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Do neural tube defects lead to structural alterations in the human bladder?

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Summary. Purpose: Anencephaly is the most severe neural tube defect in human fetuses. The objective of this paper is to analyze the structure of the bladder in anencephalic human fetuses. Methods: We studied 40 bladders of normal human fetuses (20 male and 20 female, aged 14 to 23 WPC) and 12 bladders of anencephalic fetuses (5 male and 7 female, aged 18 to 22 WPC). The bladders were removed and processed by routine histological techniques. Stereological analysis of collagen, elastic system fibers and smooth muscle was performed in sections. Data were expressed as volumetric density (Vv-%). The images were captured with Olympus BX51 microscopy and Olympus DP70 camera. The stereological analysis was done using the software Image Pro and Image J. For biochemical analysis, samples were fixed in acetone, and collagen concentrations were expressed as micrograms of hydroxyproline per mg of dry tissue. Means were statistically compared using the unpaired t-test ($p<0.05$). Results: We observed a significant increase ($p<0.0001$) in the Vv of collagen in the bladders of anencephalic fetuses (69.71%) when compared to normal fetuses (52.74%), and a significant decrease ($p<0.0001$) in the Vv of smooth muscle cells in the bladders of anencephalic fetuses (23.96%) when compared to normal fetuses (38.35%). The biochemical analyses showed a higher concentration of total collagen in the bladders of anencephalic fetuses (37354 μg/mg) when compared to normal fetuses (48117 μg/mg, $p<0.02$). Conclusions: The structural alterations of the bladder found in this study may suggest the existence of functional alterations in the bladder of anencephalic human fetuses.

Key words: Anencephaly, Bladder, Embryology, Human fetuses

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Introduction

Neural tube defects are one of the commonest congenital malformations of the central nervous system, with an average prevalence at birth of 1 per 1000 (Blatter et al., 1994). Anencephaly is the most severe of fetal neural tube defects, resulting from failure of the neural tube to close at the base of the skull in the third or fourth week (day 26 to 28) after conception, leaving the skull bones that usually surround the head unformed. Thereby, the brain lacks part of the entire cerebrum, and the remaining brain tissue is often exposed to injury from amniotic fluid (Cook et al., 2008).

Anencephaly invariably is lethal, but some anencephalic infants are born alive with a rudimental brain. The absence of a functional brain makes the anencephalic incapable of consciousness or feeling pain, but the brainstem reflexes can cause actions like breathing and, occasionally, they respond to sound and touch. Anencephalic newborns are not viable or tractable and the survival is measured in hours or days (Müller et al., 2006).

The pathogenesis of anencephaly is still controversial. Either a failure of closure of the neural tube or reopening after closure has been hypothesized (Hern, 1984; Mercer et al., 1987; Byrne, 2005). Several studies have suggested that anencephaly arises from exencephaly, in which the cerebral tissue not covered by the meninges, the cranium and skin is progressively destroyed within the uterus (Calzolari et al., 2004).

Despite ethical conflicts, the literature shows some reports about the use of anencephalic fetuses' organs for transplantation (Davis, 1988; Salaman, 1989; Byrne, 2005; Milliez, 2008). The organ structure of anencephalic fetuses and children is almost unknown. Recently the structure of anencephalic fetal kidneys was studied (Kalaycioğlu et al., 2010). Morphology of the bladder in anencephalic fetuses are unknown. The

Abbreviations: WPC: weeks post-conception; Vv: volumetric density

objective of our study is to analyze the bladder structure in anencephalic human fetuses.

Material and methods

The present work received institutional review committee and parent approval. This work was carried out in accordance to the ethical standards of the responsible institutional committee on human experimentation.

We studied 40 bladders obtained from 40 normal human fetuses (20 male and 20 female) and 12 bladders from anencephalic human fetuses (5 male and 7 female) that died of causes non-related to the genitourinary tract (Fig. 1). The fetuses were macroscopically well-preserved and there was no evidence of congenital malformation. The gestational age of the fetuses was determined in weeks post-conception (WPC), according to the foot-length criterion. Presently, the foot-length criterion is considered the most acceptable parameter used to calculate the gestational age (Mercer et al., 1987; Hern, 1988; Platt et al., 1988; Costa et al., 2002; Favorito et al., 2004). The fetuses were also evaluated regarding crown-rump length and body weight immediately before dissection, and all measurements were taken by the same observer.

After the measurements, the fetuses were carefully dissected with the aid of a stereoscopic lens with 16/25 x magnification. The fetal bladder was carefully removed, together with kidneys and ureters.

The bladder was separated from the other structures and fixed in 10% buffered formalin, and routinely processed for paraffin embedding, and 5 μm thick sections were obtained at 200 μm-intervals. Smooth muscle, connective tissue, elastic system fibers and collagen were studied by histochemical, immunohistochemical and biochemical methods.

Sections were stained with haematoxylin-eosin to assess the integrity of the tissue. We performed the following staining: Masson's trichrome, in order to quantify connective tissue and smooth muscle; Weigert Resorcin Fucsin with previous oxidation in order to observe elastic system fibers; and Picro-Sirius Red with polarization for observation of different collagen types. Connective tissue, smooth muscle and elastic system fibers were quantified by a stereological method (Cavalcanti et al., 2007).

Five sections were stained, and five fields of each section were selected. All selected fields were photographed and the images were captured with Olympus BX51 microscopy and Olympus DP70 camera. Images were transferred to Image Pro software. The fibers were quantified using software Image J in order to determine the volumetric density (Vv) of each component (Fig. 2).

The immunohistochemical analysis of the collagen type III (mouse monoclonal collagen III ABCAM) and collagen type I (mouse monoclonal collagen I ABCAM) fibers used the avidin biotin (ABC) method with positive and negative controls. The slides were previously treated with poly-L-lysine for better adherence of the sections.

For the biochemical analysis of the collagen, tissue samples were fixed in acetone. The concentration of total collagen in the bladder tissue was determined by a colorimetric hydroxyproline assay. Thus, 5 to 14 mg of dry, defatted bladder tissue was hydrolyzed in 6N HCl for 18 hours at 118°C, as previously described (Cabral et al., 2003). The assay was then carried out in the neutralized hydrolysates using a chloramine T method (Bergman and Loxley, 1963). Results were expressed as micrograms of hydroxyproline per milligram of dry, defatted tissue.

Means were statistically compared using the Unpaired T test ($p<0.05$) with Graph Pad Prism

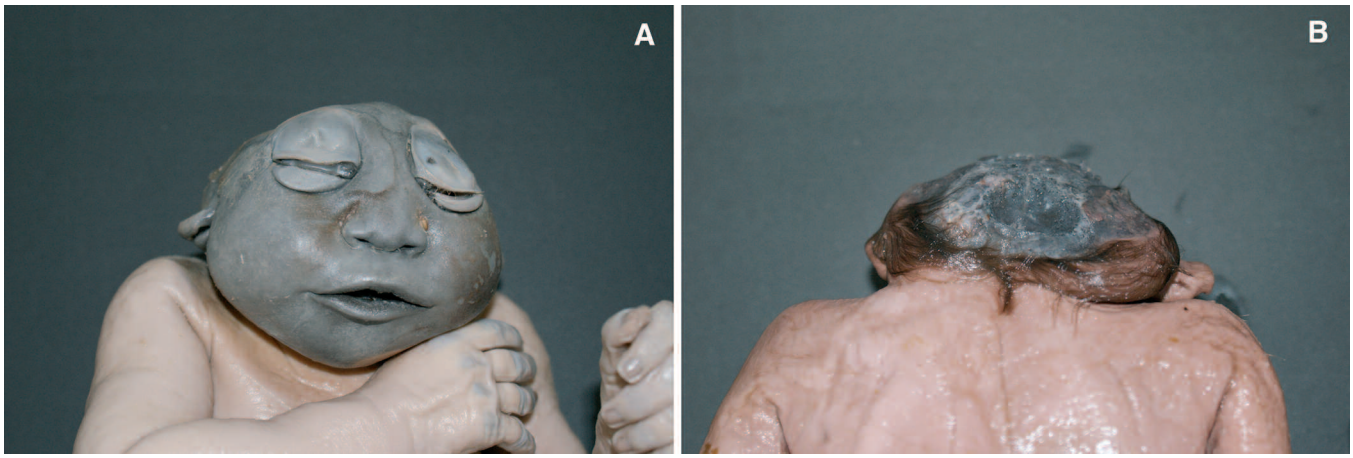

Fig. 1. The figure shows a 23 WPC well-preserved anencephaly male fetus. **A.** Anterior view of the fetus with anencephaly. **B.** Posterior view of the fetus with anencephaly.

software.

Results

The age of the normal fetuses studied ranged from 14 to 23 WPC they weighed between 125 and 950 g and had crown-rump length between 13 and 26.5 cm (Table

Table 1. The table shows the age and the fetal parameters of our sample in normal fetuses.

NORMAL HUMAN FETAL BLADDER				
AGE (WPC)	SEX	WEIGHT (g)	LENGHT (cm)	CRL (cm)
14.2	M	125	19	13
14.7	M	165	18.5	13
15.5	M	190	13	20
15.7	F	260	25.5	15.5
15.9	M	185	21.5	14.5
16.3	M	195	20	15
16.5	F	300	24.8	16.2
16.6	M	150	21.5	14.5
16.6	F	225	22	16
17.3	F	140	31	14
17.4	F	290	23.5	16
17.5	M	245	21	15
17.6	M	190	24	16
17.8	F	285	22	15.5
17.8	F	280	23	15.5
18.2	F	405	26.5	18
18.2	F	285	24.5	15.3
19.4	F	400	28.5	18
21	M	580	31	20.5
23	F	950	35.5	24

The fetuses studied ranged in age between 14 to 23 WPC, weighed between 125 and 950g, and had crown-rump length between 13 and 26.5 cm. M: Male; F: Female; WPC: age in weeks post-conception; g: grams; CRL: crown-rump length; cm: centimeters.

Table 2. The table shows the age and the fetal parameters of our sample in anencephalic fetuses.

ANENCEPHALY HUMAN FETAL BLADDER				
AGE (WPC)	SEX	WEIGHT (g)	LENGHT (cm)	CRL (cm)
18	F	265	22	13
18.8	F	170	22.5	14
18.9	F	230	22	14
19	M	230	22	14
19.3	F	245	24	14
19.6	F	330	24	14.5
19.6	M	280	21.5	14
20.1	F	280	23	14.5
20.2	M	340	26	16.5
21.2	M	420	26.4	15.5
21.2	F	320	25	14.5
21.9	M	340	26.5	16.5

The fetuses studied ranged in age between 18 to 22 WPC, weighed between 170 and 420g, and had crown-rump length between 13 and 16.5 cm. M: Male; F: Female; WPC: age in weeks post-conception; g: grams; CRL: crown-rump length; cm: centimeters.

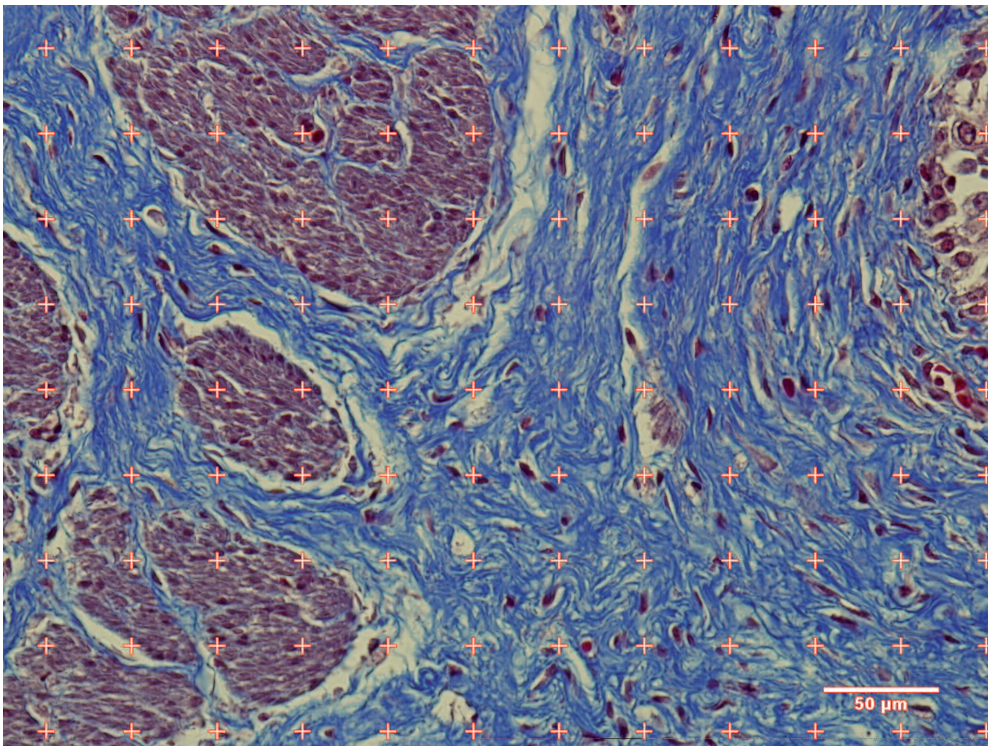

Fig. 2. Photomicrography showing the morphometric analysis of the fetal bladder. Quantification of smooth muscle cells of the bladder in a fetus with 15 WPC using the software Image J Test grid. Masson's trichrome, x 400

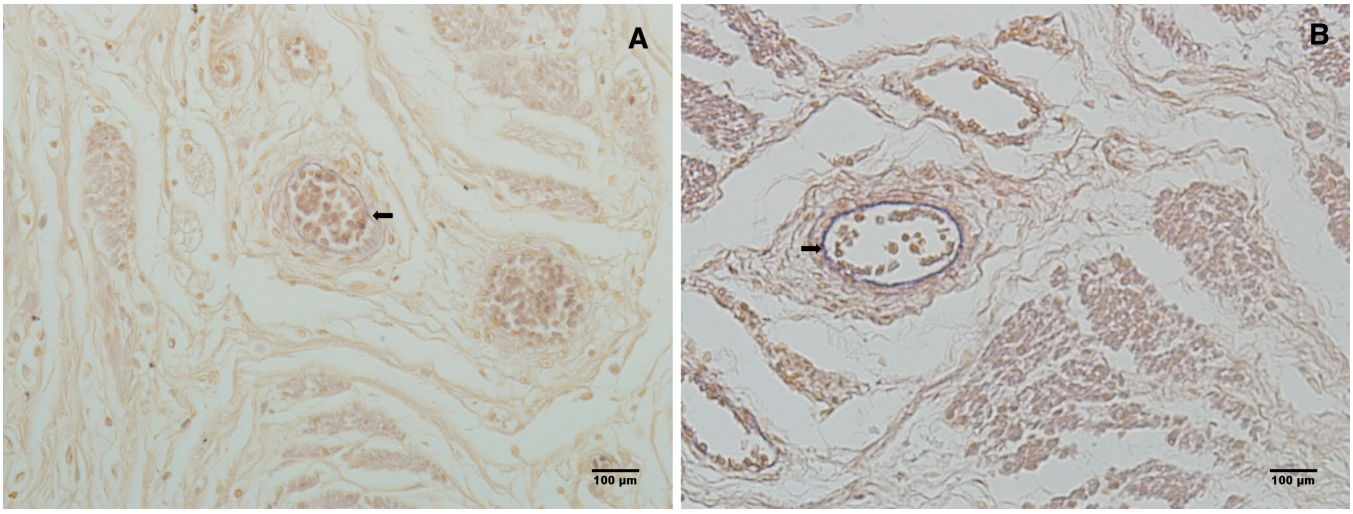

Fig. 3. Photomicrographies showing the elastic system fibers. **A.** Normal fetal bladder with 13 WPC. **B.** Anencephaly fetal bladder with 20WPC. In both cases these fibers were only observed in vessels (arrow head). Weigert, x100

1). Anencephalic fetuses ranged in age between 18 to 22 WPC, weighed between 170 and 420 g and had crown-rump length between 13 and 16.5 cm (Table 2). After dissection we did not observe any macroscopic anomalies in the urogenital system of the anencephalic fetuses.

Elastic System Fibers

We did not observe elastic system fibers in any fetal bladder analyzed. In Figure 3 we can observe bladders of normal and anencephalic fetuses stained by Weigert's Resorcin-Fucsin with oxidation. Elastic system fibers were well visualized only on the arterial wall.

Connective tissue

Stereological analysis documented a statistically significant increase ($p<0.0001$) of connective tissue in anencephalic bladder (69.71 %; 95%CI: 68.02±71.39) as compared to normal (52.74 %; 95%CI: 48.90±56.57). When we compared the gestational age with connective tissue, we did not find any correlation either in normal (r=-0.5520; 95% CI: -35.48±39.58) or in anencephalic fetal bladder (r=0.5125; 95% CI: 21.11±26.80). In figure 4 we observe the comparative graphs of connective tissue and smooth muscle in the studied groups.

Smooth muscle

Stereology analysis documented a statistically significant decrease ($p<0.0001$) of smooth muscle in anencephalic bladder (23.96%; 95% CI: 21.11±26.80) as compared to normal (38.35%; 95% CI: 35.96±40.75).

When we compared the gestational age with smooth muscle, we did not find any correlation either in normal

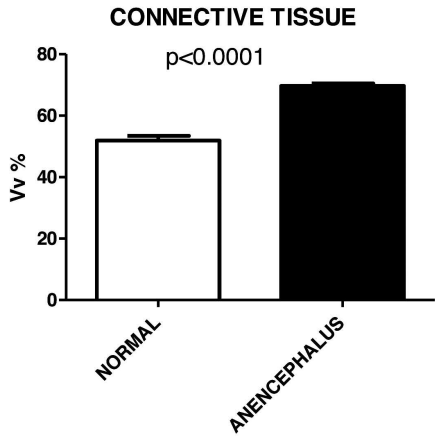

Fig. 4. Comparative graph of connective tissue (Vv) between normal and anencephaly fetal bladder. There was more connective tissue in fetus with anencephaly than in normal fetal bladder and this difference was statistically significant ($p<0.0001$)

(r= 0.3211 95% CI: -0.1416±0.6687) orr in anencephalic fetal bladder (r=-0.17724; 95% CI: -0.6818±0.4418). In figure 5 we observe photomicrographies when comparing the smooth muscle arrangement in normal and anencephalic fetus bladders.

Collagen

Biochemical analysis showed a statistically significant increase ($p<0.02$) in total collagen concentration in the anencephalic group (48117 μg/mg; 95% CI: 32.38±42.33) as compared to the normal group (37354 μg/mg; 95% CI: 39.71±56.52). In figure 6 we

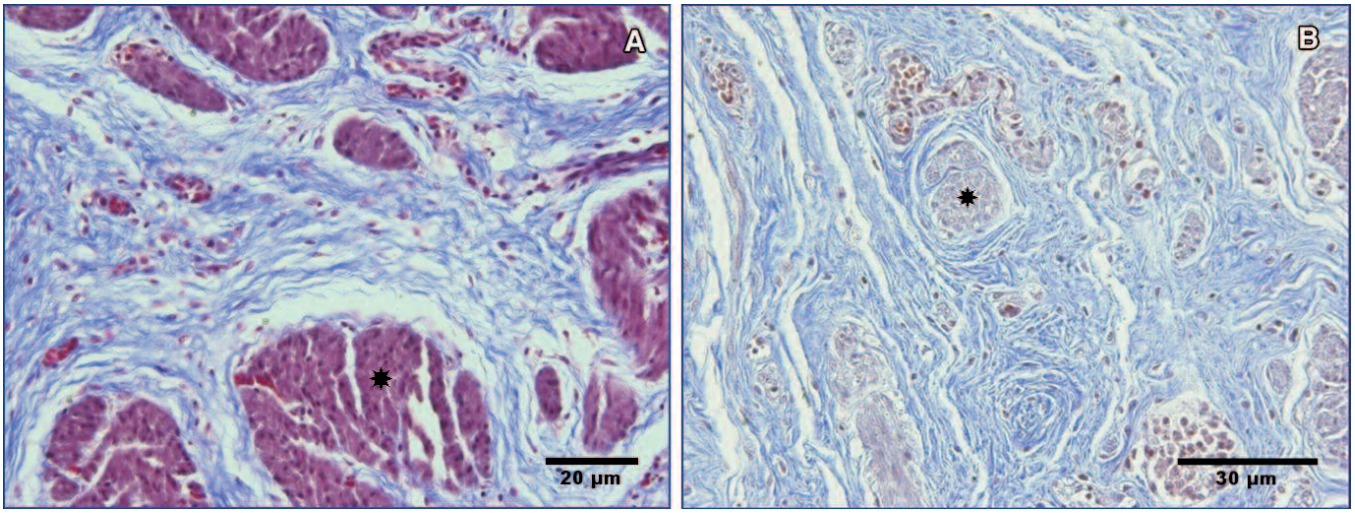

Fig. 5. Photomicrographies showing connective tissue and smooth muscle. **A.** Normal fetal bladder with 18 WPC. **B.** Bladder of fetus with anencephaly with 18 WPC. We can observe a reduction of smooth muscle(*) in anencephaly fetal bladder. Masson's trichrome, x 200

observe the comparative graph between normal and anencephalic groups.

In qualitative analysis, (immunohistochemistry) type III collagen was observed in both groups, although anencephalic bladder fetuses showed a higher quantity (Fig. 7A,B).

Regarding type I collagen, a small quantity was observed in both groups. Picro Sirius Red with polarization photomicrographies presented a high difference in colors between groups. This difference could suggest changes in the collagen fiber organization of anencephalic fetus bladders (Fig. 7C,D).

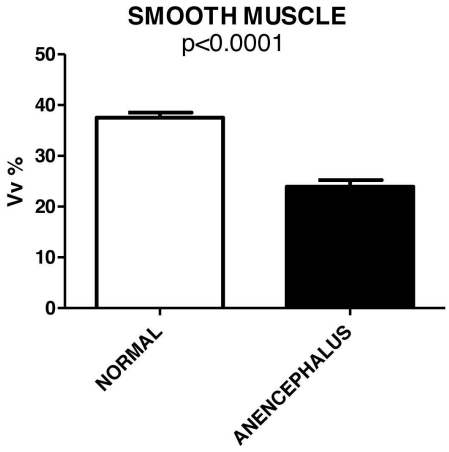

Fig. 6. Comparative graph of smooth muslce (Vv) between normal and anencephaly fetal bladder. There was less smooth muscle in anencephalus than in normal fetal bladder and this difference was statistically significant (p<0.0001)

Discussion

Knowledge of the structure of the bladder in an anencephalic fetus is of great importance, as there are reports of an anencephalic fetus transplant donor with chronic renal failure where the bladder was used to divert urine (Laberge, 1987).

Fetal bladder is identified in the tenth week post conception due to the beginning of urine production, but opinions about this beginning vary from the 11th to the 16th weeks post conception, based on ultrasounds (Patten et al., 1990; Bronshtein et al., 1993; Wilcox and Chitty, 2001). The bladder is formed from mesenchimal and endodermal cells (Ersoy et al., 2005). The most part of the urinary bladder originates from the vesical part of the urogenital sinus, while the trigone results from the absorption of the caudal region of the mesonephric duct in the development of the bladder (Moore and Persaud, 2003).

Human fetal bladder undergoes a series of vital developmental changes during 13-21 weeks of gestation, finally acquiring the typical urothelial lining and a well-developed muscular coat. Until the 11th week of gestation the remaining bladder wall consists of mesenchyme, gradually maturing to lose connective tissue. Collagen becomes apparent by the 13th WPC (Newman and Antonakopoulos, 1989).

Collagen and elastin are important components of the bladder wall, which affect bladder function. Collagen provides tensile strength, although an over accumulation may inhibit bladder contractility and the conduction of electrical impulses through the wall. Smooth muscles, which have no tendons, require more collagen. Elastin provides tissue elasticity and could help compliance (Kim et al., 1991).

Collagen increases in chronic bladder obstruction in

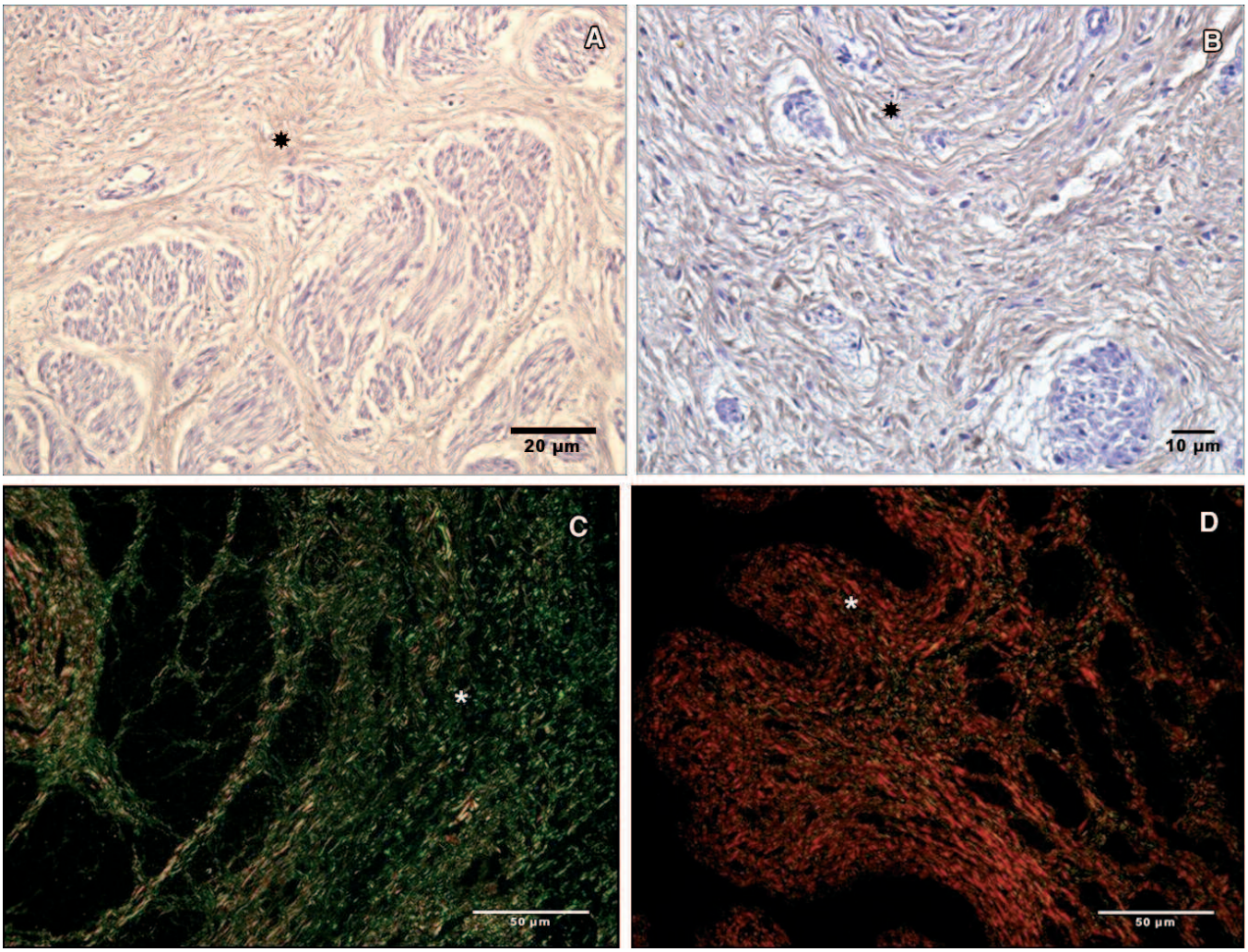

Fig. 7. Photomicrographies showing fetal Bladder collagen. **A.** Immunohistochemistry showing type III collagen (brown) in a normal fetal bladder with 17 WPC. Anti-collagen type III antibody. **B.** Immunohistochemistry showing type III collagen (brown) in anencephaly female fetal bladder with in a fetus with 16 WPC. Anti-collagen type III antibody. **C.** The photomicrography shows a predominance of green in anencephaly fetal bladder, suggesting collagen Type III presence in fetus with 19 WPC. Picro Sirius Red with polarization. **D.** The photomicrography shows a predominance of red in normal fetal bladder, suggesting collagen type I presence in fetus with 20 WPC. Picro Sirius Red with polarization. A, B, x 400; C, D, x 200.

human adults. However, a study of the obstructed human fetal bladder found that although the total amount of collagen was increased, this was proportionate to the amount of muscle, which also increased in response to obstruction. If all collagen is removed from the smooth muscle, its active force will actually be decreased. Interestingly, the abnormally large amount of collagen found in obstructed bladders is also believed to decrease muscle contractility, in addition to affecting bladder compliance (Kim et al., 1991).

In our study, we observed a significant increase of total collagen in anencephalic bladder fetuses with type III collagen predominance.

The detrusor muscle of the bladder is one of the

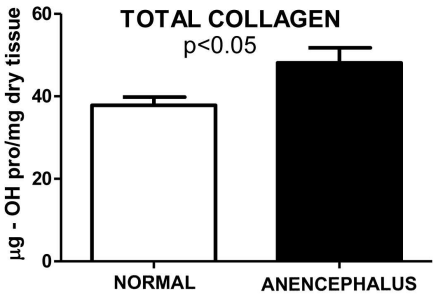

Fig. 8. Comparative graph of total collagen concentration (μg-OHpro/mg dry tissue) between normal and anencephaly fetal bladder. There was more total collagen in anencephalus than in normal fetal bladder and this difference was statistically significant ($p<0.02$).

thickest smooth muscle layers in the body. It is responsible for the main function of the bladder, namely the storage and evacuation of urine. In certain pathological conditions, such as benign prostatic hyperplasia, posterior urethral valves, spina bifida and spinal cord injury, the detrusor muscle organization and function are profoundly altered. These alterations lead to abnormal bladder compliance and subsequent high intravesical pressure, which, if left untreated, result in renal damage (Baskin et al., 2001). In anencephalic bladder fetuses, we observed a significant decrease of the smooth muscle.

The histological analysis of the developing bladder reveals that the smooth muscle is initially developed in the periphery of the bladder adjacent to the serosa surface. The signal from the epithelium must, therefore, be able to cross the submucosa mesenchyme, which to reaching the outer mesenchyme. Another explanation is that the embryonic submucosa mesenchyme that eventually will be the lamina propria of the bladder, acts as an intermediary signaling mechanism. In other words, the submucosa is able to transmit the epithelial signal to the periphery of the bladder without undergoing differentiation into smooth muscle. The connective tissue and/or basement membrane of the bladder may act as signaling channels or mediators of differentiation at their discretion (Liu et al., 2000).

Even as the bladder matures and the smooth muscle layer increases in size, a well-defined submucosa separates the urothelium from the muscle layer. The developmental architecture of the bladder implies that the signal must transverse the submucosa (Liu et al., 2000). In the bladder of anencephalic fetuses we observed a greater amount of connective tissue than in the normal fetuses' bladders. It could suggest an alteration in the complete development of the smooth muscle layer.

Elastic system fiber alterations are involved in fibrotic tissue formation; however, in our samples, we did not observe the presence of elastic fibers in the bladder. This may indicate that this extracellular matrix component appears only in the third gestational trimester in fetal bladder. Previous studies showed the elastic system fibers in other human fetal genitourinary organs (Bastos et al., 2004).

Bastos et al. (2004) observed scarce and fine elastic system fibers in the homogeneous and intense cellular tissue of the corpus spongiosum, in a fetus with 15 weeks of gestation; in a fetus with 36 weeks of gestation, there was evidence of the trabeculae of the corpus spongiosum delimitating large vascular spaces. The elastic system fibers are plentiful and organized in older fetuses (Bastos et al., 2004). This study indicates that the elastic system fibers in the genitourinary fetal system are more evident and developed in the third gestational trimester. Our sample was composed of fetuses in the second gestational trimester, probably the period where elastic system fibers are still being formed in fetal bladder.

Structural bladder alterations in anencephalic fetuses were significant in our study. The lesion in the nervous system with consequent alteration in bladder nerve regulation could be a plausible hypothesis to explain these structural changes. Anencephalic fetuses have cerebral exposition, usually with spinal cord preservation. Bladder nerves in anencephalic fetuses could be modified due to cerebral lesions with consequent brain control damage in bladder nerves. This could lead to structural alterations in anencephalic bladder fetuses.

The bladder development is significantly impaired without neural integration the structural bladder alterations cited in this paper may be of great interest to the tissue engineering.

Conclusions

The bladder in fetuses with Anencephalia showed more connective tissue, less smooth muscle and more type III and total collagen concentration. These differences could indicate that anencephalic fetal bladder has significant structural alterations when compared to normal fetal bladder.

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