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EXPRESSION OF CONNEXINE 43 IN THE SEMINIFEROUS EPITHELIUM OF WHITE MICE DURING TESTICULAR ONTOGENESIS: IMMUNOHISTOCHEMISTRY AND IN SITU HYBRIDIZATION

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ABSTRACT

Immunohistochemistry and in situ hybridization were used to elucidate the distribution of connexine43 (Cx43) protein and its mRNA in mice seminiferous epithelium during testicular ontogenesis. Cx43 protein expresses itself in mice male gonads as early as the premordia germ cells reach the gonadal blastema at 12 days postcoitum (dpc) indicating its importance in testicular development. It was expressed mainly in the basal portions of testicular cords throughout pre- and postnatal periods of gonadal development. Cx43 mRNA was expressed more strongly in germ cells than in supporting cells. Cx43 protein expression was found to be stage dependant being expressed strongly in stage VII (which is characterized by retraction of the main bulk of Sertoli cells to basal locations allowing better visualization of the inter Sertoli cell junction complexes which represent the main site of Cx43 expression).

INTRODUCTION

Connexins are the protein subunits of gap junction channels through which the direct exchange of ions, metaboloites and second messengers occurs ***(Saèz et al., 1989; Elfgang et al., 1995; Kumar and Gilula, 1996).***

The role of Cx43 in testicular development has also been documented. The number of germ cells in fetal testis of CX43 Knock-out mice is significantly reduced ***(Juneja et al., 1999)***. In addition the junctional intercellular communications between Sertoli cells and between Sertoli cell and spermatogonia is considered to play a key role in regulation of both proliferation and differentiation of germ cells ***(Fischer et al., 2005)***. From the earliest stage of gonadal development, Cx43 is expressed in principal cell types that participate in the control of male fertility.

During postnatal development, Cx43 has been reported to express itself in basal portions of Sertoli cells of newly born mice. It shifts to an adluminal location during the first two weeks, but then it relocalizes itself peripherally ***(Brovo-Moreno et al., 2001)***.

Several evidences have been presented for the requirement of Cx43 in the control of fertility. For example, it has been shown that decreased Cx43 expression in adult Sertoli cells is associated with disturbances in spermatogenesis in both human ***(Steger et al., 1999)*** and mouse ***(Batias et al., 1999).***

At mRNA level, the cellular localization of Cx43 has been determined by in situ hybridization only during postnatal development ***(Batias et al., 2000)***. Investigations at this level during prenatal development was not conducted so far. The present study aims to visualize the expression of CX43 in different cellular components of the seminiferous epithelium during pre- and postnatal development using immunohistochemical and in situ hybridization.

MATERIALS AND METHODS

Animals and tissue:

This study was conducted on 54 white mice embryos aging 12 to 20 dpc, 6 embryos for each of the above 9 prenatal ages. Each embryo was cut transversely just caudal to the thoracic limbs into two halves. The caudal half was fixed in Bouin's fixative for 24 hours then processed for paraffin embedding. Cosecutive 5 µm thick sections were cut from each block, mounted on superfrost slides (Menzel-Glaser, Germany) and dried for 24 hours at 37°C. In addition three mice aging 5, 10 days and an adult one were used for the postnatal study. The testis were collected from these animals, fixed in Bouin' fixative and processed for paraffin sectioning as above.

Cx43-IHC:

Immunohistochemical staining for Cx43 were performed on consecutive paraffin sections. Briefly, sections were microwave treated for 30 min at 1000 watts in sodium citrate buffer (pH 6.0), blocked with 5 % BSA for 30 min and incubated with the polyclonal anti-cx43 primary antibody (1:250; Zytomed, Berlin, Germany) over night at 4 °C. Sections were then exposed to the secondary antibody (1:50; mouse anti-rabbit IgG; DAKO) followed by the third antibody (1:50; rabbit-anti-mouse IgG; DAKO) and finally mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) antibody complex (1:100, DAKO) for 30 min each. The immunoreaction was visualized using HistoMark Red (KPL). For each immunoreaction, control incubations were performed by substituting buffer for the primary antibody. Cx43 IHC was repeated at least twice.

Production of Digoxigenin (DIG)-labeled cRNA probes for ISH DIG-labeled cRNA-probes were generated as described previously by ***Brehm et al. (2006)***. The DNA sequence of the human Cx43 gene ***(Accession***

AF151980) was generated using a touch-down PCR with primers Cx43F and Cx43R (MWG, Ebersberg, Germany [y]). PCR conditions were as follows: 1x 95 °C for 3 min, 15x [95 °C for 1 min, 66 °C for 1 min, 72 °C for 2 min], 45x [95 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min], 72 °C for 10 min. The 138 bp PCR-product of the human Cx43-gene was subcloned in pGEM-T vector (Promega, Mannheim, Germany). Plasmids were transformed in the XL1-Blue E. coli strain (Stratagene, Heidelberg, Germany) and extracted by column purification, according to the manufacturers instruction (Qiagen, Hilden, Germany). After sequencing, vectors containing the Cx43-insert were digested with NcoI and NotI (New England Biolabs, Frankfurt, Germany) for the production of sense-cRNA (NcoI) and antiSense-cRNA (NotI), respectively. Subsequently, in-vitro transcription was performed using the 10x RNA-DIG Labeling-Mix (Boehringer Mannheim, Mannheim, Germany) and RNA-polymerases T7 and SP6 (Promega).

Y = Cx43 5'- cca tct cta act ccc atg cac agc - 3' (F)

5'- tgg cac gac tgc tgg ctc tgc tt - 3' (R) 138 bp

Cx43-ISH:

ISH was performed on consecutive sections as described previously. [x] Briefly, deparaffinized tissue sections were incubated in active DEPC water for 2 x 12 min at 40 °C, postfixed in 4 % paraformaldehyde for 10 min, exposed to 20 % acetic acid and prehybridized in 20 % glycerol for 30 min. Sections were then incubated with the DIG-labeled sense or antisense cRNA probes. Both cRNAs were used at a dilution of 1:25 in hybridization-buffer containing 50 % deionized formamide, 10 % dextran sulfate, 2x standard saline citrate (SSC), 1x Denhardt´s solution, 10 µg/ml salmon sperm DNA (Sigma-Aldrich, Taufkirchen, Germany) and 10 µg/ml yeast t-RNA (Sigma). Hybridization was performed overnight at 40 °C in a humidified chamber containing 50 % formamide in 2x SSC after posthybridization washes. Subsequently, sections were incubated with the anti-DIG Fab-

antibody conjugated to alkaline phosphatase (Boehringer) overnight at 4 °C. Staining was visualized by developing sections with NBT/BCIP (nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate; KPL, Gaithersburg, MD, USA) in a humidified chamber protected from light. Finally, sections were rehydrated for 5 min in deionized water and then dehydrated through successive baths of ethanol and xylol, and mounted in Eukitt resin (Merck, Darmstadt, Germany). For each test, negative controls were performed using DIG-labeled cRNA sense probes. ISH was repeated at least twice.

RESULTS

Immunohistochemistry:

Cx43 was expressed in mice testis as early as 12 dpc. It appeared in the form of deeply stained small dots surrounded by irregular shadow. The reaction included the areas of contact between some supporting cells as well as in contact areas between some supporting and germ cells (Fig. 1).

By the appearance of testicular cords, Cx43 was localized mostly in basal locations of these cords. The reaction was limited to junctional contacts between supporting cells and between supporting and germ cells. The adluminal expression was demonstrated to sporadic locations (Fig. 2).

By advancement of fetal ages, Cx43 expression became more intensified in basal portions of testicular cords (Fig. 3). In full-term fetuses, the cellular components in testicular cords seemed to be relatively decreased, possibly due to increased gonadal size and this became reflected on Cx43 reaction which showed a slight down regulation (Fig. 4).

During the early postnatal period, Cx43 expression was up regulated in testicular cords and included almost all inter-Sertoli cell junctions (Fig. 5). On reaching maturity, Cx43 displayed variable reactions in tubular compartment of the testis. The seminiferous tubules in stages prior to spermiation (stages VII and VIII), showed the strongest reactivity. However tubules with younger deeply inserted spermatids (in stage XII) showed little or even no Cx43 expression (Fig. 6).

In situ hybridization:

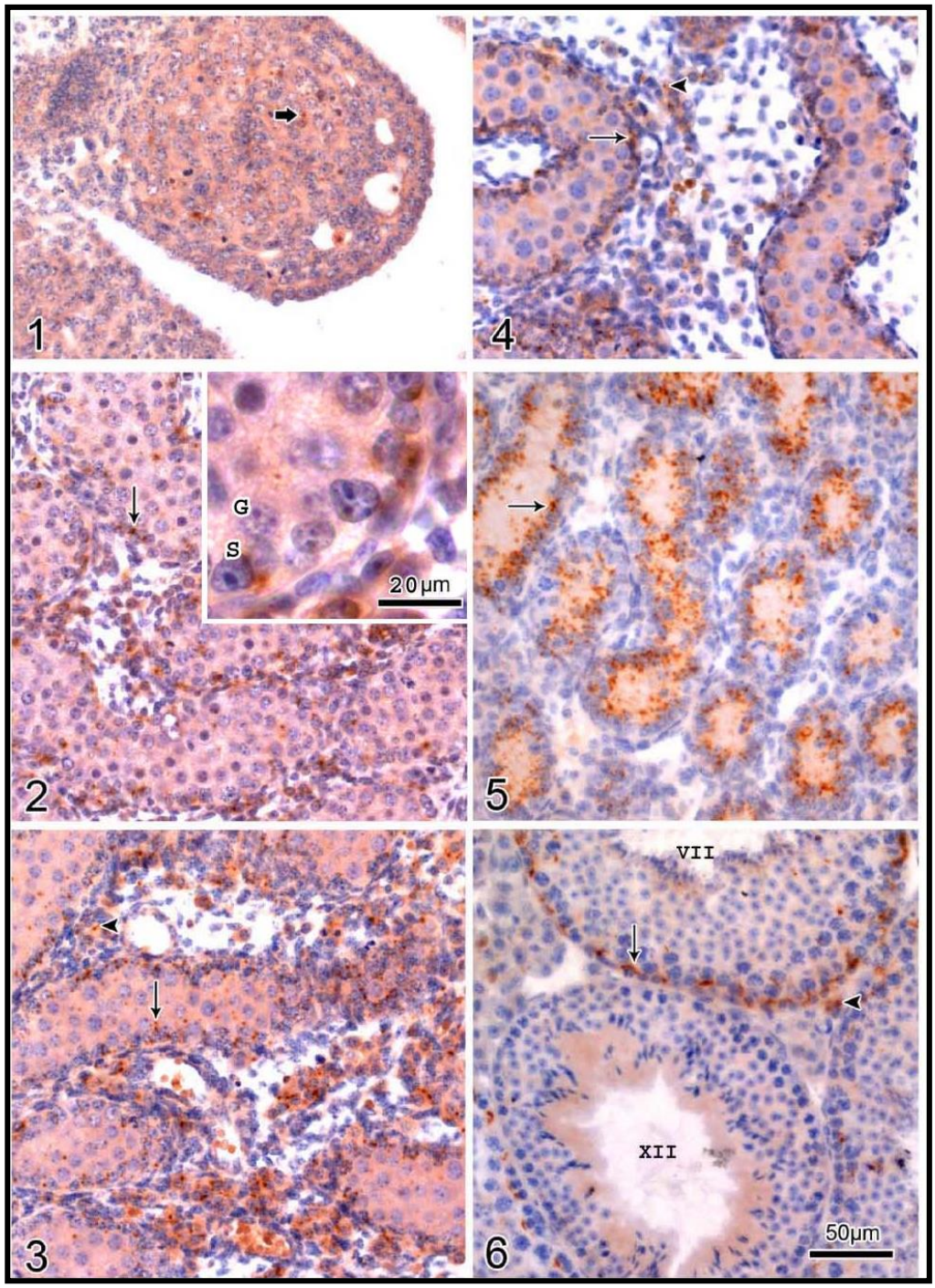
Cx43 mRNA was detected in testicular cords as early as 14 dpc. Its expression in germ cells cytoplasm was more evident than in Sertoli cells throughout pre- and postnatal development. The whole germ cell cytoplasm stained positively with Cx43 mRNA. It was demonstrated in the beginning in both basal and adluminal (Figs. 7 & 8), then after, it shifted to basal portions in late prenatal (Figs. 9) and prepuberal periods (Figs. 10 & 11). In mature testis, Cx43 mRNA was investigated in spermatogonia, primary spermatocytes and basal portions of Sertoli cells particularly in stage VII of spermatogenic cycle (Fig. 12).

LEGENDS

Figs. 1-6: Localization of Cx43 protein in mice testis during pre- and postnatal development. Cx43 can be detected as early as 12 dpc in the form of dark dots surrounded by irregular shadow between some blastemal cells (thick arrow) as seen in fig.1. After appearance of testicular cords at 14 dpc (Fig. 2), the punctuate expression of Cx43 (arrow) is demonstrated mainly in basal tubular locations. The inset in fig. 2 demonstrates

expression of Cx43 between supporting (S) and Germ cells (G). By advancement of fetal ages and at 17 dpc (Fig. 3), the Cx43 expression becomes up regulated and includes almost all inter Sertoli cell junctions. In full term fetuses at 20dpc (Fig. 4), the Cx43 expression seems to be down regulated. During the early postnatal period, Cx43 expression was up regulated in testicular cords and included almost all inter-Sertoli cell junctions at 10 dpc (Fig. 5). On reaching maturity (fig. 6), Cx43 showed the strongest expression in stage VII of spermatogenesis (arrow) and is nearly not expressed in stage XII.

Fig. 7-12: Expression of Cx43 mRNA in mice testis during pre- and postnatal development. It starts at 14 dpc (Fig. 7) in the form of light brown to brown cytoplasmic staining. It is demonstrated mainly in germ cell cytoplasm which is restricted firstly in adluminal locations at 14 dpc and 15 dpc (Fig. 8). It then starts to occupy basal locations at 20 dpc (Fig. 9), 5 days postpartum (Fig. 10) and 10 days postpartum (Fig. 11). In mature testis (Fig. 12), Cx43 mRNA was investigated in spermatogonia, primary spermatocytes and basal portions of Sertoli cells particularly in stage VII of spermatogenic cycle.


1
4
2
G
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VII
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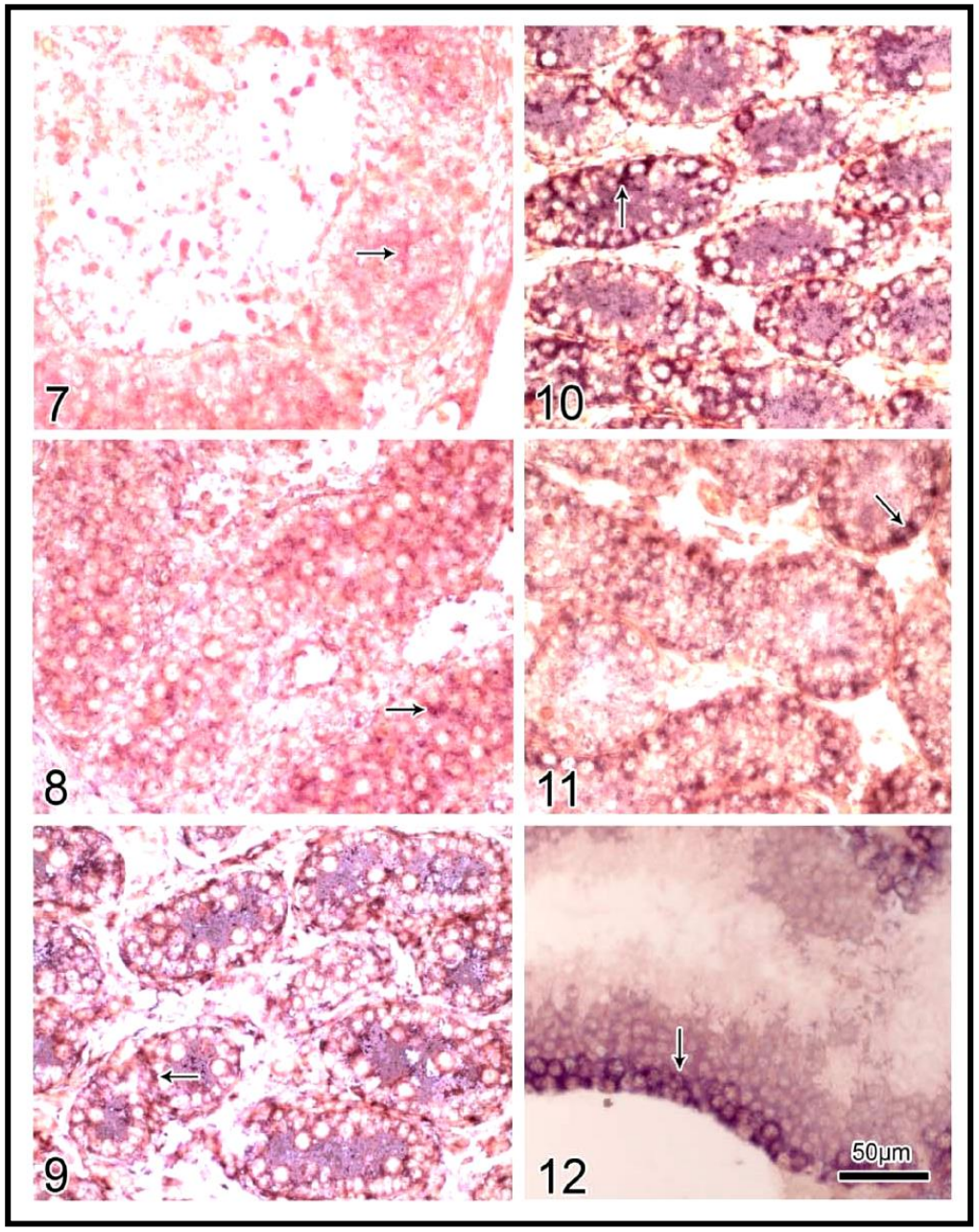
7
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50µm

DISCUSSION

The present study showed for the first time a full coverage of the expression of Cx43 protein and its mRNA in mice testis during the pre- and postnatal periods of development. In agreement with ***Perez-Armendariz et al. (2001)*** Cx43 protein is detectable in mice male gonad as early as the primordial germ cells reach the gonadal blastema (11.5-12 dpc). In the current study, Cx43 protein was demonstrated in the form of sporadic deeply stained dots in contact areas between some blastemal cells which were not yet organized to form testicular cords. This very early expression points to an important role of Cx43 in testicular development. This is supported by the statement of ***Brovo-Moreno et al. (2001)*** that Cx43 plays other roles in gonadal development besides control of spermatogenesis. This raises the possibility that Cx43 may be included in the control of germ cells proliferation and survival. In this concern, ***Juneja et al. (1999)*** have reported that Cx43 deficient mice experience a significant decrease in germinal cells throughout embryonic life.

The present work emphasized that Cx43 protein was located mainly in the basal compartment of testicular cords throughout both pre- and postnatal development, the adluminal expression was sporadic in some cords. Cx43 protein was investigated mainly in gap junctional complexes between adjacent supporting cells as well as between supporting and germ cells. The basal location of the former cells explains the basal expression of Cx43 throughout testicular ontogenesis. ***Unlikely, Batias et al. (2000)*** have mentioned that Cx43 protein is first detected in the adluminal compartment of the growing seminiferous tubules before it becomes progressively located in the basal compartment at latter ages. This inconsistence in their results could be attributed to pictures obtained from tangential sections in testicular cords which may give false adluminal expression of Cx43.

In situ hybridization revealed that Cx43 mRNA was expressed more strongly in germ cells than in the supporting cells. The presence of Cx43 ISH signals in germ cells is consistent with previous observations of ***Batias et al. (2000)*** and supports the presence of gap junctions between supporting and germ cells reported by ***Enders (1993)***. Our findings enable us to postulate that Cx43 mRNA is primarily synthesized in germ cells before being transferred to the neighbouring supporting cells. This is supported by the description of homotypic, heterotypic or heteromeric channels containing Cx43 ***(Bukauskas et al., 1995; Elfgang et al., 1995; Elenes et al., 1999; Oh et al., 1999)*** and in situ dye transfer ***(Batias et al., 2000)*** between supporting and germ cells.

The present investigation supports previous reports that Cx43 protein and its mRNA were dependant on the stage of spermatogenesis. Our results agree partially with those of ***Risley et al. (1992); Tan et al. (1996); Lablack et al. (1998) and Batias et al. (2000)*** that Cx43 protein and mRNA were expressed strongly in stage VII, i.e. prior to spermiation. However, our work emphasized that after spermiation, Cx43 was down regulated to be nearly absent in stage XII. It is not clear why Cx43 expression increases in stage VII of spermatoigenesis. The late spermatids appear to be a major regulator of Sertoli cell function in adult mammalian testis ***(Jegou, 1993)***. A concomitant reduction in the expression of Cx43 and the number of elongated spermatids has been reported in Ebourrife mutant house mice ***(Lalouette et al., 1996)***. This has raised the possibility of the implication of elongated spermatids, existing in stage VII, in modulation of Cx43 synthesis ***(Batias et al, 1999)***. In our opinion, stage VII of spermatogenbesis is also characterized by retraction of the main bulk of Sertoli cells masses to basal locations that may allow better visualization of the inter Sertoli cell junctions which represent the main site of Cx43 expression.

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ظهور الكونكسين 43 فى النسيج الطلائى المنوى للفأر الأبيض أثناء مراحل تطور الخصية

أحمد الزهرى زايد ، منى عبد الفتاح على ، أمين حسانين ،
أنى هلد ، راف بريم ، كلاوس شتيجر

أستخدم فى هذه الدراسة كل من الكيمياء النسيجية المناعية والتهجين المحلى لإستيضاح توزيع بروتين وال ر ن ا الناقل للكونكسين 43 فى النسيج الطلائى للانيبيبات المنوية لخصية الفأر الأبيض أثناء مراحل تطورها المختلفة حيث ظهر بروتين الكونكسين فى خصية أجنة الفأر عند عمر أثنى عشر يوما للحمل مما يدل على أهميته فى تطور الخصية. ولقد تمركز بشكل خاص فى الجزء القاعدى من الأحبال الخصوية طوال مراحل ما قبل وبعد الولادة من التطور. أما ال ر ن ا الناقل فقد عبر عن نفسه بشكل أكثر قوة فى الخلايا الجرثومية أكثر منها فى الخلايا الدعامية. كذلك فإن وجود بروتين الكونكسين فى الأنيبيبات المنوية للفئران البالغة تركز بشكل أقوى فى المرحلة السابعة للدورة المنوية والتى تتميز بإنكماش خلايا سارتولى بإتجاه قاعدة الانيبيبات المنوية مما يسمح برؤية أوضح لمناطق الإتصال لهذه الخلايا مع بعضها البعض والتى تمثل المناطق الرئيسية لظهور الكونكسين 43.