

Full Length Research Paper

Histochemical determination of glycosaminoglycans (GAGs) in normal and ethanol-induced chick embryo during neural tube development

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Alcohol as a teratogenic agent inhibits cell growth, function, proliferation and migration by affecting macromolecules, and can induce cell death. Prenatal ethanol exposure causes neural tube defects (NTD) and growth deficiency in experimental animals. NTDs are a group of malformations that result in failure of neural tube (NT) closure in early embryonic development and are among the most common congenital malformations in humans. NTDs are also associated with a number of other central nervous system malformations. Basal layers are the most densely stained structures with Alcian blue which determines glycosaminoglycan (GAG) types. While all sulphated GAGs were observed in the basal layers of NT of the embryos in control and saline-injected groups, hyaluronic acid was dominant in the 10% alcohol-administered embryos. It was reduced in the 15% alcohol-administered embryos and keratan sulphate was significantly low in 20% samples. Especially in the control and saline-injected groups, chondroitin sulphate and dermatan sulphate were highly expressed around cells migrating from the NT, while the same were reduced in 10% alcohol-administered embryos. In 15% alcohol-administered embryos, while the heparine and heparane sulphate were dense around cells migrating from the NT, staining specificities were decreased in 20% alcohol-administered embryos in same regions. Increased alcohol degrees cause decrease of the GAG types in both areas.

Key words: Neural tube development, alcohol, glycosaminoglycans (GAGs), chick.

INTRODUCTION

During embryonic development, the neural tube (NT) is the first organ to develop and it comprises the nervous system in adult stage in vertebrate (Dickinson et al., 2004; Massa et al., 2009). Development and formation of the NT occurs in five stages: induction, elevation of neural plate, bending of neural folds toward the midline, migration of neural crest cells and fusion of neuroectoderm and surface ectoderm. It is indicated that neural cell adhesion molecule (N-CAM), fibronectin, laminin and proteoglycans, especially sulphated glycosaminoglycans (S-GAG), play a role in elevation of neural plate and conjunction of neural folds (Newgreen et al., 1997; Hall et al., 2001). GAGs, located in the membrane and on the surface of the neuro-epithelial cells, are implicated in the formation of NT, proliferation

and formation of the notochord and neurons (for example, motor neurons, astrocytes, oligo-dendrocytes) and differentiation and occurrence of somites that form several tissues and organs (Soulintzi and Zagris, 2007).

Some chemical agents (such as rifampicine, talidomide, fenitoin, retinoic acid and alcohol), heavy metals (for example, Zn, Cr,) (Asmatullah and Shakoory, 1998), physical factors (high or low temperature and radiation) (Wang, 2001) or some infectious diseases which cause developmental anomalies (Colla et al., 2001) are accepted as a teratogen in fetus and embryos. Living things are most responsive to teratogens in the embryonic stage during development as central nervous system, heart, sense organs and other organs develop during the said stage and defects in this stage are permanent and can lead to death (Ornoy, 2007).

It has been declared that especially alcohol causes failure of migration and integration of neurons and glial cells during embryogenesis, frontonasal tissue loss,

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Table 1. Staining specificities of AB-PAS sequences at different electrolyte concentrations.

Stain	Interpretation of reaction	Reference
AB pH 5.8 in 0.025 M MgCl ₂	Sulphated GPs, all acid mucins	Scott and Dorling (1965), Bancroft et al. (1994) and Scott (1996).
AB pH 5.8 in 0.06 M MgCl ₂	All acid mucins	
AB pH 5.8 in 0.3 M MgCl ₂	Weakly and strongly sulphated mucins	
AB pH 5.8 in 0.65 M MgCl ₂	Heparin, Heparan sulphate and Keratan sulphate	
AB pH 5.8 in 0.9 M MgCl ₂	Keratan sulphate	
PAS	GPs with oxidizable vicinal diols and /or glycogen	Yamabayashi (1987) and Bancroft et al. (1994).

AB, Alcian blue; GPs, Glycoproteins; PAS, periodic acid Schiff's.

cranial neural crest cell death, inhibition of receptor proteins [for example, sonic hedgehog (Shh) receptor protein] and GAGs which have a significant role in cell differentiation (Sari and Gozes, 2006). However, it has been thought that alcohol impairs cell function by affecting signal transduction through receptors associated with G protein and tyrosine kinase and ionotropic receptors during embryonic development and leads to apoptosis (Newton and Messing, 2006). Furthermore, it triggers cell death by destabilizing Ca²⁺ homeostasis in neurons (Webb et al., 1997). Apart from these basic mechanisms, it is indicated that alcohol changes conformation and quality of N-CAM, a member of immunoglobulin super family which is a cell adhesion molecule, and also increases expression of N-CAM in polysialated or high molecular weight form (Hirai et al., 1999). This distinct form of N-CAM increases homophilic binding in cell-cell adhesion and leads to accumulation of cells. Thus, migration of the cells is inhibited.

The aim of this study was to determine alteration and distribution of GAGs located on the surface of the cell in normal and alcohol-induced chick embryos during neural tube development.

MATERIALS AND METHODS

In this study, specific pathogen free (SPF) chicken eggs were used. Eggs were divided into two groups which were the control (n: 30) and exposure (saline, 10, 15, 20% alcohol) (n: 30) and were incubated in 60 to 80% humidified atmosphere at 37.5°C. At 33 h of incubation (HH-stage-9-10), (Hamburger and Hamilton, 1951) eggs were rinsed with 70% alcohol and a piece of plastic tape was placed close to the air cavity of the eggs, and a small hole was opened for injections. 100 µl of 10, 15 and 20% ethanol solutions and 100 µl saline were injected under the embryo discs with a 30-gauge syringe in the test groups and sham group respectively. Then, in all the groups, the eggs were closed with a sterile tape and incubation was continued for 55 to 60 h. The control group did not undergo any procedure. After the incubation (HH-stage-16-17), all eggs were reopened and embryos were fixed in Saint-Marie's solution (99 ml 96% ethyl alcohol and 1 ml glacial acetic acid) at +4°C, dehydrated in graded ethyl alcohols, passed through xylene (Riedel-de Haën), and embedded in paraffine (Isolab) blocks.

Sections were stained with Mayer's hematoxylin-eosin, sodium acetate buffer (pH 5.8) (Merck) and 0.1% Alcian blue (AB) at different MgCl₂ concentrations (Merck) (0.025, 0.06, 0.3, 0.65, 0.9 M) (Scott and Dorling, 1965; Tuckett and Morriss-Kay, 1988; Bancroft et al., 1994; Scott, 1996) and were combined with periodic acid-schiff (PAS) (Sigma) (Bancroft et al., 1994) to demonstrate the general structure and GAG's of the NT respectively. GAGs were identified by critical electrolyte concentrations at which the polyanions changed from binding AB to MgCl₂²⁺. In this method, at low molarities of MgCl₂²⁺ (for example, 0.06 M) both carboxylated (that is, hyaluronic acid) and sulfated acid mucins (that is, chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate) were stained, whereas at higher molarities (for example, 0.3, 0.65, 0.9 M) only sulfated acid mucins were stained (Table 1). While they did not show the total amount of GAG, the change in dyeing provides a subjective assay regarding the type of GAGs (Bancroft et al., 1994; Scott, 1996).

Stained sections were observed under light microscope (Olympus BX40) and photographed. Results were compared with other histochemical reactions in the literature. Distribution of GAGs were scored from less (+) staining to dense (+++++) staining (Table 2).

RESULTS

Light microscopic results of neural tube development

At HH-stage-16-17, it was observed that closure of the neural tube was completed and neuroepithelium and surface ectoderm fused to differentiate into two distinct epithelia; and this stage was accompanied by nascent neural crest cells migrated ventrally from the dorsal edges of the currently closed neural tube in trunk region. Neuroepithelial cells, radially located around the lumen of the NT and having prominent cytoplasm and spheric and oval shape nuclei, formed a thick pseudo-stratified epithelium. Additionally, the notochord had developed beneath the neural tube, and a pair of somites also formed along the right and left side of the neural tube (Figure 1A).

Embryos of alcohol exposure and sham groups were similar to the control group, and any abnormal appearance were not observed (Figure 1B, C and D).

Table 2. Graduation of staining with AB prepared in critical electrolyte concentrations (CEC) of GAGs in the basal layer of the neural tube and the notochord luminal surface of the neural tube and around the cells migrating from the neural tube in 2.5 days chicken embryos of the control, sham and test groups.

Experimental group	Area	CEC				
		0.025 M MgCl ₂	0.06 M MgCl ₂	0.3 M MgCl ₂	0.65 M MgCl ₂	0.9 M MgCl ₂
Control/ sham	Basal layer of neural tube	+++++	++++	++++	+++	++
	Luminal surface of neural tube	++++	+++	+++	++	±
	Basal layer of notochord	+++++	+++++	+++++	++++	+++
	Around the migrating cells from neural tube	+++++	++++	++++	++	+
10% Alcohol	Basal layer of neural tube	+++++	++++	+++	++	++
	Luminal surface of neural tube	+++	+	+	±	±
	Basal layer of notochord	+++++	++++	++++	++++	+++
	Around the migrating cells from neural tube	++++	+++	+++	++	+
15% Alcohol	Basal layer of neural tube	++++	+++	+++	++	++
	Luminal surface of neural tube	++	+	±	±	±
	Basal layer of notochord	+++++	++++	++++	++++	+++
	Around the migrating cells from neural tube	+++	++	++	++	+
20% Alcohol	Basal layer of neural tube	+++	++	+	+	±
	Luminal surface of neural tube	+	±	±	±	—
	Basal layer of notochord	++++	+++	+++	+++	++
	Around the migrating cells from neural tube	++	++	++	+	±

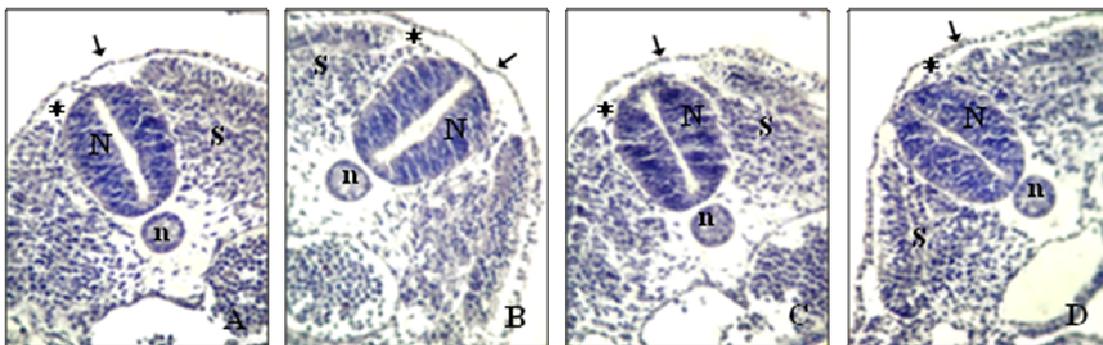


Figure 1. Control + Sham (A), 10 (B), 15 (C) and 20% (D) alcohol-injected 2.5 days embryos. N, Neural tube; →, surface ectoderm; n, notochord; s, somite; *, migration of neural crest (H+E; x 40).

Histochemical results of neural tube development

AB staining was considered in the basal layer and luminal surface of the NT, basal layer of the notochord and cells migrating from the NT. Staining density of the types of GAGs were scored in (HH stage) 2.5 days embryos of the control, sham and exposure groups by staining with AB prepared in different critical electrolyte concentrations (CEC) as summarized in Table 2.

Areas stained with AB of the control and test groups were compared. In the control and sham groups, all

sulphated-GAGs were present in the basal layer of the NT, with chondroitin sulphate (Ch-S), dermatan sulphate (DS), heparine (HP) and heparane sulphate (HS) dominant in the luminal surface of the NT. Keratan sulphate (KS) was strongly expressed in the basal layer of the notochord, and Ch-S and DS were dense around the cells migrating from the NT (Figure 2 A1 to 5). In 10% alcohol-injected embryos, it was observed that hyaluronic acid (HA), Ch-S, DS and KS were dominant in the basal layer of the NT, glycoprotein (GP), Ch-S and DS were dominant in the luminal surface of the NT, KS was

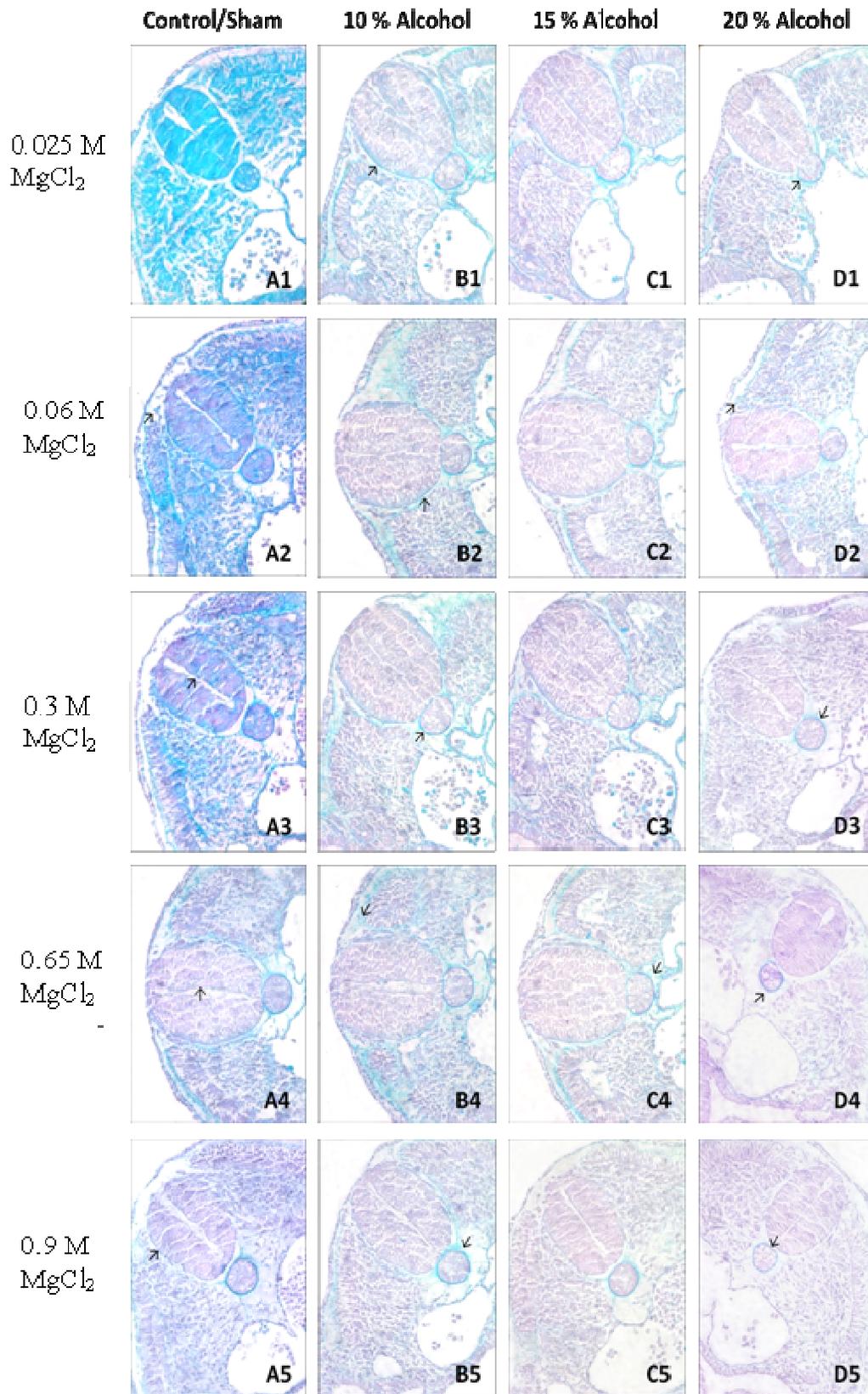


Figure 2. Staining of AB prepared in CECs and PAS in the control and sham (A1-5), 10 (B1-5), 15 (C1-5) and 20% (D1-5) alcohol-injected 2.5 days embryos. GAG specific blue staining by AB (→) (x 40).

dominant in the basal layer of the notochord and Ch-S, DS and KS were dominant around the cells migrating from the NT (Figure 2 B1 to 5). In 15% alcohol-injected embryos, Ch-S, DS and KS were dominant in the basal layer of the NT, HA was dominant in the luminal surface of the NT, KS was present in the basal layer of notochord and KS and HP, HS and KS dominated around the cells migrating from the NT (Figure 2 C1 to 5). In 20% alcohol-injected embryos, HA, HP and HS were dominant in the basal layer of the NT, GP was dominant in the luminal surface of the NT, KS was dominant in the basal layer of the notochord along with HP and HS and Ch-S, DS, HP and HS were observed around the cells migrating from the NT (Figure 2 D1 to 5).

AB staining of the control and test groups were compared with each other. In control and sham groups, all S-GAGs were observed in the basal layer of the NT while HA and KS were dominant in 10% alcohol-injected embryos, and HA and KS were decreased in 15 and 20% alcohol-injected embryos respectively. In the control and sham groups, GP, Ch-S, DS, HP and HS were dense in the luminal surface of the NT and both GP and types of GAGs were significantly diminished in 10, 15 and 20% alcohol-injected embryos. The dominant type of GAG in the basal layer of the notochord both in the control and test groups was KS. All of the S-GAGs, especially Ch-S and DS were predominantly found around the migrating cells from NT in both groups, while the Ch-S and DS reactivity were decreased in 10% alcohol-injected group, HP, HS and KS were dense in the 15% alcohol-injected group and Ch-S and DS intensities were dominant in 20% alcohol-injected group (Figure 2).

Eventually, staining specificity of all areas was identical both in the control and sham groups. Basal layers were the ones most densely stained. It was observed that increased alcohol concentration caused decrease of GAG intensity in all areas of administrated embryos. In conclusion, while the GAG intensity was strongly decreased in the luminal surface of the NT, GAG specificity was altered according to administrated alcohol concentrations in other regions.

DISCUSSION

In vertebrates, the neural tube is the embryonic structure that forms the central nervous system in adults. Notochord is an essential formation during normal vertebrate development, it supports embryos and is a source of inductive signals for regulation of the surrounding tissues. It has been reported that notochord has a major role in proliferation, differentiation, recognition, and migration of cells, an anomaly of notochord inhibited neurulation and thus development of the central nervous system (Sugimoto et al., 2005).

It is known that during the developmental process, type of teratogen and its dosage, as well as the developmental

period of fetus which was affected are important (Tuormaa, 1996). There are several studies reporting that teratogens (rifampicine, talidomide, fenitoin, diazepam, retinoic acid and alcohol) and other factors (high temperature, radiation, some infectious diseases) impair NT during organogenesis. Alcohol, well accepted as a teratogen, causes malformations such as fetal alcohol syndrome (FAS), impaired heart and musculoskeletal development, head-face and nervous system abnormalities and other malformations that have various unwanted outcomes such as physical and mental damage (Tuormaa, 1996).

Teratological studies using alcohol have shown that cell death occurs in the primitive neural and facial tissue. In particular, cell death was observed at the neural crest cells in chick embryo, which leads to reduction in size of the embryos (Smith, 1997; Ahlgren et al., 2002). Furthermore, alcohol can damage the growth, function and life span of neurons by harming the developing brain and also can impact migration of these cells (Hirai et al., 1999).

Bannigan and Burke (1982) injected 25% ethanol to mice on day 8 of pregnancy and after histopathological examination, they reported that the neural tube had been damaged due to intensive necrosis within the neuroepithelium. Sari and Gozes (2006) applied an alcohol-containing diet to mice from day 7 to day 14 of pregnancy, and reported a detriment in the brain on embryonic day 15 (E15). It was suggested that size of brain reduction was due to inhibition of cell proliferation and migration along with induction of cell death due to alcohol toxicity.

The most striking feature of organisms is development of a highly complex structure in working order. The myriad of cells that make up living things have internal structures composed of complex molecules. Embryonic life progresses by having cells capable of proliferation and differentiation that build tissues and is ensured by continuous differentiation through the final stage of development. This process is completed with cooperative function and interaction of various extracellular matrix glycoproteins (Nakanishi et al., 1986; Davies, 1996), ECM receptors, PGs (Elisabeth et al., 1998), cell adhesion molecules (Buxton and Magee, 1992; Davies, 1996), intracellular cytoskeleton proteins, growth factors, hormones and their receptors (Adamson, 1993), ECM enzymes and their inhibitors (Kleiner and Stetler-Stevenson, 1993). The relationship among these distinct macromolecules is a common trait of epithelial-mesenchymal interactions during development of organ systems (Elisabeth et al., 1998).

Macromolecules that affect neurulation are proteoglycans, cytoskeleton proteins and non-identified proteins from human breast milk. Glycosaminoglycans are unbranched repeating disaccharide units that contain negative charges, coupled with a protein core form proteoglycans (Gill et al., 2010; Skandalis et al., 2003). Not all GAGs are covalently bound to PGs; hyaluronan.

The hyalectin family of CSPGs bind HA, an association stabilized by link protein. It has been indicated that GAGs have a role in morphological alterations, migration, proliferation and act as adhesion molecules in the ECM and on the surface of the cell membrane (Skandalis et al., 2003). Moreover, GAGs have functions in many morphogenetic mechanisms that are critically important in embryonic development (Morriss-Kay and Tuckett, 1989).

In this study, GAGs were identified by critical electrolyte concentrations at which the polyanions shifted from binding AB to $MgCl^{2+}$. AB stains polyanions had increasing selectivity as the $MgCl^{2+}$ concentration in the staining solution increased (Table 1). This method enables molecular recognition because of the conspicuous alteration from a fully "painted" polyanion to "non-painted" polyanion (Scott, 1996). While the total concentration of GAG was not shown, the change in dye intensity allows a subjective opinion about the type of GAGs.

In our study, we applied this method and found that basal layers of NT and notochord were the most densely stained regions in which regional differences were observed. Several studies have shown that the amount of GAGs is high in basal layers during fetal and neonatal periods and that their distribution exhibits regional and transient differences during stages of growth and, moreover, that GAGs are fundamental regulators in cell-ECM interactions (Toole, 1991; Soto-Suazo et al., 1999).

Our results demonstrate that in the control and sham groups, all S-GAGs were dominant in the basal layer of the NT, KS was dominant in the basal layer of the notochord, and Ch-S and DS were prevalent in the luminal surface of the NT and around the cells migrating from the NT. In 10% alcohol-injected embryos, HA was dominant in the basal layer of the NT, GPs were especially dominant in the luminal surface of the NT and Ch-S and DS were low around the cells migrating from the NT. In 15% alcohol-injected embryos, HA was also low in the basal layer of the NT, all types of GAGs were reduced in the luminal surface of the NT and HP and HS were dominant around cells migrating from the NT. In 20% alcohol-injected embryos, HP and HS were the most densely stained GAGs in the basal layer of the NT, KS was clearly reduced in the basal layer of the notochord and all GAGs were reduced in the luminal surface of the NT.

There is an inverse relationship between amount of HA and Ch-S along migratory pathways during cell migration during embryonic development. Previously, it was reported that HA establishes a suitable environment for cell migration (Toole, 1991; Soto-Suazo et al., 1999; Gall, 2010; Vabres, 2010) and plays an effective role in regulating morphogenetic movements (Spicer and Tien, 2004) while Ch-S is important in controlling cell migration (Toole, 1991; Henderson and Copp, 1997; Soto-Suazo et al., 1999). Cranial neural crest cells that form facial

structures are released from the surface ectoderm and migrate into HA-rich and cell-absent region (Greenberg and Pratt, 1977; Erickson, 1988). In another study, an autoradiographic analysis was performed by injecting ^{35}S -sulphate to chicken embryos and it was ascertained that the amount of HA and S-GAGs was enhanced during gastrulation stage in which the initial cell migration begins (Skandalis et al., 2003). This finding suggests strongly that GAGs have a role in cell migration and differentiation. As a result of autoradiographic analysis with chicken embryos during HH 9 of development, it was found that HA was predominant while Ch-S was synthesized less (Greenberg and Pratt, 1977).

HS, having a key role in stimulating events during growth, provides a interaction between two cells and/or a cell and its substratum (Sudey et al., 1991; Kuure et al., 2000; Kruegel and Miosge, 2010). Furthermore, it is indicated that HS also stimulates the growth of neurons on laminin (Dow et al., 1991). Trasler and Morriss-Kay (1991) confirmed that Ch-S and HS proteoglycans are related with the closure of the NT, migration of neural crest and formation of the notochord, and splotch mutation on chromosome 1 cause a detention of the processes. It is known that Ch-S plays a role in proliferation, differentiation and adhesion of the cells (Vasan et al. 1983).

In a study conducted on chondroitinase ABC administered 10 day rat embryos, Trasler and Morriss-Kay (1991) indicated that chondroitinase ABC inhibited migration of cranial crest cells migrating from neural tube and delayed neural tube closure.

In embryonic development of the nervous system, there is a mutual interaction between the neural tube and the notochord. While the vertebrae take shape in response to substances that are produced by notochord, vertebral arch are formed in response to substances produced by neural tube (Keynes and Stern, 1988; Theiler, 1998).

Previous experimental studies suggest that Ch-S molecules secreted from the notochord are strong stimulants to form cartilage of the sclerotome (Dias et al., 1998). In addition, intervertebral discs in notochord of *Xenopus*, chick, and human fetal cartilage contain proteoglycans carrying keratan sulphate chains. This information is consistent with our work. However, in another study, it was found that KS was absent in the human fetal notochord and this finding was explained as the difference between the species (Salisbury and Watt, 1988). Therefore, it is important to recognize where and how much Ch-S and KS are attached to the core protein of proteoglycans; may be variable according to the anatomical area.

Disruption of morphological development was observed in studies about prevention of sulphated PGs or binding of GAGs chains to a core protein (Liu et al., 1991). These studies showed that PGs perform many of their biological activities via GAG chains (Elisabeth et al., 1998). It has been stated that this feature of GAGs may be associated

with composition of sugar and O-sulphation, but it is not connected with the length of chain and charge density (Platt et al., 1990).

As a result of our study, we demonstrates that moderate alcohol application led to both quantitative decreases and changes in the distribution of GAGs during neural tube development. It must be emphasized that changes that occur for each GAG is intimately associated with regulation by other proteins and genes and should be further investigated at the molecular and organelle level.

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