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Effect of Vincristine on Steroidogenic Pathway of Male Wistar Rat

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ABSTRACT

Vincristine is major player in front line combination chemotherapy for treatment of cancer and shows decrease in testosterone after treatment contributing to reproductive toxicity. Hence, the present research was aimed to study the effect of vincristine on steroidogenic pathway which is responsible for testosterone production in males. Vincristine was intraperitoneally injected to adult male Wistar rats of proven fertility with a dose of 40µg/kg/day dissolved in 0.5 ml of physiological saline for 30 days. Animals were sacrificed at the end of treatment period; their testes were removed and used for further studies. No significant changes were observed in body weight, testis weight and mean relative testis weight. Expression studies of enzymes involved in steroidogenic pathway showed that there was decrease in expression of Steroidogenic acute regulatory (StAR) protein which might be responsible for altered testosterone production.

Keywords: Vincristine, Steroidogenic Pathway, Combination chemotherapy, Western blotting.

Introduction:

Vincristine (VCR), an alkaloid is isolated from *Vinca rosea* Linn. The property of VCR to inhibit cell division through prevention of polymerization of tubulin of the microtubules which constitute the spindle fibre, and dissociation of the existing microtubules leads to the extensive use of vincristine in the chemotherapy against several kinds of cancers [1]. Treatment with combination chemotherapeutic regimens for cancer leading to severe male reproductive dysfunction including azoospermia/oligospermia, gynaecomastia and lack of sexual desire and libido has already been reported [2] and vincristine is one of the constant ingredients in most of such regimens. Vincristine as a causative agent of male reproductive toxicity, particularly affecting Spermatogenesis as well as androgenesis of the testis, has already been studied [3-5]. Spermatogenesis is required for the formation and maturation of sperms which is controlled by the male sex hormone i.e. testosterone. Testosterone is produced by the steroidogenesis which utilizes Cholesterol and different enzymes act on it to form testosterone [6, 7]. StAR is the protein which is involved in transport of cholesterol from outer mitochondrial membrane to inner mitochondrial membrane. The mitochondrial cholesterol side chain cleavage enzyme (P450scc) converts this cholesterol into pregnenolone which in turn is converted to testosterone by enzymes in the smooth endoplasmic reticulum; 3β-hydroxysteroid dehydrogenase (3β-HSD), 17 hydroxylase/C-17,20 lyase (17 OHase /C17,20-lyase) and 17- hydroxysteroid dehydrogenase (17β- HSD). Aromatase is the terminal

enzyme in the steroidogenesis pathway and is a key enzyme responsible for the conversion of testosterone to estradiol [8]. The androgen receptor on binding to testosterone gets activated and regulates the transcription of several genes required for the process of spermatogenesis [9, 10]. Vincristine affects the spermatogenic compartment in a dose dependent as well as duration Dependent manner [11]. Studies have been reported that VCR affects the structural and functional integrity of Sertoli cells through the effect on microtubules [3, 12]. Leydig cell atrophy has also been observed, which results in the decrease in the circulating level of androgens [13].

Vincristine has been shown to disrupt the sperm's axoneme and dense fibrils [14] which are important component of the sperm's flagellum resulting in compromised motility in sperms [15]. It was already investigated that VCR treatment results in the decrease in the testosterone production resulting in the faulty spermatogenesis causing male infertility [13, 16]. Though it is known that VCR treatment results in decreasing level of testosterone there is no possible mechanism to elucidate the reason of decrease testosterone level. The possibility of Steroidogenic pathway might have hampered upon vincristine treatment cannot be ruled out. Hence present study aims to study the expression of genes involved in steroidogenic pathway which includes StAR protein, P450scc, 3β HSD, P450C17, 17 β- HSD and aromatase. Further the expression of receptors that is androgen receptor which is required for mediating the effect of testosterone on treatment with VCR is also studied.

Materials and methods:

Animals

Adult male Wistar rats of proven fertility weighing 150-200 g were obtained from Bharat Serum and Vaccines Ltd, India and were handled according to the Institutional legislation regulated by the committee for the purpose of control and supervision of experiments on animals and acclimatized to laboratory conditions for 15 days. Animals were assigned to treatment and control groups (5 rats per group) and were allowed free access to food and water *ad libitum* (Amrut feed Ltd).The room temperature of 250C and the light/dark cycle of 12hrs were maintained.

Drug treatment

Vincristine sulphate (Cytocristin; Goa Cipla Ltd, India) was purchased from a local drug house. The control group received a daily dose of 0.5 ml of physiological saline while the experimental rats received (five rats) 40µg VCR/kg/day through the intraperitoneally in constant volume of 0.5 ml of physiological saline for 30 days via intraperitoneal route.

Extraction of RNA

Total RNA was extracted from the testes using the RNeasy Mini Kit obtained from QIAGEN, USA. All the general chemicals and reagents were obtained from Sisco Research Laboratory Pvt. Ltd., Mumbai, India and were of analytical grade. The quality of the RNA samples was evaluated by the determination of the ratio

260nm/280 nm and their integrity was checked by electrophoresis on 1.5% agarose gel. RNA was stored at -200C until use.

Reverse transcription polymerase chain reaction

Semi-Quantitative One Step RT-PCR was performed using 500 ng RNA template, in which RNA was first reverse transcribed to cDNA and then Polymerase chain reaction was carried out in a final volume of 25 µL. For Primer sequence and conditions for amplification are shown in Table 1 **(See Tables & Figures Section)**. Primers was obtained from (Sigma Aldrich Chemicals Pvt. Ltd, INDIA). RPS 16 was used as internal control. The amplified PCR product of respective gene were run on 2% agarose gel stained with ethidium bromide, visualized under Gel Doc and analysed with Densitometer Software.

Extraction of Protein and Western Blotting

Protein was extracted using around 500 mg of testicular tissue and homogenised in 5 ml of lysis buffer (50mM Tris-Cl, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1% Triton X-100). The homogenate was allowed to stand on ice for 15minutes and then was centrifuged at 10,000 rpm at 4°C for 40 minutes. The supernatant containing proteins were used for further study. The concentration of protein was determined by Lowry (1951) [17] method. 50µg of protein samples were separated on 12% SDS-PAGE and then transferred to PVDF membrane. Nonspecific binding was blocked with 5% skimmed milk in 0.1 % Tris-buffered saline for 1 hr with shaking. The membrane was then incubated overnight at 40C with 1:200 diluted rabbit polyclonal StAR antibody

in 1.5% Non fat skimmed milk prepared in T-TBS.After washing with T-TBS, the membrane was incubated with 1:1000 diluted horseradish peroxidise-conjugated goat anti rabbit IgG for 1 hr with shaking and then washed 3 times with T-TBS for 20 mins of each after that membrane were washed once with TBS for 20 min in order to remove Tween. An enhanced chemiluminescence detection reagent was used to visualize proteins on PVDF membrane.

Statistical Analysis

The data was represented as the mean ± standard error of the mean (S.E.M) and was analysed by using independent Student t test. The differences in the control and treated groups were considered significant at $P<0.05$.

Results

Body and testicular weights

Vincristine treatment at a dose of 40µg/kg/day for a period of 30 days was studied. The body weights of control and treated groups were monitored on a weekly basis during the treatment period for assessment of effects on the general health. Although there was a decreasing trend seen in the body weight each week, the body weight of the rats at the end of the treatment was not significantly altered as compared to the control rats (Table 2.) **(See Tables & Figures Section)**. However, there was 15% decrease in the body weight of the treated rats at the end of the treatment period. The organ weight as well as the relative organ weight of the treated rats did not show any significant change with respect to control (Table 3) **(See Tables & Figures Section)**.

Expression of genes involved in Steroidogenic pathway

The A260/ A280 ratio obtained for the RNA sample extracted from each testis of the two groups was in the range of 1.8-2.0, indicating that the RNA obtained was comparatively free of protein and DNA contamination. Testicular expression of genes involved in steroidogenic pathway after the treatment with vincristine at the dose of 40µg/kg/day was analysed by semi quantitative one- step RT-PCR, by using gene specific primers [Fig 1 (A) – 7 (A) **(See Tables & Figures Section)**]. There was a significant decrease of 25% in StAR mRNA of vincristine treated rats with respect to control but no significant changes were observed in any of the enzymes involved in steroidogenic pathway. Androgen receptor also showed no significant changes in the expression pattern when compared with control [Fig 1 (B) – 7 (B) **(See Tables & Figures Section)**].

Western blot analysis

Fig. 8 (A) **(See Tables & Figures Section)** shows the western blot of StAR protein. From testis tissue a band of approximately 33Kd was detected as StAR proteins. Quantitative analysis of western blot was performed by scanning densitometry and significant decrease of 54 % were observed in the expression of StAR protein of treated rats when compared with control ones as shown in Fig.8 (B) **(See Tables & Figures Section)**.

Discussion

Vincristine, a potent anticancer drug has been shown to have adverse effect on the male reproductive system. In the present study, Vincristine administration did not alter the testis weight significantly although a slight reduction in the body weight was observed. The body weight reduction can be attributed to the general toxicity of vincristine.

The alterations in the level of testosterone could be probably due to the effect of the vincristine on the key proteins and enzymes of the steroidogenic pathway. Steroidogenic pathway involves the delivery of the substrate, cholesterol from the outer to the inner mitochondrial membrane, by StAR [18, 19], which is the rate limiting step in steroidogenesis [20]. The cholesterol side chain cleavage enzyme P450scc converts cholesterol to pregnenolone, the first committed step in the process [21, 22] which is subsequently catalysed to progesterone by 3β hydroxysteroid dehydrogenase (3β-HSD) [23]. Progesterone is then converted to androstenedione by cytochrome P450 C17-20 lyase, and then to testosterone by 17β hydroxysteroid dehydrogenase (17β HSD). Leydig, Sertoli and germ cells further express low levels of aromatase, which converts testosterone originating from the Leydig cells into estradiol, a step that appears to be necessary for the successful initiation of spermatogenesis and mitosis of spermatogonia [24]. Testosterone synthesis is regulated by LH which binds to LH receptors and induces cAMP signalling pathway. The cAMP in turn binds to cAMP responsive element in the promoter [25] and

regulates the expression of enzymes involved in steroidogenic pathway that is StAR, P450scc, 3β-HSD, P450C17, 3β-HSD and Aromatase. Semi quantitative RT-PCR analysis in present study revealed that chronic exposure of rats to 40μg/kg/day vincristine resulted in significant decrease in the level of StAR mRNA and protein expression but no alterations was observed in other genes involved in steroidogenic pathway. Compelling evidence documenting the critical role of StAR in the regulation of steroidogenesis has been obtained from both basic and clinical studies [26, 27]. Decrease in the expression of StAR results in reduced cholesterol transfer to P450scc within the mitochondria which leads to suppression of steroid biosynthesis [28, 29]. The decrease in the level of StAR protein on vincristine treatment might affect the transfer of cholesterol from outer to inner mitochondrial membrane, thus leading to reduction in testosterone level as reported earlier [30, 31, 32]. In males, testosterone levels are maintained by feedback mechanism, wherein the LH secreted by the pituitary binds to LH receptor [33] and activates the cAMP signalling pathway for synthesis of androgen [34]. The high levels of LH on treatment with vincristine [35, 36] should have activated the cAMP signalling pathway thereby inducing the expression of genes involved in the process of steroidogenesis. However in the present study, there is decrease in StAR mRNA and protein on vincristine treatment which probably could be due to altered cAMP signalling because of low levels of LH receptors, as seen on treatment with the cytotoxic drug, cis-platin [37]. Some studies have also demonstrated that the inhibition of StAR mRNA or protein levels results in

a dramatic decrease in steroid biosynthesis which is incongruence with present study [38, 39]. Once testosterone enters inside the mitochondria, P450scc converts cholesterol into pregnenolone which is further converted into progesterone by 3β-HSD6. There are no significant changes in mRNA expression levels of P450scc and 3β-HSD on vincristine. A similar observation was also reported by Namasivayam and his coworkers[40] in cyclophosphamide treated male rats.

The lack of significant alterations in P450scc and 3β-HSD expression levels could be possibly due to the fact that they are chronologically regulated and possess long half life [41]. Unaltered expression of P450c17 and Aromatase indicates that probably an alternative pathway or other factors apart from the cAMP signalling pathway regulates the transcription of this gene. Testosterone mediates its effect by binding to the androgen receptor [42]. In the rat testis, AR protein has been localised to Sertoli cells, peritubular myoid cells and Leydig cells [43]. Several studies have showed that androgens may auto regulate the expression of their own receptor mRNAs [44], yet estrogen down regulates the AR mRNA [45]. Previous studies in which rats were pretreated with ethane dimethane sulfonate, specifically inhibited testosterone production while the AR mRNA level in the testis was unchanged [46]. This report is in agreement with present result which also shows low level of testosterone and unaltered expression of AR mRNA. The unaltered mRNA expression of P450scc, 3β-HSD, P450c17, 17- β-HSD and Aromatase in the current investigation suggests that

steroidogenic pathway after transfer of cholesterol by StAR is normal suggesting the toxic effect of vincristine on StAR gene.

Conclusion

Down-regulation of StAR expression and protein level can be one of the reasons for decrease level of testosterone on vincristine treatment. Further study, focusing on various paracrine and autocrine modulators of steroidogenic pathway should be carried out to identify the precise targets of this drug.

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Declaration of conflicting interest

All authors declared no conflict of interest.

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Tables & Figures Section

Table 1. Primers used for RT-PCR: *Primer sequence and conditions for amplification*

Target mRNA	Primer sequence	Product size (bp)	No of cycle	Annealing temp. (˚C)
StAR	Forward primer- TTGGGCATACTCAACAACCA Reverse primer- ATGACACCGCTTTGCTCAG	389	35	59°C
P450scc	Forward primer- AGGTGTAGCTCAGGACTT Reverse primer- AGGAGGCTATAAAGGACACC	399	35	50°C
3β-HSD (type I)	Forward primer- TTGGTGCAGGAGAAAGAAC Reverse primer- CCGCAAGTATCATGACAGA	547	28	55°C
P450C17	Forward primer- GACCAAGGGAAAGGCGT Reverse primer- GCATCCACGATACCCTC	302	32	59°C
17 β-HSD (type III)	Forward primer- TTCTGCAAGGCTTTACCAGG Reverse primer- ACAAACTCATCGGCGGTCTT	653	28	61°C
Aromatase	Forward primer- GCTTCTCATCGCAGAGTATCCGG Reverse primer- CAAGGGTAAATTCATTGGGCTG	290	35	62°C
AR	Forward primer- TACCAGCTCACCAAGCTCCT Reverse primer- ACACTGGGCCACAAGAAGAT	496	32	59°C
RPS 16 (internal control)	Forward primer- AAGTCTTCGGACGCAAGAAA Reverse primer- TTGCCCAGAAGCAGAACAG	196	The Cycles and annealing temperatures were the same as the gene with which it was amplified.	

Table 2. *Vincristine treatment at a dose of 40µg/kg/dayX30 days: Analysis of weights in control and treated rats*. The body weight of control and treated rats after treatment with vincristine for 30 days (weekly bases). The number in parentheses indicates the number of rats in each group. The values are expressed as mean ± SEM ($P<0.05$).

Group	Week 1	Week 2	Week 3	Week 4	Week 5
Vincristine Control (5)	282±7	321±12.39	346.6±13.36	370±12.45	391±13.31
Vincristine Treated (5)	257±12.81	291±13.17	286±17.13	310±21.68	331±22.26

Table 3. *Vincristine treatment at a dose of 40µg/kg/dayX30 days: Organ weight analysis of control and treated rats*. The testis weight and relative percentage weight of testis of control and vincristine and treated rats. The number in parentheses indicates the number of rats in each group. The values are expressed as mean ± SEM.

	Testis weight	Relative testis weight
Control Rat(5)	1.575±0.093	0.397±0.093
Treated Rat (5)	1.501±0.062	0.463±0.030

Figure 1:

Fig1A. Agarose gel electrophoresis showing co-amplification products of StAR and RPS16 cDNA of control and vincristine treated samples.

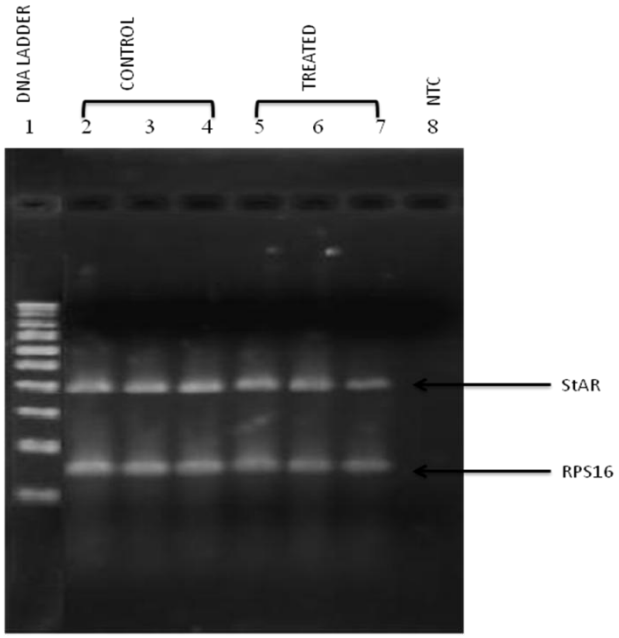
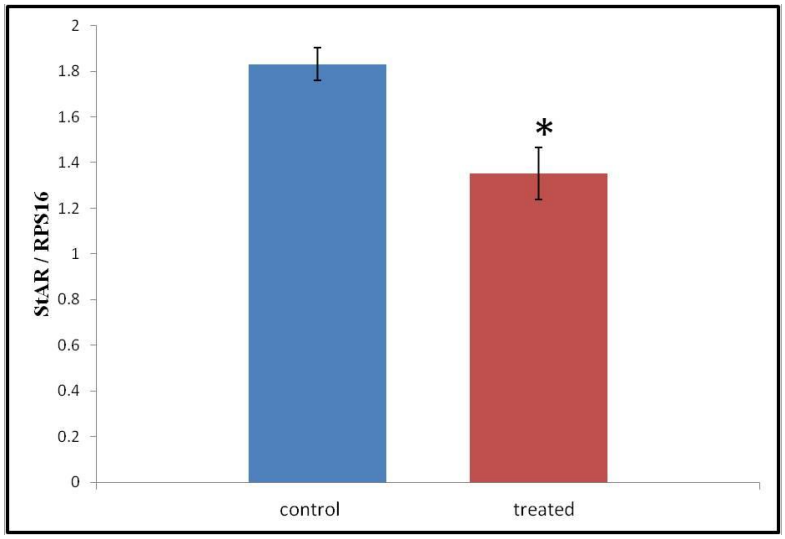


Fig.1B. mRNA level expression of StAR gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of StAR and RPS16.



*** Statistically significant differences. The values are expressed as mean ± SEM (P<0.05).**

Figure 2

Fig.2A. Agarose gel electrophoresis showing co-amplification products of P450scc and RPS16 cDNA of control and vincristine treated samples.

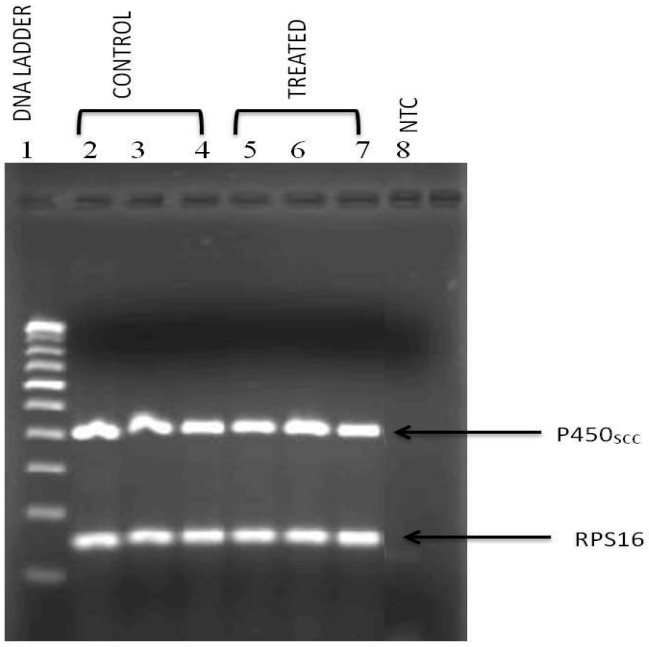


Fig.2B. mRNA level expression of P450scc gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of P450scc and RPS16. The values are expressed as mean ± SEM ($P<0.05$).

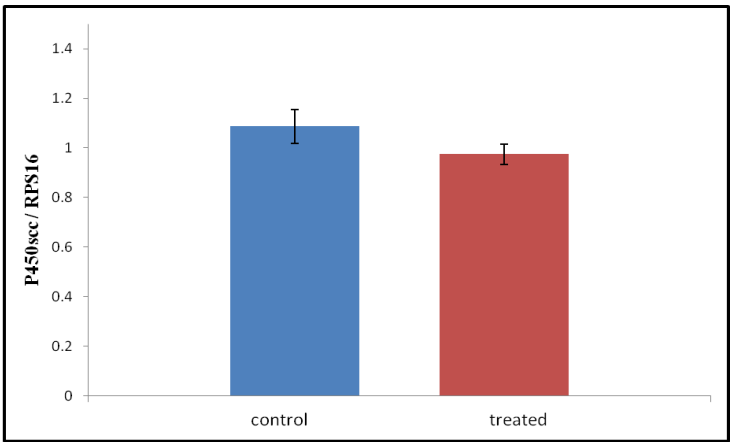


Figure 3

Fig.3A. Agarose gel electrophoresis showing co-amplification products of 3βHSD and RPS16 cDNA of control and vincristine treated samples.

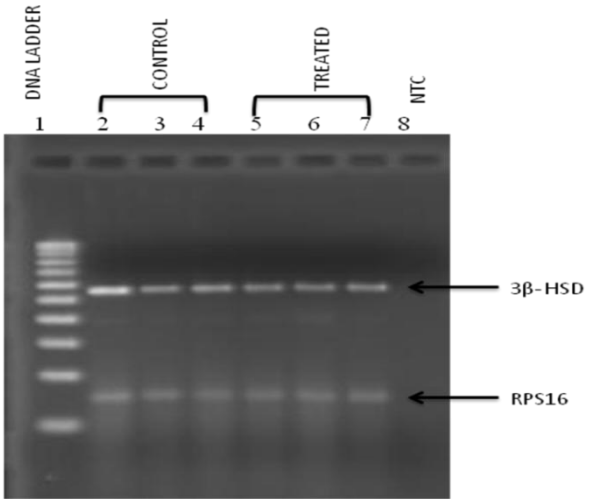


Fig.3B. mRNA level expression of 3βHSD gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of 3βHSD and RPS16. The values are expressed as mean ± SEM (P<0.05).

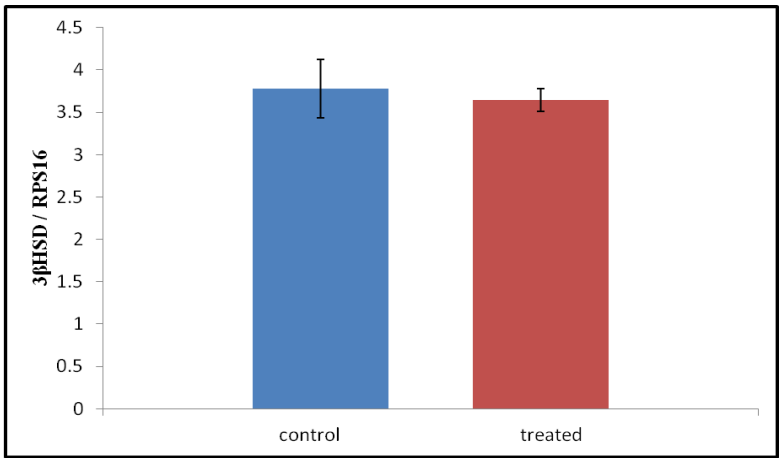


Figure 4

Fig.4A. Agarose gel electrophoresis showing co-amplification products of $P450_{C17}$ and RPS16 cDNA of control and vincristine treated samples.

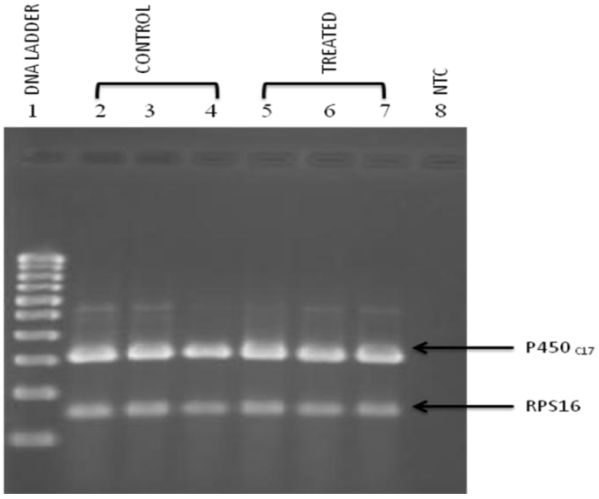


Fig.4B. mRNA level expression of P450scc gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of P450scc and RPS16. The values are expressed as mean ± SEM ($P<0.05$).

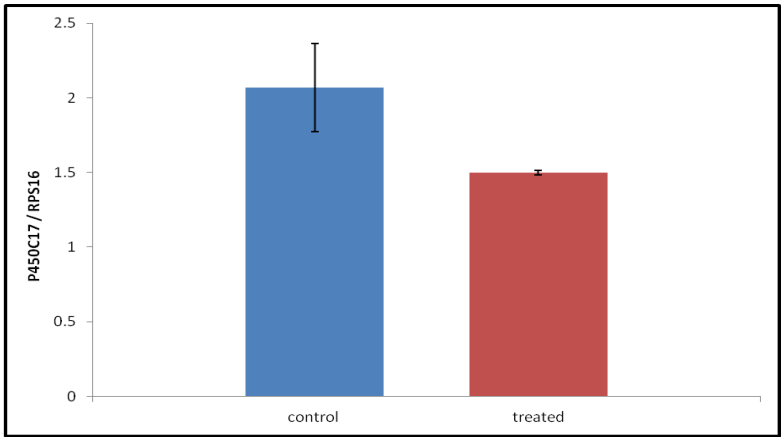


Figure 5

Fig5A. Agarose gel electrophoresis showing co-amplification products of 17βHSD and RPS16 cDNA of control and vincristine treated samples.

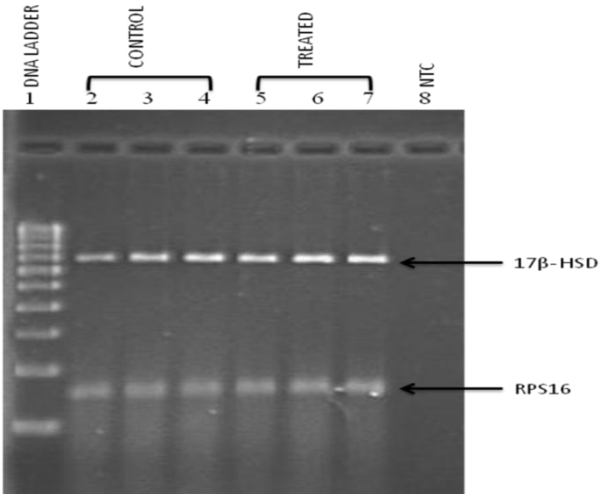


Fig5B. mRNA level expression of 17βHSD gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of 17βHSD and RPS16. The values are expressed as mean ± SEM (P<0.05).

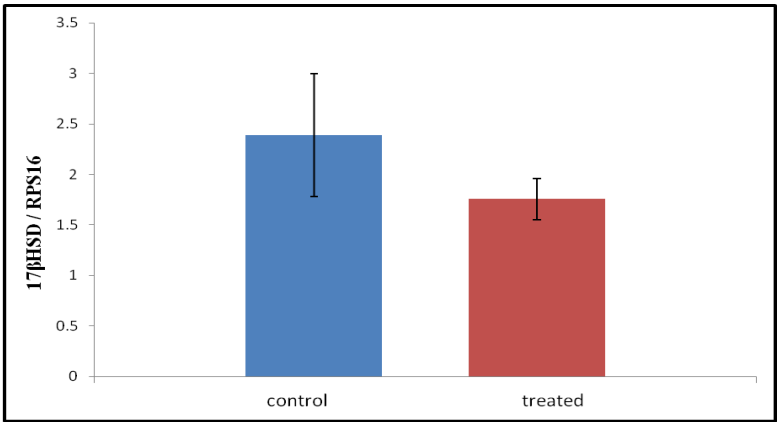


Figure 6

Fig6A. Agarose gel electrophoresis showing co-amplification products of Aromatase and RPS16 cDNA of control and vincristine treated samples.

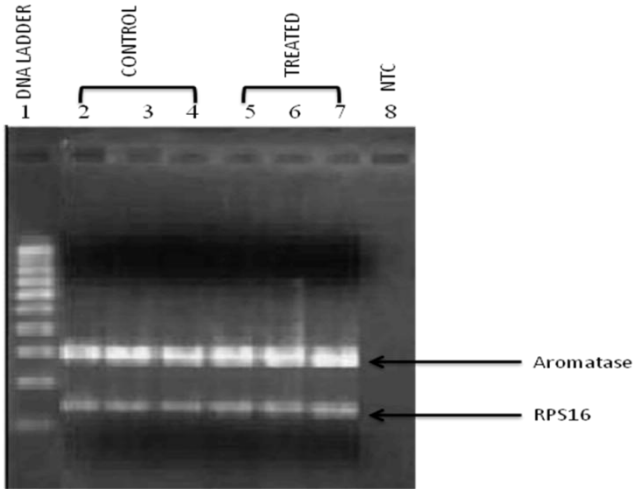


Fig6B. mRNA level expression of Aromatase gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of Aromatase and RPS16. The values are expressed as mean ± SEM ($P<0.05$).

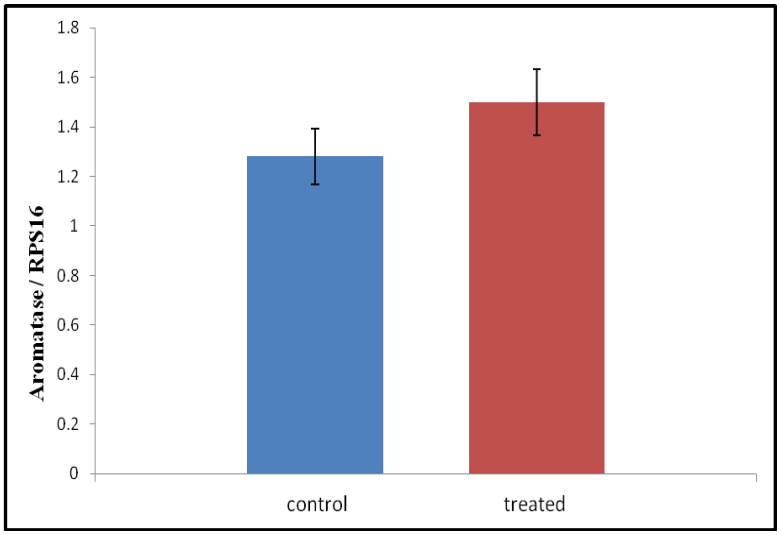


Figure 7

Fig7A. Agarose gel electrophoresis showing co-amplification products of AR and RPS16 cDNA of control and vincristine treated samples.

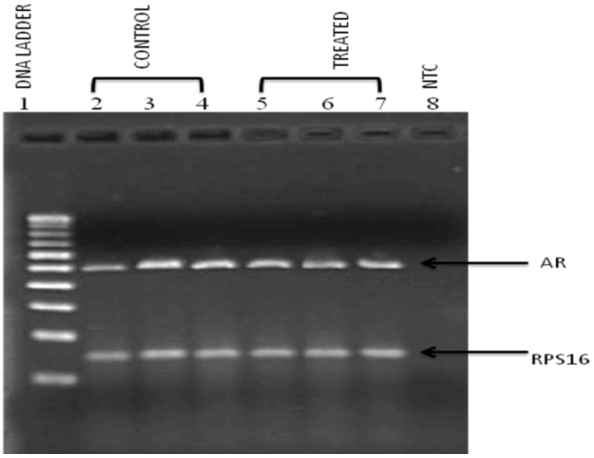


Fig7B. mRNA level expression of Aromatase gene normalized with respect to RPS16. Gene expression levels are graphed as relative mRNA expression by taking the ratio between the signal intensities of Aromatase and RPS16. The values are expressed as mean ± SEM ($P<0.05$).

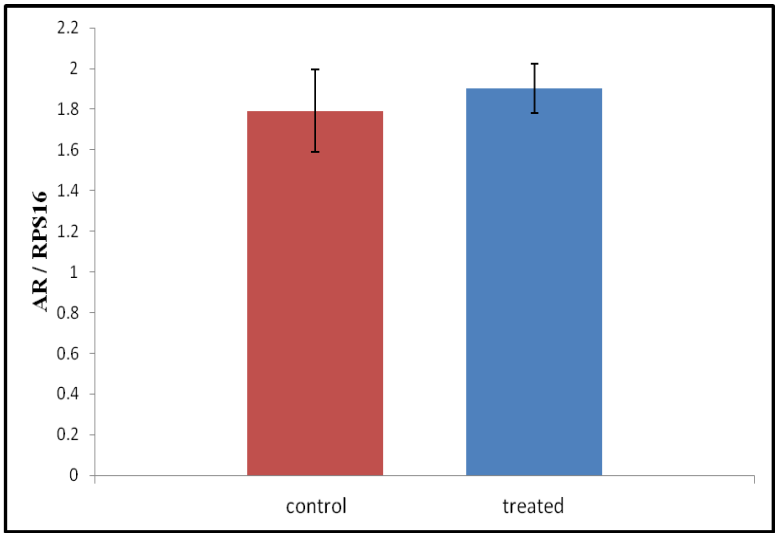


Figure 8

Fig8A. Western Blot of StAR protein.

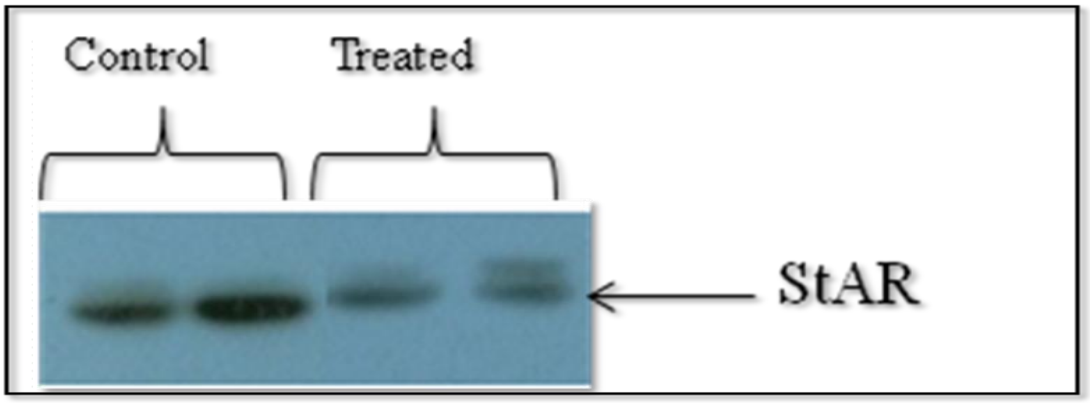


Fig8B. Quantitative analysis of western blotting by scanning densitometry.