figure 4.5.7 Bcl-2 gene expression in testicular tissue after the correction in expression pattern by lycopene in albino male rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test' Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

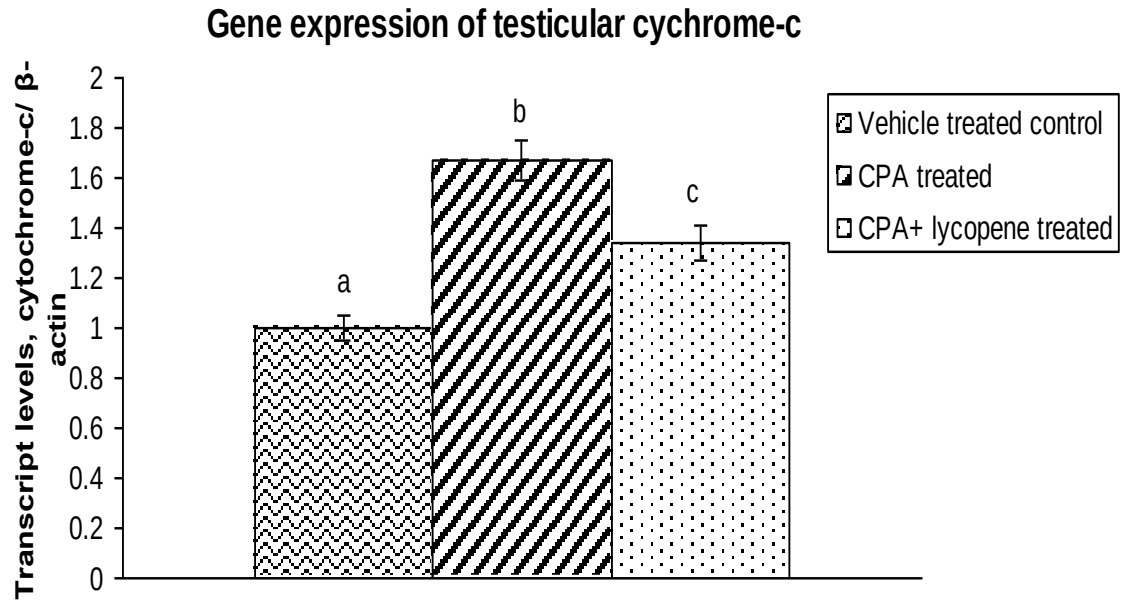


Figure 4.5.8 Rectification in the cytochrome-c gene expression in testicular tissue after oral administration of lycopene to the CPA induced infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

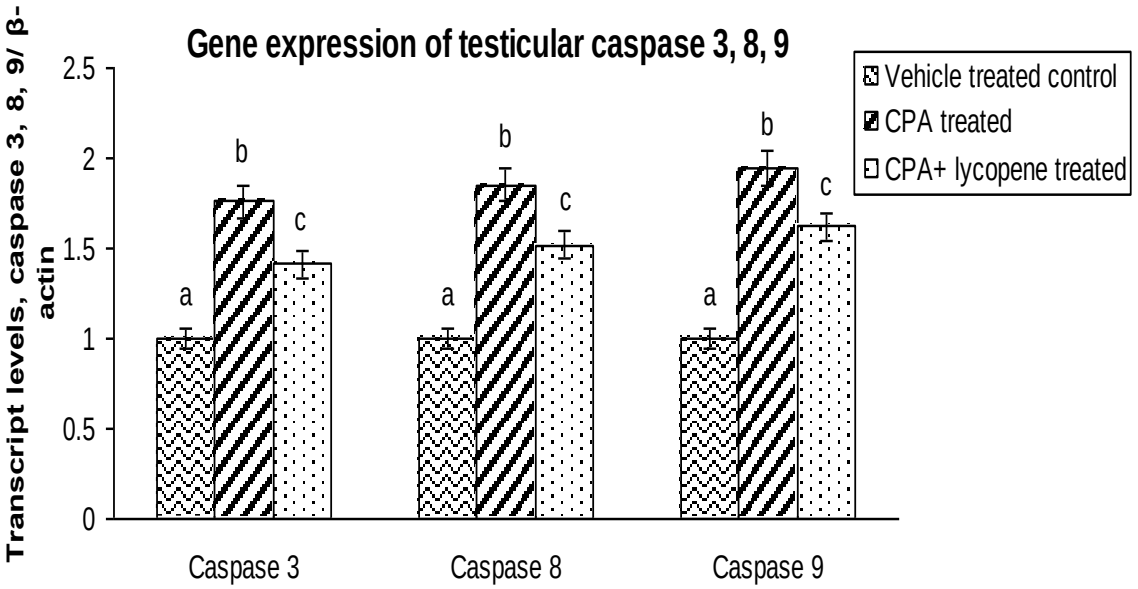


Figure 4.5.9 Corrective efficacy of lycopene on the gene expression pattern of testicular caspase 3, 8 and 9 in testicular tissue after oral administration of lycopene to the CPA-induced infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

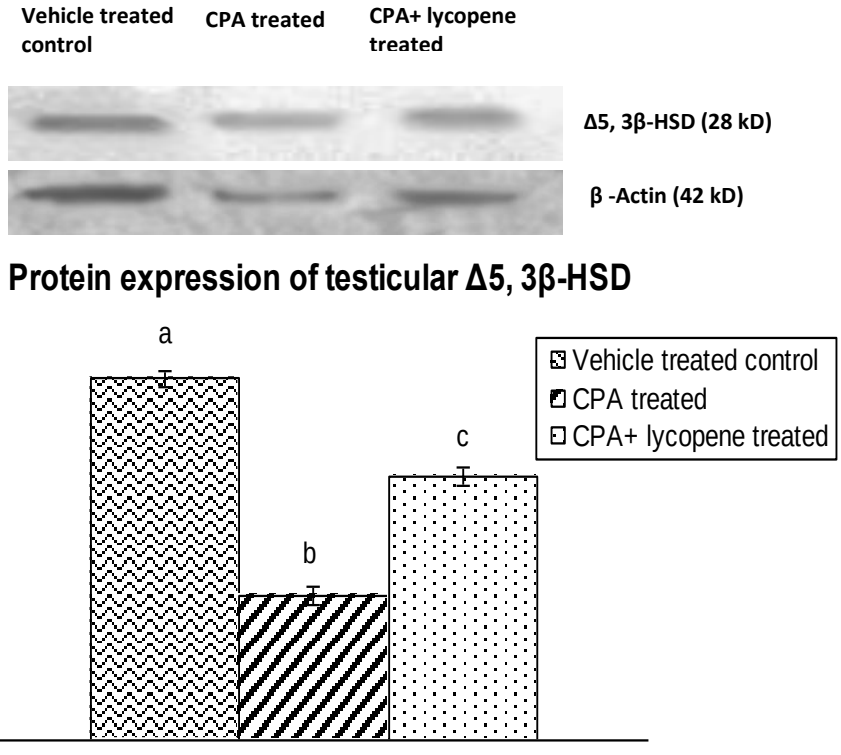


Figure 4.5.10 Western blot analysis of testicular Δ5, 3β-HSD protein expression after lycopene administration in CPA treated infertile rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test' Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

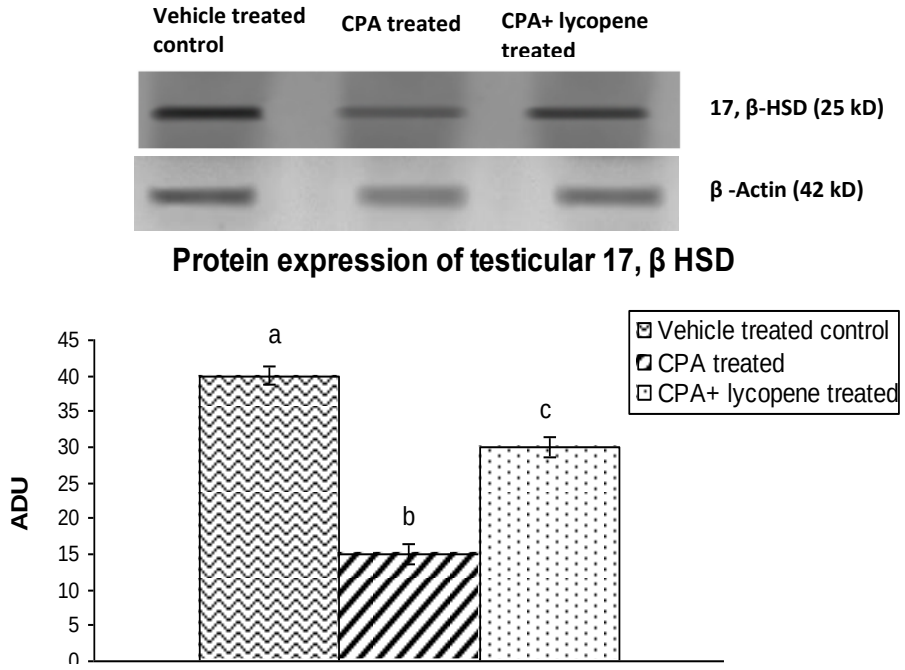


Figure 4.5.11 Ameliorative efficacy of lycopene on testicular 17β-HSD protein expression on CPA treated infertile male rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

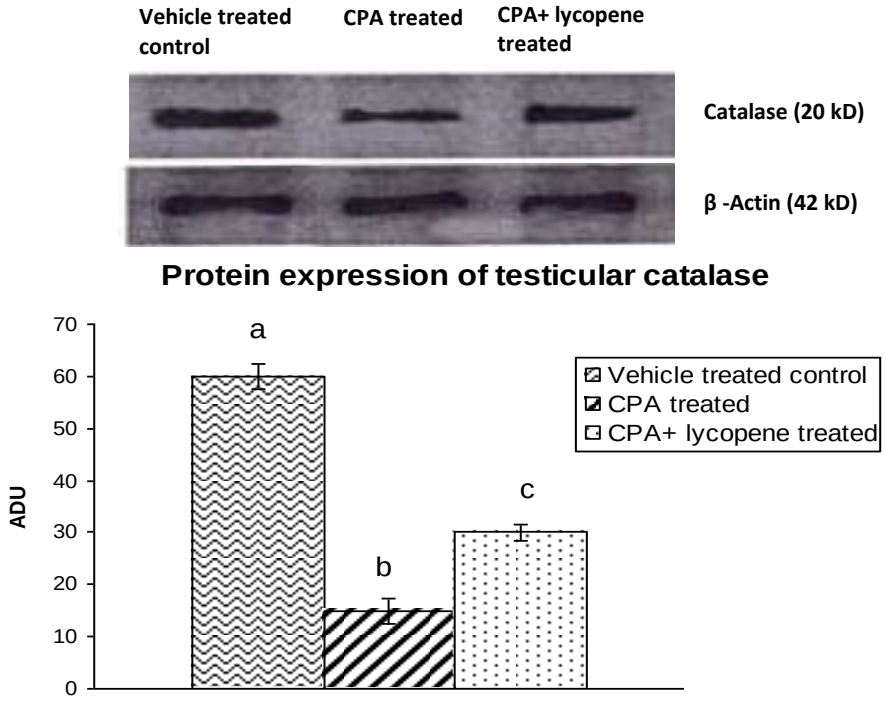


Figure 4.5.12 Rectification in the protein expression of testicular catalase after administration of lycopene in CPA pre-treated albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

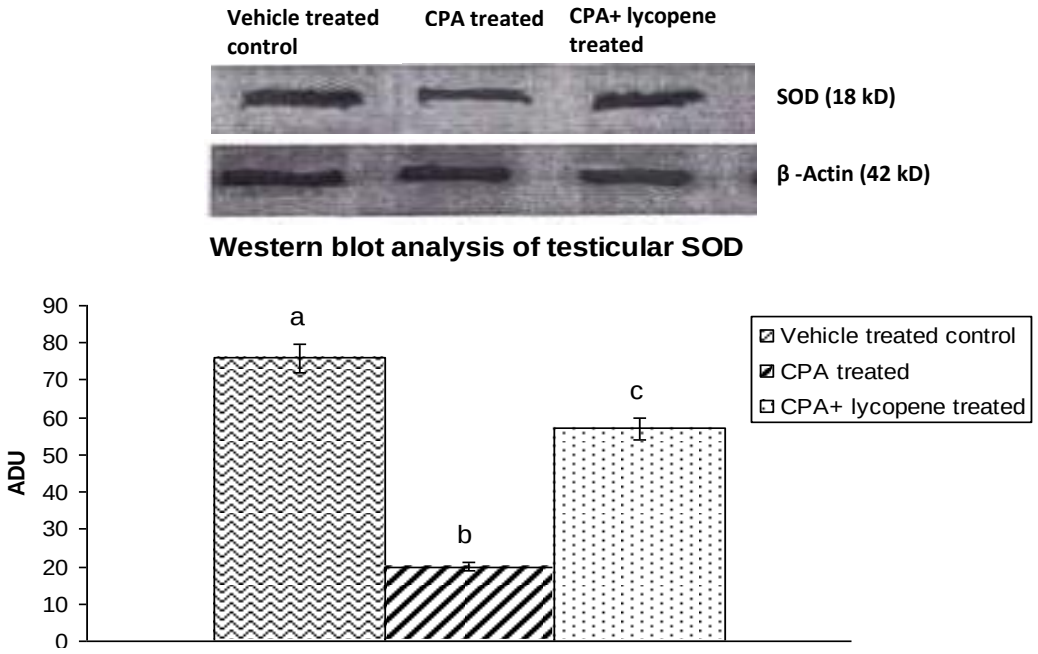


Figure 4.5.13 Rectification in the protein expression of testicular SOD after administration of lycopene in CPA pre-treated albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

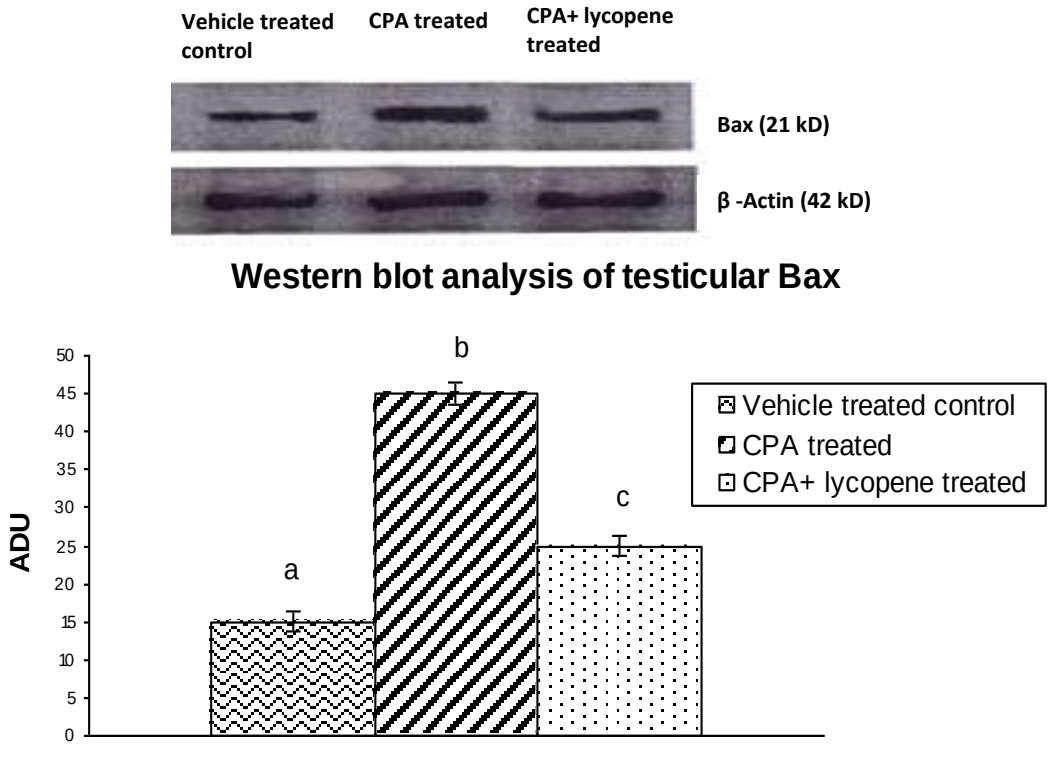
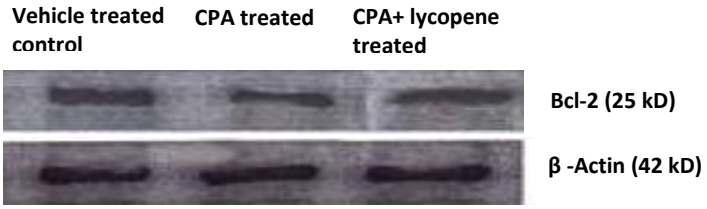


Figure 4.5.14 Western blot analysis of testicular Bax protein expression after lycopene administration in CPA pre-treated albino rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.



Western blot analysis of testicular Bcl-2

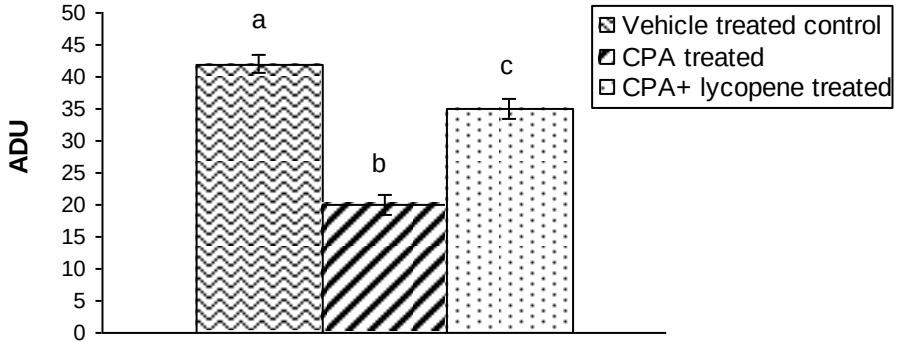
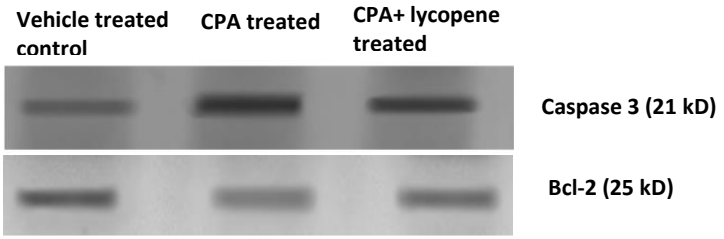


Figure 4.5.15 Attenuation of testicular Bcl-2 protein expression by lycopene in CPA treated infertile male rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test' Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.



Protein expression of testicular caspase-3

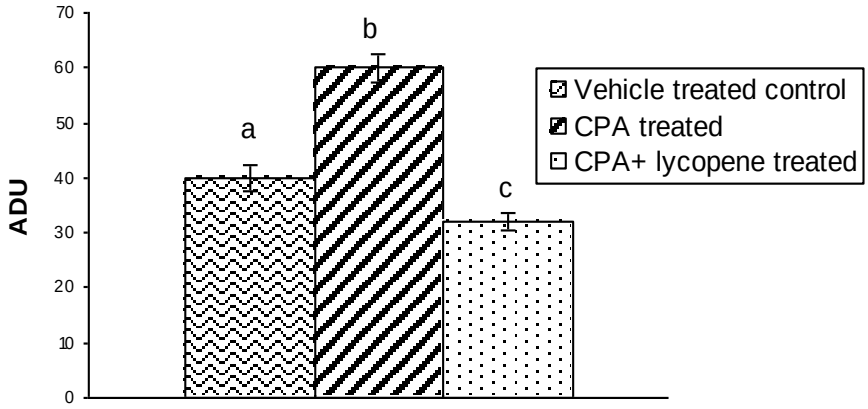


Figure 4.5.16 Correction of testicular Bcl-2 protein expression by lycopene in CPA pre-treated infertile male rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

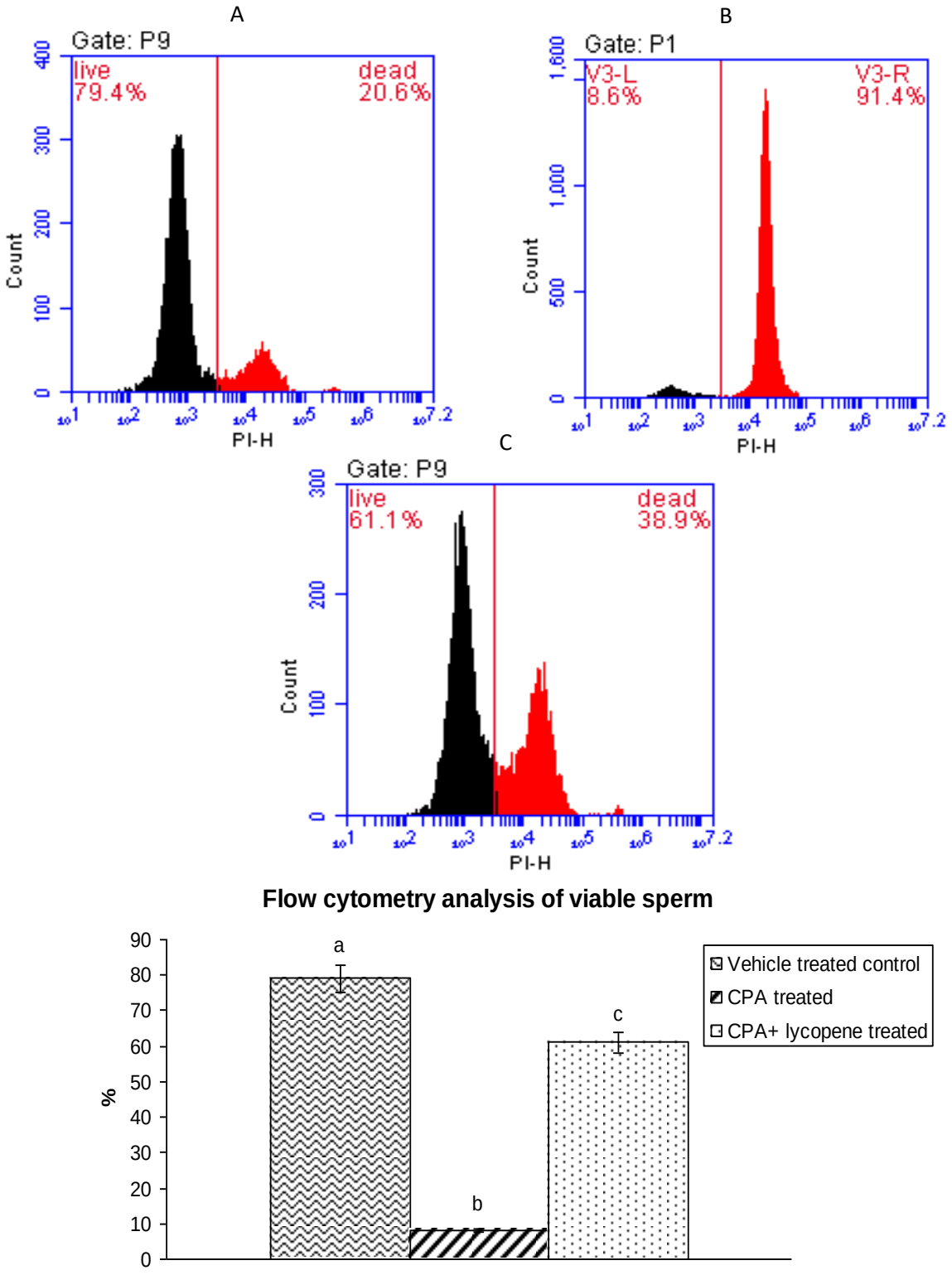


Figure 4.5.17 Flow cytometry analysis of viable sperm in A. Vehicle treated control, B. CPA treated rat, C. CPA+ lycopene treatment. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

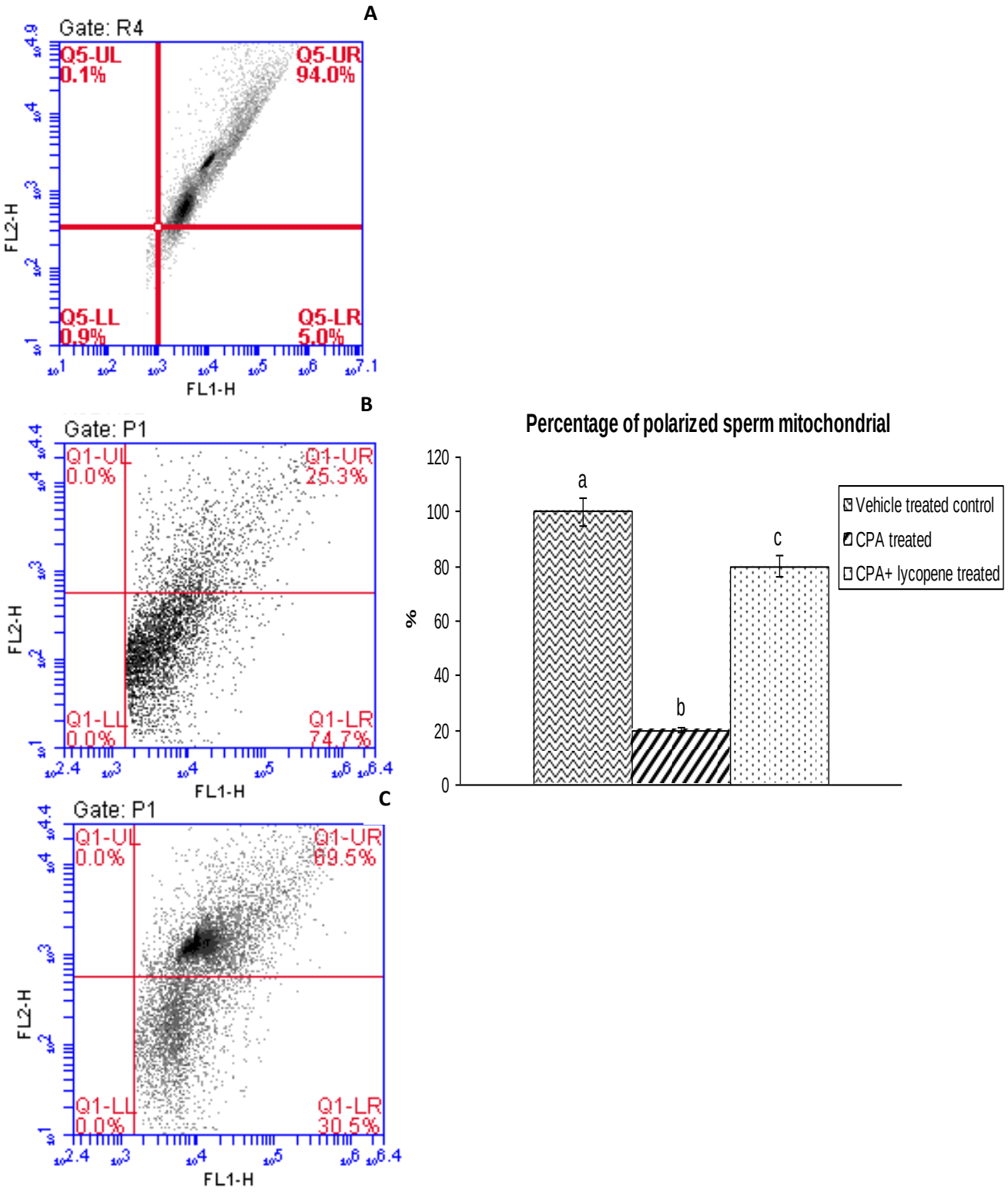


Figure 4.5.18 Effect of lycopene on sperm mitochondrial integrity in A. vehicle treated control, B. CPA treated group and C. CPA+ lycopene treated group. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

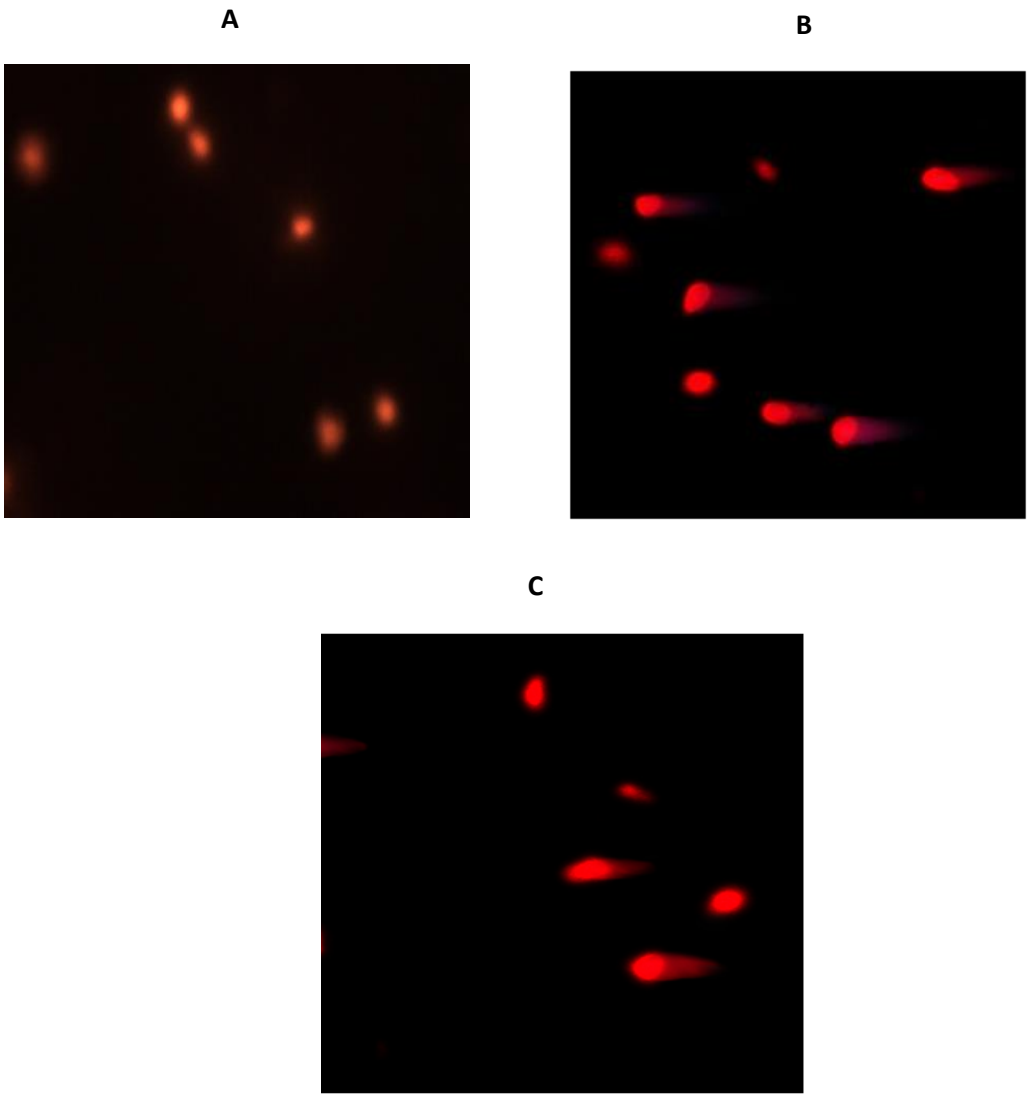


Figure 4.5.19 Remedial effect of lycopene on sperm DNA breakage through comet assay in A. vehicle treated control, B. CPA treated group and C. CPA+ lycopene treated group. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

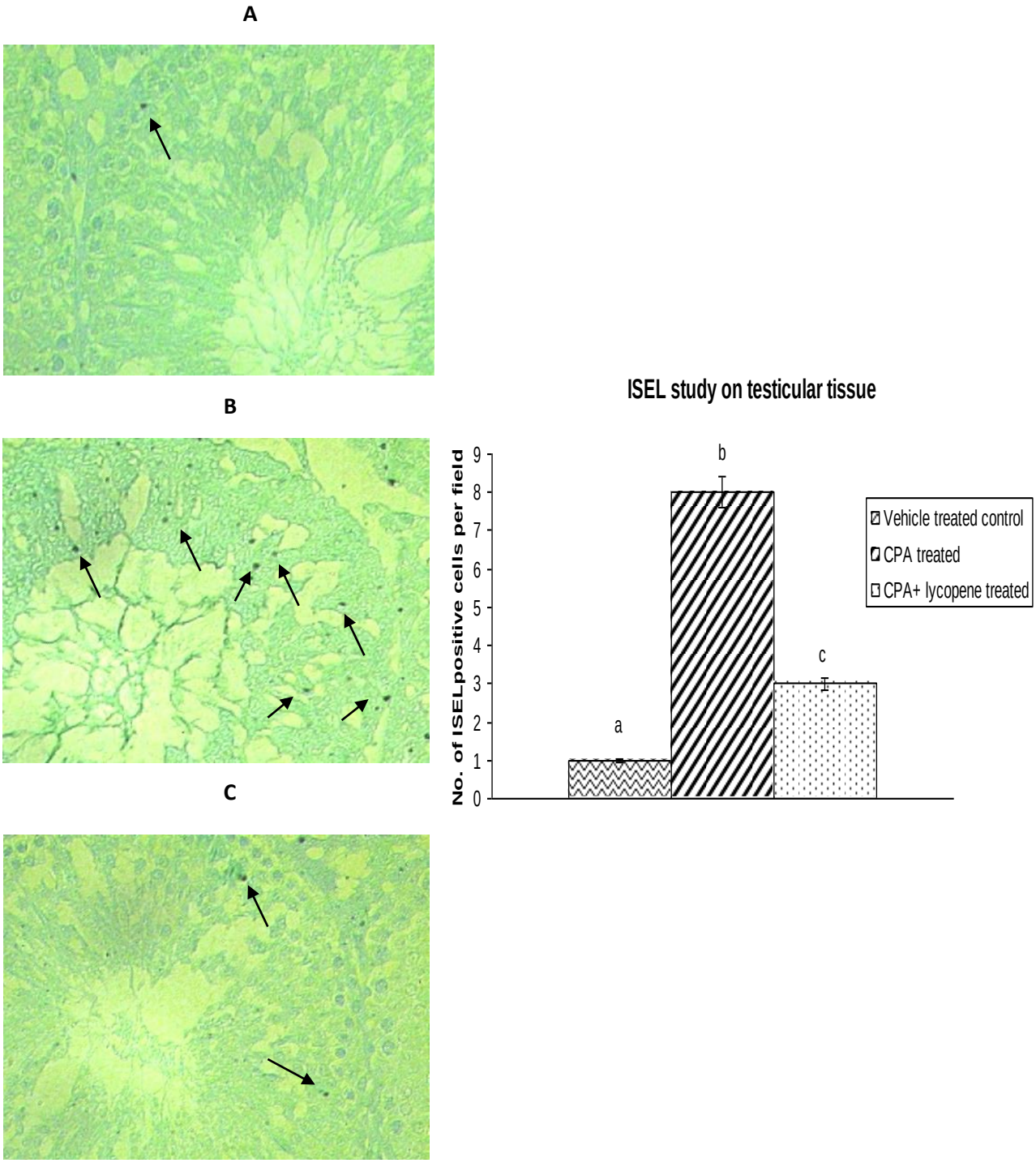


Figure 4.5.20 Immunohistochemical study of testicular tissue for the detection of apoptosis in A. vehicle treated control, B. CPA treated group and C. CPA+ lycopene treated group. Arrow denoted the ISEL positive cells. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.

4.6. Experiment No. VI

Role of lycopene on testicular androgenic key enzymes and antioxidant enzymes in cyproterone acetate induced male infertile rats: An *in vitro* study

4.6.1 Significance of the study

Remedial effect of lycopene on the concerned testicular enzymes, androgenesis and antioxidant enzymes activity which are directly responsible to minimise the burden of the CPA induced testicular damage and oxidative stress has already been established *in vivo*. The main purpose of the present experiment was to focus on the direct effect of lycopene on androgenic key enzymes such as testicular Δ^5, 3β-HSD, 17β-HSD and antioxidant enzymes i.e. testicular SOD, catalase, peroxidase, GST in connection to the rectification from the CPA induced hypo-testicular dysfunction.

4.6.2 Experimental design

To conduct the in vitro experiment, total sixteen rats were taken. All the rats were obtained from the university enlisted vendor, Shah Enterprise, Kolkata, West Bengal. Experimental animals were maintained like other experiments as before. The body weight of the animals was 120± 10 g. All the experimental albino rats were kept in central animal house of our university. Clean, sterilized and dry polypropylene cages were used in this purpose. Room temperature and humidity were 25 ± 2º C and 40-60% respectively. Before the initiation of the experiments, all the animals were allowed for acclimatization for 15 days to make them habituated with human contact and the concerned environment to reduce their stress during handling. Animals were supplied with standard food and water. Prior approval was taken from Institutional Ethic Committee (IEC) [IEC/3/C-4/14, dated 03/11/2014] to conduct the experiment and all the conditions were maintained as per the guideline provided by CPCSEA

(The Committee for the Purpose of Control and Supervision of Experiments on Animals) regulation, Govt. of India.

Total eight albino rats out of sixteen were pre-treated with CPA at the dose of 3.0 mg/ 0.5 ml distilled water/ 100 g body weight/ day for 30 days orally. The protocol of CPA administration followed by feeding time was maintained as previous experimental protocol.

After 30 days of CPA treatment regimen, all the CPA pre-treated and untreated animals were sacrificed by using euthanasia. Before exposing lycopene to the CPA pre-treated and vehicle treated control animals, sperm count was measured to confirm the achievement of infertile condition. For this, cauda epididymis was dissected for collecting the sperm and the protocol was followed by standard method **(Pant and Srivastava, 2003**). It was observed that the sperm count was reduced to 43.3% to the animals treated with CPA compared to the vehicle treated control.

Animals were categorised in four groups i.e., vehicle treated control, Lycopene treated control, CPA treated group and CPA + lycopene treated group. Testes of the animals from each group were dissected out. Then incision was made at both the poles of each testis to conduct the *in vitro* experiment. Two testes of each animal were kept in each test tube that contains 10 ml Kreb's Ringer Solution (KRB) in 5% Co_2 and 95% O_2 gaseous mixture and 0.5 ml tween-80.

The treatment regimen of *in vitro* experiment was as follows:

Group I: Vehicle-treated control: This group contains four animals. Testes of each animal were allowed for direct exposure of tween-80 at 0.5 ml along with KRB solution with CO_2 5% and O_2 95% gaseous mixture.

Group II: Lycopene treated control: Another four animals were included in this group. Testicular tissues of the animals were placed in *in-vitro* media where 1.5 mg lycopene was added in 0.5 ml tween-80 along with KRB solution and above mentioned gas mixture.

Group III: CPA treated: CPA pre-treated four infertile animals were grouped as CPA treated group. Testes of all the animals were incubated in KRB solution along with 0.5 ml tween-80 and then processed in gas mixture as other groups.

Group IV: CPA+ lycopene treated: Testes of another four CPA pre-treated proven infertile rats were exposed with lycopene directly at the potent dose i.e., 1.5 mg/ 0.5 ml tween-80 in KRB solution followed by *in vitro* incubation gas mixture as followed in other groups.

All the test tubes were incubated at the temperature of 37ºC for 2 hours and during this period, continuous supply of gaseous mixture was provided in the form of gas bubbles at 30/ minutes. After 2 hours of incubation, testes were separately kept in the other test tube which contains 1 ml of sodium phosphate buffer solution having pH 7.4 for washing purpose **(Brady, 1951)**. Finally, the tissues were allowed for the enzyme activity assessment.

4.6.3 Parameters and methods

Effect of direct exposure of lycopene on CPA induced infertile rats was assessed by measuring the antioxidant enzyme activity in testicular tissue such as catalase **(Beers & Sizer, 1952)**, peroxidase **(Sadasivam & Manickam, 2008)**, superoxide dismutase **(Marklund & Marklund, 1974)** and glutathione-S-transferase **(Hebig et al., 1974)**. *In virto* efficacy of lycopene was also analysed by considering another domain known as androgenic domain. This domain highlights the measurement of androgenic key enzyme activities such as Δ^5, 3β-HSD **(Talalay, 1962)**, 17β-HSD (**Jarabak et al., 1962)** in testicular tissue which were exposed to lycopene in *in vtro* manner. Simutaneously, direct effect of lycopene on toxicity marker of testis was assessed by measuring the activity of acid phosphatase **(Vanha-pertulla & Nikkanen, 1973)** and alkaline phosphatase **(Horecker, 1966).** Statistical analysis of all the data was performed by following the standard method **(Sokal and Rohle, 1997)**.

4.6.4 Results

4.6.4.1 Testicular Δ5, 3β-HSD and 17β-HSD

Significant ($p < 0.05$) diminution was noted in testicular Δ^5, 3β-HSD activity in CPA treated infertile rats compared to that of vehicle, and lycopene treated controls. Direct exposure of lycopene to the testes of the CPA+ lycopene treated group showed a non-significant ($p > 0.05$) difference in the rectification of these enzyme activities compared to the CPA treated group. Simultaneously, non-significant ($p > 0.05$) difference was observed in between the vehicle and lycopene treated control groups **(Figure 4.6.1).**

On the same manner, activity of testicular 17β-HSD was significantly ($p < 0.05$) diminished in CPA treated group in comparison to the vehicle, and lycopene treated control groups. A non-significant ($p > 0.05$) difference was observed in the enzyme activity after direct exposure of lycopene at a dose of 1.5 mg/ 0.5 ml tween-80 dose to the CPA + lycopene treated rats. Non-significant ($p > 0.05$) difference was observed in between vehicle, and lycopene treated control groups.

4.6.4.2 Antioxidant enzyme activities

Testicular SOD activity of was significantly ($p < 0.05$) decreased in CPA treated infertile rats compared to that of vehicle, and lycopene treated control groups. On the other side, a non-significant ($p > 0.05$) difference was observed in the activities of the concerned enzyme in between the vehicle, and lycopene-treated control groups. Direct exposure of lycopene to the testes of CPA+ lycopene treated group who were previously treated with CPA, exhibited a significant ($p < 0.05$) recovery in the enzyme activity towards the vehicle-treated control **(Figure 4.6.2).**

A significant ($p < 0.05$) diminution was observed in testicular catalase activity in CPA-treated rats when compared with the vehicle, and lycopene-treated control groups. Direct

exposure of lycopene to CPA+ lycopene-treated rat at 1.5 mg/ 0.5 ml tween-80 dose focused a significant ($p < 0.05$) alteration in enzyme activity in positive direction towards the vehicle treated control. Non-significant difference ($p > 0.05$) was noted between vehicle, and lycopene treated control groups in connection with the catalase activity **(Figure 4.6.3)**.

Significant ($p < 0.05$) diminution was observed in testicular peroxidase activity after administration CPA to the CPA treated group in comparison with the vehicle, and lycopene-treated control groups. Peroxidase activity was not significantly ($p > 0.05$) differ in between the vehicle, and lycopene treated control groups. Significant ($p < 0.05$) rectification of this enzyme activity was observed in CPA + lycopene- treated group compared to the CPA treated group **(Figure 4.6.4)**.

Cyproteone acetate treated group focused a significant ($p < 0.05$) decrease in testicular GST enzymes activity in respect to the vehicle, and lycopene treated control groups. The enzyme activity was not significantly ($p > 0.05$) differ between the vehicle and lycopene-treated control groups. *In vitro* treatment of lycopene to CPA + lycopene treated group resulted a significant ($p < 0.05$) rectification in the enzyme activity towards the vehicle treated control **(Figure 4.6.5)**.

4.6.4.3 Activities of acid phosphatase (ACP) and alkaline phosphatase (ALP)

Activities of ACP and ALP in testis were significantly ($p < 0.05$) increased in CPA treated infertile animals in comparison with the vehicle, and lycopene treated control groups. Non-significant ($p > 0.05$) difference was observed in between the vehicle, and the lycopene treated control groups. Lycopene exposure at 1.5 mg/ 0.5 ml tween-80 to the CPA + lycopene treated group focused a significant recovery ($p < 0.05$) in the both of the enzyme activities towards the vehicle treated control **(Figure 4.6.6)**.

4.6.5 Discussion

Experiment VI was performed to focus on the direct effect of lycopene the testicular tissue of CPA pre-treated infertile rats in connection to rectification in the andrological sensors, anti-oxidant sensors and toxicity domain. Testes of all the animals belonging to the above mentioned groups were directly exposed to the lycopene at the concerned dose in order to assess the activities of Δ5, 3β-HSD, 17 β-HSD along with catalase, GST, peroxidase, SOD. Testicular ALP and ACP activities were also evaluated to search out the recovery in toxicity that took place due to CPA administration.

In vivo experiment established that lycopene has promising effect on the rectification of androgenic key enzyme activities which was decreased after CPA administration **(Tripathy et al., 2016)**. But, the direct exposure of lycopene on CPA pre-treated proven infertile rat did not focus any remedial efficacy further in the concerned enzyme activities. This contradictory result in connection with the rectification in the enzyme activity between *in vivo* and *ex vivo* may be due to the lack of processing time for initiating genomic effect by lycopene for such purpose in *in vitro study* or it has no direct effect on Δ5, 3β-HSD, 17 β-HSD activities.

Remarkable recovery in the antioxidant enzyme activities such as peroxidase, SOD catalase and GST were observed after direct exposure of lycopene to the CPA + lycopene treated group probably due to the potent antioxidant activity of lycopene which involves two mechanisms such as rapid destruction of free radical **(Tripathy et al., 2016)** by minimising oxidative stress due to its remarkable free radical-quenching ability **(Mein et al., 2008)** and activation in the anti-oxidant enzymes after the binding of this nutraceutical to the enzyme.

On the other hand, in the in *vitro study,* the activities of ACP and ALP in testicular tissue were evaluated where direct exposure of lycopene showed its remedial potentiality from toxic effect of CPA. This phenomenon took place may be because of the direct binding of lycopene

with ACP and ALP that diminished the activities of the enzymes or lycopene may stabilize the lysosomes that reduces the release of ACP and ALP to the cytosolic compartment of the exposed cell **(McCarty, 2013)**.

4.6.6 Conclusion

It may be concluded that, direct exposure of lycopene at the dose of 1.5 mg/ 0.5 ml tween-80 to the CPA pre-treated infertile rat may rectify the male reproductive hypo-function either by improving antioxidant status by scavenging free radical which were produced by or by activating antioxidant enzymes those can manage stress-induced male reproductive hypo-functions.

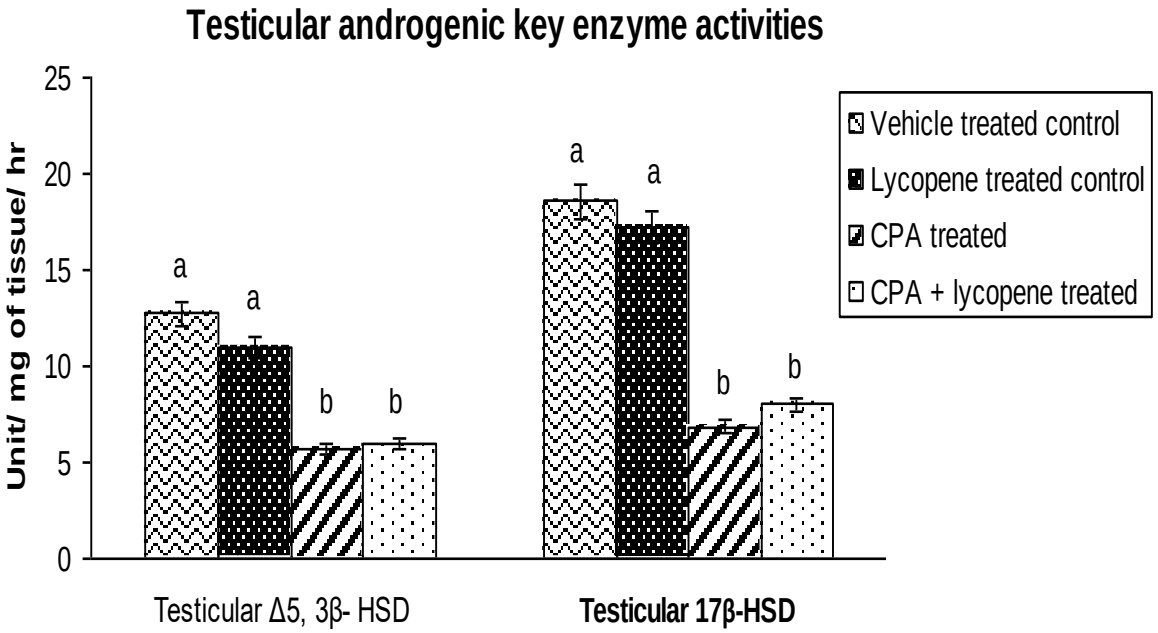


Figure 4.6.1 Direct effect of lycopene exposure on testicular Δ5, 3β-HSD, 17 β-HSD activities in CPA treated infertile male rats. Bars were expressed as mean ± SEM (*n* = 8). ANOVA followed by "Multiple Comparison Two-tail *t* test" was performed. Bars with different superscripts (a, b) differ from each other significantly at $p < 0.05$

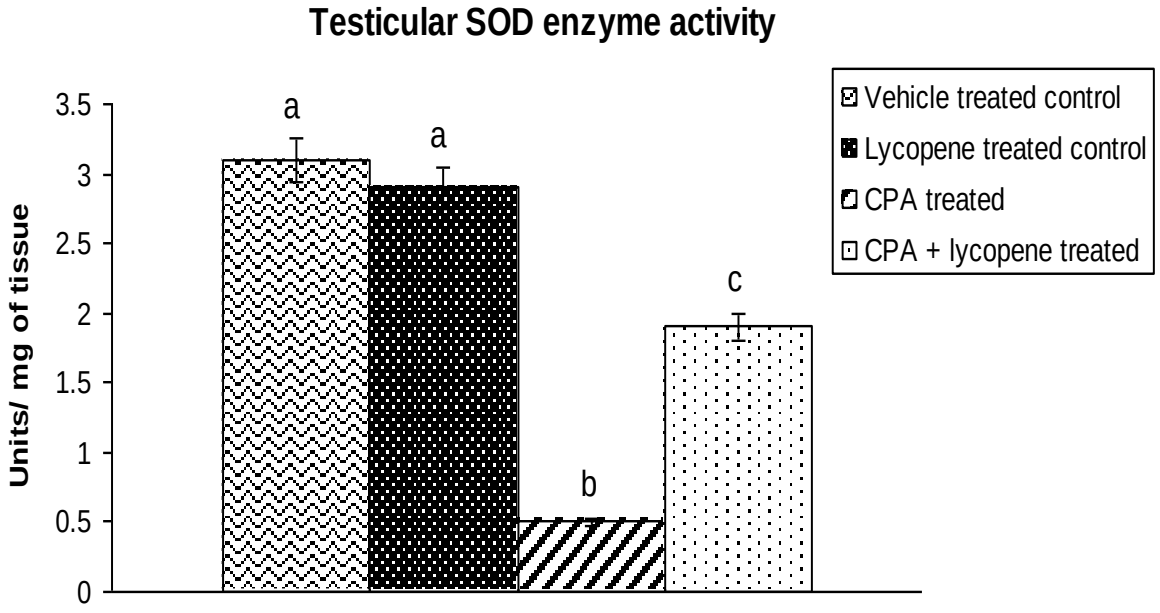


Figure 4.6.2 Remedial effect of lycopene after its direct exposure to the testis of CPA pre-treated infertile rats. Bars were expressed as mean ± SEM (*n* = 8). ANOVA followed by "Multiple Comparison Two-tail *t* test" was performed. Bars with different superscripts (a, b, c) differ from each other significantly at $p < 0.05$

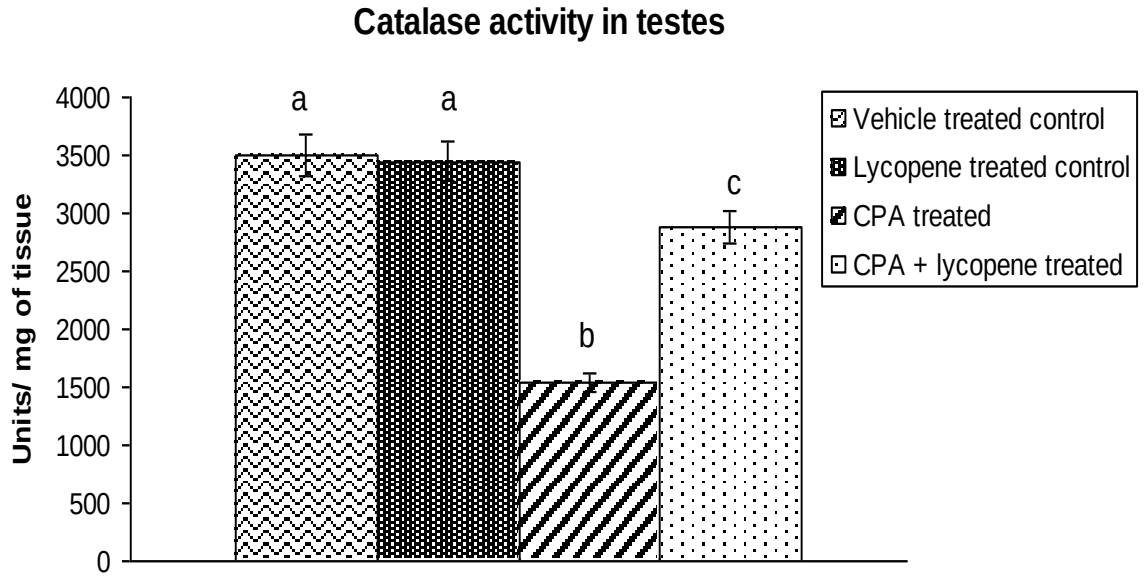


Figure 4.6.3 Rectification in the catalase activity after the direct exposure lycopene at the dose of 1.5 mg/ 0.5 ml tween-80 CPA pre-treated infertile rats. Bars were expressed as mean ± SEM (*n* = 8). ANOVA followed by "Multiple Comparison Two-tail *t* test" was performed. Bars with different superscripts (a, b, c) differ from each other significantly at $p < 0.05$

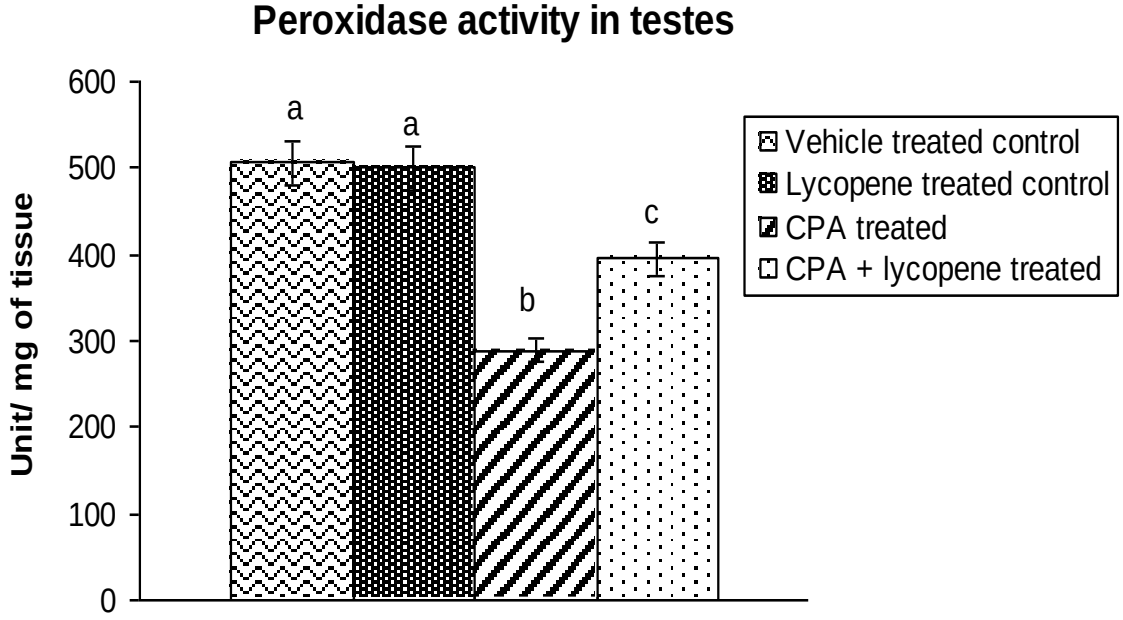


Figure 4.6.4 Rectification in the catalase activity after the direct exposure lycopene at the dose of 1.5 mg/ 0.5 ml tween-80 CPA pre-treated infertile rats. Bars were expressed as mean ± SEM (*n* = 8). ANOVA followed by "Multiple Comparison Two-tail *t* test" was performed. Bars with different superscripts (a, b, c) differ from each other significantly at $p < 0.05$

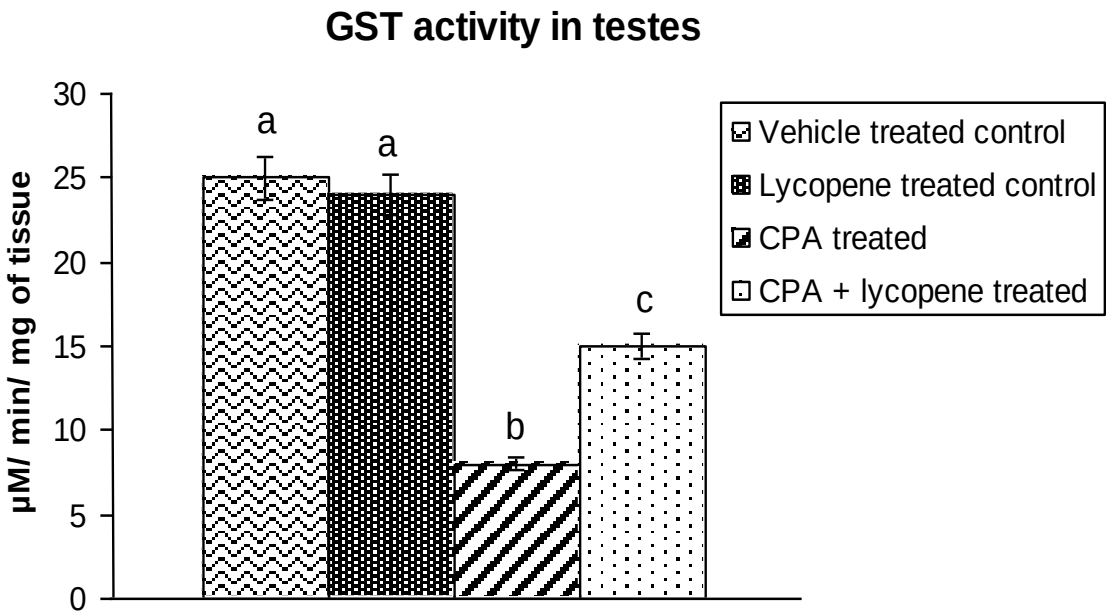


Figure 4.6.5 *In vitro* study on effectiveness of lycopene on GST activity in testes of the animals treated with CPA. Bars were expressed as mean ± SEM (*n* = 8). ANOVA followed by "Multiple Comparison Two-tail *t* test" was performed. Bars with different superscripts (a, b, c) differ from each other significantly at $p < 0.05$

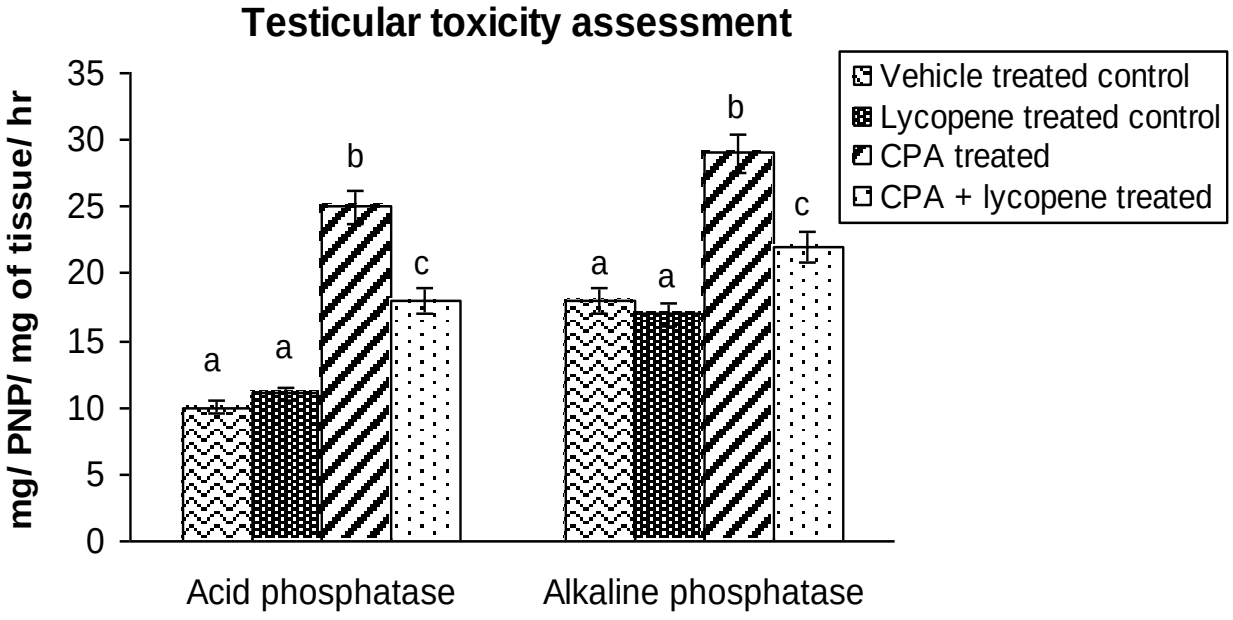


Figure 4.6.6 Ameliorative effect of lycopene on acid phosphatase and alkaline phosphatase activities in testes after direct exposure of lycopene on the testes of CPA pre-treated infertile rats. Bars were expressed as mean ± SEM (*n* = 8). ANOVA followed by "Multiple Comparison Two-tail *t* test" was performed. Bars with different superscripts (a, b, c) differ from each other significantly at $p < 0.05$

4.7. Experiment No. VII

Assessment of toxicity profile of the potent dose of lycopene in cyproterone acetate-induced testicular impairement in male infertile Wistar strain albino rat

4.7.1 Significance of the study

The present experiment has been carried out to confirm the toxic effect of lycopene if any at its potent dose i.e., 1.5 mg/ 0.5 l tween-80/ 100 g body weight on adult, healthy, fertile Wistar strain albino rats.

4.7.2 Experimental design

Twelve rats were taken for this experiment. Experimental animals were procured from the Shah enterprise (Kolkata, West Bengal), university enlister vendor. All the animals were proven fertile and weighing about 120 ± 10 g. They were kept in the dry, ventilated and properly sterilized animal house. Room temperature was maintained within 25± 2ºC and humidity of the room was within 40-60%. Clean and sterilized polypropylene cages were used to keep the animals. Acclimatization phase was continued for 15 days before initiation of the experiment. Acclimatization was needed to accustomed the animals with laboratory environment and make them familiar with the human contact so that the stress during drug administration or animal handling can be minimised. Standard rat chew and water were supplied to the rat. Institutional Ethic Committee (IEC) [IEC/3/C-4/14, dated 03/11/2014] has approved the experiment and total protocol of animal experiment from animal handling to after sacrifice protocol was maintained as the guideline of CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) regulation, Govt. of India.

Experimental animals were categorised in two groups as follows:

Group I: Vehicle treated control: Healthy, fertile albino rats were orally administered with tween-80 at 0.5 ml/ 100 g body weight for 30 days of the total experimental period.

Group II: Lycopene treated group: Animals of this group was treated with lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight for 30 days.

Animals belongs to the lycopene treated group were treated with lycopene at the potent dose at 8 A.M. in fasting condition. After two hours of lycopene administration, food was provided to the animals. Then in the evening at 8 A.M. animals were again provided with the food. Animals belonging to vehicle treated control group were also supplied with food at the same time followed in lycopene treated group.

After 30 days of the lycopene treatment regimen, all the animals of the vehicle treated control group and lycopene treated group were sacrificed by using euthanasia. Blood was collected from dorsal aorta and allowed for centrifugation at 3000 x g for 3 min in order to separate the serum. Metabolic organs like kidney and liver were dissected out and stored in -20ºC.

4.7.3 Parameters and methods

Toxicity of lycopene (if any) was assessed by analysing GOT and GPT activity in serum **(Henry et al., 1960)**. Some of the important sensors of the protein profile such as total protein, albumin, globulin in the serum were assessed by kit method **(Reinbold, 1980)**. Levels of urea, blood urea nitrogen and creatinine in the serum was analysed by the standard protocols **(Tiffany et al., 1972; Kabasakilian et al., 1973; Junge et al., 2001)**. Some of the parameters belongs to the lipid profile such as triglyceride **(Desai et al., 2002),** low density lipoprotein (LDL)**,** very high density lipoprotein (VLDL) **(Friedewald et al., 1972),** high density lipoprotein cholesterol (HDLc) **(Waenic and Albers, 1978)** were also analysed in serum level. Activity of acid phosphatase **(Vanha-Pertula and Nikkanen, 1973)** and alkaline phosphatase **(Malamy and Horecker, 1996)** in hepatic and renal tissue were assessed

following the standard protocol. Statistical analysis of the data was conducted following the standard protocol **(Sokal and Rohle, 1997).**

4.7.4 Results

4.7.4.1 Activity of GOT and GPT in serum

Lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight for 30 days did not show any significant ($p > 0.05$) difference in the activity of GOT and GPT in the renal and hepatic tissue when comparison was made with the vehicle treated control (**Figure 4.7.1)**.

4.7.4.2 Activity of renal and hepatic ALP and ACP

Non significant ($p > 0.05$) alteration was observed in the activities of hepatic and renal ACP and ALP in lycopene treated group in comparison with the vehicle treated control group **(Figure 4.7.2 and 4.7.3).**

4.7.4.3 Serum level of total protein, albumin, globulin

Normal, healthy, proven fertile albino rats treated with potent dose of lycopene for 30 days showed a non-significant ($p > 0.05$) variation in the levels of total protein, albumin and globulin in the serum when comparison was made with the vehicle treated control (**Figure 4.7.4)**.

4.7.4.4 Levels of urea, BUN and creatinine

Serum levels of urea, BUN and creatinine exhibited a non-significant ($p > 0.05$) alteration after administration of lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days in comparison to the vehicle treated control (**Figure 4.7.5)**.

4.7.4.5 Serum levels of triglyceride, LDL, VLDL and HDL

There was a non-significant ($p > 0.05$) alteration noted in the serum levels of triglyceride, LDL, VLDL and HDL after administration of lycopene at the concerned potent dose when comparison was made with the vehicle treated control (**Table 4.7.1)**.

4.7.5 Discussion

Experiment VII was performed to assess whether lycopene administration at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight for 30 days can develop sub acute toxicity or not. In this concern, toxicity enzyme profile, serum protein profile and serum lipid profile was assessed to determine the toxicity of lycopene if any.

Result showed a non-significant difference in GOT and GPT activity as lycopene prevents the cellular damage and as a result these cytoplasmic enzymes could not release in to the blood stream **(Recknagel et al., 1989; Berry et al., 1992; Romeo et al., 1998).** Similar result was observed in the levels of serum urea, BUN and creatinine. These markers are nitrogenous constituent present in the blood. Kidney plays a vital role to filtrate the blood. Elevation in the levels of these enzymes indicated the renal injury. Non-significant alteration in the levels of the above said markers denoted the protective efficacy of the nutraceutical on kidney **(Cosola et al., 2018)**. Serum total protein, albumin and globulin levels were also measured to find out the effect of lycopene on protein metabolism. The albumin is maximally available in liver and it mainly helps to prevent the leakage of blood from blood vessel. The result was further strengthening by the outcome of alkaline phosphatase. This enzyme is generally found in the liver. Elevation in the activity of this enzyme denoted liver damage **(Maldonado et al., 1998)**. In this experiment, activity of the ALP was significantly unaltered after lycopene treatment compared to the vehicle treated control. This mechanism denoted the protective effect of lycopene on liver function **(Jiang et al., 2016)**.

The non-toxic property of lycopene was further strengthened by the analysis of levels of triglyceride, LDL, VLDL and HDL. In experiment IV, it has been observed that the cyproterone acetate altered the levels of the parameter in negative direction which were recovered by lycopene. In this experiment, all the above mentioned parameters were remained unchanged after lycopene treatment. No further deterioration was observed after lycopene

treatment on healthy fertile albino rats. This is may be due to the defensive effect of lycopene on minimising the hyperlipidemia which is associated with the low sperm count and ultimately leads towards the infertility in male **(Liu et al., 2017)**.

Cellular toxicity was assessed by measuring the activity of acid phosphatase. ACP is a lysosomal enzyme and mostly remains in the latent form and the remaining free form of this enzyme is available in the cytoplasm. Experiment showed statistically unaltered activity of this enzyme after lycopene administration at the concerned dose for 30 days which indicated the defensive mechanism of lycopene on cellular environment **(Mattei et al., 1985)**.

4.7.6 Conclusion

From the present experiment, it may be concluded that lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight/ day for 30 days did not create any cellular, hepatic and renal toxicity. This non-toxicity property of lycopene may help in conducting any preclinical study for disease management especially hypo-testicular dysfunction management among male in future.

Table 4.7.1: Effect of lycopene treatment on serum tryglyceride, VLDL, LDL, HDL profile in albino rat.

Groups	Tryglyceride (mg/ dl)	VLDL (mg/ dl)	LDL (mg/ dl)	HDL (mg/ dl)
Vehicle treated control	90 ± 2.49^{a}	16.95 ± 1.03^{a}	20.43 ± 1.32^{a}	40.12 ± 1.56^{a}
Lycopene treated	88.85 ± 2.84^{a}	18.32 ± 1.64^{a}	19.21 ± 1.10^{a}	42.03 ± 1.34^{a}

Data were expressed as mean ±SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Values with same superscript (a) in each vertical column did not differ from each other significantly $p > 0.05$.

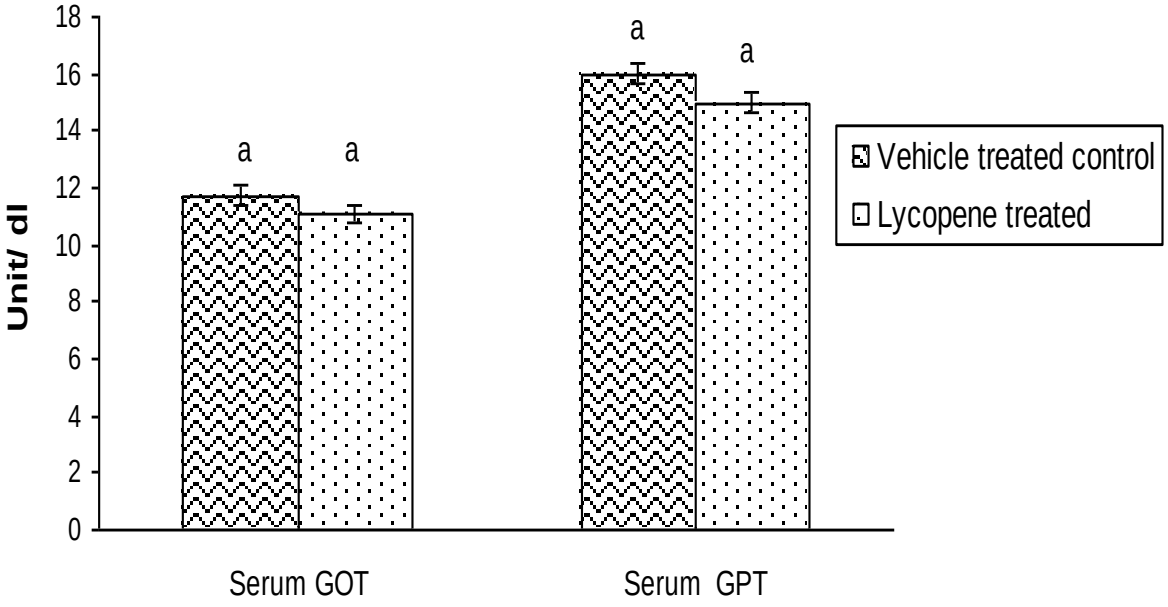


Figure 4.7.1 Serum GOT and GPT enzyme activities after administration of lycopene at the potent dose to the albino rats and its comparison to the vehicle treated control. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with same superscript (a) did not differ from each other significantly, $p > 0.05$.

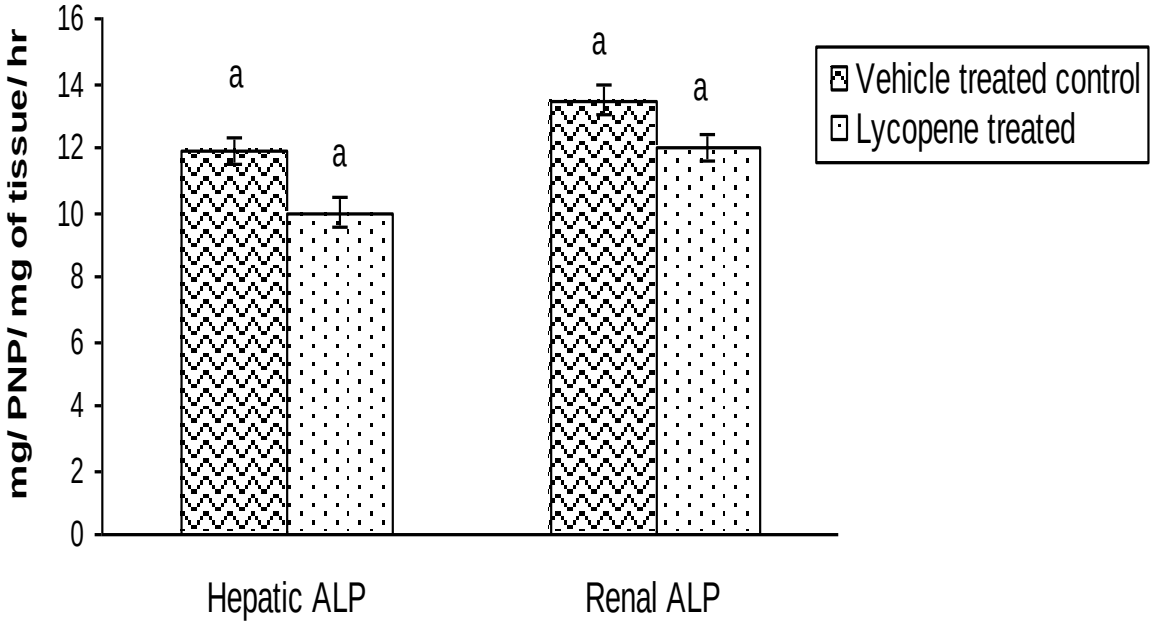


Figure 4.7.2 Activities of hepatic and renal ALP after the administration of lycopene at 1.5 mg/ 0.5 ml tween-80/ 100 g body weight to the experimental rat. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with same superscript (a) did not differ from each other significantly, $p > 0.05$.

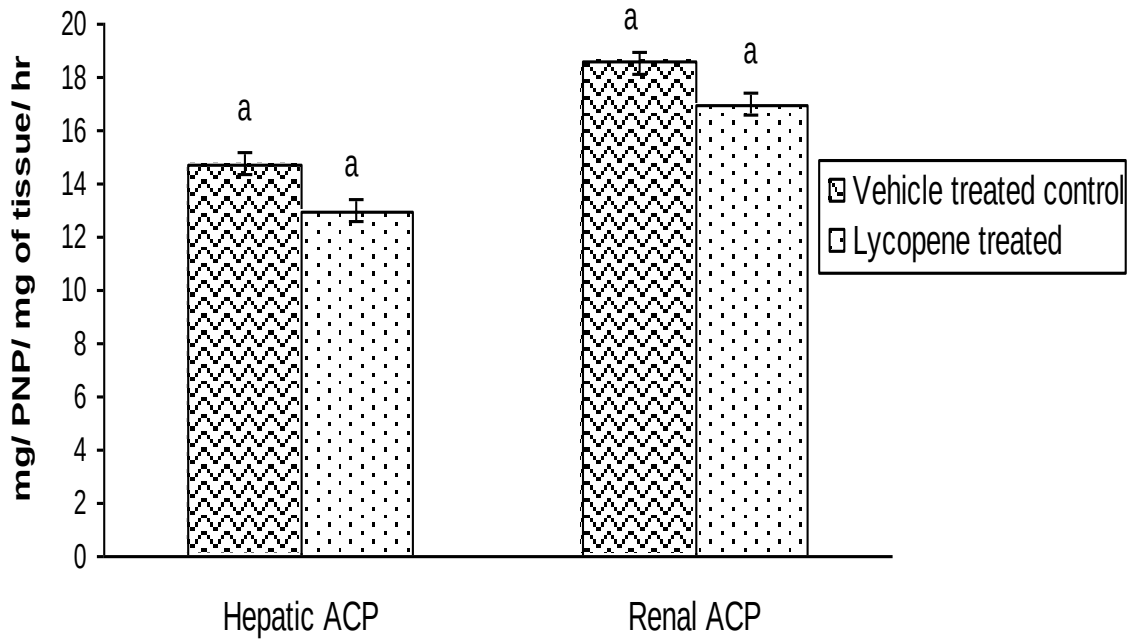


Figure 4.7.3 Effect of lycopene on renal and hepatic ACP activity in albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with same superscript (a) did not differ from each other significantly, $p > 0.05$.

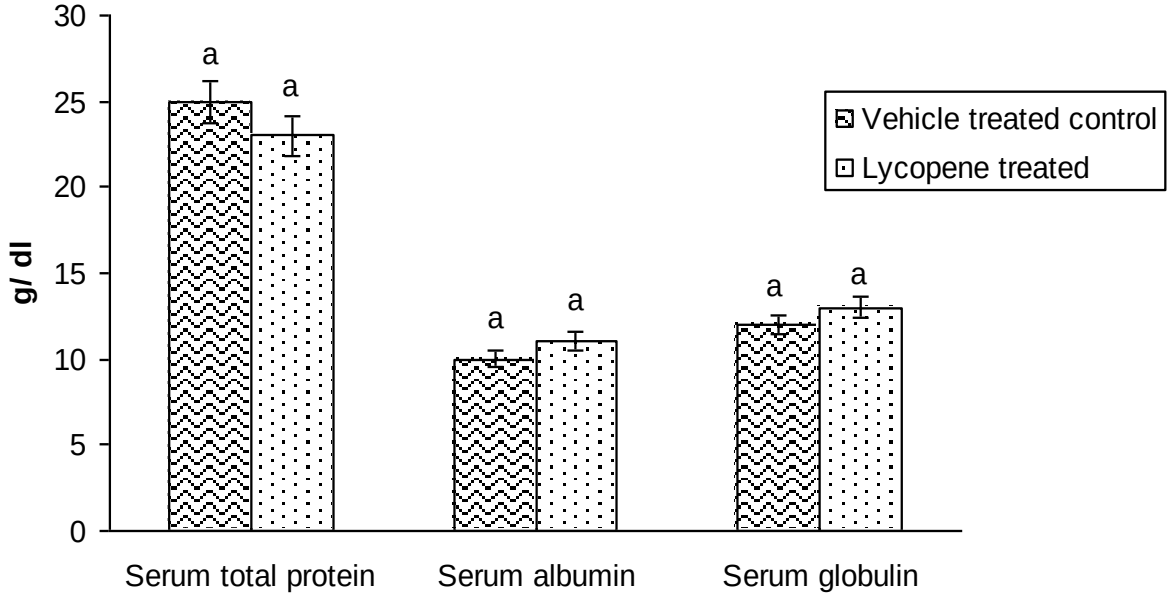


Figure 4.7.4 Serum total protein, albumin and globulin levels after treatment with lycopene at the potent dose. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test' Bars with same superscript (a) did not differ from each other significantly, $p > 0.05$.

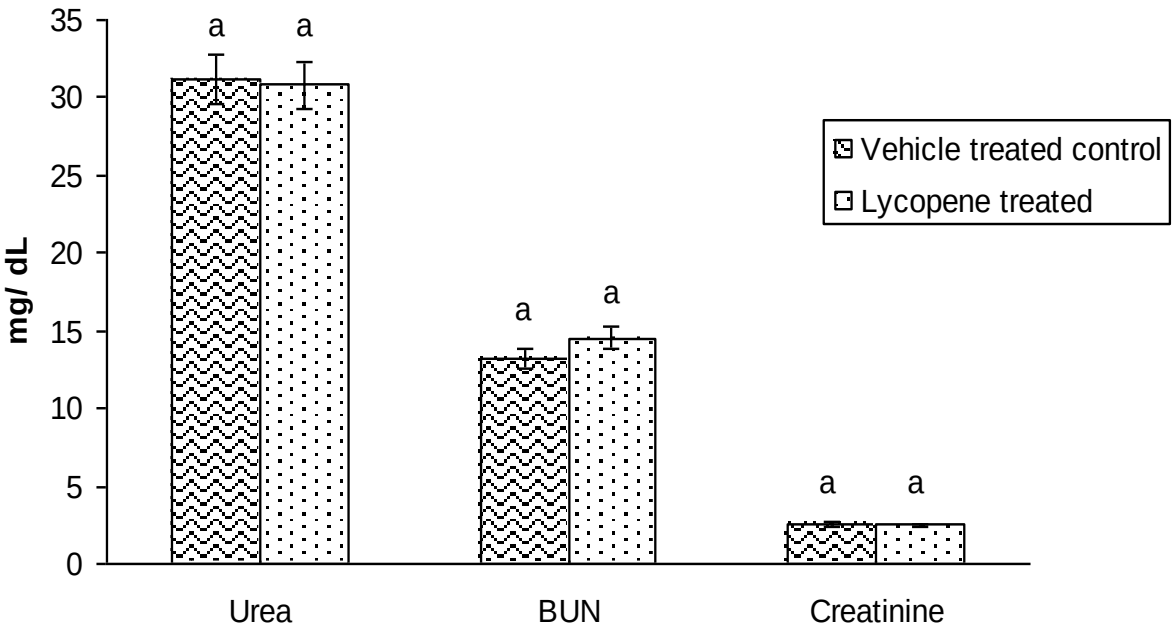


Figure 4.7.5 Effect of oral administration of lycopene on urea, BUN and creatinine levels in Wistar strain albino rats. Bars were expressed as Mean ± SEM (n = 6). ANOVA followed by 'Multiple Comparison Two-tail *t*-test'. Bars with same superscript (a) did not differ from each other significantly, $p > 0.05$.

4.8. Experiment No. VIII

Assessment of fertility ability after treatment with lycopene to the cyproterone acetate-induced infertile Wistar strain albino rat: An approach through mating study

4.8.1 Significance of the study

The experiment was carried out to assess the recovery of the fertility ability of the CPA treated proven infertile rats after oral administration of lycopene at the dose of 1.5 mg/ 0.5 ml tween -80/ 100 g body weight/ day. In this concern, male rats were allowed for mating with the fertile female. Implantation site was assessed for the confirmation of successful mating.

4.8.2 Experimental design

Twenty-seven adult fertile and healthy female rats and about nine male fertile animals were considered for the conduction of this experiment. All the male and female albino rats were purchased from the university enlisted vendor, Shah Enterprise, Kolkata, West Bengal. All the procured animals were weighing an average of 120± 10 g. Both the males and females were kept in the clean, sterilized, dry, well ventilated room. Temperature and the humidity of the room was maintained about 25 ± 2ºC and 40-60% respectively. 12h: 12h day and night cycle was also maintained. Prior the initiation of the experiment, animals were allowed for adaptation of laboratory environment so, that during the drug administration and human handling, no other stress can be generated. Animals were provided with standard rat chew and water ad libitum. Approval was taken prior the initiation of the experiment from the Institutional Ethic Committee (IEC) [IEC/3/C-4/14, dated 03/11/2014] and all the protocol during animal treatment was maintained by following the protocol of CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) regulation, Govt. of India.

In this experiment, at first the male rats were brought in to the animal house to develop the infertile model and then lycopene administration along with CPA was continued. The total duration of the experiment was for 60 days and the feeding time and drug administration time was maintained as per the previous experiment. At the 45 days of the experimental regimen, female rats were brought in the animal house to acclimatize them with the environment. Just after the end of the treatment protocol, male animals were allowed for mating with the virgin female rats having 4 days estrous cycle. During the acclimatization period vaginal smear was collected prior one week of the mating to identify the estrous phase. Estrous phase was confirmed by identifying the existence of irregular, non nucleus containing cornified cells in the vaginal smear collected from the female rats. At the estrous phase of female rats, both male and female rats were kept in the same cage having ratio male 1: female 3 as follows:

Group I: Vehicle treated control: Male animals of this group were orally administered with Tween-80 for the last 30 days of the treatment regimen. After that rats were allowed for mating at the above mentioned ratio for 7 days.

Group II: CPA treated group: CPA treated infertile male rats were kept in the same cage with the female rats at the ratio of male 1: female 3 for 7 days for mating.

Group III: CPA+ lycopene treated: CPA pre-treated and lycopene treated animals were subjected for mating with fertile female rats at the above mentioned ratio for 7 days.

Mating was confirmed by the presence of spermatozoa in the vaginal smear and that day was considered as the zero day of gestation. After 10th post coital day, all the female rats were sacrificed by using euthancia and then are allowed for the counting of implantation sites.

4.8.3 Parameters and methods

Vaginal smear was collected by a specific protocol **(Marcondes et al., 2002)**. Confirmation of estrous phase of the female rat was conducted by standard protocol **(Marcondes et al., 2002)**. Implantation sites were counted by standard process **(Hamilton et al., 1994)**. Total

procedure of mating study was counted as per the protocol of Yama et al. **(Yama et al., 2011)** Statistical analysis of the data was performed by a standard method **(Sokal and Rohle, 1997)**.

4.8.4 Results

4.8.4.1 Presence of spermatozoa in vaginal smear

Vaginal swab was collected from the female rat after mating. The percentage of sperm present in the vaginal secretion of the female rat mated with the CPA treated rat was significantly ($p < 0.05$) low comparison to the female mated with vehicle treated control rats. The percentage of the spermatozoa present in the vaginal swab of the female mated with the lycopene treated male rat reflected a significant ($p < 0.05$) recovery in percentage of spermatozoa and the rate of recovery was towards the vehicle treated control **(Table 4.8.1)**.

4.8.4.2 Implantation site

No implantation site was present in the uterine horn of the female rat mated with the CPA treated male infertile rat when comparison was made with the female rat mated with the vehicle treated control. Significant ($p < 0.05$) recovery in the number of the implantation site of the uterine horn of the female rat mated with lycopene treated male rat that is towards the vehicle treated control **(Figure 4.8.1)**.

4.8.5 Discussion

Previous experiments revealed that CPA administration for 2 months resulted significant diminution in the sperm count, sperm motility and sperm viability which are the prime parameters for the analysis of semen quality. Low sperm count is one of the major causes of infertility **(Durairajanayagam, 2018)**. Present investigation was carried out to evaluate the resettlement in the fertility ability after lycopene administration to the CPA treated infertile rat. In this concern, healthy, fertile female rats were allowed for mating with the male rats grouped under vehicle treated control, CPA treated group and lycopene treated group.

Result focused that the percentage of spermatozoa present in the vaginal swab of the female rat mated with the CPA treated rat was significantly low. This is may be due to the role of CPA that inhibits the spermatogenesis **(Moltz et al., 1980)**. Simultaneously, due to huge amount of free radical generation by CPA treatment, percentage of viable sperm was reduced which was proved in the earlier experiment. Percentage of spermatozoa in the vaginal smear of the female rat mated with the lycopene treated group showed significant elevation in the number of spermatozoa which proved that the lycopene has the capability to improve the spermatogenesis (**Durairajanayagam et al., 2014**). The result was further confirmed by quantifying the implantation sites in the uterine horn of the female rat. No implantation site was observed among the female rats mated with the CPA treated group. Beside the low sperm count or viability caused by CPA, another vital cause which is responsible for the onset of the infertility is lack of testosterone. As CPA is an antiandrogenic agent, it inhibits testosterone synthesis. As a result low testosterone causes interruption in spermatogenesis. Experiment VII also focused the role of CPA on down-regulation of anti-oxidant enzyme, androgenic key enzyme protein and up regulation of apoptotic markers that causes DNA damage and ultimate leads towards the infertility. As a result the fertility power of the CPA treated rat was decreased that causes no implantation site on the uterine horn in the female rat mated with CPA treated rat. Simultaneously, significant elevation in the implantation sits was noted in the female rat mated with lycpoene treated rat which may be due to the potentiality of the lycopene to stimulate the androgen receptor for the synthesis of testosterone **(Raj et al., 2014)**. Side by side being a potent antioxidant, lycopene stimulate the antioxidant enzyme synthesis by which redox balance can be maintained and free radical induced apoptosis can be hindered.

4.8.6 Conclusion

From the above experiment, it an be concluded that lycopene at the dose of 1.5 mg/ 0.5 ml tween-80/ 100 g body weight for 30 days duration treatment has the capability to regain the fertility ability which was lost after CPA administration.

Table 4.8.1: Quantification of the implantation site in both of the uterine horn of the female albino rats mated with the vehicle treated control, CPA treated group and CPA+ lycopene treated rat.

Group	**No. of implantation sites in both uterine horns**
Vehicle treated control	4 ± 0.04^{a}
CPA treated	0^{b}
CPA+ Lycopene treated	2 ± 0.02^{c}

Data were expressed as mean ± SEM (n = 9). ANOVA followed by 'Multiple comparison two-tail *t*-test'. Values with different superscripts (a, b, c) in each vertical column differ from each other significantly $p < 0.05$.

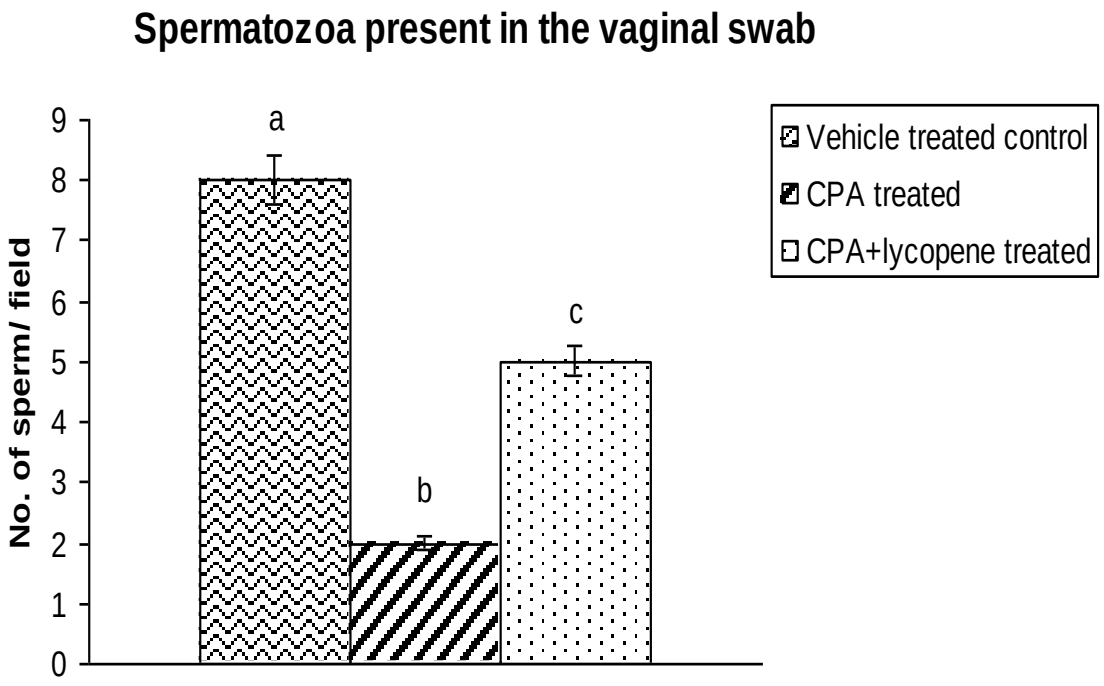


Figure 4.8.1 Diagramatic presentation of the number of spermatozoa present in the vaginal swab of the female rat mated with the male rats of different groups. Bars were expressed as Mean ± SEM (n = 9). ANOVA followed by multiple comparison two-tail *t*-test. Bars with different superscripts (a, b, c) differ from each other significantly, $p < 0.05$.