Effects of alcohol during secondary neurulation in chick embryos

Alkoli̇n tavuk embriyolarında sekonder nörilasyon üzerine etkileri

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ABSTRACT

Objective: Alcohol continues to be consumed even though its harmful effects are well established. One of the most common damage of alcohol consumption is fetal alcohol syndrome, characterized by craniofacial anomalies, cardiac anomalies and neural tube defects. Therefore, understanding the molecular mechanisms underlying the alcohol-induced toxicity that occur with time and dose dependent manner is very important. Most of the studies in order to understand the effects of alcohol have been carried out on early neurulation, however its effects on late neurulation are still unknown. Therefore in this study, effects of alcohol on secondary neurulation were investigated in chick embryos.

Methods: Leghorn breed of embryonic chicken eggs were used. At 50 h of incubation, 100 μL 50% ethanol solution was injected. Depending on the period of exposure to alcohol, varying degrees of pathological disorders were detected in E3, E7 and E10 days.

Results: Developmental delay, structural abnormalities, morphological abnormalities in the heart and face and especially presence of two spinal cord cavities were found. In addition, we also detected delays in the closure of the neural tube, cellular deformities and the structural abnormalities in notochord. While eNOS, iNOS, and TUNEL levels increased, while laminin levels decreased.

Conclusion: In this study during late development, significant alcohol-induced morphological and histopathological changes were observed. We also determined Increased level of oxidative stress caused by alcohol was accompanied with the changes in matrix composition. Better understanding of these mechanisms which affect the cell behavior is important and will allow learning of harmful effects of alcohol.

Keywords: Alcohol, neural tube defect, secondary neurulation

ÖZ


Yöntem: Leghorn cinsi embryyonik tavuk yumurtalarını kullandı. Kuluçka işleminin 50. saatinde, 100 μL %50 etanol çözeltisi enjekte edildi. Alkole maruz kalan embriyonların embrional gelişiminin en doğru etkisi edildi. Alkol maruz kalma süresine bağlı olarak, E3, E7 ve E10 günlerinde değişik derecelerde patolojik bozuluklar belirlendi.

Bulgular: Gelişim gerilgi, yapışal anomaliler, kalp ve yüzeyeki morfolojik anomaliler ve özellikle iki spinal kord boşluğunun varlığı saptandı. Bunun ek olarak, nöral tüpün kapanmasında gecikmeler, hücreSEL deformasyonlar ve notokordun oluşumunda anomaliler belirlendi. ENOS, iNOS ve TUNEL düzeyleri arttı, laminin önemli ölçüde azaldığını gösterdi.

Sonuç: Bu çalışmada mevcut verilerde alkolün yol açtığı, önemli ve hücreli morfolojik ve histopatolojik değişiklikler gözlemendi. Alkolden kaynaklanan oksidatif stres düzeyindeki artışa matris kompozisyonundaki değişiklikler eşlik etti. Hücre davranışı etkileyen bu mekanizmalar daha iyi anlaşılmasını önemlili olup, alkolün zararlı etkilerinin öğrenilmesine izin verecektir.

Anahtar kelimeler: Alkol, nöral tüp defekti, sekonder nörilasyon

Araştırma

Alındığı tarih: 02.11.2017
Kabul tarihi: 05.11.2017

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INTRODUCTION

Neural tube is formed by primary neurulation ventrally and secondary neurulation dorsally at the structural overlap between the caudal end of the neural plate and cranial end of the tail bud (1). Full and complete neurulation is very important for the development of brain and spinal cord. Neurulation has to be successfully completed for a normal structural and functional development of various tissues and organs associated with the central nervous system. It is known that some diseases and physical abnormalities can occur in later stages of life due to exposure to agents with teratogenic effects during neural tube development (2). Alcohol, which is a chemical agent, can easily pass the placenta barrier, reach the fetus and cause developmental abnormalities such as seen in fetal alcohol syndrome (FAS). FAS was first described by Jones and Smith in 1973 and characterized by prenatal and postnatal growth retardation, craniofacial anomalies, central nervous system dysfunction, and anomalies involving the musculoskeletal system, heart, eyes and kidneys as reduced proliferation, disrupted DNA and protein synthesis, and apoptosis contribute to the effects of alcohol on growth retardation (2,3). However, the exact molecular pathways leading to FAS are still unknown.

NO is a potent molecule that plays an important role in intra-cellular and inter-cellular messaging systems (4). It exhibits antioxidant effects via detoxification of reactive oxygen species (ROS). Studies on ROS have argued that increased oxidative stress disrupted the functions of the mitochondria, resulting in neuronal disorders and caused embryonic malformations characterized by high levels of apoptosis (5). Peunova et al. (6) argued that NO altered the cell behavior via kinases, cytoskeleton, scaffold proteins and epigenetics. Ron and Messing (7) also lend support to Peunova et al. (6) by showing that alcohol caused NTD through similar pathways.

During the development of the neural tube, mesenchymal cells become polarized to form the neural tube epithelium and the basement membrane is formed by accumulation of laminin and fibronectin especially from large glycoproteins. Laminin is involved in adhesion of the cells to the basement membrane. Further, it communicates with intracellular skeleton and guides the cellular function. Neuronal NO synthase (nNOS; NOS-1) and endothelial NO synthase (eNOS; NOS-3) are constitutively expressed and do not vigorously respond to extracellular stimulation. In contrast, inducible NO synthase (iNOS; NOS-2) actively responds to extracellular changes, with a marked upregulation in expression and activity of laminin. It has been thought that the presence of laminin in the neural tube and mesenchyma was not altered much during secondary neurulation and that it was there to form a boundary. Fibronectin, on the other hand, has been found in abundance especially in regions where neural crest cells were populated. It has been shown that mesenchymal cavitations which occur during neurulation but not related to neural tube contain copious amounts of fibronectin which has been considered as an adjunct to cell-to-cell adhesion (8). When the oxidative stress is increased in biological tissues, the cells cannot fulfill detoxification, resulting in destruction of the cytoskeleton, reduction in adhesion capacity, cell degeneration and, subsequently, cell death (9). Loss of cell matrix and adhesion capability affects cell cycle, inhibits growth and induces apoptosis (5).

Even though there is a debate as to whether secondary neurulation in chick is similar to posterior neural tube development in humans, it is highly possible that similar molecules are used. Defects in this region are also believed to occur similarly (10). It has been shown that laminin is present in mesenchymal-epithelial transition zones throughout HH-18 and 20 stages and that it provides polarization of cells there and contributed to the development of the basement membrane. This environment with these cells produces secondary neurulation (11). However, exact effect of laminin in this period is yet to be established.

Neural tube defect is one of the central nervous system disorders that cause very important social, economical and medical problems. Although there have been many studies on primary neurulation, studies on the function of secondary neurulation and caudal region are limited. In the present study, effects of ethanol application on secondary neurulation in
chick embryos were investigated. Possible developmental anomalies and mechanisms underlying these anomalies have been investigated with e-NOS and i-NOS staining with regard to oxidative stress, laminin α1 with regard to matrix molecules and TUNEL staining with regard to apoptosis.

MATERIAL and METHODS

We used Leghorn breed of embryonated chicken eggs which were supplied by Republic of Turkey Ministry of Agriculture and Rural Affairs, Bornova Veterinary Control and Research Institute. Eggs were divided into three groups (each n=10) as Control, Sham and ethanol treated groups. They were incubated in about 60-80% humidified atmosphere at 37.5°C. At 50th h of incubation which corresponds to HH-stage-13-14 (12) they were rinsed with 70% ethanol and a piece of plastic tape was placed close to the air cavity of the eggs, and a small hole was opened for injections. Hundred μL 50% ethanol solution and 100 μL saline (sham) were injected under the embryo discs with a 30-gauge syringe while the control group did not undergo any procedure. Then, in all the groups, the eggs were closed with a sterile tape. Samples were taken at 1st (E3 days group), 5th (E7 days group) and 8th (E10 days group) days after the injection. Embryos were fixed in 10% buffered formalin solution, dehydrated in graduated ethyl alcohol and were passed through xylene (Riedel-de Haën, Germany) and embedded in paraffin (Isolab, U.K.) blocks. All samples of 5 μm-thick serial sections were taken on normal and poly-l-lysine coated slides (Sigma, U.K.). Sections were stained with Mayer’s haematoxylin-eosin (HE) (Sigma U.K.) to demonstrate the histological structure. Also eNOS (RS-654, CA, USA), iNOS (RS-651, CA, USA), laminin α1 (SC-5582, CA, USA) and TUNEL (Millipore-s7101, CA, USA) immunohistochemistry stainings were performed in order to detect possible effects of proteins thought to be in the mechanism (15). Sections were viewed under Leica (DM 4000B) light-field microscope at various magnifications and images were acquired by Olympus (DP 71) camera.

Immunohistochemistry: The sections were incubated at 60°C overnight then dewaxed in xylene for 30 minutes. After rehydrating through a decreasing series of ethanols, sections were washed in distilled water and PBS for 10 minute. They were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37°C for 15 minutes and washed again with PBS. Sections were delineated using a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H2O2 for 15 minutes to inhibit endogenous peroxidase activity. After this procedure, sections were washed with PBS and incubated with primary antibodies to iNOS (1:100 dilution; Zymed, 61-7700 South San Francisco, CA) and eNOS (1:200 dilution; Biomol, SA-258, Hamburg, Germany) for 18 hours. After washing, the sections were incubated with biotinylated IgG and then with streptavidin-peroxidase conjugate (Histostain-Plus Bulk Kits; Zymed, South San Francisco, CA, according to kit instructions). Then, the sections were washed with PBS, incubated with a solution containing 3-amino-9-ethylcarbazole (AEC) for 5 minutes to visualize immunolabelling, and finally counterstained with Mayer’s haematoxylin. The negative controls were treated as above, except incubation with the primary antibody was replaced by incubation with rabbit IgG or mouse IgG. Control samples were processed in the same manner except that the primary antibodies were omitted. All dilutions and thorough washes between stages were performed using PBS unless otherwise stated (14).

TUNEL method: An in situ apoptosis detection kit (Dead End Colorimetric) TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick endlabelled System, Promega) was used to detect apoptosis and all reagents listed below were included, unless otherwise stated. The sections were deparaffinized in xylene, rehydrated as above, incubated with 20 μg/ml proteinase K for 10 minutes, and rinsed in distilled water. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide (H2O2) for 5 minutes. The sections were then incubated with equilibration buffer for 10-15 seconds and TdT enzyme, and prepared according to kit instructions, in a humidified atmosphere, at 37°C, for 60 minutes. They were subsequently placed in pre-warmed working strength stop/wash buffer at room
temperature for 10 minutes, and incubated with anti-
streptavidin-peroxidase, at a 1:500 dilution in PBS,
for 45 minutes. Each step was separated by careful
washing in PBS. Labelling was revealed using DAB/
H$_2$O$_2$, nuclei were counterstained with Mayer’s hae-
matoxylin, and sections were mounted as described
previously (15).

Statistical evaluation: Immunohistochemistry was
evaluated semiquantitatively utilizing the H-score
technique by a histologist with blinded manner.
H-score (0-300) was calculated by multiplying stai-
nling intensity (0, negative; 1, weak; 2, moderate; 3,
strong) with the positively stained area (0-100%). For
TUNEL staining, each section was counted for 100
cells from randomly chosen fields by a histologist
with blinded manner. The percentage of apoptotic
cells to total number of cells was indicated as apop-
totic index (16).

RESULTS

Embryos from the shams and alcohol administra-
tion groups were observed and photographed both
macroscopically and microscopically. There was no
infected embryos among these samples. Numbers
and percentages of normal and abnormal embryos
after incubation with physiological saline and alcohol
%50 are seen in Table 1 Macroscopic evaluation:
Growth retardation, deformations in the heart and
limbs, deterioration in vesicles in brain regions, flat-
tening of the head and distortions in vascularization
were observed in E3 embryos. In addition, vascular
disorders that affect the embryos were observed.
Growth retardation in all organs, the small head, con-
tour distortion and flattening in the facial region were
observed in E7 embryos. Growth retardation, distur-
bances in the body symmetry, deformation in the
layer and pigmentation of the eyes were observed in
E10 embryos (Figure 1).

Microscopic evaluation: HE staining in E3 day
group (Stage 20) of sham group as control group;
medullary cord and ventricular layers which were
formed by neuroepitelial cells, dermomyotome, scler-
rotome and notochord were seen. The surface ecto-

Table 1. Numbers and percentages of normal and abnormal
embryos after incubation with physiological saline and.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Embryos n (%)</th>
<th>Lethal n (%)</th>
<th>Observed n (%)</th>
<th>Growth Retardation n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham 3rd, 7th, and 10th days</td>
<td>10 (100%)</td>
<td>0 (0)</td>
<td>10 (100%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>50 % 3rd days</td>
<td>10 (100%)</td>
<td>0 (0)</td>
<td>10 (100%)</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>Alcohol 7th days</td>
<td>10 (100%)</td>
<td>3 (30%)</td>
<td>7 (70%)</td>
<td>6 (85%)</td>
</tr>
<tr>
<td>10th days</td>
<td>10 (100%)</td>
<td>7 (70%)</td>
<td>3 (30%)</td>
<td>3 (100%)</td>
</tr>
</tbody>
</table>

Figure 1. Compared to sham group, ethanol administration
caus ed growth retardation, reduction of the vascularization
and, in particular, structural abnormalities of the heart or
head in E3 days. In E7 days, brain vesicles expansion, eye and
retinal pigmentation abnormalities can be considered. In E10
days, moderate growth retardation and disorders were obser-
v ed.

Figure 2. In Sham group, normally closed neural tube, nor-
mal surface ectoderm and neural ectoderm and notochord
were seen. However, in E3 days ethanol administration
group, reduction in the notochord and open neural tube were
detected. Bar: 20 μm.
derm and the neural tube was properly settled, the cavity was formed with a central channel and surrounding mesenchymal cells with the normal appearance was established and also neural tube closure was observed. However, in alcohol treated group, delay in neural tube closure, notochord shrinking, reduction in neuroepithelial thickness and differences in the cell shape and layout were observed. Although in sham group, normal basal level of e-NOS and i-NOS staining were present, these staining were more frequently seen in alcohol treated group. We observed that e-NOS staining was relatively more intense than i-NOS staining. In both sham and alcohol-treated groups, e-NOS staining was darker than i-NOS staining. Laminin α1 immunoreactivity was lower in 50% alcohol-administration group. In sham group; TUNEL staining which demonstrates the apoptosis of the neuroepithelial tissue was very low. However, in alcohol-treated group TUNEL staining was more strong than sham group (Figure 2).

On E7 day of alcohol treated group; varying degrees of disorders were revealed in ventricular layer, motor columns and dorsal root ganglia (DRG). Significant changes were seen at medullary cord maturation center channel, white-gray matter, dorsal, ventral and lateral horns (Figure 3). The main finding of this group was the presence of the double center channel and one of the channels was surrounded by ciliated ependymal cells. Also, deformation at notochord was observed (Figure 4). In sham group, normal basal level of e-NOS and i-NOS staining were present. However, these stainings were more strong in alcohol treated group. We observed that e-NOS staining was relatively higher than i-NOS staining. In both sham-, and alcohol-treated groups, e-NOS staining was darker than i-NOS staining. Laminin immu-
noreactivity was decreased. In addition, apoptotic cells were increased especially in gray matter (Figure 3).

In E10 day of alcohol-treated group; pathology was observed in mantle layer, ventral and dorsal horns of vertebral body, vertebra curves and DRG. The spinal cord and notochord were quite smaller depending on the decrease in the density of cells. Also, we observed that, the spinal cord channel disappeared, cell loss was widely seen, and cells were collected at the edge. Corruption and irregularities were found in the cells. The e-NOS and i-NOS reactivities were increased where i-NOS reactivity was more than e-NOS reactivity. Reactivity of the laminin was increased clearly. Intense apoptosis was observed in the spinal cord and notochord (Figure 5).

Microscopic examination demonstrated that decrease in pathological disorders in E7 day- samples was statistically significant (p<0.01). However, pathological disturbances were more frequent in E3 and E10 day-samples. iNOS was more frequently seen than eNOS which was statistically significant (p<0.01). iNOS was found more frequently in subsequent days however it was less significant. Laminin expression decreased in following days and this decrease was statistically significant (p<0.01). Whereas, increase in apoptotic cells were found but it was less significant (Figure 6).

DISCUSSION

Neurulation is the embryonic process that forms the brain and spinal cord. This process includes the formation of the neural plate, rise of the lateral neural folds, and eventually the fusion of these folds to create the neural tube (17). Secondary neurulation begins with the formation of medullary cord at HH stage 16 or 51-56 h of incubation. Multiple cavities develop inside the medullary cord. These cavities coalesce to form single lumen, which becomes continuous with the neurocele of the primary neural tube and finally secondary neurulation is completed by HH stage on 35th or 8th-9th days of incubation (18).

Several animal models such as chick embryos, Japanese medaka, mice, zebrafish have been used successfully to investigate the effects of ethanol on developing central nervous system (1,18). However, chick embryos, which do not metabolise alcohol to acetaldehyde until day 9 of gestation permits investigating the effects of ethanol in the absence of its primary metabolite. This is the reason why we used chick embryos in this study. In this study the relation between primary and secondary neurulation was shown, experimental analysis on this process was performed morphologically and organizations and behaviors of cells were defined. In optimization experiments, it was observed that 10%, 25% and 50%, alcohol applications caused neural tube defects when applied before neurulation (19). Although there are many studies on the primary stage of neurulation, secondary neurulation which has gained an importance due to its possible role in manipulating neural tube formation is still a mystery. In our study, 50% ethanol treatment was applied at 50th h of incubation supposing that this would interrupt or affect secondary neurulation. There was clear effect of alcohol on the secondary neurulation which also affected later development.

In addition to exposure to ethanol and many chemical agents can affect the neural tube development of chick embryos. For example, Lee and Nagele (20) administered local anesthetics under in vivo (100-200 μg/ml) and Güney et al. (21) administered diazepam (400 μg/ml) in vitro culture conditions to the chicks at the same developmental stage and found that these caused neural tube defects by affecting microfilament function in neuroepithelial cells. In a study by Greenaway and Fantel (22) rifampin (100 μg/ml) was injected to embryos and it was observed that rifampin impaired the metabolism of cytochrome p450 enzyme and thus caused neural tube defect. Some studies have reported that heavy metals and enviromental pollutants cause defects in neural tube, heart, brain and sensory organs of chick embryos where the most frequent factor FAS + (17).

Ethanol-induced anomalies are closely related to the timing of exposure. Namely, in chick embryos for example, ethanol exposure at early gastrulation through neurulation stages induces cranial defects (24,25). However, exposure at post neurulation stages causes significant growth retardation. In the studies conduc-
ted using alcohol, cell death was observed in the facial region and neural crest in chick embryos, which caused a loss in the size of chick embryos with growth retardation (26). Moreover, alcohol impairs development, function and life of nerve cells by affecting brain and prevents cell migration (27). In these studies, findings were associated with the dose and duration of alcohol exposure. Similar to these studies, in the present study, growth retardation in all organs, disturbances in the body symmetry, contour distortion and flattening in the facial region were observed macroscopically due to defects in secondary neurulation.

During secondary neurulation, defining two cell groups as central and surrounding cells and observing cavity formation in central cells point out to the importance of the secondary neurulation phase. Relations among central cells having such different cell behaviour show that the factors playing a role in making the cell stay at the centre during migration have a role in the formation of secondary neurulation (28). The fact that cells stop migrating or proliferating, but die to form a cavity for the canal or attach to the developing neural tube increases the importance of such factors. Cell behaviour mentioned here is mediated through different factors such as genetic information, environmental factors, growth factors and adhesion molecules (29). In this study, we determined significant changes at medullary cord maturation, center channel, white-gray matter, dorsal, ventral and lateral horns in E7 embryos. The main finding of this group was the presence of the double center channel and one of the channels was surrounded by ciliated ependymal cells. However, we observed that, the spinal cord channel disappeared in E10 embryos. Therefore, alcohol treatment affected all secondary neurulation processes which induced moderate abnormalities during later stages of development.

It has long been known that developmental defects occur due to increased NOS. In a related study, Ron and colleagues reported that L-N6-(1-iminoethyl)-lysine, which is used to inhibit NOS2, reduced the neural tube defects in babies of diabetic mothers. This treatment resulted in alleviation of endoplasmic reticulum stress, decline in apoptosis and reduction in congenital defects (9). Plachta and coworkers noted NTD when apoptosis was inhibited and they attributed this finding to increased NOS as well as inhibition of apoptosis (32). In the present study, increased defects as a result of alterations in NOS immunohistochemistry and concurrent apoptosis were similar to previous studies.

Other factor which is as important as cell behaviours such as migration, proliferation and differentiation (28) in the secondary neurulation of developing embryo is the behaviour of the cell. This behaviour of the cell to produce normal development depends on the ability of the cell to die at the right place, at the right time and in right number besides having an ability to die for orientation. Although the cells have the same nutrition and have no pathological reason to die, there is a programmed cell death leading to cavitation. Cell death occurs at tail bud morphogenesis in chick embryo (31). In terms of cell death and apoptosis, cell formation studies are carried out most commonly on tail region. Tail region incubated between 2-5 days contain many structures along with neural tube, notochord, somits and mesenchyme. The role of cell death is thought to be reshaping embryonic tail (32). Pyknotic nuclei were observed in studies on cell proliferation (33) and cell death was observed at 18th-22nd stages according to HH stages (12). It was observed based on the appearance of the cells at tail bud morphogenesis and TUNEL staining. Signs of apoptosis at tail bud are mostly observed at medullar cord (31). In our study, in E10 day-embryos, the spinal cord and notochord were quite smaller depending on the decrease in the density of cells. TUNEL staining revealed, disappearance of the spinal cord channel, diffuse, cell loss and accumulation of cells at the edge due to apoptosis. Intense apoptosis was also observed in the spinal cord and notochord.

Extracellular matrix proteins play significant roles in cell growth, cell differentiation, migration, polarization and the formation of basement. Laminin is a matrix molecule which participates in the formation of basement membrane. It also helps organization of epithelial cells and adhesion of these cells to the basement membrane. O’Shea (8) carried out a study on mice and showed that laminin staining was more
prominent on the neuroepithelium facing the noto-
chord and basement membrane of the side surface. In
another study, the researchers noted that laminin sta-
nining was weak in neural crest migration region and
non-existent in certain regions (34). Their finding is in
agreement with our findings. This happens possibly
to enable free migration of neural crest cells. Changes
in the amount of laminin with time have been shown
to affect cell differentiation (11). Sometimes the lumen
has been observed to develop even from a cavity.
Changes in the presence of laminin with respect to
location and time throughout neurulation may be an
indicator of its importance in the formation of base-
ment membrane and cell migration. Formation of
cavity and lumen by confluence of cavities is coinci-
dental and do not occur in an array (18). In one of the
experimental models, small amount of fibronectin
and very small amount of laminin have been identifi-
ed at the distal end of the tail bud in mouse embryos
on day 10.5, during the process of secondary neuru-
lation. It has been shown that laminin was present in
the lateral aspect of the basement membrane of neu-
roepithelium but not in the dorsolateral region in an
11 day-old embryo. Presence of copious amounts of
fibronectin in these regions on that day has been
demonstrated (8). In the present study, we found that
immunohistochemical staining of laminin became
weaker with development of the embryo and diffe-
rntiation. Decrease in staining intensity of laminin
became more prominent with alcohol application. This
can be attributed to the response of the matrix molec-
ules disruptions due to increased oxidative stress.

All of these observations demonstrated that,
secondary neurulation is an important step and alco-
hol caused moderate pathology in this process. These
findings point out that, problems are likely to develop
during the ongoing life of embryos that affect the
quality of life in the future. Ex ovo monitoring and
explanation of these effects at the molecular level,
will be helpful to understand adverse impacts of alco-
hol intake on the development.

Acknowledgement: We thank the Celal Bayar
University for support of this research thought grant
number 2005/FEF/058.

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