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Research Article

Expression Profile of Sox5 and Sox6 in Sertoli and Spermatogonial Cells in Growing Mice Testis

Zia Ur REHMAN [1,3,a] Qing LIU [1,2,b] Dinesh BHATTARAI [1,c] Aixin LIANG [1] Farman ULLAH [4,d] Rahim DAD [5,e] Sher Bahadur KHAN [3,f] Hira Sajjad TALPUR [5,g] Sher Ali KHAN [6,h] Liguo YANG [1,i]

[1] Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction, Education Ministry of China, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR CHINA
[2] College of Animal Sciences and Technology, Yandzhou University, Yanzhou, 225000, PR CHINA
[3] Department of Animal Health, Faculty of Animal Husbandry and Veterinary Science, The University of Agriculture, Peshawar, 2500, PAKISTAN
[4] Faculty of Animal Husbandry and Veterinary Science, Lasbela University of Agriculture, Water and Marine Sciences (LUAWMS), 90150, PAKISTAN
[5] Faculty of Animal Husbandry and Veterinary Science, Sindh Agriculture University, Tandojam, 70060, PAKISTAN
[6] Sericultural and Agri-food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, 510640, PR CHINA
[a] ORCID: 0000-0002-4238-575X; [b] ORCID: 0000-0002-2452-1121; [c] ORCID: 0000-0002-9653-4044; [a] ORCID: 0000-0003-1163-5004
[e] ORCID: 0000-0001-7097-6146; [f] ORCID: 0000-0002-0866-3971; [g] ORCID: 0000-0003-2597-0292; [h] ORCID: 0000-0002-6422-2951
[i] ORCID: 0000-0002-7677-1448

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Abstract

SRY box genes are peculiar to animal kingdom. They are involved in many processes particularly sex determination and testes development in male embryo. Although Sox family genes have been identified in various cells, their expression pattern and role is not entirely recognized in Sertoli cells. In this research, we focused on the expression of SoxD group Sox5 and Sox6 genes in Sertoli cells of mice during pre- and post-pubertal testicular development ranging from one-week-old to eight-week-old mice. The expression was studied by immunohistochemistry on whole testes, and qPCR to determine the mRNA level of all age groups, while immunocyto-chemistry was performed for localization in specific age groups. qPCR results of Sertoli cells from first week to eight week showed different levels of expression. The mRNA level of Sox5, during pre-pubertal age, was significantly high (P<0.001), but as the age progressed, the expression became low. Conversely, Sox6 was initially expressed faintly, but at the pubertal age, the expression rose significantly (P<0.001). Furthermore, the expression signals of both genes on spermatogonial cells were also found strong. The study shows that Sox5 and Sox6 are expressed during postnatal and pubertal periods and may play a vital role in the maturation of spermatozoa. In addition, they overlap to regulate multiple functions like spermatogenesis and steroidogenesis in testes.

Keywords: *Sertoli cells, Spermatogenesis, Sox5, Sox6, Testis*

Gelişme Dönemindeki Fare Testislerinde Sertoli Hücreleri ve Spermatogonial Hücrelerde Sox5 ve Sox6 Ekspresyonu

Öz

SRY gen grubu hayvanlar alemine özgüdür. Erkek embriyoda cinsiyet tayini ve testis gelişimi başta olmak üzere birçok sürece dahildirler. Sox familyası genleri çeşitli hücrelerde belirlenmiş olsa da, Sertoli hücrelerindeki ekspresyon özellikleri ve rolleri tam olarak tanımlanmamıştır. Bu araştırmada, puberta öncesi ve sonrası bir ila sekiz haftalık yaştaki farelerde testis gelişimi sırasında Sertoli hücrelerindeki SoxD grubu Sox5 ve Sox6 genlerinin ekspresyonu araştırıldı. Ekspresyon, tüm testis örneklerinde immünohistokimya ve tüm yaş gruplarının mRNA seviyesini belirlemek için qPCR ile incelenirken, spesifik yaş gruplarında lokalizasyonun belirlenmesi için immünositokimya yapıldı. Sertoli hücrelerinin qPCR sonuçlarında, ilk haftadan sekizinci haftaya kadar farklı ekspresyon seviyeleri gözlendi. Puberta öncesi yaşlarda Sox5'in mRNA seviyesi anlamlı derecede yüksekti (P <0.001), ancak yaş ilerledikçe ekspresyon azaldı. Tersine, Sox6 expresyonu başlangıçta zayıfken, puberta ile birlikte anlamlı şekilde arttı (P <0.001). Ayrıca, spermatogonial hücrelerde her iki genin ekspresyon düzeyleri de güçlü bulundu. Çalışma, Sox5 ve Sox6'nın postnatal ve pubertal dönemlerde eksprese edildiğini ve spermatozoanın olgunlaşmasında hayati bir rol oynayabileceğini göstermektedir. Bunun yanı sıra, söz konusu genler testislerde spermatogenez ve steroidogenez gibi birden fazla işlevin düzenlenmesinde rol oynamaktadır.

Anahtar sözcükler: *Sertoli hücreleri, Spermatogenez, Sox5, Sox6, Testis*

İletişim (Correspondence)
+86 27 87281813. Fax: +86 27 87281813
ylg@mail.hzau.edu.cn

INTRODUCTION

Sertoli cells (SC) are the primary cells to differentiate and play a crucial role in testis development and spermatogenesis. These cells differentiate in the male fetal gonads and have a task in mullerian duct regression with the help of Anti-mullerian hormone (AMH) [1]. These cells have originated from the early somatic ancestor cells that are considered to have grown from the coelomic epithelium. Furthermore, the coelomic epithelium develops from meso-nephros which differentiates after thickening, a process implied to be resulting in the expression of Sry gene. Coelomic epithelium also enwraps gonadal primordium, the development of which depends on certain transcription factors [2,3]. As gonadal primordium thickens, the coelomic epithelium and the expression of Sry get enhanced to play a crucial role in the development of Sertoli cells. A quarter of Sertoli cells are required to express Sry for enabling the testes to differentiate in gonad [4].

Sox gene group is the Sry family-related group contained in HMG box, a DNA binding domain having features of the Sox family transcription factors. This group, which is found only in animal kingdom, consists of twenty genes which are further divided into eight groups ranging from A to H according to the degree and level of similarity or conserved regions [5]. Sox group of protein allocate a high degree of homology (usually 70-95%) within the same group both in and outside the HMG region, but the Sox proteins among different groups have limited homology (>46%) within HMG region and not any outside this region. While majority of Sox genes present up to three exons and combine to form a single protein, SoxD and SoxH genes are divided into compound exons and result in different variants having distinct characteristics. In mammals, the determination of male sex is determined by Sry gene expression [6,7]. These genes are briefly expressed in premature Sertoli cells during embryonic days ED (10.5) and (ED) 12.5. Their function in the Sertoli cells is yet unclear and they are supposed to either repress or nourish male sexual growth [8]. In addition, Sry gene expression also triggers the commencement of another Sox9 gene [9]. The history of Sry research shows that it belongs to a group of transcription factors having a role in the architectural cluster of steriospecific nucleuicprotein complex which are crucial for right gene expression [10]. It is observed that Sry related genes, sub grouped in DNA binding protein, are expressed in testis DNA having specific overlapping characteristics with Sry [11].

Sox D group transcription factors are the largest in all sox family. The members of this group are highly identical and occupy a large conserved region in HMG box domain. The HMG box is located at the C-terminal of the protein. The protein of this group shows a short extension outside from these domains. There is about 87% identity among this group and <60% with other Sox genes. SoxD has different characteristics of expression, that is, only the long form expresses N-Terminal, composed of Lucine zipper, coiled coil domain, and a glutamine containing regions called Q box, which shows their hemodimerization or herterodimerization quality with other SoxD members.

Due to common conserved region, Sox5 and Sox6 are closer to one another than Sox13. They have overlapping functions and seem to play an important role in gene expression during spermatogenesis in mice [12,13]. Both Sox5 and Sox6 are known for their lack of transactivation domain. The twin genes can be expressed in short form, i.e., 2kb and 3kb respectively. But they can also be expressed as long transcripts 6kb and 8kb in other tissues including testes. Sox6 is expressed in full length but Sox5 appears in short and long form in testis. Sox5 gene was reported earlier and later on its isoforms were also identified [13,14]. These genes are involved in different pathways including chondrogenesis and development of nervous system [13-15].

The DNA binding short form of Sox5 bears a specialized function in development and is expressed in testes, lungs and brain [16], while long form of Sox5 expression is found in different cells of lungs, kidney, brain and heart. Sox5, along with Sox6 genes, also play role in bone formation including tooth and muscle development. Due to its different isoforms, researchers have always shown more interest in Sox5 [17,18]. On the other hand, Sox6 has different expression patterns. It appears in the central nervous system, otic vesicle, somites, thymus, branchial arches, craniofacial mesenchyme, notochord, liver and limb buds [12,19-21]. Using blotting technique mRNA, Sox6 has been detected in tissues of heart, brain, lungs, spleen, pancreas, liver, kidney, skeletal muscles and whole testes [22,23]. The extensive expression of Sox6 has also been reported in adult tissues of rainbow trout [24]. Moreover, Sox6 is involved in the chondrogenesis and erythropoiesis and its absence in mice results in quick death after birth, most probably from deformed development of heart [25,26]. There are no recognized regions for trans-suppression or trans-activation, but several cofactors are involved in the above-mentioned process to regulate the proliferation, differentiation, and continuous existence in ectoderm, mesoderm and endoderm originated cell-lineage [27].

In a nutshell, this family has a distinctive and overlapping function in the development of the vertebrates which poses the main challenge. Nevertheless, the knowledge concerning the function of SoxD in the vertebrate development is improving with every passing day. Although soxD group is found in various tissues of mammals, twin genes expression and role in maturing testes is still unknown. Therefore, in this study we focused on the expression patterns of Sox5 and Sox6 in different cells of testes to make a gateway and to explore their roles in spermatogenesis and steroidogenesis.

MATERIAL and METHODS

Experimental Animals

Male pathogenic free Kunming mice ranging from week-1 to week-8 were obtained from Central Animal Laboratory of Hubei Province, Wuhan, China. All procedures were performed under the protocols approved by Ethical Committee of Hubei Research Centre and Huazhong Agriculture University bearing the ID HZAUMO-2017-042 and in accordance with the guidelines established by the NIH Lab. Animal Care Committee.

Isolation and Culture of Primary Sertoli Cells

Isolation and culturing of primary Sertoli cells (SC) were performed according to previous reports with slight modifications. The procedure normally results in >90% minimal contamination of Sertoli cells [28,29]. Briefly, testes from five Kunming mice were aseptically removed in petri dish having 1xHBSS. Testes were extensively washed with 1xHBSS 3-4 times. After removing the tunica albuginea, tubules were chopped and transferred to 15 mL tube containing 7-10 mL DMEM F-12 (Dulbecco's Modified Eagle's Medium/Nutrient F-12 medium) enriched 0.5 mg/mL Collagenase IA (Sigma-Aldrich) and 200 µg DNaseI (Sigma, USA) incubated at 37°C for 20 min. The suspension was layered over 5% percol (Pharmacia, Sweden). The supernatant was discarded in order to remove the Leydig cells and bottom layer was further digested by trypsin 0.25% and 0.02% EDTA (1:1) (Gibco, USA) for 15 min at 37°C including 200 µg DNaseI (Sigma-Aldrich, USA). The digestion process was halted by adding equal quantity of 10% FBS cultural media (Invitrogen, USA). The mixture was passed through 70 µm and 50 µm (BD Bioscience USA) cell strainer, centrifuged at 1500 rpm/5min and washed twice. Later on the cells were poured in Lectin DSA coated cell culture plate (Lectin DSA at 5 µg/mL in 1x HBSS lectin from Datura stramonium that selectively binds Sertoli cells; Sigma, USA) which was already prepared and incubated at 37°C in 5% CO_2 for 1 h. Lectin coated plates were washed twice before use. The cells were transferred to lectin coated plates and incubated for at least 1 h at 37°C to achieve maximum attachment of Sertoli cells. Cultural media was changed by hypotonic shock solution (0.3x HBSS) and the plates were maintained under standard cell culture conditions at 37°C with 95% air and 5% CO_2 in a humidified chamber to lyse the unnecessary germ cells. Hypotonic solution was changed and Sertoli cells were enriched by 10%FBS, 1%penicillin/streptomycin and fungizone at 0.5 µg/mL in DMEM F-12 by fresh culture media for 2 days.

Immunohistochemistry (IHC)

The regional localization of sox5 and Sox6 was examined by IHC method with little modification [30]. The expression of Sox5 and Sox6 were checked in the testes of two-week-old and adult (42 days) male mouse and three mice were used in each age group. A standard protocol of streptavidin biotin-peroxidase complex was followed. In short, testes from the respective ages were collected and dehydrated in different concentrations of alcohol. The paraffin embedded 4-5 µm tissue section was cleaned with xylene, dewaxed and incubated with blocking solution at room temperature for 30 min (1% BSA solution (bovine serum albumin), Wuhan, China). The sections were incubated with Sox5 and Sox6 anti-rabbit and anti-goat polyclonal antibodies (Cat. sc-20091, Sc-17332, Santa cruz, USA) overnight at 4°C. Next day, at room temperature, the sections were washed 3 times with PBST and incubated with HRP conjugated with anti-rabbit or anti-goat secondary antibody and peroxidase-conjugated streptavidin for 1 h (Boster, Wuhan China). In negative control, primary antibody was not used. Expression of Sox5 and Sox6 was defined with brown color.

Extraction of RNA and cDNA, qPCR

The cultured cells were rinsed with 1xHBSS twice and total RNA was extracted by Trizol kit from Sertoli cells of all ages of mice (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity and purity of RNA were measured using Nanodrop Spectrophotometer absorbance at optical density 260/280. cDNA was synthesized using first strand cDNA kit (Toyobo Co., Japan), the manufacturer's instruction was followed step by step. Primers for the respective genes were designed by primer 5 software and confirmed by UCSC Bioinfo. software. For qPCR, the implication was performed (Bio-Rad iQ5 Real Time PCR System, CA, USA), in reaction mixture consisting of specific primers *(Table 1)*, the master mix and RNA free water. The mRNA level was normalized with GAPDH and RNAse free water was used as control. For precision, melting curve analysis was used to check the PCR purity as prescribed previously [31]. Experiment was conducted in triplicates.

Immunocytochemistry (ICC)

The localization of Sox5 and Sox6 at cellular level were examined after 48 h in cultured mouse sertoli cells from five mice aseptically. The ICC results show the location of both proteins inside the Sertoli cells. The cells were transferred on cover-glass (in 6 well plates) and washed with PBS three times and fixed by 4% formaldehyde (Beyotimes,

Table 1. *Quantitative real time polymerase chain reaction primers*

S.No.	Gene Name	Primers	Tm °C
1	SOX5	ATGCTTACTGACCCTGATTTACCT TCCACTTTCTGTCTGCTTGTCAC	58
2	SOX6	TGGGCAAAGGACGAAAGGAG GCCTGTTCTTCATAGTAAGGTTGCT	58
3	GAPDH	TCAACGGCACAGTCAA CTCGCTCCTGGAAGAT	

China) at room temperature for 30 min. After rinsing, cells were exposed to 0.5% Triton X-100(PBS preparation) for 20 min. Cells were again washed with PBS thrice each time for three min. Normal goat and rabbit serum was used drop by drop on the cells before they were incubated for 30 min. at room temperature. Furthermore, each slide was saturated with sufficient primary antibodies of Sox5 and Sox6 (Santa Cruz Laboratories, CA, USA), diluted 1:150 in 5% BSA, in respected slide and incubated at 4°C overnight in dark moist chamber. Next day, cells were stained with Cy3 labeled secondary antibodies (goat anti-rabbit IgG, rabbit anti-goat IgG, Boster China at 1:100) in a wet box for 1 h at 37°C, then rinsed each time for 5 min. For nuclear staining, cells were stained with DAPI (1:5000) for 5 min, and extra staining was removed by washing. Cells were then dried with absorbent paper. In control, primary antibodies were not used, only PBS was added. The slides were finally mounted with 95% glycerin. Each plate was finally analyzed with confocal laser scanning microscope (LSM 510 Meta instruments Zeiss, Germany).

Statistics Analysis

All the experiments were repeated three times for each section and the data were presented as ±SEM. Multiple group data were analyzed byone way ANOVA followed by LSD test using SPSS-16 system software. For individual comparison P<0.001 was considered as highly significant.

RESULTS

In order to check the expression of Sox5 at both stages, i.e., pre-pubertal and post-pubertal age of mice testes, the immunohistochemistry result showed that the expression of both genes in post-pubertal age was higher as compared to pre-pubertal age only shown by brown color. The expression was also high in spermatogonial cells (black arrows) followed by Sertoli cells (red arrows) *(Fig. 1)*. So this indicates that this gene is closely related to the spermatogenesis. In adult age, the spermatogonial cells are more active in the way of sperm development. Sox6 gene expression is clear in both per pubertal and post pubertal Sertoli cells (red arrows) as well as spermatogonial cells (black arrows). Spermatogonial cells have stronger expression *(Fig. 1)*. The same finding was also reported that the sox5 expressed in the postmeiotic and round spermatids and sox6 (LZ) was found in adult testes [6].

In order to observe the research potential of Sox5 and Sox6 in various developmental ages of Sertoli cells and spermatozoa production, we checked the expression level by qPCR. The results showed variation among the expression level in different ages of mice. At second, third and fourth week of postnatal age, the level of Sox5 was significantly high (P<0.001) but in adult age the expression level was low *(Fig. 2A)*. But in case of Sox6, expression level in adult age was significantly high (P<0.001) *(Fig. 2B)* as compared to pre pubertal age which indicates that sox5 and sox6 seem to have overlapping characteristics. It means that they boost the role of one another and helps in functions of Sertoli cells. Furthermore, it also refers to the role that Sox5 and Sox6 may have in the spermatogenesis.

Immunocytochemistry shows the localization of Sox5 and Sox6 in Sertoli cells at one week of age. As Sox5 (strong signals in nucleus) and Sox6 (nucleus and cytoplasm) are transcription factors so this experiment showed that they localized in the nucleus of Sertoli cells. The immunocytochemistry results show the translation of gene and indicated by red color (Cy3 stained), show the presence of Sox5 and Sox6 protein in the nucleus and cytoplasm as in *Fig. 3*. The SoxD proteins previous information has shown that they critically modulate cell fate in major lineages [13].

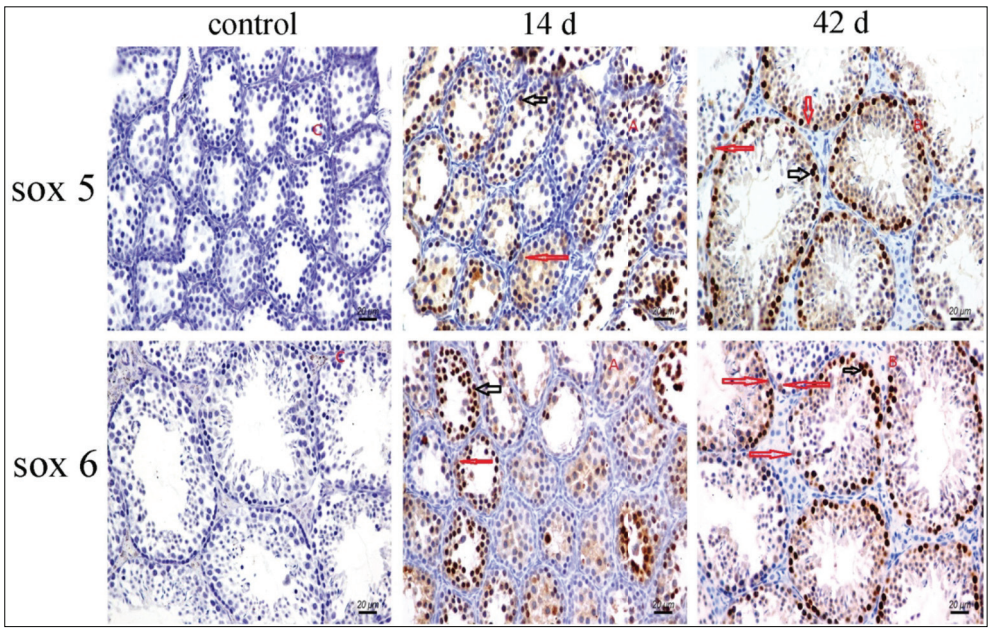

Fig 1. The expression of Sox5 and Sox6 in seminiferous tubules of mouse testis. Red arrows indicate positive Sertoli cells while black arrows indicate spermatogonial cells. Control is indicated by C without antibody. The letters A & B indicates 14 & 42 days mouse testes sections, respectively. Bar is 20 µm for each slide and brown color shows the positive cells

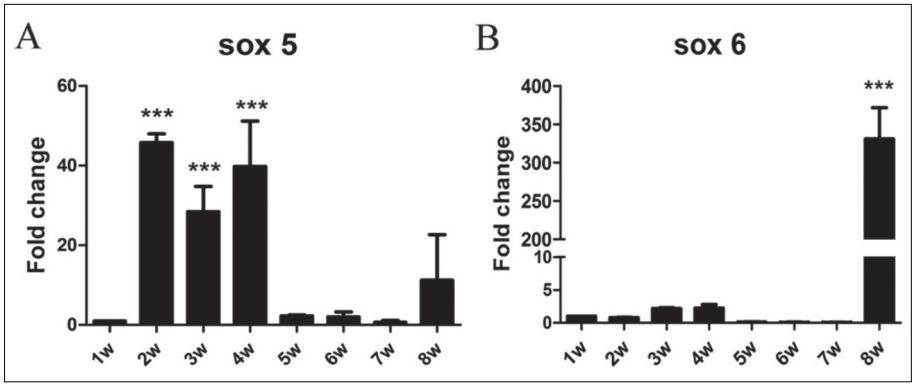

Fig 2. Relative expression (by quantitative polymerase chain reaction) of Sox5 and Sox6 in Sertoli cells of mouse in different growth stages. Both pre- and post-pubertal ages showed highly significant difference *** (P<0.001) in both genes

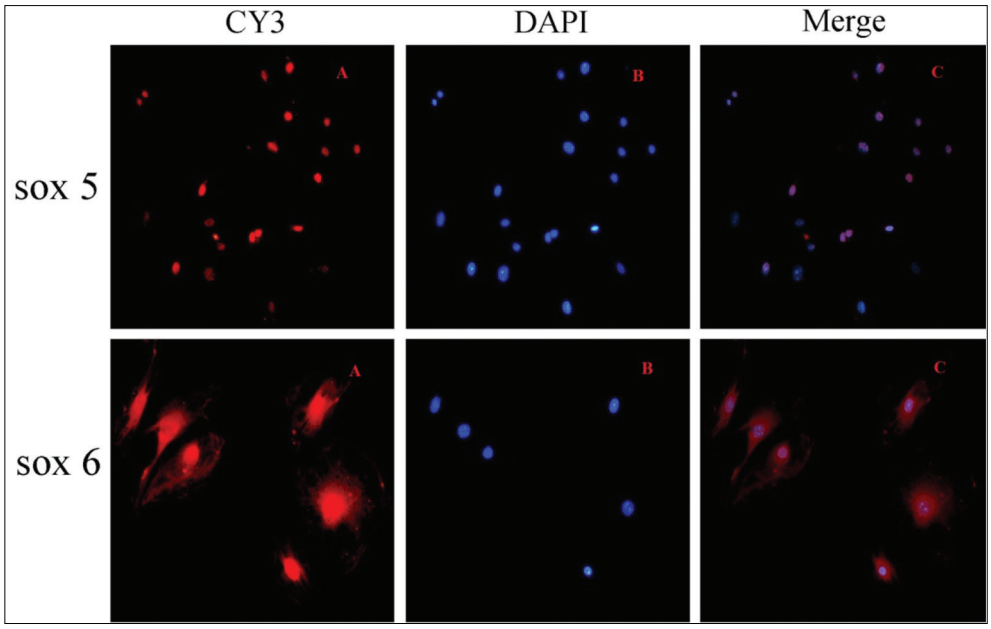

Fig 3. Localization of Sox5 and Sox6 by immunocytochemistry analysis. Cy3(A) stained cytoplasm is shown by red color whereas the DAPI stained nucleus (B) is indicated by blue color and (C) shows the merged image. The images are taken by laser microscope using 200 and 400 folds magnification

DISCUSSION

This study shows the expression of Sox5 and Sox6 at mRNA and protein level in mice testes both in pre- and post-pubertal periods. Although the expression of Sox5 and Sox6 is reported vastly in other organs, the area concerning its expression and roles in the testes has relatively received less attention. The immunocytochemistry result showed that both Sox5 and Sox6 transcription factors are expressed at the pre pubertal age in Sertoli and spermatogonial cells. As they are transcription factors, so they give stronger signals in the nucleus *(Fig. 3)*. The mRNA levels in Sertoli cells showed that the expression of Sox5 and Sox6 varies at different developmental stages. At the pre pubertal level, Sox5 expression was significantly high (P<0.001) *(Fig. 2A)*, but as the adolescence neared, its expression became fainter and this finding is similar to the previous works [16]. On the contrary, Sox6 expression at the pre pubertal age was significantly low. But at the 8th week, its expression became highly significant (P<0.001) *(Fig. 2B)* all in line with the previous findings [12]. In addition, immunohistochemistry results showed that Sox5 and Sox6 are strongly expressed in spermatogonial cells of both the respective ages. Their expression exists in Sertoli cells but fainter than spermatogonial cells. These results are consistent with the previous researches [12,14,16]. It means that these genes at adult age are more functional and assists in sperm maturation.

The expression of SoxD family genes are reported in various tissues and cells in testis, neuron, oligodendrocytes, chondrocytes and palatogenesis [16,32,33]. A member of this family (Sox5) has been reported in the postnatal period in testes [14], which concede with our findings in spermatogonial cells. The S-Sox5, a short transcript, in human and mouse having 48kD size was also found in testes in ciliated/flagellated cells; the short isoform of Sox5 is deficient in N-terminus and have a length of half of the long transcript (6kb). The long form was discovered earlier than the short form [34]. The expression of L-Sox5 is high in chondrocytes and muscles which prove that it has function in the cartilage and development of muscles [35]. Sox5, besides in testes, is also strongly expressed in human brain as high expression of S-Sox5 was found in brain and some ciliated tissues, which indicates that it might have role in the sperm development. Long Sox5 84kD, on the

other hand, is expressed in brain, kidney, heart and skeletal muscles; meanwhile, it has also been reported that another short Sox5 of 25kD is expressed in spleen, liver and testis which seems to be a new translated protein reported by some databases [34]. At mRNA level from the results of Sox5 expression varies in Sertoli cells (2A), as age increases its expression level also increases, reaching the highest level at the adult age. According to immunohistochemistry, which show the presence of its protein and it exists at both pre pubertal and adult age but the expression in Sertoli cells is fainter than spermatogonial cells. The molecular structure of sox protein is unique and shows various motifs. They are flexible and versatile in DNA binding sequencing are capable of using various mechanisms to either enhance or repress transcription [13]. The immunocytochemistry result at the same age shows that Sox5 is localized in the nucleus and show the expression.

Sox6 is also a member of SoxD group family. It appears in variety of mammalian tissues. The role of Sox6 in testicular development is not known, the large transcript of Sox6 is expressed in skeletal tissues but its short form, about 3kb, appears in the testis. It is also observed in central nervous system during embryogenesis. The expression of Sox6 in cardiac and skeletal muscles shows crucial role in the muscle development [26]. The Sox6 ortholog is also found in the fish (i.e. trout) in both forms about 10kb and 3kb form. This indicates that there are conserved regions in the pattern so it might have a significant function in different tissues [36]. Sox5 and Sox6 along with sox9 are expressed during embryogenesis at early stage and have functional role in embryo development. These transcripts are fully detectable in the mesenchymal cells at early stage of cartilage formation up to the bone formation. Sox5 and Sox6 are homologous proteins and the persistent presence of sox transcripts shows that they have crucial role in the development of bone formation and cell differentiation pathway and they also take part in the activation of a group of gene matrix. The initial data also shows that the twin (Sox5 and Sox6) genes are paramount for proper cartilage and bone formation in vivo [37]. The short form of Sox5 and Sox6 are detectable in the adult testes which may have function in the spermatozoa maturation pathway [13]. Sox genes are mostly involved in the architectural structure, so their mutation or deletion may interfere with birth or cause developmental defects [23]. Sox6 is important transcription factor for cell endurance as it contributes to cell survival. The deficiency of Sox6 causes inhibition of neural differentiation in P19 cells [38]. Sox6 has also been identified to have a unique tumour suppression role, as it has HMG domain using p53 stabilization mediated channel [39].

LSox5 and Sox6 are highly similar and are co-expressed in many types of cells. Mice born with both null Sox5 and Sox6 gene die soon after birth because of generalized chondrodysplasia while mice with single null gene had been born with mild abnormality in skeletal. Moreover, deficiency of both genes causes poor development in skeletal tissues [40]. Sox5 and Sox6 have been reported to be only expressed at adult age and restricted to post meiotic spermatogonial cells [12], but our experiment showed that these genes are expressed in other cells like Sertoli cells, which are crucial for sperm development. Several published research have observed that these structurally unique proteins (sox) are biologically very important in the cellular development. They are highly flexible in selecting DNA-binding sequences and are capable of using various mechanisms to either enhance or repress transcription. They thereby modulate such varied processes as cell proliferation, survival, differentiation, and terminal maturation in a number of cell lineages [13].

In conclusion the expression of soxD genes shows that their role is important in the development in cells and tissues. From our study, it is clear that Sox5, Sox6 and their different isoforms exist at various stages of testicular development, and the maturation of spermatozoa, although the functions of these two genes in testes have still not been elucidated. After this identification, further pathways studies through RNAi or overexpression experiments are needed to know their potential function in spermatogenesis or steroidogenesis. Moreover, our study has implications for the further role which Sox6 may play in the sustenance of tissue. The area has been left rather undiscovered, though ready for further explorations.

Conflict of Interest

All authors do not have any potential conflict related to this research.

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