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Original Article

In vitro exposure to triclosan regulates hydroxysteroid dehydrogenase activity in epididymal sperm of goat

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ABSTRACT

Triclosan, an antibacterial and antimicrobial agent, is widely used in pharmaceuticals, personal care and household products. The present study evaluated the effects of triclosan on sperm functional parameters and steroidogenic enzyme activity in goat epididymal sperm *in vitro*. Epididymal sperm of mature fertile goat were incubated with triclosan in Ringer's phosphate solution. Experiments were divided into three groups, such as Group I: Triclosan at different concentrations (50, 100, 200 and 300 µM) for 5 h, Group II and III: Triclosan at 200 and 300 µM concentrations for different durations (1, 2, 3, 4 and 5 h). Epididymal sperm incubated with triclosan showed decline in sperm motility from 77 to 46% and sperm viability from 78% to 47% after 5 h of incubation in concentration-dependent manner. Activities of 3β-HSD decreased significantly at 200 and 300 µM, and 17β-HSD decreased significantly at 100, 200 and 300 µM of triclosan incubation for 5 h. Sperm when incubated with triclosan at 200 and 300 µM concentrations decreased the activities of 3β-HSD and 17β-HSD only after 3 h of incubation. In conclusion, triclosan affects the sperm functions as well as the male fertility by down regulating the activities of testicular steroidogenic enzymes.

Key Words: Triclosan, sperm motility, sperm viability, 3β HSD, 17βHSD, goat

INTRODUCTION

Triclosan, 5-chloro-2-(2, 4-dichlorophenoxy) phenol or 2, 4, 4′-trichloro-2′-hydroxydiphenyl ether is a widely used antibacterial or antimicrobial agent in a variety of pharmaceuticals, personal care and household products [1]. As an antibacterial agent, triclosan is added to numerous consumer goods and are commonly found in detergents, dishwashing liquids, kitchen sponges, soaps, deodorants, cosmetics, lotions, antimicrobial creams, acne medications, skin cleansers, toothpaste, mouthwashes, various plastics including children's toys, paint, wallpaper, flooring, textiles, curtains, sandal foot beds, public railings, keyboards, countertops, faucets and even dog bowls.

It is being added to an increased number of consumer products including kitchen utensils, cutting boards, socks, and trash bags [2]. Owing to the earlier belief of its efficacy and safety in use has led to the widespread use of triclosan in personal hygiene products such as soaps, deodorants and skin cleansing preparations. More recently it has been added to dentifrices to prevent plaque and gingivitis and it is sometimes incorporated into solid matrices, such as specialized clothing and in work surfaces used in food preparation [3, 4].

Official acceptance of the wide usage of triclosan by approved regulatory authorities permitted for the continuous marketing until the reports of harmful effects in humans were observed. Pharmacokinetics and toxic effects of triclosan have been extensively studied in humans as it is known to be absorbed from the gastrointestinal tract and across the skin in humans [5]. Triclosan has been shown to affect cytogenetic, physiological, biochemical, immunological, endocrine, developmental and reproductive systems at varying concentrations in different period of exposures. Triclosan was also known to enter into the biological fluids such as blood plasma, breast milk, urine, nails, and skin and even known to cross blood-brain barrier, blood-epididymal barrier and placenta. However, its mode of action is

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controversial, different studies indicated triclosan as estrogenic, weak androgenic or anti-androgenic and has been experimentally proved in various animals [6, 7, 8]. Epididymis, an important male accessory reproductive tissue, is an elongated coiled duct suspended within the mesorchium and firmly or loosely bound to the testicular tunica albuginea. The long length of the epididymis provides storage space for spermatozoa and allows them to mature before they are released. Therefore, epididymis promotes sperm maturation, facilitates the transport of spermatozoa along the duct, stores spermatozoa and protects from xenobiotics. These functions are coordinated in precise manner to ensure the production of fully viable spermatozoa [9].

Reproductive health involves all reproductive processes, functions and systems collectively involved to reproduce and maintain the healthy generations. Recent researches have focused on the exposure to environmental contaminants potentially contributed to disorders in male reproductive system. It includes hypospadias, cryptorchidism, reduced anogenital disorders in infants and decrease in sperm counts, semen quality, sperm motility and function, testicular and prostate cancer, testicular dysgenesis syndrome and infertility in adults [10]. Triclosan has been shown to inhibit the androgen production by depressing the expression of steroidogenic acute regulatory protein (StAR) and finally by the down-regulation of several key steroidogenic enzymes [7]. It is well known that the inhibition of steroidogenesis by reducing StAR protein widely contributes to the reproductive dysfunction in animals. The present study was therefore, aimed to investigate the effect of triclosan on male infertility by evaluating the epididymal sperm parameters and the activities of key steroidogenic enzymes in epididymal sperm of goat *in vitro*.

MATERIALS AND METHODS

Chemicals: Triclosan (5-chloro-2-(2,4 dichlorophenoxy)phenol) of 97 % purity, NAD, NADPH were obtained from HiMedia Laboratories, Mumbai, India. DHEA and testosterone were obtained from Sigma Chemicals, USA. All other chemicals were of analytical grade and obtained from local commercial sources.

Collection and incubation of epididymal sperm: Testes were obtained from the adult fertile goat from local slaughter house, from which epididymal sperm were collected as described by Gangadharan et al. [11]. Briefly, epididymis was cut into several pieces with a sharp razor blade and dispersed in a modified Ringer's phosphate solution (RPS) at pH 6.9 with gentle stirring. Sperm samples dispersed in media were adjusted to obtain a final concentration of 10^8 sperm/ 2 ml (in 100% DMSO) for 5 h at 32 °C.

Epididymal sperm count: Epididymis was chopped into several pieces in modified RPS medium, incubated for 5 min at 32°C with gentle stirring. Then 10 µl of sperm was diluted with 90 µl of modified RPS medium. 10 µl of the diluted sperm suspension was transferred to counting chamber of the hemocytometer and was allowed to stand for 5 min in a humid chamber to prevent drying out. Complete spermatozoa are counted using a microscope at 40X magnification.

Epididymal sperm motility: Fluid from the cauda epididymides was obtained using a pipette tip and diluted to 2 ml in modified RPS medium at 32°C. 10 µl of this solution was placed in Neubauer-type hemocytometer and counted for motile and non-motile sperm. First non-motile sperm was counted followed by motile sperm. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted

Epididymal sperm viability: Equal volume of sperm suspension is mixed with 0.05% Eosin Y, incubated for 2 min at room temperature and viewed under a microscope for live and dead sperm cells. Dead sperm appear pink and live are not stained. About 200 sperm cells were scored for each sample and viability is expressed in percentage (%).

Experimental design: After several washings of epididymal sperm in RPS medium, the samples were equally dispersed in RPS medium and incubated with triclosan at various concentrations at 32ºC.

The present study was designed as follows:

Group I: Single duration at different concentrations

Triclosan at 50, 100, 200 and 300 µM concentrations were suspended in culture media and incubated at 32ºC for 5 h. The culture medium consists of 10 µL of epididymal sperm sample maintaining 10^8 sperm cells in 2 ml of modified RPS medium. Toxicant-free controls namely, epididymal sperm in modified RPS medium, and epididymal sperm in modified RPS medium with DMSO were maintained along with treatment groups at 32ºC for 5 h.

Group II: Single concentration (200 µM) at different durations

Triclosan at 200 µM concentration was incubated with epididymal sperm suspension in RPS medium at 32ºC for different time intervals as 1, 2, 3, 4 and 5 h. Two control groups such as toxicant-free

epididymal sperm in modified RPS medium, and epididymal sperm in modified RPS medium with DMSO were maintained along with treatment groups at 32ºC for 1, 2, 3, 4 and 5 h.

Group III: Single concentration (300 µM) at different durations

In this experiment, culture media containing epididymal sperm suspension in RPS medium were treated with 300 µM concentration of triclosan and incubated at 32ºC for 1, 2, 3, 4 and 5 h. Two control groups such as toxicant-free and solvent-free group, and toxicant free but with solvent (DMSO) were maintained separately along with treatment groups at 32ºC for 1, 2, 3, 4 and 5 h.

At the end of respective hour of incubation in all experiments, the sperm cell suspensions, after washing with Ringer's phosphate medium, were homogenized in a glass teflon homogenizer for ten seconds and centrifuged at 800*g* for 10 minutes and the supernatant was used for biochemical assays.

Testicular steriodogenic enzymes: The activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase were assayed by the method described in Bergmeyer [12].

Statistical analyses: Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $p<0.05$ against control groups. Data are presented as mean ± SD for n=6. All biochemical estimations were carried out in triplicates.

RESULTS AND DISCUSSION

There has been an increased concern regarding the male infertility and environmental pollutants. In the present study, the effects of triclosan on epididymal sperm of goat were studied *in vitro*. Triclosan was incubated along with epididymal sperm at different concentrations as well as in different durations. Motility and viability of epididymal sperm in goat remained above 50% up to 5 h in laboratory condition and therefore, the time interval was maintained up to 5 h. The concentration of 300 µM was the maximum concentration chosen in the present study because the viability and motility of epididymal sperm remained above 45% for 5 h. Adult fertile male goats were used in the present study maintaining the epididymal sperm count in the range of 1.5-2.5 X 10^8 sperm/ ml where 2 X 10^8 sperm/ ml is considered as normal sperm count in goat [13]. Epididymal sperm incubated to different concentrations (50, 100, 200 and 300 µM/ L) at different durations (1 to 5 h) was maintained along with positive (DMSO) and negative (Buffer-RPS medium alone) control groups.

The quality of semen is determined by several sperm parameters as sperm morphology, sperm count, sperm motility and sperm viability. Sperm morphology was observed along with motility and viability and it was found normal. Motility of sperm is classified as non-motile, progressively motile or non-progressively motile. A progressively motile sperm swims forward in an essentially straight line, whereas a non-progressively motile sperm swims, but with an abnormal path, such as in tight circles. In the present study, epididymal sperm of controls showed progressively motile sperms with motility at 90%. Sperm when incubated with buffer containing RPS medium and DMSO showed 87% and 86% motility, respectively for 5 h. However, when the epididymal sperm is incubated with triclosan at different concentrations showed decline in sperm motility in concentration-dependent manner from 77 to 46% when observed for 5 h (Table 1).

Sperm viability or vitality is an important measure of fertility, where the percentage of living and non-living sperm can be analysed. The percentage of live spermatozoa is determined by identifying sperm with an intact cell membrane. This is usually done by using a dye exclusion method where dye enters a non-vital (non-living or dead) cell due to the damaged plasma membrane. Therefore, viable cells will not appear stained, but non-viable cells will take up the stain. Usually sperm viability test is performed only in low percentage of motile sperms, less than 30-40%. But in the present study viability is assessed on both controls and treatment groups and it was observed that sperm viability at 91% initially and when incubated with buffer or DMSO, the viability noted was 89% and 87%, respectively for 5 h (Table 1). On the other hand, when the epididymal sperm is incubated with triclosan at different concentrations for 5 h significantly ($P<0.05$) decreased the sperm viability from 78% to 47% in concentration-dependent manner.

Sperm motility and sperm viability are the two important characteristics of sperm function. The quality of the sperm is considered as more important than the quantity of the sperm and therefore, the potential of male reproduction is determined by these sperm parameters. In the present study it was observed that triclosan affect the sperm parameters, as sperm motility and viability, thereby signify that triclosan influence the sperm functions and also induces infertility in goat.

It has been observed that epididymal sperm incubated with triclosan decreased the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) at 200 and 300 µM concentrations whereas no significant

changes were noted at 50 and 100 µM concentrations when compared to the controls (Figure 1). Whereas the activity of 17 β-hydroxysteroid dehydrogenase showed significant ($P<0.05$) decrease at 100, 200 and 300 µM concentrations of triclosan incubation than the control groups (Figure 2). Sperm samples when incubated with triclosan at 200 and 300 µM concentrations showed the significant ($P<0.05$) decrease in the activities of 3β and 17β-hydroxysteroid dehydrogenase only after 3 h of incubation (Figures 3 and 4).

In mammals, steroid hormones play a crucial role in mediating a variety of physiological processes, including electrolyte and water balance, carbohydrate metabolism, and for synthesis of sex steroids. The sex steroids are principally produced in the gonads and all steroid hormones are synthesized sequentially from cholesterol by a number of steroidogenic cytochrome P450s (CYPs) and hydroxysteroid dehydrogenases (HSDs) [14]. 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) plays a pivotal role in the synthesis of all classes of steroid hormones [15]. According to literature, environmental contaminants exert its effects either by direct up/ down regulation of steroidogenic enzymes and consequently the testicular steroidogenesis can be affected at several levels. The decrease in the activities of the key steroidogenic enzymes has been reported when triclosan was exposed to male albino rats for 60 days [7]. The observation from the present study evidently reveals that triclosan affect the male fertility in goat by down regulating the activities of testicular steroidogenic enzymes.

To brief, epididymis is very sensitive and a potential target for the toxic effect of any pollutants, which may then influence male fertility. The fertility of male is evaluated through semen quality, sperm concentration, motility and morphology. In the present study, triclosan when incubated to the goat epididymal sperm was observed to reduce the motility and viability of sperm, which indicates triclosan as an endocrine disruptor adversely affect the male reproduction of goat. Triclosan also alter testicular steroidogenesis as evidenced by the decrease in the activities of key steroidogenic enzymes as 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase. Therefore, the effect of triclosan on testicular steroidogenesis and its adverse effects on male fertility of goat were demonstrated.

CONCLUSION

The present study demonstrates that triclosan exposure alters sperm functions and regulates steroidogenesis thereby disrupting male reproduction of goat.

Table 1: Body weight, weights of testes and epididymis of goat; and the percentage of goat epididymal sperm motility and viability incubated with triclosan

Body weight (Kg)	**Weight of testis (g)**	**Weight of epididymis (g)**
16 ± 2	70.5 ± 4	10.78 ± 1.03
	Epididymal sperm motility (%)	**Epididymal sperm viability (%)**
Control (before 1 h)	90 ± 1.1	91 ± 0.47
Buffer (at 5 h)	87 ± 1.2	89 ± 0.7
DMSO (at 5 h)	86 ± 0.88	87 ± 0.64
Triclosan 50 µM/ L (at 5 h)	77 ± 0.92	78 ± 0.53
Triclosan 100 µM/ L (at 5 h)	70 ± 0.75	72 ± 0.61
Triclosan 200 µM/ L (at 5 h)	62 ± 0.55*	63 ± 0.82*
Triclosan 300 µM/ L (at 5 h)	46 ± 0.63*	47 ± 0.75*

Values are expressed as Mean ± SD for triplicates. Asterisks (*) denotes significance at $P<0.05$ against control groups.

Figure 1

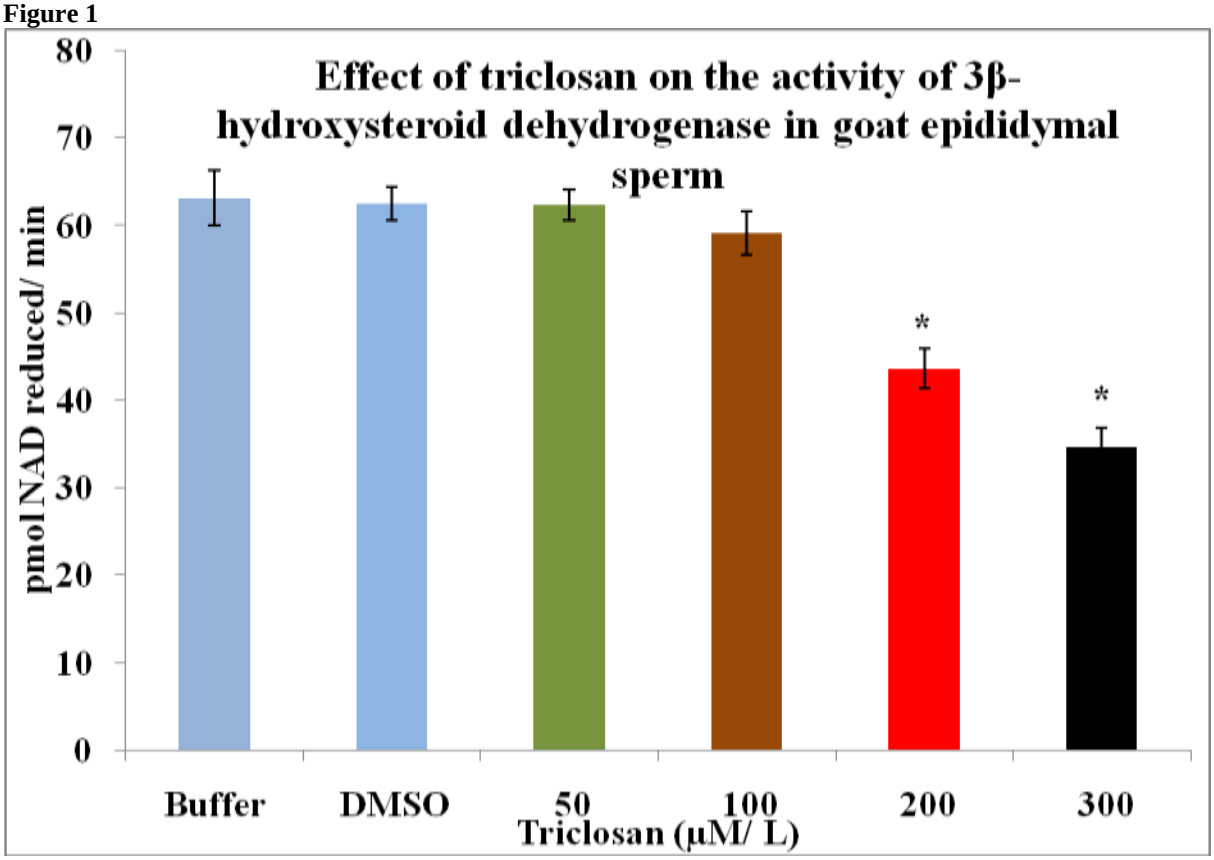


Figure 2

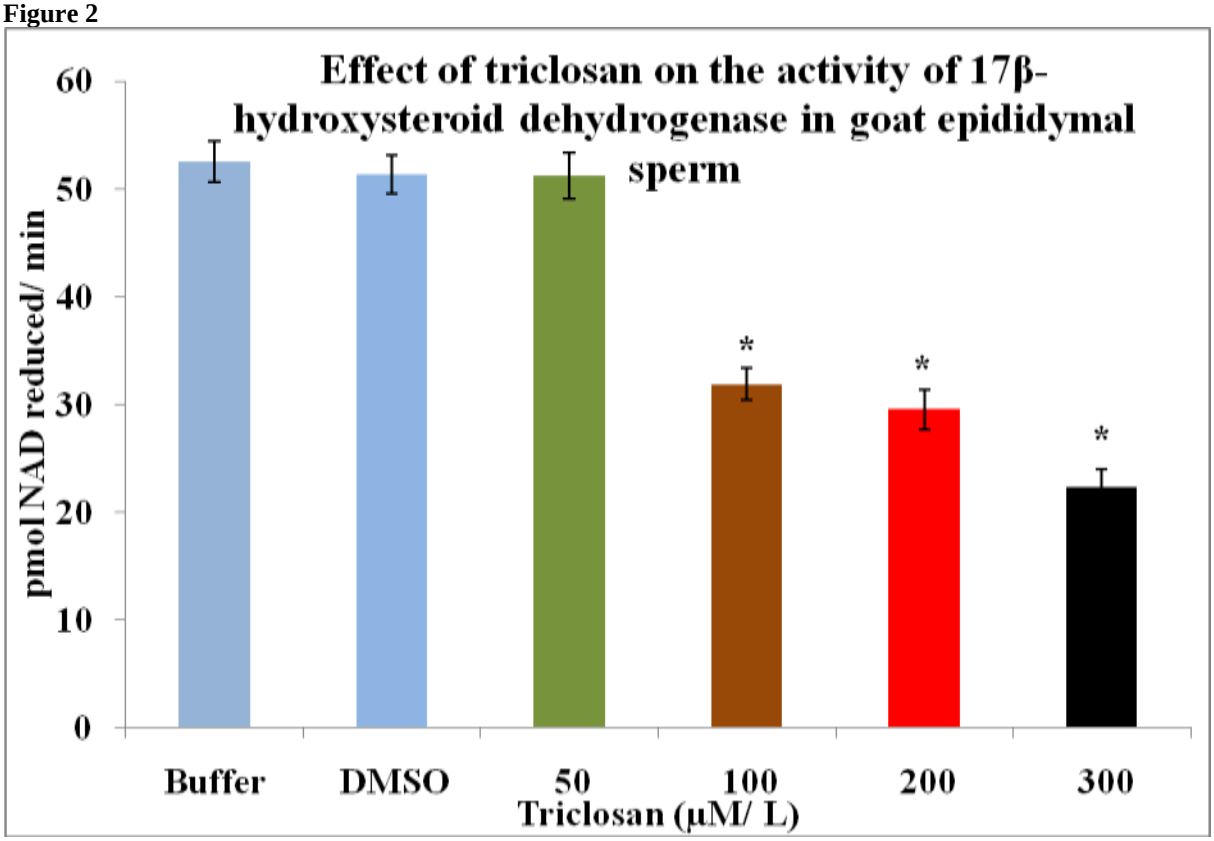


Figure 3

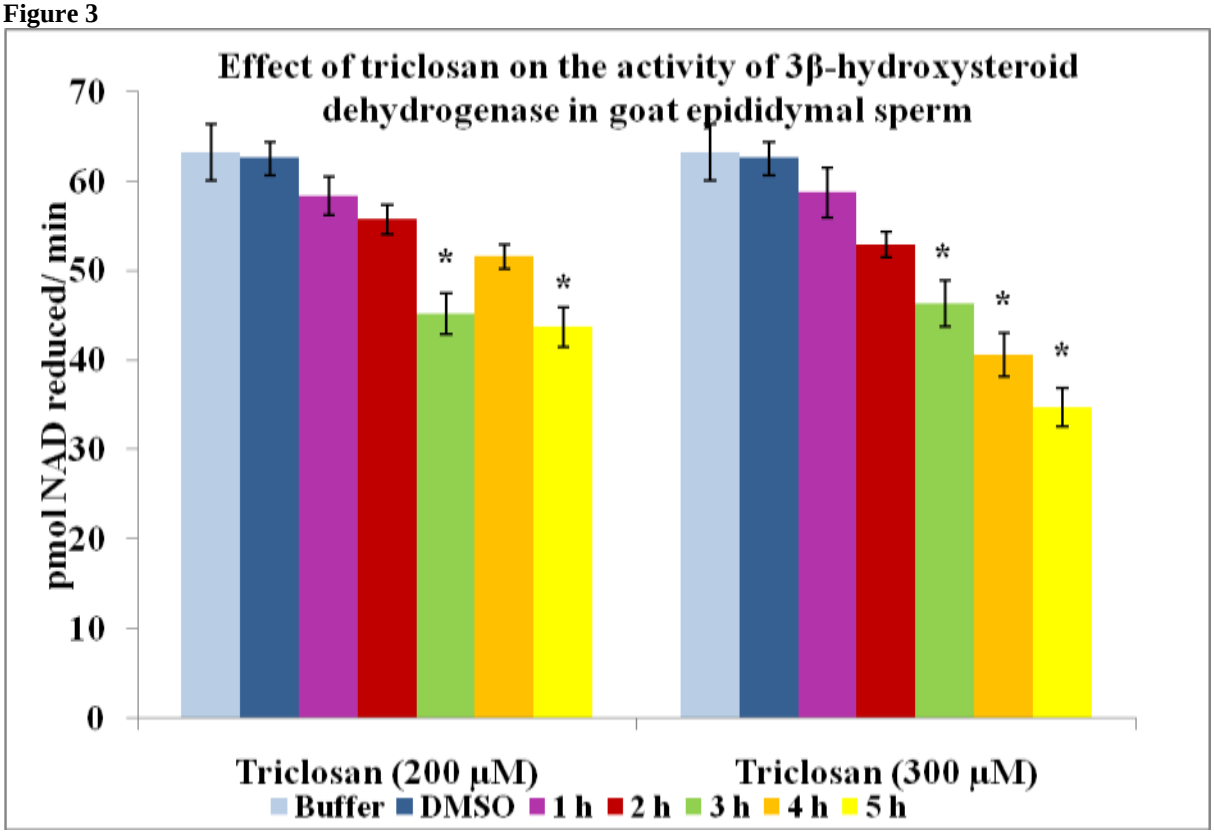
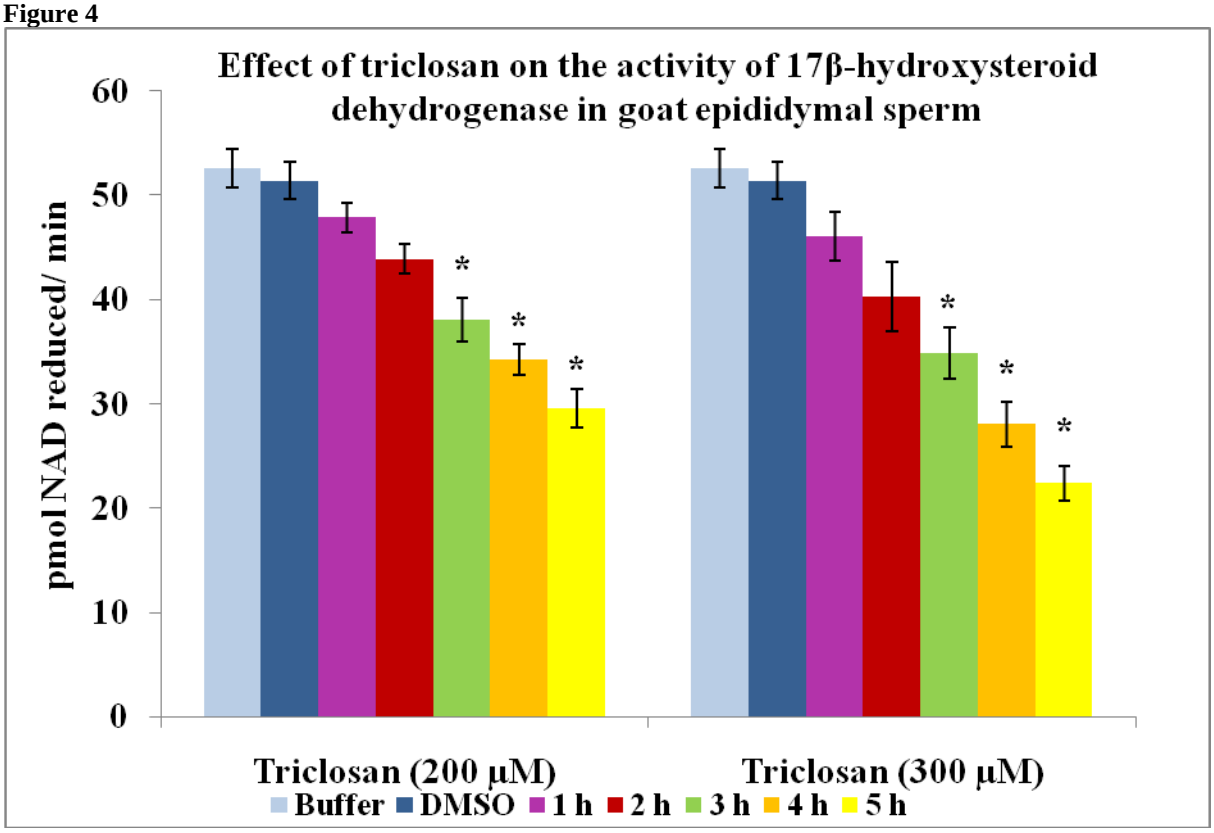


Figure 4



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