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EFFECT OF NIMODIPINE ON STEROIDOGENESIS IN MALE RATS

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ABSTRACT
The purpose of the present study was to investigate whether treatment of male rats with the calcium antagonist nimodipine, interferes with the steroidogenesis .Thirty male rats were allocated into five groups .Control (D.W.) (n=6) and test groups that received (20mg, 40, 80 mg/kg/day) of nimodipine by oral gavage, each one (n=6) and sulfasalazine (500mg/kg) for 30 days .Animals were kept in standard conditions. At day thirty of experiment the testis tissue of rats in whole groups was removed. The effects of nimodipine on steroidogenic acute regulatory protein (StAR) mRNA expression also assessed by using reverse transcription (Reverse Transcription)-polymerase chain reaction analysis .Testosterone and Pituitary hormones serum concentrations were also measured using human ELISA apparatus.The results of the present study indicated that nimodipine in a dose-dependent pattern (20, 40, 80 mg/kg) significantly inhibit the (StAR) mRNA expression and have greater effect at dose of (80 mg/kg body weight) that is significantly higher than all of the effects produced by other doses of drug. The result showed that nimodipine, in a dose-dependent pattern, was effective in attenuating steriodogensis production through its inhibitory effects on StAR protein gene expression in rats.

KEY WORDS: Nimodipine, steroidogenesis, male rats.

INTRODUCTION:

Calcium ion is implicated in diverse cellular functions in both germ cells and somatic cells in the testis, particularly, mediating the responses to endocrine hormones and local regulators in genital tracts.[1,2] A common belief is that the Ca^{2+} influx and efflux should be tightly regulated to maintain the intracellular Ca^{2+} homeostasis, and an alteration in the Ca^{2+}transport across the cell membrane could result in a drastic impact on spermatogenesis and steroidogenesis.[3,4]Leydig cell production of testicular androgens is tightly controlled by endocrine interactions among the pituitary gland and the testis, as well as through the paracrine and autocrine regulation within the testis.[5,6,7]Leydig cells secrete testosterone responsible for the onset of both spermatogenesis and male sexual development.

Endocrine control of Leydig cell steroidogenic activity by luteinizing hormone (LH), follicle-releasing hormone (FSH) or human chorionic gonadotropin (hCG) has been exerted through their respective receptors coupled to the cAMP- or the Ca^{2+}- mediated signaling pathway.[8,9,10] The rate limiting step in steroid hormone synthesis has long been recognized to be the conversion of cholesterol to pregnenolone, Cholesterol must transverse the aqueous space between the cholesterol-rich outer mitochondrial membrane and cholesterol-poor inner mitochondrial membrane to reach the P450 side chain cleavage enzyme cytochrome P11A (CYP11A).[11] The enzyme associated electron transport proteins, which reside on of the inner mitochondrial membrane, convert cholesterol into pregnenolone.[11]

Orme-Johnston and colleagues [12-20] identified a group of mitochondrial 30 kDaphosphoproteins that appeared in adrenal cells stimulated with ACTH and gonadal cells stimulated with LH. The 30 kDa proteins were shown to be derived from a 37 kDa precursor synthesized in the cytoplasm and then imported into mitochondria and processed to the 30 kDa forms. Purification of the 30 kDa protein from MA-10 cells and amino acid sequence analysis allowed the cloning of its cognate cDNA and the identification of a novel protein in the mouse and human , named the steroidogenic acute regulatory protein (StAR).[21]Nimodipine is a calcium channel blocker. The contractile processes of smooth muscle cells are dependent upon calcium ions, which enter these cells during depolarization as slow ionic transmembrane currents. Nimodipine inhibits calcium ion transfer into smooth muscles cells and thus inhibits contractions of these cells. Nimodipine had a greater effect on cerebral arteries than on arteries elsewhere in the body perhaps because it is highly lipophilic, allowing it to cross the blood-brain barrier; concentrations of nimodipine as high as 12.5 ng/mL have been detected in the cerebrospinal fluid of nimodipine-treated subarachnoid hemorrhage (SAH) patients.[22] The aim of this study is to show the *effects of nimodipine on Steroidogenic acute regulatory (StAR) protein using Reverse transcription PCR (RT-PCR) analysis and on serum gonadal hormones including LH and FSH and testosterone.*

MATERIAL AND METHODS

Animals

Sprague-Dawly male rats weighing 180-250 gm and 8 weeks old were obtained from the Animal House of the College of Pharmacy/University of Baghdad. The animals were maintained on normal conditions of temperature, humidity and light/dark cycle. They were fed standard rodent pellet diet and they have free access to water.

Preparation of nimodipine solution

Nimodipine tablet (60mg) (Bayer company, Germany) was dissolved in (30ml) distilled water to produce a solution with concentration of 2mg/ml .which is used as a standard solution for the preparation of different doses.

The study design

Thirty rats were used in the present study, the study groups were divided into 5 groups:

First group: (control) 6 rats were administered distilled water for 30 successive days by oral gavages.

Second group: 6 rats were used in which (20mg/kg BW) of nimodipine was given for 30 successive days by oral gavage.

Third group: 6 rats were used, in this group (40 mg/kg BW) dose of nimodipine were used for 30 successive days by oral gavage.

Fourthgroup: 6 rats were used, in which (80 mg/kg BW) nimodipine was used for 30 successive days by oral gavage.

Fifth group: 6 rats were given a dose of (500mg/kg BW) of sulfasalazine for 30 successive days by oral gavage as a positive control (in this group, sulfasalazine represent standard steroidogenisis inhibitory agent.)

cDNA synthesis and purification of the StARcDNA using reverse transcription-polymerase chain reaction (RT-PCR)

Isolation of rat tissue RNA

Total RNA was isolated from testis of male rat tissues utilizing Total RNA Mini Kit.[23]

Estimation and electrophoresis of Total RNA[24]

The concentration of RNA was calculated by spectrophotometer method using UV-visible spectrophotometer.

First strand synthesis

First strand cDNA was synthesized according to the manufacturer's instructions using a reverse transcription system kit. For each reaction the following Reagents and their volumes were used for PCR amplification.

Amplification Conditions:

The PCR cycle was set up as the following according to (Jae-Ho Lee *et al.,* 2010).[25] The PCR product was electrophoresed in 2% agarose gel (Sambrok *et al*., 1989)(24), 10 µl of each PCR product was added to each well.5 µl of molecular marker (100-2000bp ladder) was mixed with 1 µl of loading dye and added at the first well. Then product was detected by examined under UV Trans illuminator.

TABLE 1: PCR condition for StAr and B-actin cDNA

Step	Temperature	Time	No. of cycle
Reverse transcription	50°C	30 min.	1
Denaturation	95°C	1 min.	
Annealing	55°C	1 min.	28
Elongation	72°C	1 min.	
Final Elongation	72°C	3 min.	1

Amplification of StAR gene

TABLE 2: Oligonucleotide primer sequences used for PCR amplification of StAR gene (Genbank: Access No. BC060970)

Primer	Sequences	TA
Forward primer	LP5 - GAC CTT GAA AGG CTC AGG AAG AAC-3	57
Reverse primer	RP5-TAG CTG AAG ATG GAC AGA CTT GC-3	53

Amplification of B-actin gene

TABLE 3: Oligonucleotide primer sequences used for PCR amplification of B-actin gene (Genbank: Access No. NM007393).

Primer	Sequences	TA
Forward primer	LP5 - GAC CTT GAA AGG CTC AGG AAG AAC-3	57
Reverse primer	RP5-TAG CTG AAG ATG GAC AGA CTT GC-3	53

The PCR products from amplification of StAR and B-actin cDNA fragment was electrophoresed on an ethidium bromide-stained (2%) agarose gel. The presence of bands in 980 and 223bp fragment respectively.

Analysis of PCR Products

PCR products can be easily and quickly analyzed and resolved using a 2% agarose gel run in either TBE (89 m*M*Tris-borate, 2 m*M* EDTA) or TAE (40 m*M* Tris-acetate, 2 m*M*EDTA, pH approx 8.5). The resolved DNA bands are detected by staining the gels with either approx 0.5 g/mL of ethidium bromide, followed by destaining with water. Finally photographed under UV illumination. Use a 1-kilobasepair (kbp) ladder as a convenient marker for size estimates of the products.[24]

Determination of serum testosterone concentration

Serum concentrations of testosterone were measured by ELISA reader using commercial assay kits according to the manufacturer's protocols. (CSB-E05100r).[26]

Determination of serum Follicle-Stimulating (FSH) concentration

Serum concentrations of Follicle-Stimulating Hormone (FSH) were measured by ELISA reader using commercial assay kits according to the manufacturer's protocols. (CSB-E06869r).[27]

Determination of serum luteinizing hormone (LH) concentration:

Serum concentrations of luteotropic hormone (LH) were measured by ELISA reader using commercial assay kits

according to the manufacturer's protocols. (CSB-E12654r).[28]

Statistical analysis

All the results were expressed as mean± standard error (SEM). The data were analyzed by using computerized statistical package for the social sciences (SPSS) program. Paired T-test was done for each group pair includes "at zero time and after seven days of treatment. The significance of difference among the studied groups was determined using one-way analysis of variance (ANOVA). *P*-values< 0.05 were considered to be statistically significant.

RESULTS

Effects of different doses of nimodipine on gene expression of StAR protein by utilizing cDNA analysis (RT-PCR) in male rat testis

From the result of cDNA microarray analysis (RT-PCR), it was observed that in the, nimodipine-treated rat testis the cellular StAR protein gene expression was reduced marledly at doses of (40mg/kg) and (80mg/kg) nimodipine compared to the control as shown in figures (1,3,4). The overall StAR mRNA expression was shown to decrease in a dose-dependent pattern compared to controls, with maximum effect produced by nimodipine 80mg/kg figure (4) and a lower effect at 20mg/kg of nimodipine figure(2). However inhibitory effects of nimodipine on mRNA expression of StAR protein at the dose of (80mg/kg body weight) were the same when compared with sulfasalazine treated group (500mg/kg body weight). The StAR protein was present at (980bp) in the (RT-PCR) analysis in the control group, meanwhile it was starting to disappear as the dose increase till it completely disappeared at the dose of (80mg/kg of nimodipine).B-actin protein expression was present in all treated groups at (228bp) and not affected by nimodipine because, it was used as indicator for accurate mRNA extraction of StAR protein.

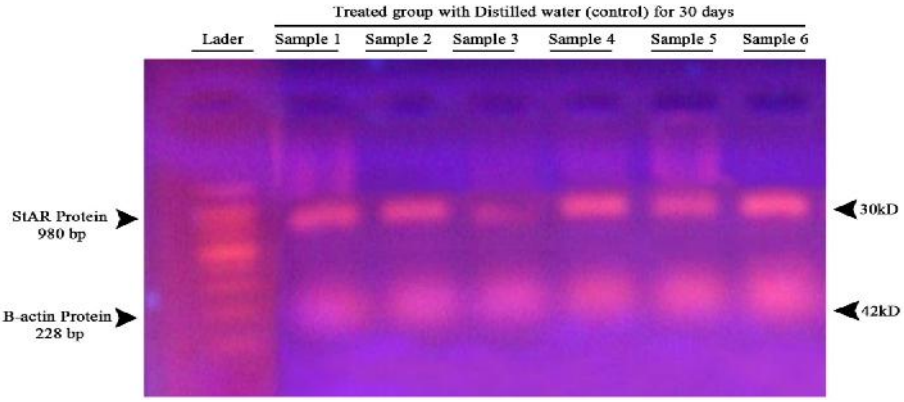

FIGURE 1: Agarose gel analysis of PCR amplification RT-PCR analysis of StAR mRNA expression in the rat testis .The figure shows that treated control group (D.W.) StAR mRNA expression. Numbers on the left side of the figure are the base pair sizes .We have directly sequenced the PCR products from molecular weight markers which were run on the same gel. The arrow indicates the position of the (980) bp PCR sequence is detected in product amplified in testis .The blots were stripped and rehybridised with a rat b-actin cDNA (bottom) at (228)**.**

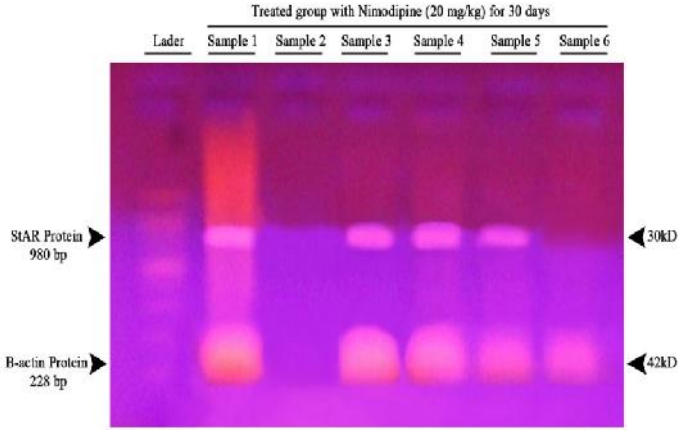

FIGURE 2: Agarose gel analysis of PCR amplification RT-PCR analysis of StAR mRNA expression in the rat testis .The figure shows that treated group (20mg/kg nimodipine.) StAR mRNA expression. Numbers on the left side of the figure are the base pair sizes .We have directly sequenced the PCR products from molecular weight markers which were run on the same gel. The arrow indicates the position of the (980) bp PCR sequencewhich is start to disappear and presence of faint line instead of it.

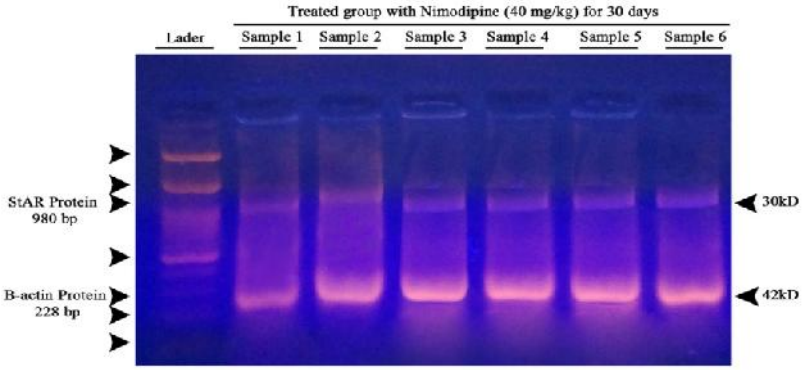

FIGURE 3: Agarose gel analysis of PCR amplification RT-PCR analysis of StAR mRNA expression in the rat testis .The figure shows that treated group (40mg/kg nimodipine) StAR mRNA expression. Numbers on the left side of the figure are the base pair sizes .We have directly sequenced the PCR products from molecular weight markers which were run on the same gel. The arrow indicates the position of the (980) bp PCR sequence is detected in product amplified in testis .The blots were be more faint suggesting more decrease in the mRNA expression of StAR protein .

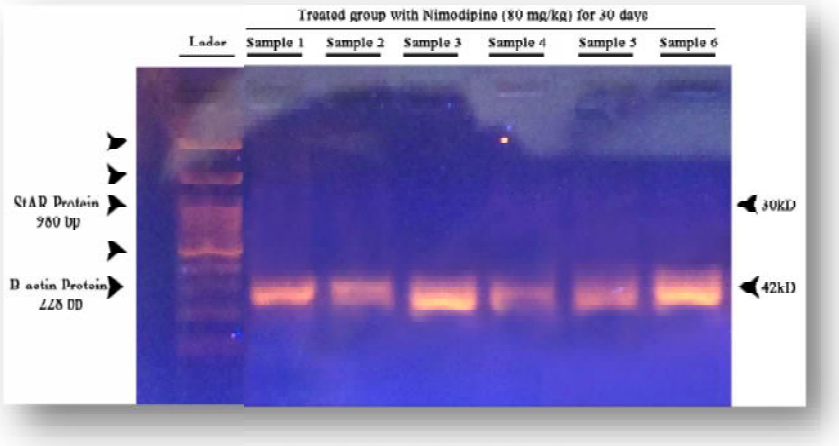

FIGURE 5: Agarose gel analysis of PCR amplification RT-PCR analysis of StAR mRNA expression in the rat testis .The figure shows that treated group (500mg/kg sulfasalazine.) StAR mRNA expression. Numbers on the left side of the figure are the base pair sizes. We have directly sequenced the PCR products from molecular weight markers which were run on the same gel. The arrow indicates the position of the (980) bp PCR sequence is detected in product amplified in testis .The blots were be more fainted similar to the effect of nimodipine (80mg/kg) figure (4).

Effects of different doses of nimodipine on testosterone serum concentration in rats

Effect of different doses of nimodipine and 500mg/kg (sulfasalazine) shown in table-4. All nimodipine doses (20, 40, and 80 mg/kg body weight) significantly reduced (P<0.05) serum testosterone conc. (In a dose-dependent pattern) compared to controls, with maximum effect produced by 80 mg/kg. In addition 500mg/kg of sulfasalazine significantly decrease (P<0.05) serum testosterone conc. compared to controls. Also we observed significant differences between sulfasalazine and nimodipine doses except 40and 80mg/kg and also significant differences between each one of nimodipine doses except between 40mg and 80mg/kg.

TABLE 4. Effects of different doses of nimodipine on serum concentration of testosterone in rats

Control	8.3 ±0.3
20mg /kg nimodipine	5.3±0.2^{*a}
40mg /kg nimodipine	2.0 ±0.6^{*b}
80mg /kg nimodipine	1.5 ±0.1^{*b}
500mg /kg sulfasalazine	1.6±0.2*

Data are expressed as mean ± standard error of mean; n=6 animals in each group; *significant different compared to control (P<0.05)

*	: sig at P<0.05 as compared with Control values
A	: sig at P<0.05 as compared with 500mg /kg salazopyrine values
B	: sig at P<0.05 as compared with 20mg /kg nimodipine values
C	: sig at P<0.05 as compared with 40mg /kg nimodipine values

Effects of different doses of nimodipine on LH serum concentration in rats

Effects of different doses of nimodipine and 500mg/kg (sulfasalazine) on rat's serum LH concentration were shown in table (5).

All nimodipine doses (20, 40, and 80 mg/kg body weight) significantly reduced (P<0.05) serum LH conc. (in a dose-dependent pattern) compared to controls, with maximum effect produced by 80 mg/kg. Meanwhile, 500mg/kg of sulfasalazine significantly decrease (P<0.05) serum LH conc. compared to controls. Also we observed significant differences between sulfasalazine and nimodipine doses except 40mg/kg and also significant differences between each one of nimodipinedoses .

TABLE 5. Effects of different doses of nimodipine on serum concentration of LH in rats

Control	12.0 ±0.5
20mg /kg nimodipine	$9.6\pm0.4^{*a}$
40mg /kg nimodipine	$5.2\pm0.3^{*b}$
80mg /kg nimodipine	$2.6\pm0.5^{*abc}$
500mg /kg sulfasalazine	$4.5\pm0.3^{*}$

Data are expressed as mean ± standard error of mean; n=6 animals in each group; *significant different compared to control (*P*<0.05)

*	: sig at P<0.05 as compared with Control values
A	: sig at P<0.05 as compared with 500mg /kg salazopyrine values
B	: sig at P<0.05 as compared with 20mg /kg nimodipine values
C	: sig at P<0.05 as compared with 40mg /kg nimodipine values

Effects of different doses of nimodipine on FSH serum concentration in rats

Effect of different doses of nimodipine and 500mg/kg (sulfasalazine) on rats serum FSH concentration was shown in table (6). All nimodipine doses (20, 40, and 80 mg/kg body weight) significantly reduced (P<0.05) serum FSH conc. (in a dose-dependent pattern) compared to controls, with maximum effect produced by 80 mg/kg. Meanwhile, 500mg/kg of sulfasalazine significantly decrease (P<0.05) serum FSH conc. compared to controls. Also we observed significant differences between sulfasalazine and nimodipine doses except 80mg/kg and also significant differences between each one of nimodipine doses except between (40mg/kg and 80mg/kg).

TABLE 6. Effects of different doses of nimodipine on serum concentration of FSH in rats

Control	6.9 ±0.6
20mg /kg nimodipine	$5.0\pm0.3^{*a}$
40mg /kg nimodipine	$3.1\pm0.3^{*ab}$
80mg /kg nimodipine	$2.7\pm0.3^{*b}$
500mg /kg sulfasalazine	$2.2\pm0.2^{*}$

Data are expressed as mean ± standard error of mean; n=6 animals in each group; *significant different compared to control (*P*<0.05)

*	: sig at P<0.05 as compared with Control values
a	: sig at P<0.05 as compared with 500mg /kg salazopyrine values
B	: sig at P<0.05 as compared with 20mg /kg nimodipine values
c	: sig at P<0.05 as compared with 40mg /kg nimodipine values

Effects of different doses of nimodipine on testes weight in rats

Effect of different doses of nimodipine and 500mg/kg (sulfasalazine) on rat's testis weight was shown in table (7). All nimodipine doses (20, 40, and 80 mg/kg body weight) significantly reduced (P<0.05) testes weights (in a dose-dependent pattern) compared to controls, with maximum effect produced by 80 mg/kg. In addition 500mg/kg of sulfasalazine also significantly decrease male rats testes weight compared to controls. Also, results of table (7) showed that there were significant differences (P<0.05) between sulfasalazine and each of nimodipine dose except 40mg/kg. Besides, significant differences (P<0.05) were observed between each of nimodipine doses except between 40mg and 80mg/kg.

TABLE 7. Effects of different doses of nimodipine on testes weight in rats

Treatment groups	Mean Testes weight
Control	2.0 ± 0.1
20mg /kg nimodipine	$1.6\pm0.1^{*a}$
40mg /kg nimodipine	$1.2\pm0.1^{*b}$
80mg /kg nimodipine	$1.0\pm0.1^{*ab}$
500mg /kg sulfasalazine	$1.2\pm0.0^{*}$

Data are expressed as mean ± standard error of mean; n=6 animals in each group; *significant different compared to control (P<0.05)

*	: sig at P<0.05 as compared with Control values
A	: sig at P<0.05 as compared with 500mg /kg salazopyrine values
B	: sig at P<0.05 as compared with 20mg /kg nimodipine values
C	: sig at P<0.05 as compared with 40mg /kg nimodipine values

DISCUSSION

Two different stimulation protocols have routinely induced steriodgensis in leydig cells a trophic hormone stimulation with LH and FSH and direct stimulation with cAMP down steam second messengers.[29,30]The content of StAR mRNA expression profiles strongly appear as clear beam in control group in RT-PCR analysis then gradually reduced to be present as faint beam until it disappears at the higher doses of nimodipine, figures(1,2,34).Ca^{+2} ions which are responsible in part for StAR protein gene expression possibly through transcription factor mediated gene expression regulation effects.[31]

StAR protein abundance in steriodogenic cells primarily governed by the rate of StAR gene expression. StAR protein gene transcription activated by cAMP signal transduction cascade.[32]As Ca^{+2} ions required for several steps of steroidogensisnimodipne as L-type Ca^{+2} channel blocker would be expected to have an effect on StAR protein gene expression.This strongly agreed with previous studies that used primary culture of rodent leydig cells, which incubated with nifidipine or nimodipine in which the inhibitory effects of L-type Ca^{+2} channel blocker on cAMP stimulated steriodogensis was mediated by transcriptional repression of StAR gene.[30,31]The serum level of testosterone was significantly reduced after treatment with nimodipine with corresponding reduction in the gene expression of StAR protein ; this observation confide with the role of StAR protein in steriodgensis in which cholesterol delivered into the mitochondrial inner membranes to be converted to pregnolone and then to testosterone,table(4). One of the most important findings in this study is that nimodipine reduced gonadal steroid hormones in treated male rats. It was generally observed that using different doses of nimodipine (20, 40, 80 mg/kg BW) for 30 successive days result in decrease serum levels of testosterone,table(4).The decrease in testosterone serum levels caused by corresponding rise in the dose of nimodipine to reach its maximum effect at the higher dose of nimodipine (80mg/kg); these results indicate that the administration of nimodipine has negative effect on the sexual reproduction function in the male rats. This reduction concomitant with reduced the expression of StAR protein gene which is necessary for synthesis of testosterone hormone. The observation are mostly consistent with the interpretation that blockade of L-type Ca^{+2} channel by nimodipine may lead to an impairment of normal spermatogenesis and steriodogensis . Collectively the decrease in serum conc. of LH might result in corresponding decrease in testosterone production by leydig cells this because Ca^{+2} ions implicated in process of LH secretion.Nimodipine was effective in attenuating LH serum conc. as showed table (5). LH serum levels were reduced in treated groups with different doses of nimodipine compared to the control .GnRH regulates the gonadotropins release by means of rapid increase in [Ca^{+2}] ions. After its binding to GnRHr through G-protein coupled signal transduction, GnRH rapidly activates phosphor lipase c(PLC) , which produceinsitol triphosphate (IP3) anddiacyl glycerol (DAG) fromphosphatidylinsitoldiphosphate (PIP2) which in turn rapidly mobilize transient intracellular Ca^{+2} to trigger burst initiation of exocytosis causing rapid LH secretion within 10 seconds and lasting for 100 seconds[32-37]. In the process of gonadotropins secretion the second phase of Ca^{+2} increase resulting from extracellular influx of Ca^{+2} through voltage gated calcium channels (VGCCs) has a role in the sustained phase of GnRH induced LH secretion rather than in the initial rapid phase.[38,39]. The present study also showed that treatment of male rats with different doses of nimodipne produce a significant reduction in the serum conc. of FSH table (6).The reduced FSH serum level was in dose -dependent profile figure producing its offensive effect at the dose of 80mg/kg compared to the controls .The inhibitory effect of nimodipine on serum level of FSH might be attributed to the intervention of Ca^{+2} ions in the signal transduction pathways responsible for FSH secretion from pituitary gland.It is acknowledge that protein kinase c (PKC) and Ca^{+2} ions influx differentially control the expression of LH and FSH subunit genes. The mechanism by which GnRH causes such control within gonadtropes is unknown but may involve the activation of different transcription factors and second messengers including Ca. Intracellular Ca^{+2} directly modulate insitol triphosphate (IP3) receptor activity and may also act through modulation of IP3binding.[40] The initial GnRH induced increase in the [Ca^{+2}] produce further elevation of [Ca^{+2}] whereas the higher conc. of [Ca^{+2}] attained in the second phase of intracellular Ca^{+2} decrease IP3 receptor activity.[41,42] initial phase of intracellular Ca^{+2} responses is independent of [Ca^{+2}] whereas the second phase depends on Ca^{+2} influxes through VGCCs.So activation of L-type Ca^{+2} channels has been suggested for GnRH induce influx of extracellular Ca^{+2} in gonadotropes which is necessary to induce FSH secretion.[43,44,45,46] Thus, by blocking Ca^{+2} channels, nimodipine could result in decrease in Ca^{+2} influx necessary for triggering FSH secretion. The weight of the testis is one of the markers of a possible alteration in androgen status. A decrease in testicular weight and epididymis a better way to assess the damage to the testes in relation to the body in experimental rats is most likely due to decreased level of serum testosterone, as androgen exerts its major role in sex organs. One of the possible causes of drop in the weight of the testes and epididymis by the effect of nimodipine that needs to be explored is suppressed spermatogenesis. In the absence of any known pathology, testis weight is highly related to daily sperm production. Literature review strongly points towards the importance of pituitary hormones in maintaining testicular, size and weight. Pituitary FSH has been shown to increase the testicular size.Table(7) showed significant reduction in

serum FSH level in nimodipine treated rats. Hence, lack of pituitary FSH can serve as a reason of decrease absolute testicular weight and epididymis weight in nimodipine treated rats. It can be concluded thatnimodipine, in a dose-dependent pattern, was effective in attenuating steriodogensis and testosterone production through its inhibitory effects on StAR protein gene expression in rats.

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