

INJECTING THE PREPUBERTAL LABORATORY MICE WITH A MIXTURE OF SERINE AND THREONINE AND ITS EFFECT ON SPERMATOGENIC FUNCTION

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ABSTRACT

The aim of this study is to determine the effect of injecting a mixture of Serine and Threonine on spermatogenic function within testes of prepubertal mice (QS strain). Therefore, the mice were injected with Saline (Control) or a mixture of Serine and Threonine (Test) for 5 days. Following the last injection, the testes were removed and dehydrated then stained with PAS (Periodic Acid Schiff). The diameter of tubules, diameter of Leydig cells and the numbers of round spermatids were measured on sections of the testes. The number of stage round spermatids was decreased by Serine and Threonine injection ($P < 0.05$). The total numbers of round spermatids per testis for control and test groups were detected as 1.66 ± 0.32 and 1.06 ± 0.05 ($\times 10^6/\text{mm}^3$) respectively. This experiment shows that Serine and Threonine injection reduced the number of round spermatids while the diameters of Leydig cells and the diameters of tubules were not affected. The reason for reduced number of round spermatids might be a result of increased expression of TGF- β s which might increase the apoptosis of the spermatogonia and this probably resulted with the reduction in the number of round spermatids.

Key words: Mice, serine, threonine, spermatogenic function

SERİNE VE THREONİNE AMİNO ASİTLERİNİN PREPUBERTAL LABARATUAR FARESİNE ENJEKTE EDİLMESİNİN SPERMATOGONİK FONKSİYONUNA ETKİSİ

ÖZET

Bu çalışmanın amacı; farelerde Serine ve Threonine amino asitlerinin birlikte enjekte edilmesinin spermatogenik fonksiyonlar üzerine etkisini araştırmaktır. Bu amaçla prepubertal labaratuvar faresine (QS türü) 5 gün süre ile Salina (Kontrol) veya Serine ile Threonine amino asit karışımı (Test) enjekte edildi. Son enjeksiyonu takiben testisler alındı ve dehidrasyona tabi tutuldu. Tesetislerden kesitler alınarak PAS (Periodic Acid ve Schiff) boyası ile boyandı. Boyanan kesitler üzerindeki Leydig hücrelerinin, tubulus Seminiferous contortusların çapları ölçüldü ve aynı anda her kesit üzerinde yuvarlak spermatid sayısı tespit edildi. Alınan sonuçlara göre Serine ve Threonine enjeksiyonu; tubulus contortusların ve Leydig hücrelerinin çaplarında bir değişikliğe neden olmadı fakat yuvarlak spermatidlerin sayısını azalttı ($P < 0.05$). Kontrol ve test guruplarına ait testislerdeki yuvarlak spermatid sayısı, sıra ile 1.66 ± 0.32 ve 1.06 ± 0.05 ($\times 10^6$) olarak bulundu. Yuvarlak spermatid sayısındaki azalmanın nedeni amino asit enjeksiyonu sonucu testis içerisinde TGF- β benzeri faktörlerinsentezinin artmış olması olabilir. Bu faktörlerin etkisi ile spermatogonialar apoptosize bağlı olarak dejenere olabilirler. Bu dejenarasyon nedeniyle yuvarlak spermatidlerin sayısı azalmış olabilir.

Anahtar kelimeler: Fare, serine, threonine, spermatogenik fonksiyon

INTRODUCTION

Serine and Threonine amino acids are substituted within the intracellular proteins involved in signal transduction. Signal transduction involves reversible phosphorylation, regulated through protein kinases and phosphatases, occurring predominantly on Serine, Threonine residues in intracellular proteins (Naz, 1999). Cyclin-dependent kinases and mitogen activated protein (MAP) kinases are two major superfamilies of Serine/Threonine kinases and both of kinase families are of critical importance in modulating various aspect of cellular processes, such as proliferation, differentiation and apoptosis (Ibets *et al.*, 1994; Lodish *et al.*, 1995; Shinkai *et al.*, 2002). Phosphorylation of Serine and/or Threonine residues in intracellular proteins taking role in gene transcription may cause expression of new proteins. Nuclear transcription factors, with a role in transcription of genes, such as cAMP-responsive enhancer elements binding protein (CREB) and cAMP-responsive enhancer element modulator (CREM) are generally phosphorylated on Serine and Threonine amino acids. CREM proteins regulate expression of mRNA for TGF- β like peptides (Potchinsky *et al.*, 1997). Therefore, Serine and Threonine phosphorylation of intracellular proteins may influence spermatogenic potential within the testes due to the changes in steroid secretion and the production of new proteins, such as TGF β_1 , β_2 and

inhibins which have local inhibitory effects on spermatogenesis (Olaso *et al.*, 1998). Recently it has been reported that, TGF- β s reduce the number of gonocytes by decreasing their proliferation or reducing their survival. TGF- β acts on Leydig cells or gonocytes rather than Sertoli cells because Sertoli cells do not have receptors for TGF- β s (Olaso *et al.*, 1998). Serine and Threonine amino acids may exert an indirect effect on spermatogenesis through the expression of TGF- β like proteins within the testes.

Increase in tubular diameters and Leydig cell diameters are used an indirect measure of spermatogenic function (Berndtson and Jones, 1989). Because, increased surface area of the spermatogenic epithelium can support more Sertoli cells and spermatogonia. Increased surface area of the basement membrane is generally related to higher testosterone production (Berndtson and Jones, 1989). Leydig cell produce testosterone in response to LH. Increased surface areas of Leydig cell may facilitate more receptor sites for LH and these results with more testosterone production. High testosterone production has been reported to have a negative effect on hypothalamus resulting with a decrease in gonadotrophin secretion and therefore sperm production. This was confirmed by an experiment in normal man by injecting high doses of testosterone (weekly, 200 mg), which resulted in azospermia (Anderson and Wu, 1996; Bebb *et al.*,

1996). This negative effect of high doses of testosterone on sperm production was thought to be a result of negative feedback effect of testosterone on gonadotrophin secretion and this was confirmed by hormone analysis in man which showed that, plasma concentrations of testosterone and oestradiol increased by 2.5-fold whereas plasma concentrations of LH and FSH decreased (Anderson and Wu, 1996; Bebb *et al.*, 1996).

Therefore, the aim of this work was to measure the effect of the injecting Serine and Threonine on spermatogenetic function in terms of changes in tubule diameter, diameter of Leydig cells and the number of round spermatids in mice testes.

MATERIAL AND METHODS

Animal and injection

Twenty-one days old prepubertal male mice (n=30; QS strain) were injected (intra-peritoneally) daily with 0.2 ml saline (Control) or saline containing 0.26g L-Serine and 0.13g L-Threonine (Test) between 10:00 and 12:00, for five days. Five to six mice from a litter were kept in the same box and 2 or 3 of them were treated as controls and the other 2 or 3 were treated as the test group. Sixty minutes after the last injection the mice were killed by cervical dislocation. Just before killing, the mice were weighted to see if there was any effect of the injections on body weight.

Removal of testes, its fixation and embedding

After the killing, testes were taken out, using fine forceps and placed in Bouin's fixing solution (Cat; 36087 4v, BDH laboratory supplies, Poole, England) for 24h. The testes were dehydrated and embedded in wax.

Cutting and staining

Each testis was serially sectioned at 10 µm, using a rotary microtome and sections placed on glass slides (Cat; 406/018/04, BDH laboratory supplies, Poole, England). The sections were de-waxed by placing them in a clearing reagent CMP30 (Cat; CD24, TAAB laboratory supplies, Berkshire, England) for 7 min, rehydrated by placing them in graded alcohol from 100% to 90, 50 and 30% alcohol each for 1 min and finally in distilled water for 2 min. After rehydration the sections were placed in periodic acid (Cat 104324Q; BDH laboratory supplies, Poole, England) for 5 min, washed several times with distilled water and then washed in running tap water. The sections were placed in Schiff's solution (Cat 19120 3S; BDH laboratory supplies, Poole, England) for 30 min. and washed in tap water for 8 min, placed in iron Haematoxylin, washed for 7 min in tap water, the sections were placed in acid alcohol (1 ml HCl + 100 ml 70% ethyl alcohol) for 1 min, washed in distilled water and dehydrated again. Finally the sections were placed in clearing reagent CMP 30 for 7 min, mounted with

Depex Mounting Medium (Cat; 16125 2B, BDH laboratory supplies, Poole, England) and coverslipped.

Measurements and Calculations

Differentiation of round spermatids

Differentiation of the round spermatids was detected by looking at acrosomal cap around the nucleus, which was quite clear with the red PAS stain (Figure 1a)

Diameter of Leydig cell nucleus

In every 80th section, vertical and horizontal diameters of 50 cells were measured under an oil-immersion lens at 100x10 magnifications (Figure 1b).

Diameter of tubules

Only the diameters of round tubules, with a good circular appearance were measured. Diameters were measured vertically and horizontally and the mean calculated. Only tubules whose ratio of horizontal and vertical diameters fell between 0.9 - 1.1 were included (Figure 1c). Diameters were measured under the light microscope using an ocular micrometer at 10 x 10 magnifications. Round tubules were measured in every 80th section. On every section, 5 - 20 tubules were measured.

Measurement of testis volume

The total number of sections for each testis was recorded. The mean area of every 80th section was measured using image analysis computer software (ZEISS KS 300, version 3, copyright 1997). The computer was connected to a light microscope (Olympus BH-2, Seri no; 238956, Japan) with a colour video camera (Model; KY-F55BE). Only testis was measured, the epididymis was not measured

Total number of sections for each testis = TNS

Mean area of every 80th section = MA80 (mm²)

Section thickness = ST (µm)

Testis volume (TV) in wax

TV = TNS x ST x MA80 (mm³)

Calculation of the number of round spermatids

Calculations were done according to the formulas by Vergouwen *et al.*, (1995). The number of round spermatids were calculated using the following formula:

ST= Section thickness (µm)

TV= Testis volume in wax (mm³)

Q= Number of nuclei

A= Area counted (mm²)

Ns = Number of round spermatids per testis

Ns=Q/AxSTxTV (Vergouwen *et al.*, 1995)

Statistical analysis

Data were analysed by using an unpaired t- test. Two set of slides, from each group, were counted blind, twice and the results compared for consistency by t- test. There were no statistically significant differences (P>0.05).

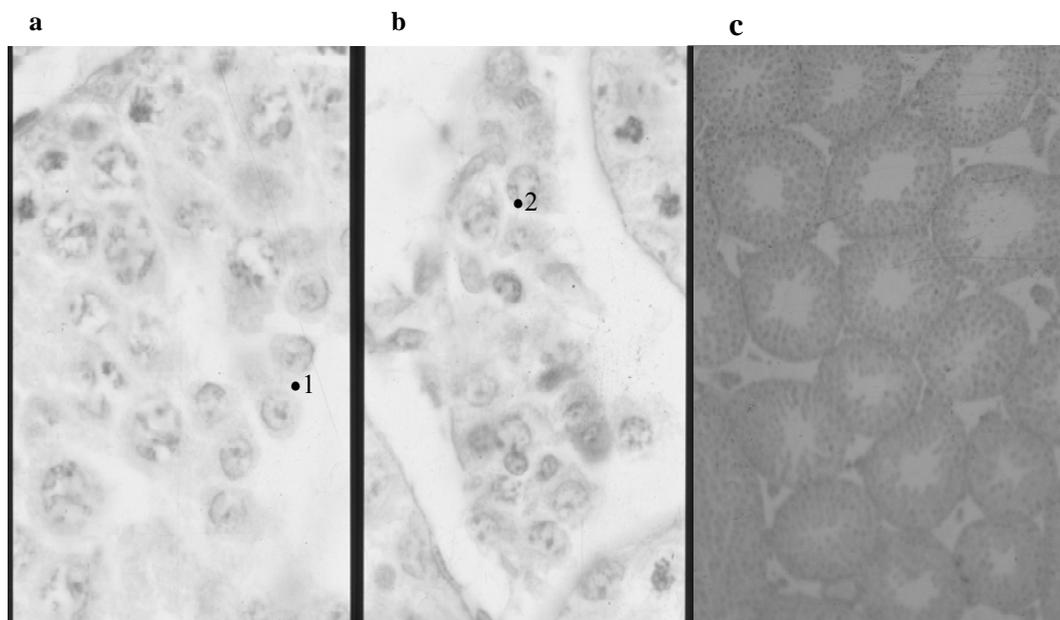


Figure 1; A micro photographic illustration of mouse testes showing round spermatids (a), Leydig cells (b) and tubules (c). Round spermatids (on PAS stained sections) were differentiated by size and colour of acrosomal granules. After staining acrosomal granule can be differentiated by the red colour around the nucleus (a1). Leydig cell are generally found in groups in intertubular area (b₂).

RESULTS

Amino acid injection did not increase tubular diameters significantly ($t= 0.48$; $P= 0.6378$). Leydig cell diameters did also not change ($t= -1.19$; $P= 0.2547$)

(Figure 2 and 3). Injection of Serine and Threonine significantly decreased the number of round spermatids in each testis ($t=1.96$; $P= 0.0714$) (Figure 4).

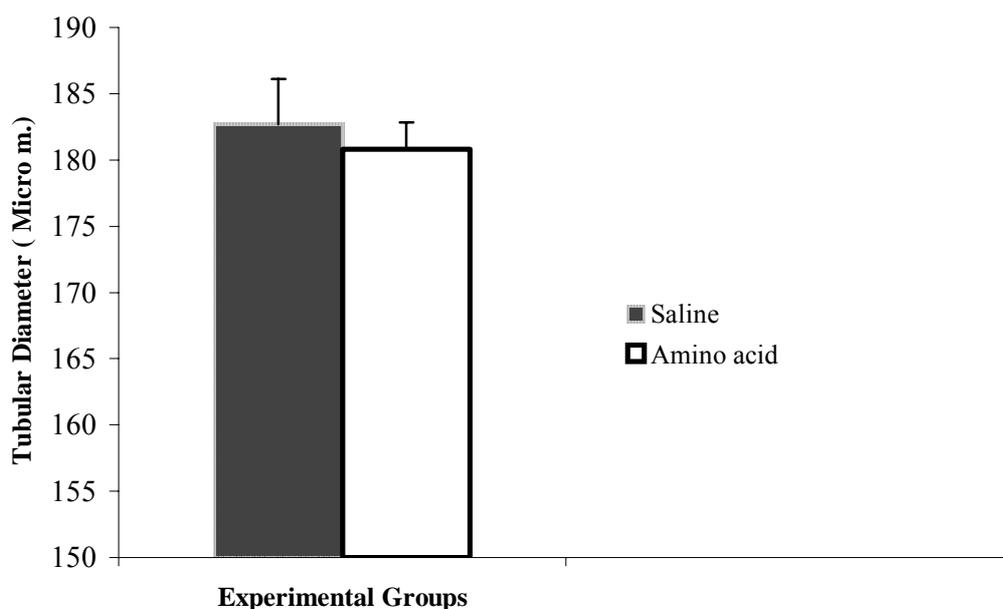


Figure 2; Tubular diameters measured on the sections obtained from the testes of saline and amino acid injected mice.

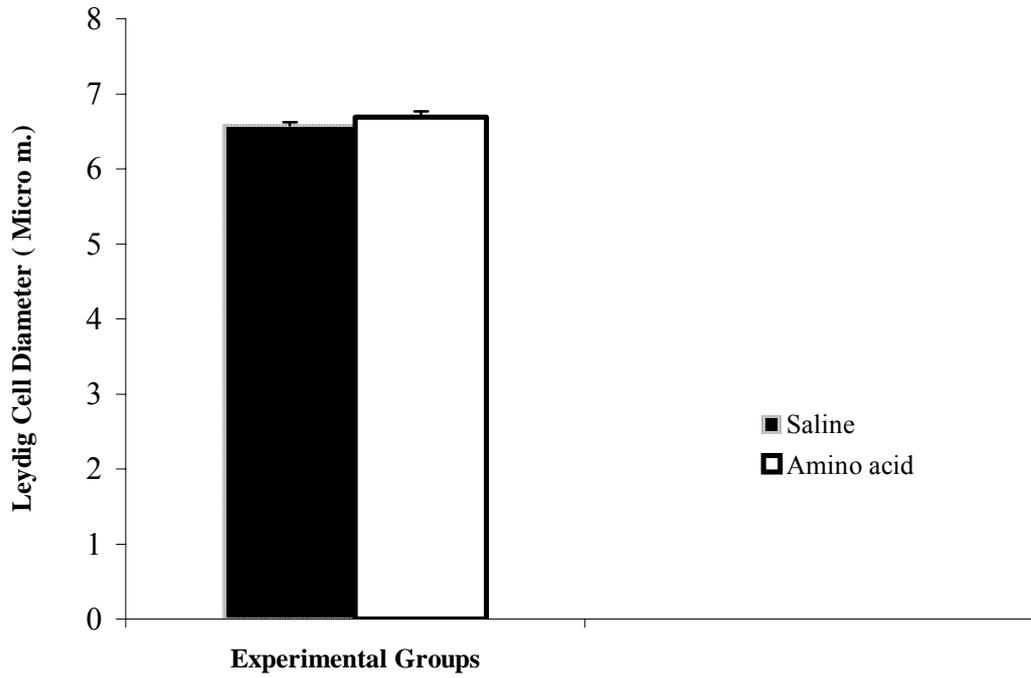


Figure 3; Diameter of Leydig cells in saline and amino acid injected groups.

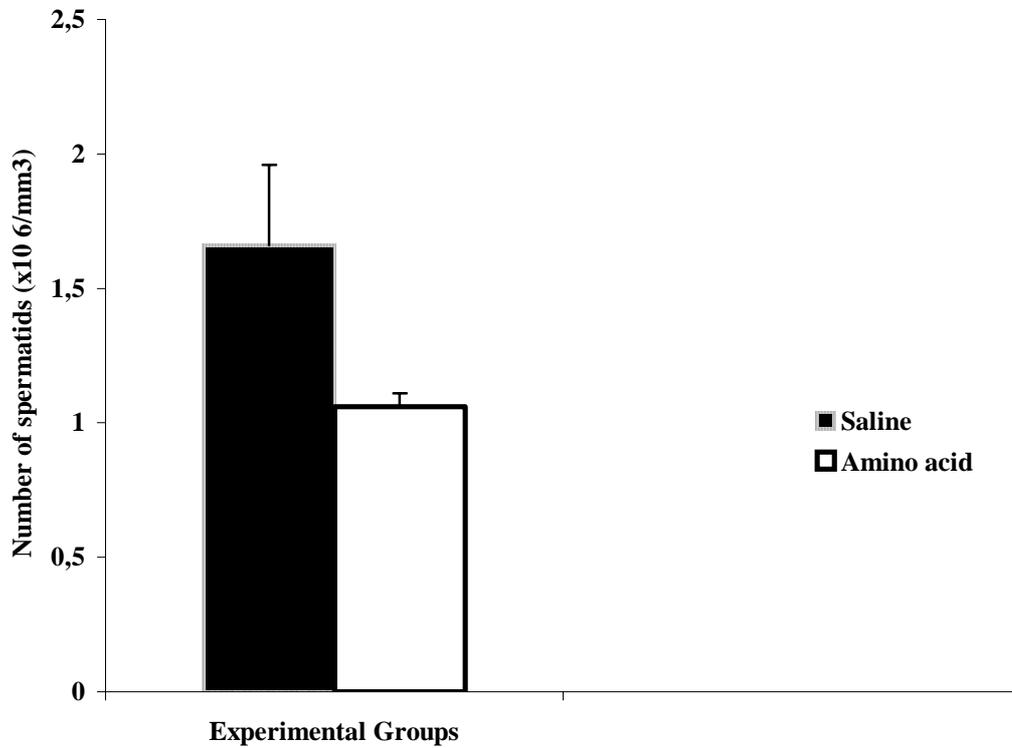


Figure 4; Number of round spermatids counted on the section of testes in saline and amino acid injected group.

DISCUSSION AND CONCLUSION

The injection of amino acids did not increase in tubular diameter and also the diameter of Leydig cells did not changed. Increased Leydig cell diameter and increased diameters of seminiferous tubules are related to increased secretion of testosterone. (Berndtson and Jones, 1989). Therefore in this study, it can not be said that the decreased number of the spermatids in amino acid injected mice was caused by testosterone while diameter of the tubules and Leydig cells did not changed. Presently, the reason for decreased number of spermatogonia due to the serine and threonine is not known. This is because, the effect of Serine and Threonine on sperm production have not been investigated. In this study, it is concluded that the reason for decreased number of spermatids might be the increase in the expression of mRNA for TGF β s. Because, in the rat, injection of L-Arginine (500ng/100g BW) resulted with an increase in mRNA expression for TGF β 1 (Kihara et al., 2001). According to an experiment, injection of the same strain of male mice with Serine and Threonine increased expression of the mRNA for TGF- β ₁, β ₂ and β ₃ (Article in press). It was reported by Olaso and colleagues (1998) that endogenous TGF- β increases the apoptosis of gonocytes and this lead to a decrease in sperm production. Therefore, lower number of spermatids, in amino acid injected mice, might be caused by the changes in TGF- β expression.

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