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The Effects of Diesel Exhaust Particles on Neural Tube Development in the Early Stage Chicken Embryo

Dizel Egzoz Parçacıklarının Erken Dönem Tavuk Embriyosunda Nöral Tüp Gelişimi Üzerine Etkileri

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ABSTRACT

AIM: Particulate matter is an important air-pollutant and its toxicity has been reported. Diesel exhaust particles (DEP) constitute a large portion of particulate matter. Therefore, we established our study to investigate the effects of DEP on neural tissue in early stage chicken embryos.

MATERIAL and METHODS: Four study groups and one control group, each of which included 24 objects were designed. Eggs were incubated for 30 hours. Solutions of DEP containing 10, 50, 100, and 200 µg/0.1 ml were prepared with serum saline. At the end of thirty hours diesel exhaust particle solutions were administered under the embryonic discs. After 72nd hour of the incubation, embryos were excised and evaluated macroscopically and histopathologically.

RESULTS: The difference between the embryos that were defined as poorly and well developed, was found statistically significant ($p<0.05$). Neural tube defects were detected in 16 of 104 embryos. Statistically significant association between the administration of DEP and development of neural tube defect was identified ($p=0.037$).

CONCLUSION: Thus, the direct neurotoxic effects of DEP, which the whole population encounters inevitably, have been shown in the early stages of embryonic development. Further studies are needed to identify the effects of these particles in the later stages of embryonic development.

KEYWORDS: Diesel exhaust particles, Embryo, Neural tube defect, Neurotoxicity, Particulate matter

ÖZ

AMAÇ: Parçacık madde, önemli bir hava-kirleticidir ve toksik olduğu bildirilmiştir. Dizel egzoz parçacıkları, parçacık maddenin önemli bir kaynağıdır. Biz de dizel egzoz parçacıklarının erken dönem tavuk embriyosunda nöral doku gelişimi üzerine etkilerini araştırmak için çalışmamızı planladık.

YÖNTEM ve GEREÇ: Her birinde 24 denek olan 4 çalışma ve 1 kontrol grubu planlandı. Yumurtalar 30. saate kadar inkübe edildi. Serum fizyolojik ile dizel egzoz parçacıklarından 10, 50, 100 ve 200 µg/0,1 ml olacak şekilde solüsyonlar hazırlandı. 30 saat sonunda dizel egzoz parçacığı solüsyonlarından embriyonik diskler altına injekte edildi. İnkübasyonun 72. saatinde embriyolar çıkarıldı ve makroskopik ve histopatolojik olarak değerlendirildi.

BULGULAR: Embriyolar arasındaki fark istatistiksel olarak anlamlı bulundu ($p<0.05$). 104 embriyonun 16 adedinde nöral tüp defekti saptandı. Tüm gruplarda dizel parçacığı verilmesiyle nöral tüp defekti gelişmesi arasında istatistiksel anlamlı ilişki bulundu ($p=0.037$).

SONUÇ: Böylece, kaçınılmaz şekilde tüm toplumun maruz kaldığı dizel egzoz parçacıklarının embriyonel gelişimin erken dönemlerinde doğrudan nörotoksik etkileri gösterildi. Bu parçacıkların embriyolojik gelişimin sonraki dönemlerindeki etkilerinin ortaya konabilmesi için ileri çalışmalara ihtiyaç vardır.

ANAHTAR SÖZCÜKLER: Dizel egzoz parçacığı, Embriyo, Nöral tüp defekti, Nörotoksisite, Parçacık madde

INTRODUCTION

In recent years, there has been a progressive increase in air pollution. Diesel exhaust, a complex mixture of gases, thousands of chemicals and particles, is a major constituent of pollution. Particulate matter (PM) is the particle component of air pollution that evidently has been found to be associated with increasing morbidity and mortality all over the world. PM is divided into three major size categories: ultra-fine (<0.1 μm), fine (<2.5 μm), and coarse (<10 μm and >2.5 μm). Ultrafine particles such as diesel exhaust particles (DEPs) which are the most toxic component of the PM consist of a carbon core and hydrocarbon compounds derived from fuel and lubricants and hydrated sulfuric acid derived from the fuel sulfur. Additionally, DEPs can adsorb polycyclic aromatic hydrocarbons (PAHs) to them. Already more than 40 PAHs have been identified to date but a number of as many as ten-fold is estimated to be adsorbed to and carried by DEPs (8,17,18,26). Once inhaled, the respirable PM (PM_{10}), owing to their small size, can enter the circulation and translocate to tissues throughout the body, including the central nervous system (CNS) probably by crossing the blood-brain barrier (BBB) (9,14,20,22-25), where ultra-fine particles are more likely to enter circulation and are associated with the major oxidative and proinflammatory effects of PM (21,28). In this regard, it is known that DEPs and ambient PM contain transition metals and other organic components that elicit reactive oxygen species (ROS) production in various cellular locations. Since the CNS is vulnerable to oxidative stress damage because of its high energy use, low levels of endogenous scavengers (e.g., vitamin C, catalase, superoxide dismutase etc.), high metabolic demands, extensive networks, and high cellular content of lipids and proteins, the possibility that the CNS might also be targeted by PM was raised. Reports showing that nanosize particles could cross the BBB (and physically enter the CNS of animals) followed this concern (15). However, the effects of DEPs on neural tissue and neural tube development in early stage embryos were not thoroughly discussed, so we established our study to investigate their effects on neural tube development in early stage chicken embryos.

EXPERIMENTAL PROCEDURE

Preparation of diesel exhaust particle suspension

DEPs (2975 Industrial Forklift) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). DEP solutions were prepared by vortexing a 200 μg/0.1 ml concentration of DEP in sterile serum saline (0.9 % NaCl) followed by sonication using water-bath sonicator (Transsonic T 460, 50-60Hz, Elma GmbH & Co KG Kolpingstr. 1-7 Singen, Germany) for 30 min to breakdown the agglomerates and achieve better suspension of DEPs. A sterile (Minisart nonpyrogenic hydrophilic filter unit, Sartorius AG-37070 Göttingen, Germany) 0.20 μm syringe filter was used to filter the sonicated DEP solution. The DEP solution was then immediately diluted to the appropriate concentrations so as to obtain 100, 50 and 10 μg of DEPs per 0.1 ml.

This study was conducted in GATA Haydarpaşa Training Hospital in cooperation with the neurosurgery and pathology departments. Fertilized, specific pathogen-free (SPF) Leghorn chicken eggs were obtained from the Manisa Chicken Research and Vaccination Facility.

Incubation and injection

One hundred twenty eggs (mean weight ± s.d.; 65 ± 2 g) were incubated at 37.2 ± 0.1 °C for 30 hours at 60% to 70% humidity. Each egg was repositioned on its axis every 3 hours. After 30 hours of incubation, randomly selected totally four eggs were sacrificed to ensure that the eggs were in stage 9 according to Hamburger-Hamilton series (4,12,13). Each egg was opened under 4X optical magnification and embryonic discs were identified. The same volume of liquid was injected under the discs with a 24-gauge syringe.

Study groups

The study was designed so as to involve four groups each of which consisted of equal number of eggs, and the rest was designated as the control group. Four eggs were sacrificed in the determination of stage and another four were found infertile during injections, therefore we obtained four groups (A,B,C,D) each of which contained 24 subjects and a control group with 16 subjects (Table I). At the end of the 30th hour of the incubation, subjects in the groups A,B,C and D were administered 0.1 ml of DEP solutions containing 10, 50, 100 and 200μg of DEPs, respectively. Each subject in the control group (n=16) was injected 0.1 ml of saline (Table I). Eggs were closed with sterile adhesive strips and incubation was continued till the end of the 72nd hour. Then, all eggs were reopened and embryos were dissected from embryonic membranes under magnification, with adherence to microsurgical rules. The harvested embryos were physically examined as to their viability, their development status, and placental vascular structure and then put into a 10% formalin solution for 24 hours and sent for histopathological evaluation thereafter.

Pathological evaluation

Photographs of the embryos were taken before fixation by formalin solution through the dissection microscope. Formalin-fixed, embryo tissue samples were then prepared to be embedded in paraffin. First, embryos were dehydrated through a graded series of ethanol solutions after washing with tap water. The embryos were incubated in xylene after 2 washes and they were transferred into a paraffin embedding mixture. Transverse serial sections (5 μm) were then taken from each group. Sections were stained with haematoxylin eosin (HE) solution. Slides were mounted using entellan and covered with glass coverslips prior to viewing and photography. Any disruption to somite or neural tube continuity was considered a neural tube closure defect.

Statistical analysis

Raw data were analyzed in SPSS for Windows 15.0 with Pearson's chi-square test, with a value of $p < 0.05$ indicating statistical significance.

Table I: Distribution of Groups at the End of the 30th Hour and Morphological Results t the end of the 72nd hour

Groups	30th hour			72nd hour		
	Sacrificed	Infertile	Injected (n)	Dead	Number of subjects evaluated	Neural tube defects
Control	4	4	16	0	16	0
$A_{(10\ \mu g)}$			24	1	23	3
$B_{(50\ \mu g)}$			24	2	22	3
$C_{(100\ \mu g)}$			24	1	23	2
$D_{(200\ \mu g)}$			24	4	20	8
Total	4	4	112	8	104	16

RESULTS

Macroscopic

Macroscopically, general development of the embryos was observed. The control group was free of the effects of DEPs and omphalomesenteric vascularization quality and quantity was detected in the physiological range. However, under the effect of DEPs, a decrease in the vascularization was observed parallel to dose augmentation from group A to D, as expected (Figure 1A-D). 7, 14, 10, and16 embryos in groups A, B, C and D, respectively were considered as poorly developed in regard to their general physical appearances (Table II), (Figure 2A-D). They were both smaller in size and had less developed brain vesicles. Growth retardation especially on the nervous system could be detected during dissection microscopy. 8 embryos were not alive at the end of 72 hours of incubation period (Table III).

HE Stain

Via HE staining, 3 neural tube defects in group A, 3 in group B, 2 in group C, and finally 8 neural tube defects (NTDs) in group D were detected (Table III). In Figure 3A-D, sample embryos with NTDs from each group are presented.

Statistical analyses

Subjects in the study are summarized in table 3. NTD was not detected in the control group. 3 subjects in group A (%13), 3 subjects in group B (%13.6), 2 subjects in group C (%8.7), and 8 subjects in group D (%40) with NTD were histopathologically displayed, where totally 16 (%18) embryos had NTD. Compared to the control group, each group had statistically significant increase in NTD incidence when the embryos were subject to DEPs ($p=0.037$, $p<0.05$). Paired Chi square test revealed statistical significance in the increase of the frequency between group D and all others except for group B, however the difference of group D was statistically significant upon total evaluation.

DISCUSSION

NTDs are the consequence of abnormal neurulation in the embryonic period and they are important congenital malformations because they can result in death and medical, financial and social problems. Genetic predisposition and environmental trigger factors play a major role in the etiology of NTDs. Although few of the environmental causes and none of the genetic factors have been fully identified in humans so far, numerous teratogens and nutritional factors have been suggested as possible causes. Exposure to chemicals is one of the known reasons for disorders related to nonclosure of the neural tube (3,5,10-12). This critical period is the first month of pregnancy and it is known as gastrulation. In humans, embryo transforms into blastocyst, thereafter gastrulation phase begins with the implantation (7th – 8th days). This is the critical stage where the three germ cell layers differentiate so as to form the organs. The early chick embryo model corresponds to the first month of embryonic development in mammals. It is well suited for the investigation of chemicals on the development of embryos. Stage eight embryos were generally chosen for these investigations since developing neural tissues exhibit a gradual variation on the degree of opening along its length that provides an excellent opportunity to study the effect of chemical agents on neural tube closure. Numerous chemical agents such as caffeine, phenytoin, alcohol, diazepam, methotrexate, meloxicam, and local anesthetics are known to cause NTDs in chick embryos (3,5,10-13). It is recently known that reactive oxygen species (ROS) are systemically increased upon certain stress factors such as excess glucose and aforementioned chemical agents which consequently interfere with cellular signaling pathways and expression of genes required for neural tube closure (6). Likewise, interest in the effects of ambient chemicals and pollutants grew in the previous decade. There

Table II: Macroscopic Evaluation of the Embryos at the End of the 72nd Hour of the Incubation

Groups	Cardiac activity, general development, vascularization	
	Good	Poor
Control $_{(16)}$	15	1 (6.3%)
A $_{(23)}$	16	7 (30%)
B $_{(22)}$	8	14 (63.6%)
C $_{(23)}$	13	10 (43.5%)
D $_{(20)}$	4	16 (80%)
Total	56	48

is growing recognition that the redox chemistry of organic chemical compounds plays a crucial role in the biological effect of ambient PM (1,7). Since then, systemic effects of PM were subject to researches, and toxicity of airborne PMs especially the ultrafine particulate matter (UPM) yielded by diesel combustion engines were accused of many illnesses including neurotoxicity (2,16). Diesel exhaust, the main particulate component of polluted air, is a worldwide health concern affecting a large number of people. Epidemiological studies suggest that susceptibility to ambient PM is variable among sensitive populations probably because of pre-existing pathologies, and exposure to UPM is dramatically increasing susceptibility to cardiovascular, respiratory, and CNS pathologies. Meanwhile, other studies focused on the fact that ambient UPM could pass the systemic circulation and even blood brain barrier and cause neurodegenerative diseases (14,22,25,27).

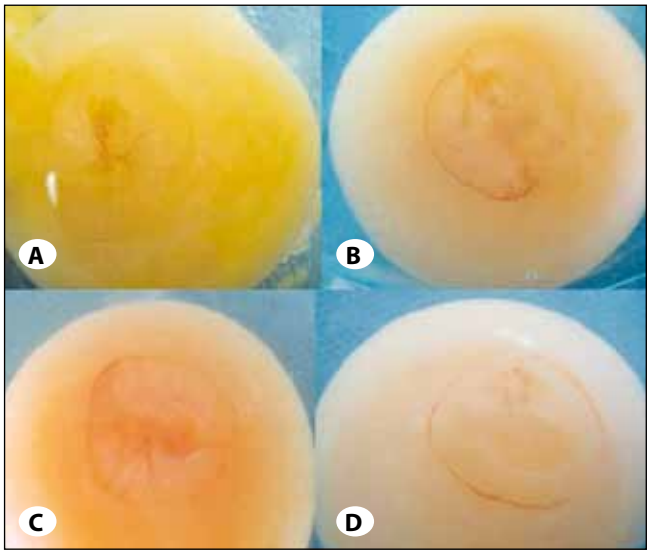

Figure 1: Vascularization was detected in physiological range. Under the effect of DEPs, a decrease in the omphalomesenteric vascularization was observed parallel to dose augmentation from group A to D. a, b, c, and d represents groups A, B, C, and D, respectively.

The present study to our knowledge is the first in ovo research questioning and demonstrating the effects of ambient diesel exhaust particles in a dose-dependent fashion on neural tube development in the early stage chicken embryo. It has been shown in various previous studies that inhalation of $PM_{2.5}$ caused oxidative stress in the airways and induces inflammatory response with upregulation of oxidative stress-sensitive pathways. Oxidative stress triggers an acute inflammatory reaction locally in the airways with the potential to up-regulate systemic inflammatory processes. Moreover, particles can cross barriers into the circulation and may directly interact with tissues to cause effects at various sites (1-7,10-13,15,16,20,24).

The results of our investigation disclosed that the application of DEPs caused retardation in general embryonic progress and further retardation in the development of the neural

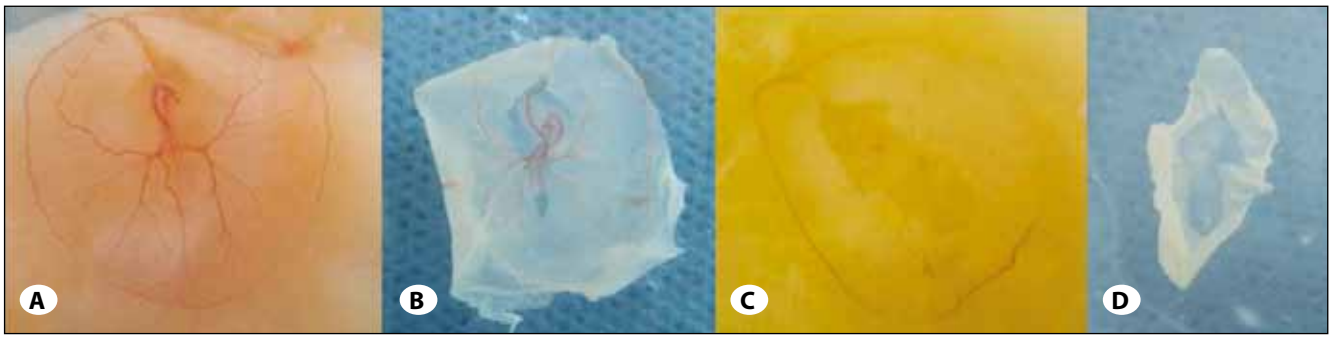

Figure 2: A) Clear and rhythmic cardiac pulsation of an embryo in the control group is observed. **B)** Cardiac activity of the same embryo lasted a long time even after dissection of the embryo with its disc. **C)** Cardiac activity of an embryo in the study group is, however, merely observed. **D)** Shortly after dissection of the embryo, cardiac pulsation ended.

Table III: Distribution of Statistical Analysis Among Groups Regarding NTD

Groups	Neural Tube Defect		Dead	Total (n)	Statistical Analysis	
	(+)	(-)			(Groups)	p
Control	0	16	0	16	(Control–D)	0.004
A	3	20	1	23	(A–D)	0.043
B	3	19	2	22	(B-D)	0.052
C	2	21	1	23	(C–D)	0.012
D	8	12	4	20		
Total	16	88	8	104	(Chi-square p= 0.037)	

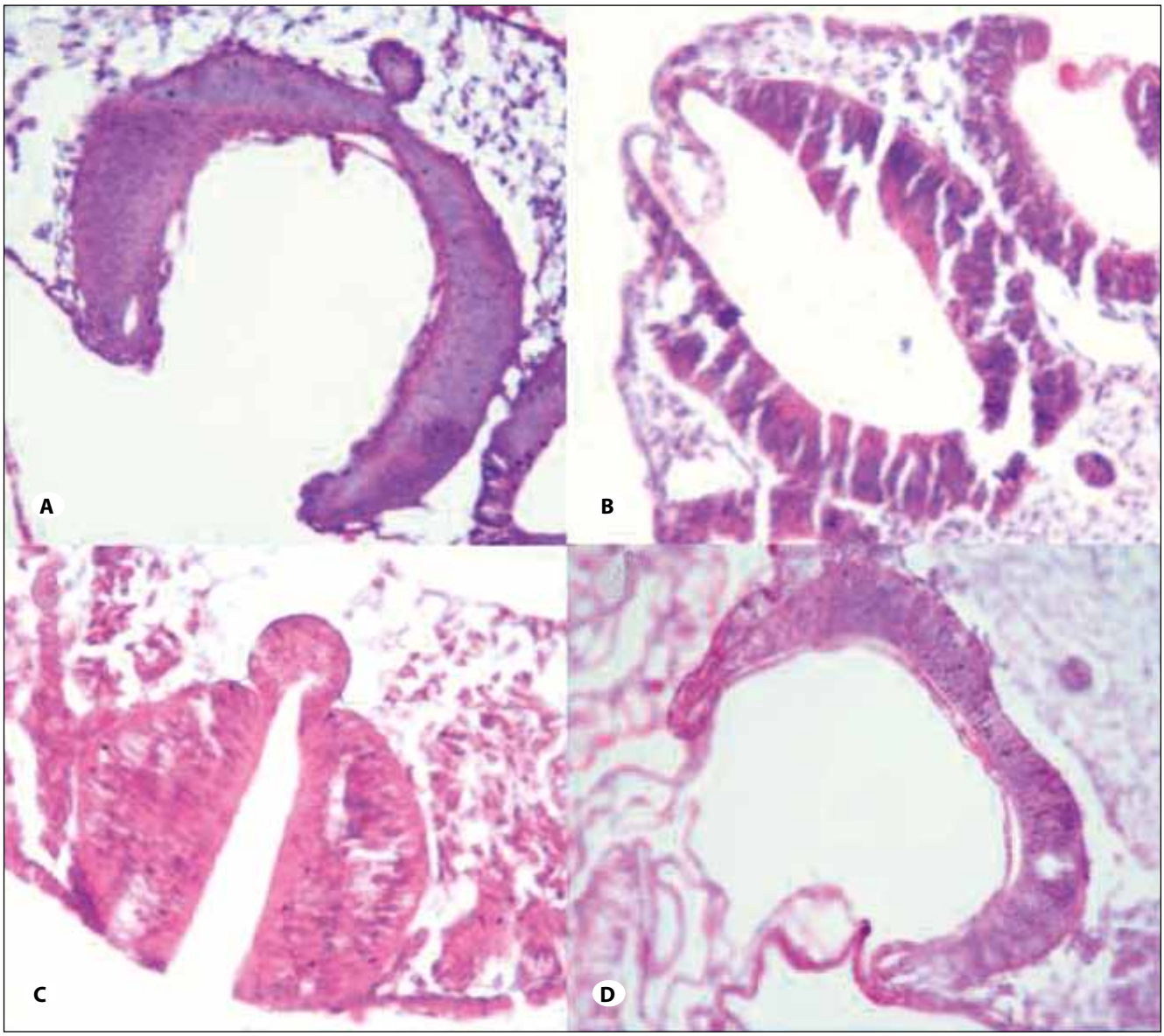

Figure 3: Embryos which were detected to have NTDs after HE staining. **A)** Group A, **B)** Group B, **C)** NTD in the caudal part of the embryo in group C, **D)** NTD in the cranial portion.

system by impairing closure probably by oxidative stress at the cellular level, which ends in an open neural tube in a dose dependent fashion. Statistical analysis discerned significant relation between exposure to ultrafine DEPs and incidence of NTDs with a maximum effect at a concentration of 200µg DEPs mL^{-1}.

CONCLUSION

Even though the relative risks are small, there is considerable public health concern because of the large number of DEP-exposed people and the existence of growing number of high risk groups. Although most of the other threats to public health can be avoided to some extent, yet we do not know the way to protect the population from the effects of DEPs in the polluted air. In our study it has been shown that exposure to DEPs in low doses could also lead to NTDs. Increased oxidant exposure due to higher doses of DEPs during embryogenesis is, as expected, relevant to higher incidence of congenital defects of the CNS. Therefore, it is our opinion that future studies directed at unraveling the possible effects of DEPs on the development of neural tissue in the further stages of embryogenesis are needed.

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