Pre- and post-pubertal Upregulation of testicular IGF-1 gene using passive immunization against inhibin α subunit in neonate rats

Jabber A. A. Al-Sa'aidi*, Mohammed-Salih and A. H. Al-Ali

الخلاصة

أجريت الدراسة الحالية لتقييم تأثير التمنيع الميسر ضد الانهبين ألفا في ذكور الجرذان حديثة الولادة على مستوى استنساخ جين IGF-1 في الخصى في مرحلتي قبل وبعد البلوغ. تم تقسيم 30 جرذا ذكرا حديث الولادة، بعمر 15 يوما ومعدل وزن 31.5 ± 1.32 غم، عشوائيا على مجموعتين متساويتين حقنت الاولى (المعاملة) بالمصل المضاد للانهبين ألفا (100 مايكرلتر من المحلول الفسلجي الحاوي على 1غم من المصل المضاد في البريتون) وحقنت الثانية (السيطرة) بالمحلول الفسلجي (100 مايكرولتر في البريتون) بعمر 15 و18 يوم. خمسة ذكور من كل مجموعة تمت التضحية بها بعمر 23 و 30 و 50 يوم، وأخذت منها الخصى لتقدير مستوى استنساخ جين IGF-1 باستخدام تقانة RT-PCR. أظهرت النتائج أن خصى ذكور جرذان المعاملة بعمر 50 يوما تضاعف فيها استنساخ جين IGF-1 بشكل ملموس احصائيا عند المقارنة مع السيطرة، في حين كانت النتائج غير معنوية بين المجموعتين في خصى الذكور بعمر 23 و 30 يوما. يستنتج من الدراسة الحالية أن التمنيع الميسر ضد الانهبين الداخلي في ذكور الجرذان حديثة الولادة يمكن أن يؤدي دورا فاعلا في مستوى وظيفة وكفاءة الخصى بعد البلوغ.

Abstract:

The present study has been conducted to evaluate the effect of neonatal passive immunization against inhibin α subunit on testicular mRNA expression level of IGF-1 gene during prepubertal and pubertal stage in male rats. Thirty male rats (weighted 21.5±1.32 g, aged 15 days) were randomly assigned into two equal groups; antiserum treated and control groups, received inhibin-α antiserum (100µl of physiological saline containing 1µg antiserum, *i.p.*) and physiological saline (100µl, *i.p.*) at the 15th and 18th days old. Five males from each group were sacrificed at the 23th, 30th and 50th days old. Testis samples were obtained for evaluation of mRNA expression level of IGF-1 gene using RT- PCR. Testis of 50d treated male rats showed higher expression level of IGF-1 gene compared with control, while 23d and 30d showed insignificant changes between studied groups. In conclusion, passive immunization against endogenous circulating inhibin during prepubertal age can perform potent role in testicular functional status and performance in the males after puberty.

Key words: IGF-1 gene, Inhibin, passive immunization, testis.

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Introduction

Inhibins and activins are functionally antagonistic members of the TGF-β family of extracellular signaling molecules (1). They were first isolated from ovarian follicular fluid of cows and pigs in the mid-late 1980s on the basis of their ability to attenuate (inhibins) or enhance (activins) synthesis and secretion of FSH by pituitary cells in vitro (2). The primary role of inhibins being the negative feedback regulation of pituitary FSH secretion. Activins are produced by a wide variety of extragonadal tissues in addition to gonads and they are primarily considered to act as local autocrine and paracrine signaling molecules. Structurally, inhibins are disulfide-linked heterodimers of an α subunit and one of two β subunits, βA or βB, forming inhibin A and inhibin B respectively. Activins A and B are disulfide-linked homodimers of two β subunits, βA and βB, respectively, while activin AB is a βA–βB heterodimer (3). Immunization against inhibin has originaly been used to study the physiological role of inhibin. Subsequently, immunoneutralization against endogenous inhibin is considered as a new paradigm to enhance gamete production and fertility. It has been method in adult cycling rats (4,5). Passive immunization against α-inhibin antigen has also been used to increases sperm production in many live stock species including rams (6), to enhance oocyte development (7), puberty in rats (8,9) and mammary gland growth and development (10).

In the male, inhibin B is produced in the testis, principally by the Sertoli cells. Inhibin B expression and secretion are positively correlated with Sertoli cell function, sperm number, and spermatogenic status and are negatively correlated with FSH. Apart from their essential role in the selective control of FSH secretion, inhibins are currently recognized as paracrine ovarian and testicular regulators and have multiple paracrine effects in the utero-placental unit, representing a promising marker for male and female infertility, gynaecological and gestational diseases (11).

In view of the possible involvement of inhibin in testicular function and performance in the males, in which detail mechanisms are not completely understood, and the potential for the application of immunization technique in male reproduction, this study aims to investigate the effect of passive immunization against endogenous circulating inhibin during prepubertal and pubertal ages on testicular IGF-1 gene expression of the male rats. The present study will provide insight into the physiology of inhibin during prepubertal stage and will give rise to potential application, primarily in the reproduction setting.

Materials and methods

Experimental animals: Fifteen days old male Wister rats (average weight was 21.5±1.32 g), were randomly assigned into two equal groups and treated as follow:

1. Antiserum group: Fifteen male rats received inhibin-α antiserum (100µl of saline containing 1µg antiserum, *i.p.*) at 15th and 18th day old postnatal (before weaning). Then they were allocated into the following subgroups:

Subgroup 1: 5 rats were sacrificed on day-23 postnatal (T-23d)
Subgroup 2: 5 rats were sacrificed on day-30 postnatal (T-30d)
Subgroup 3: 5 rats were sacrificed on day-50 postnatal (T-50d)

2. Control group: Fifteen male rats received physiological saline (100 µl, *i.p.*) at 15th and 18th day old postnatal (before weaning). Then they were allocated into the 3 subgroups as in the antiserum group (C-23d, C-30d and C-50d).

At the end of each treatment and control subgroups period, rats were sacrificed and testis from each animal was obtained, weighted and put in formalin 10% for 6 hours and then impeded in paraffin for evaluation of mRNA expression level of target gene (*IGF-1*) and housekeeping gene (GAPDH) by using reverse transcriptase Real-Time PCR technique based on Syber Green dye.

Preparation of Inhibin Antiserum 1 %: Inhibin antiserum (1µg/100µl) was prepared according to the following protocols (depending on the manufacture instructions; ABO, Switzerland).

Preparation of agarose gel (50 ml) (12)

Casting of the horizontal agarose gel (12)

Quantitative Reverse Transcriptase Real-Time PCR: was used for quantification of IGF-1 gene expression levels relative to Housekeeping (GAPDH) gene expression levels in rat testis cells treated with anti-inhibin serum. This technique was done according to the method described by (13) as follow:

1.Collection of rat testis: testicular tissues were fixed in 10% neutral-buffered formalin for 5to 6 hours. After fixation, the samples were dehydrated, incubated in xylene, embedded in paraffin, and sectioned using standard histological protocols. Paraffin-embedded tissue blocks were cut with a disposable microtome blade into 3×15 µm section and placed in eppendrof tubes. Tissue were deparaffinized by incubation in two consecutive baths of xylene for 5 minutes each, then in two consecutive baths of 100% ethanol for 5 minutes each. After deparaffinization and centrifugation, the pellets were air dried.

2.Extraction of chromosomal rat DNA: A chromosomal rat DNA was extracted to prepare genomic DNA standard curve of TaqMan-based Real Time PCR assay. DNA was extracted according to the protocol described by

the manufacturer instructions as follow: testicular tissues were transferred into 1.5 ml microcentrifuge tube. Micropestle was used to grind the tissue to a pulp. 200 µl of GT Buffer was added to the tube and tissue homogenization was continued by grinding. 20 µl of Proteinase K was added to the sample mixture and mixed by vigorous shaking. The mixture was incubated at 60C° for 30 minutes to complete lyses. During incubation, the tube was inverted every 5 min. 200 µl of GBT Buffer was added and shacked vigorously for 5 sec. Incubation was repeated at 60C° for 20 min. During incubation, the tube was inverted every 5 min. Elution Buffer was pre-heated to 60C° for DNA Elution. 200 µl of absolute ethanol was added to the sample lysate and immediately mixed by vigorous shaking for 10 min. GD column was placed in a 2 ml collection tube. All of mixture was transferred to the GD column. The mixture was centrifuged at 16000×g for 2 min. The 2ml collection tube was discarded and the GD column was transferred to a new 2ml collection tube. 400 µl of W1 Buffer was added to the GD column. The mixture was centrifuged at 16000×g for 30 sec. and discarded the flow-through. The GD column was placed back in the 2ml collection tube. 600 µl of Wash Buffer was added to the GD column. The mixture was centrifuged at 16000×g for 30 sec. and discarded the flow-through. The GD column was placed back in the 2ml collection tube. The mixture was centrifuged at 16000×g for 3min. to dry the column matrix. The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube. 50 µl of pre-heated Elution Buffer was added to the center of column matrix. The mixture was centrifuged at 16000×g for 30 sec. to elute the purified.

3.Total RNA extraction: Total RNA were extracted from rat testis cells by using (TRIzol® reagent kit) and done according to company instructions as following steps: testis tissue was homogenized by grinding in liquid nitrogen, and the tissue powder was transferred into DEPC treated Eppendorff tube contains 1 ml of TRIzol® reagent. The tubes were shaken vigorously for 30 seconds. Chloroform (200 µl) was added to each Eppendorff tube and shaken vigorously for 15 seconds. The mixture was incubated on ice for 5 minutes, spined at 12,000 rpm , 4C° , for 15 minutes. Supernatant was transferre to a new Eppendorff tube, and isopropanol (500 µl) was added. Mixture was mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes, spined at 12,000 rpm , 4C° for 10 minutes. Supernatant was discarded. Adding 80% Ethanol (1 ml), Vortex again, spined at 12,000 rpm , 4C° for 5 minutes. Supernatant was discarded and the pellet dried. RNase free water (30µl) was added to the sample with vortexing until dissolving. Extracted RNA sample was kept at -20.

4.DNase inactivation (DNase I) treatment: the extracted total RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done

according to method described by promega company, USA instructions as follow: RNA (1µl) and 10 × reaction buffer with Mgcl2 (1µl) and DNase I, RNase-free (1µl) and DEPC treated water (7µl) were added to Eppendorff tube. The mixture was incubated at 37C° for 30 minutes. 1µl EDTA was added and incubated at 65C° for 10 minutes. A volume of DNase Inactivation reagent equal to 20% of RNA sample was added to each RNA sample. The tubes vortexed to mix the DNase Inactivation Reagent with RNA sample. All RNA samples left at room temperature for 2 minutes with flicking the tubes once or twice during this period to resuspend the DNase inactivation regent. The tubes were centrifuged at (12,000 rpm) for 1 minute to allow the DNase inactivation reagent separated from RNA sample solution, then, the RNA solutions transferred to new eppendorff tube.

5.Assessing RNA yield and quality: There are three quality controls were performed on isolated RNA. First one is to determine the quantity of RNA (ng/µL) that has been isolated by used Nanodrop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS Korea), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine, and the third is the integrity of the RNA by prepared gel electrophoresis, as follow: After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA). A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH_2O onto the surface of the lower measurement pedestal. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as black solution which is same elution buffer of RNA samples. After that, the pedestals are cleaned and pipet 1µl of RNA sample for measurement. The purity of RNA, also determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, so the RNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. After that, the integrity of the RNA was determined with gel electrophoresis as by preparing 1.5% agarose gel in 0.5x TBE buffer and heating for 2 minutes until disappearing all crystals in agarose solution. After cooling, 3 µL of ethidium bromide was added to the solution, then the gel was poured in the tray and left until solidifying, then transferred into electrophoresis machine which contains same buffer. The RNA samples were prepared by mixing 5µl of RNA sample with 1µl of loading dye**.** Then, all were transferred into agarose gel wells, and running the electrophoresis power at 100 Volt for 1 hours, then the RNA bands are seen by U.V light.

7.cDNA synthesis: DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit (Bioneer company, Korea) and done by adjusting all DNase-I treatment total RNA samples at same concentrations that measured by nanodrop by DEPC water. RNA was converted to cDNA by preparing of 20 µL of Reverse transcription PreMix reaction (10 µL of total RNA with concentration of 100 ng/ µL, 2 µL of Oligo 15 primer 10 mmole, and 8 µL of DEPC water). RT PreMix was added into AccuPower RocketScript RT PreMix tubes that contains Reverse transcription enzyme in lyophilized form which dissolved by vortex and brief spinning down. The RNA converted into cDNA in two thermo cycler conditions (cDNA synthesis at 50°C for 1 hr and heat inactivation at 95°C for 5 min. Finally, the samples were stored at -20C° until performing qRT-PCR.

8.qRT-PCR based SYBER Green I Dye Detection: qRT-PCR was performed using AccuPower® Greenstar™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). According to method described by Cheon *et al.* (1999), the Syber Green based qRT-PCR PreMix reagent kit is designed for PCR amplification of cDNA for IGF-1 target gene by using IGF-1 primer and GAPDH Housekeeping gene, as well as for quantification of PCR amplification copy numbers comparatively to copy numbers of genomic DNA qRT-PCR standard curve. The Syber Green dye that used in this kit is DNA binding dye which reacted with new copies of amplification specific segment in target and housekeeping genes, then the fluorescent signals recorded in Real Time PCR thermocycler. A genomic DNA standard curve was generated from GAPDH gene of Rattus norvegicus (27.9Mbp) were taken from NCBI-Gene Bank information is approximately (~1×10^7) copies, and serial dilution representing (1×10^7, 1×10^6 and 1×10^5) gDNA copies were used as genomic DNA standard curve.

9.Experimental design of qRT- PCR: for quantification of IGF-1 gene expression in samples at duplicate, internal control gene; housekeeping gene (GAPDH), was used for normalization of gene expression levels, therefore, three qRT-PCR master mixes were prepared for gDNA standard curve, IGF-1 target gene, and GAPDH housekeeping gene as follow:

A) -qRT-PCR Master Mix for IGF-1 target gene

qPCR PreMix		**Volume**
cDNA template		10 µL
Primers	IGF-1 -F	2 µL
	IGF-1-R	2 µL
	DEPC water	6 µL
Total		20 µL

B) - qRT-PCR Master Mix for GAPDH housekeeping gene

qPCR PreMix		Volume
cDNA template		10 µL
Primers	GAPDH-F	2 µL
	GAPDH-R	2 µL
DEPC water		6 µL
Total		20 µL

After that, qPCR PreMix were added into *AccuPower* GreenStar qPCR PreMix tube. Then, real-time PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes were centrifuged at 3,000 rpm, for 2 min, then started the Exicycler™ 96 Real-Time Quantitative Thermal Block instrument and loaded the following Program according to kit instruction: pre-denaturation at 95ºC for 5 min. (1 cycle), denaturation at 95ºC for 20 sec. (45 cycle), annealing/extension and detection (scan) at 60ºC for 45 sec. (45 cycle), and melting for 1 cycle. After reaction was completed, data analysis has been performed.

10. Data analysis of qRT-PCR: the data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method (14). The relative quantification obtained from q RT-PCR experiment was normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples was considered the calibrator such as control samples, each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a reference gene was used as in following equations:

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (ref, calibrator) – CT (target, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

$\Delta\Delta CT = \Delta CT$ (test) – ΔCT (calibrator)

Fold change = $2^{-\Delta\Delta CT}$

Statistical analysis: All the values are expressed as mean ± SD. Data of each of 23d, 30d and 50d periods of the experiment were analyzed using *student t-test* to estimate the significant differences between groups. P value less than 0.05 was considered significant (15).

Results

The Concentrations and Purity of Total RNA: Total RNA concentrations and purity were assessed using Nanodrop spectrophotometer at absorbance (260/280 nm). All testicular tissue samples gave high concentrations of total RNA and appeared enough to proceed in quantitative RT-PCR, also the treatment group was significantly higher ($P<0.05$) than control during all experimental periods (figure 1). The purity of RNA samples (also assessed by agarose gel electrophoresis) of testicular tissues recorded different band thickness (figure 2). The figure shows clear 18s and 28s bands.

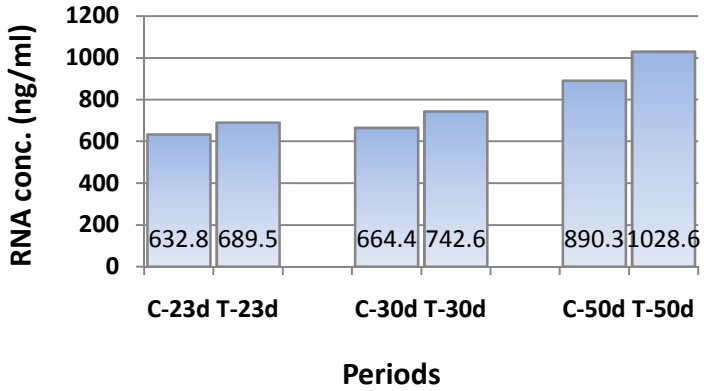

Figure (1): total RNA concentration (ng/ml) in testicular tissues of male rats passively immunized against inhibin-α subunit at 15 and 18 days.

- **Values represents M±SE.**
- **Different letters mentions to significant difference ($P<0.05$) between groups.**
- **C: male rats injected with NS (100 µl, *ip*).**
- **T: male rats injected with inhibin-α subunit antiserum (1µg, *ip*, dissolved in 100µl of NS)**

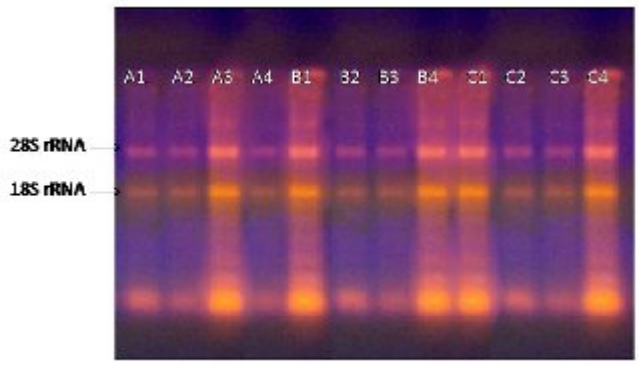

Figure (2): agarose gel electrophoresis analysis of total RNA in testicular tissues of male rats passively immunized against inhibin-α subunit during the experimental periods.

A1 & A2: T-23d, A3 & A4: C-23d, B1 & B2: T-30d, B3 & B4: C-30d, C1 & C2: T-50d, C3 & C4: C-50d.

Quantitative Reverse Transcriptase Real- Time PCR: Data analysis of SYBR®green based RT-PCR assay were divided into primer efficiency estimation and relative quantification of IGF-1 gene expression level normalized by housekeeping gene expression (GAPDH).

1. Primer efficiency estimation: The data result, threshold cycle numbers (Ct) were calculated from amplification plot of real-time PCR detection system, during exponential phase of fluorescent signals of SYBR®green primer of IGF-1 gene that react with complementary DNA(cDNA) of rat testis mRNA, where, the amount of PCR product (DNA copy numbers) in master mix reaction is approximately doubles in each PCR cycle. First prepared series dilution of testicular cDNA of control was used with the primer of different genes (IGF-1 and GAPDH) separately to draw the amplification plot of each gene (fig.4, 6, 8, and 10), and then from this plot, threshold cycle (Ct) was used to calculate a linear regression based on the data points, and inferring the efficiency of each primer from the slope (fig.3,5,7,and 9).

IGF-1 primer optimization: The resulted standard curves yielded R^2 values was 1 and amplification efficiency was 109% (fig. 3 and 4).

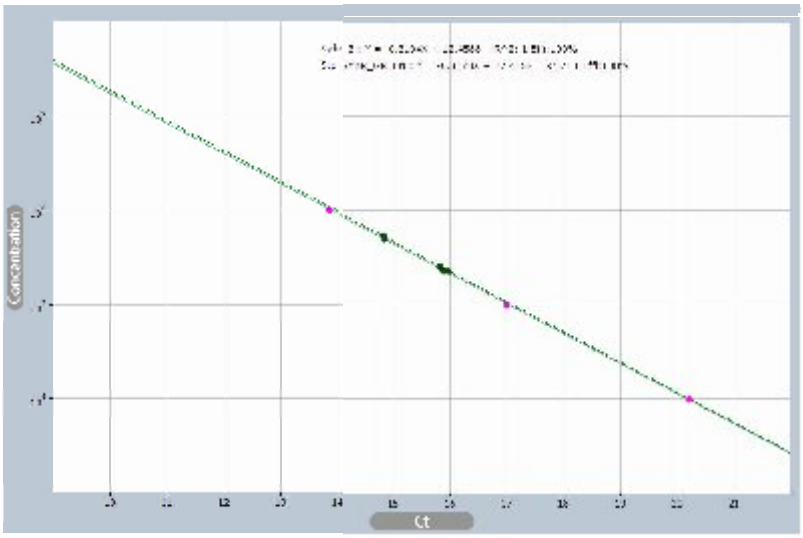

Figure (3): The IGF-1 gene primer standard curve of treated group.
IGF-1 primer Efficiency (E) = 1; IGF-1 primer Efficiency (%) = 109%

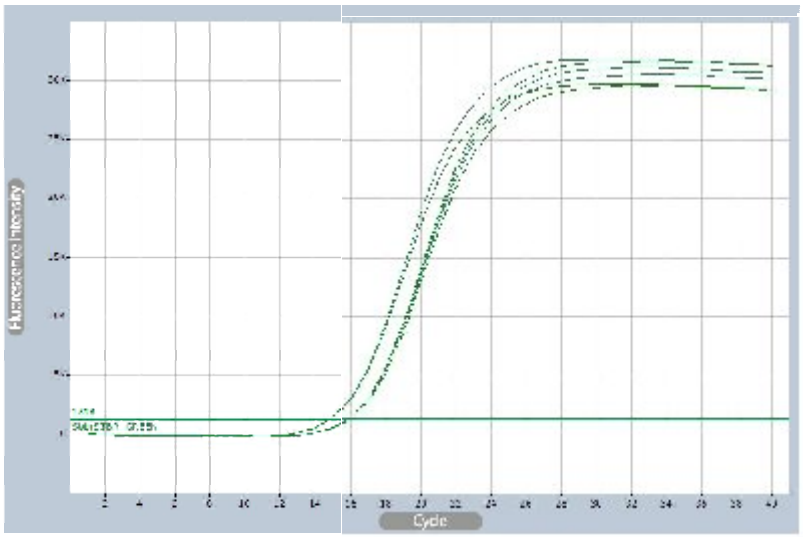

Figure (4): The IGF-1 gene primer Amplification plot of treated group.

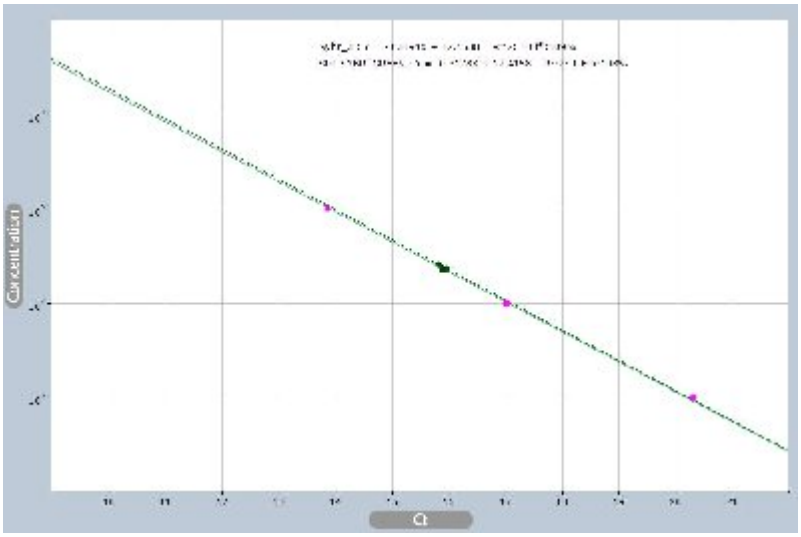

Figure (5): The IGF-1 gene primer standard curve of control group.

IGF-1 primer Efficiency (E) = 1; IGF-1 primer Efficiency (%) = 109%

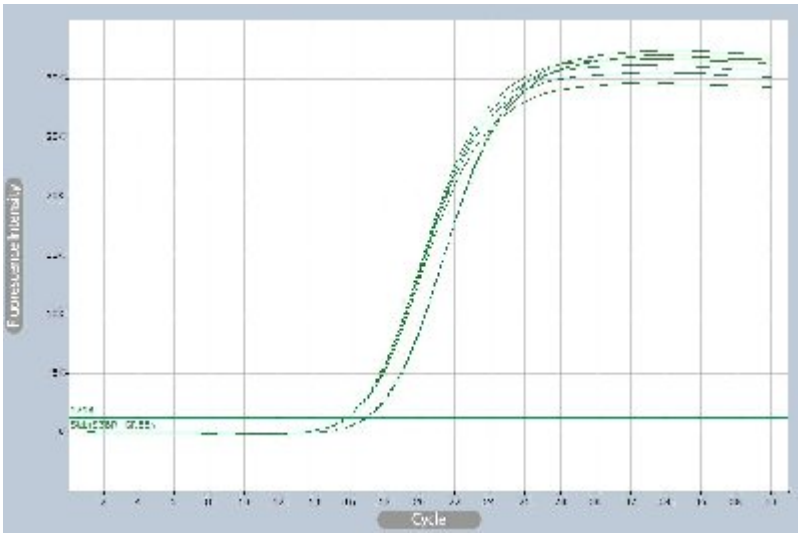

Figure (6): The IGF-1 gene primer Amplification plot of control group.

GAPDH primer optimization: The resulted standard curves yielded R^2 values was 1 and amplification efficiency was 108% (fig. 7 and 8).

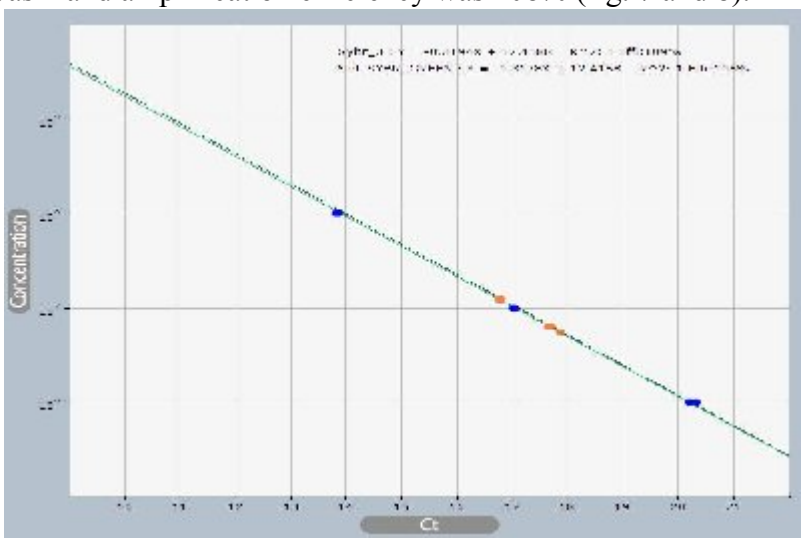

Figure (7): The GAPDH gene primer standard curve of treated group.
GAPDH primer Efficiency (E) = 1; GAPDH primer Efficiency (%) = 108%

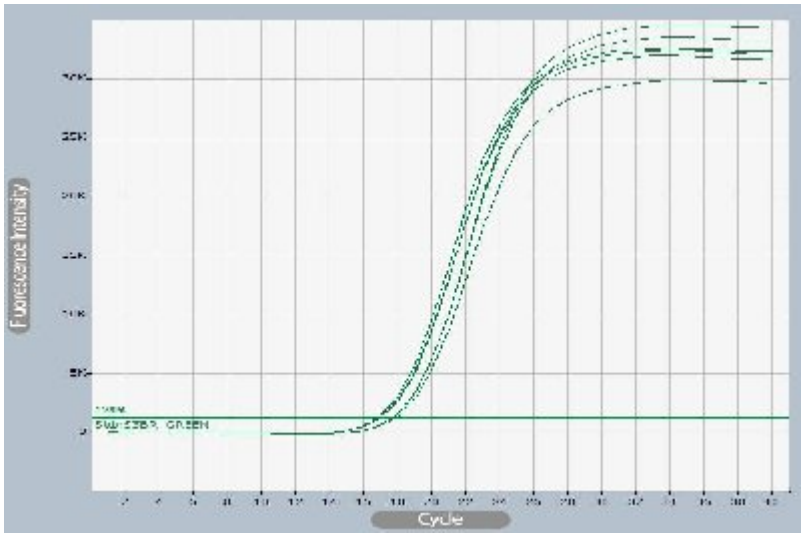

Figure (8): The GAPDH gene primer Amplification plot of treated group.

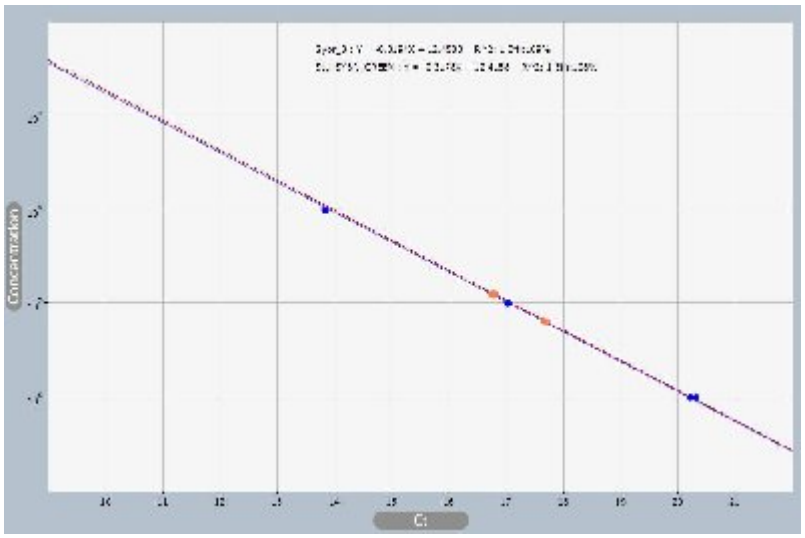

Figure (9): The GAPDH gene primer standard curve of control group.

GAPDH primer Efficiency (E) = 1; GAPDH primer Efficiency (%) = 108%

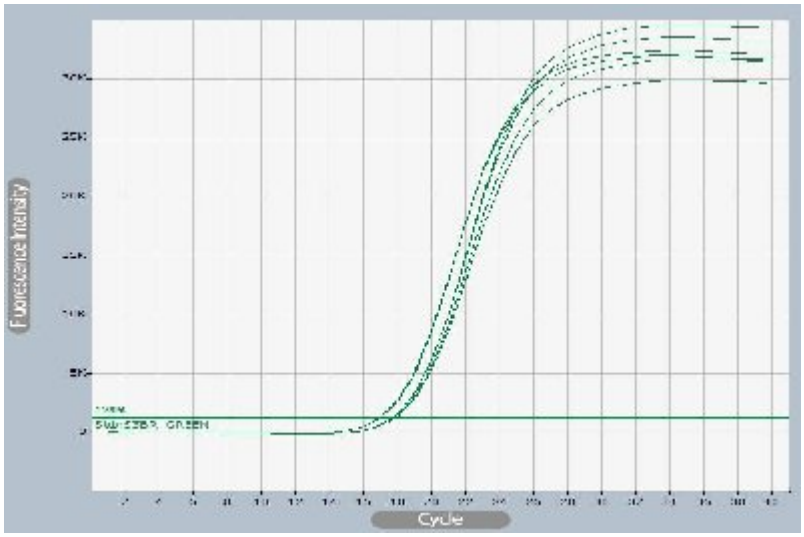

Figure (10): The GAPDH gene primer Amplification plot of treated group.

Relative quantification of target gene expression: To calculate the relative expression of target gene (IGF-1) in rat testis, the 2^-ΔΔCt (livak) method used by normalize gene expression of target gene with expression of housekeeping gene (GAPDH) as reference gene. The gene expression in control group was expressed as (calibrator) or control in both target gene (IGF-1) and reference gene (GAPDH), at first, the threshold cycle number of target gene normalized to that of reference gene in all treatment groups and calibrator (control groups). Second, the ΔCt of treatment groups normalized to the ΔCt of calibrator, and finally the expression ratio (fold change) was calculated.

Relative quantification of IGF-1 gene expression: Reverse transcriptase real-time PCR results shown that IGF-1 mRNA expression level were increased more than 6-folds in 50d aged male rats treated with anti-inhibin α antiserum compared with corresponding age of control male rats (slightly more than 1-fold) as well as 30d and 23d treated male rats (around 1-fold) (figure 11).

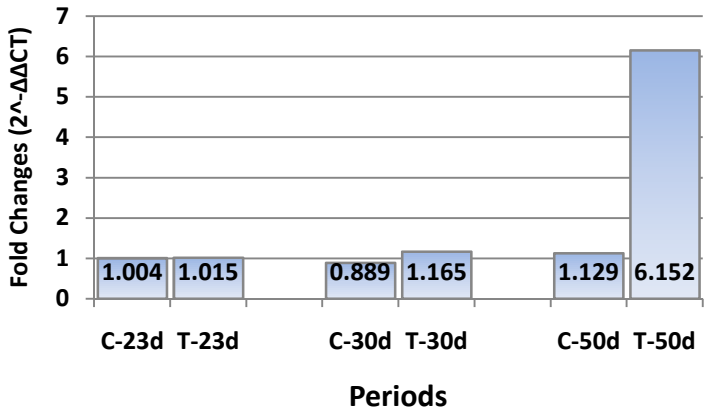

Figure (11): Effect of passive immunization against inhibin-α subunit during prepubertal stage on *IGF-1* gene expression (fold change) in testicular tissues of male rats.

- **Values represents M±SE.**
- **Different letters mentions to significant difference (P<0.05) between groups.**
- **C: male rats injected with NS (100 µl, *ip*).**
- **T: male rats injected with inhibin-α subunit antiserum (1µg, *ip*, dissolved in 100µl of NS)**

Discussion

Structural differentiation of male reproductive organs is in the direction of hormonal profile as early as from prepuberty. Major steps of this differentiation include, sequentially, formation and development of Sertoli cells, Leydig's cells as well as the activity status of germinal epithelium in the lining of semineferous tubules. Development of testicular tissues characterized by an intense synthesis and secretion of testosterone in relation to such hormones responsible for these changes including FSH, LH, inhibins and activins as well as other hormone related to the biochemical activity in testicular tissues, such as GH, which perform its action via mediators inside the testicular tissues; namely IGF-1 and IGF-2. The present observations revealed high testicular expression level in mRNA level of IGF-1 gene in male rats passively immunized against inhibin alpha during pubertal stages. IGF-I is a well-conserved growth factor that has important roles during pre and post-natal development in vertebrates, but it also controls tissue homeostasis throughout life via regulation of cell proliferation, differentiation and apoptosis. In mammalian testis, IGF-I can promote germ cell proliferation/differentiation, acting alone or as a permissive factor for testosterone. It is known that IGF-I has endocrine, paracrine and autocrine effects. As it has been mentioned that deletion of the IGF- gene resulted in reduced numbers of Leydig's cells as compared with age-matched wild-type

mice (16). In previous studies, IGF-I was shown to stimulate the proliferation of progenitor leydig's cells (PLCs) and immature leydig's cells (ILCs), and deletion of the Igf1 gene to cause reduced numbers of adult Leydig's cells (ALCs) at postnatal day (PND 35) (13,17). From the present result some questions have been answered about the possible mechanisms of IGF-I action. Based on that, we tried to reach the goal of our decision to investigate the role of IGF-I using Wistar rats as a mammalian model.

It has been found that inhibins is mostly secreted from Sertoli cells prior to puberty, while after puberty, serum inhibin levels are closely related to spermatogenic status, as shown by the direct correlation between serum inhibin B level and sperm count (18). So, passive immunization against inhibin α subunit during postnatal period counteracted the selective inhibitory action on FSH synthesis and release by circulating inhibins, as it have been demonstrated in several animal species including rodents (8,9,11). Beside the role of FSH, activin may be attributed by such away during prepubertal period of mammalian male life. From these results appeared that hormonal effects on testis size and function performed through up-regulation of Sertoli and Leydig's cells number and function.

The orchestrated increment of activin and FSH with the up-regulation of mRNA expression level of IGF-1 in testicular tissue may the possible mechanism that increase proliferation of Sertoli cells, Ledig's cells and germinal epithelium lining the semineferous tubules. It has been mentioned that the suggested mechanism whereby activin might control testis size was that activin which directly promote Sertoli cell proliferation in culture (19), and supported by other observations that the primary source for activin-A in the testis was peritubular myoid cells which acts in a paracrine fashion to promote Sertoli cell proliferation (20). In the present study we have use passive immunization against inhibin-α subunit during prepubertal stage as an alternative way to increase FSH concentration in order to increase the number of Sertoli cells which can be reflected by the increment of final reproductive capacity after puberty. During puberty, the Sertoli cell undergoes a functional maturation and takes on a functionally separate role of supporting spermatogenesis. Sperm production and reproduction of the species require the physical and metabolic support that Sertoli cells provide to the germ cells. Thus, the maturation of and number of Sertoli cells ultimately determines the sperm output of an adult mammalian testis (21).

In conclusion, the present high level of IGF-1 gene expression indicates a highly significant potency of prepubertal immunonutralization against inhibin α subunit during prepubertal and pubertal stages. This finding may explain the strong correlation between IGF-1 gene expression level and testicular growth and development. This elucidation, of its action, may lead to the development of novel strategies aimed at supporting reproduction and its future

performance, therefore, prepubertal immunoneutralization againt inhibin α subunit may play a significant potent role in animal reproduction applications.

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