The Effects of Methotrexate on the Development of Neural Tube Defects in the Chick Embryo[#]

H. Seda VATANSEVER*, A. Şükrü UMUR**, V. Sevinç İNAN*, Mehmet SELÇUKİ**
Celal Bayar University, Faculty of Medicine, *Department of Histology and Embryology,
**Department of Neurosurgery, Manisa - TURKEY

Abstract: During chick development, one of the earliest differentiated tissues is the neural tube. After 24 h of incubation, a chick egg starts to differentiate and 30-48 h after incubation the neural plate is closed from head to tail to form the neural tube. If factors controlling the neural tube's closing are disrupted, this consequently causes neural tube closure defects during this time. In this study, the effect of methotrexate on the developing neural tube was investigated during early chick development.

For this research, 40 specific pathogen free (SPF) white Leghorn type chick embryos were used. They were incubated for 30 h at 37.8 ± 2 °C. Methotrexate, which inhibits the dihydrofolate reductase enzyme by a competitive mechanism, was injected within therapeutic dosage limits (10 mg/m^2, 20 mg/m^2, 40 mg/m^2) in ovo. Ten eggs were injected with 0.9% NaCl and used as a control group. All groups, after the injection, were incubated for 48 and 72 h. They were then dissected and the embryos were fixed in 10% (v/v) formalin for 2 h. The embryos were embedded in paraffin wax and 5 µ serial sections were taken. Sections were stained with haematoxylin and then observed under light microscopy.

While 20 mg/m^2 or 40 mg/m^2 methotrexate embryos were not alive when they were opened at 48 h incubation, 10 mg/m^2 methotrexate embryos maintained normal development after 48 and 72 h incubation. However, there was developmental retardation in the methotrexate injected group when compared with the control group with development of the brain being retarded; the volume of brain vesicles was lower than in the control group.

Our results suggested that methotrexate, an antimetabolite of folic acid, caused neural tube closure defects when injected at therapeutic dosage levels. Folic acid is essential for normal development of the nervous system; therefore, folate antagonists might be more harmful to the central nervous system than to other parts of the developing body.

Key Words: Methotrexate, early chick embryo, nervous system.

Tavuk Embriyosunda Gelişen Nöral Tüp Defekti Üzerine Metotreksat'ın Etkileri

Özet: Tavuk gelişiminde en erken farklılanan doku nöral tüptür. Tavuk yumurtasının 24 saatlik inkübasyonundan sonra, farklılaşma başlar ve 30-48 saatlik inkübasyondan sonra nöral plak nöral tüpü oluşturmak üzere baştan kuyruğa doğru kapanır. Bu dönemde, eğer nöral tüp kapanmasını kontrol eden faktörler etkilendiğinde, nöral tüp kapanma kusurlarına neden olur. Bu çalışmada, erken tavuk embriyosu gelişimi sırasında metotreksat'ın nöral tüp gelişimi üzerindeki etkilerinin incelenmesi amaçlanmıştır.

Bu nedenle, 40 patojen içermeyen (SPF) beyaz Leghorn tipi tavuk embriyosu kullanıldı. Embriyolar $37,8 \pm 2$ °C'de 30 saat inkübe edildi. Kompetitif mekanizma ile dihidrofolat redüktaz enzimini inhibe eden metotreksat, terapötik dozlarda (10 mg/m^2, 20 mg/m^2, 40 mg/m^2) in ovo olarak enjekte edildi. Yumurtaların 10 tanesine ise kontrol grubu olarak % 0,9 NaCl enjekte edildi. Tüm gruplar, enjeksiyondan sonra 48 ve 72 saat inkübe edildi. Daha sonra yumurtalar açıldı ve embriyolar % 10'luk formalin solüsyonu içinde 2 saat tespit edildi. Embriyolar parafin bloklar içinde gömüldü ve 5 µ seri kesitler alındı. Kesitler hematoksilen ile boyandıktan sonra ışık mikroskobu altında değerlendirildi.

20 mg/m^2 veya 40 mg/m^2 metotreksat verilen embriyolar 48 saatlik inkübasyon sonrası açıldığında yaşamaz iken, 10 mg/m^2 metotreksat verilen embriyoların 48 ve 72 saatlik inkübasyondan sonra normal gelişimlerine devam ettikleri gözlendi. Bununla beraber, kontrol grubu ile karşılaştırıldığında, metotreksat enjekte edilen embriyolarda gelişme geriliği olduğu, aynı zamanda beyinin gelişmesinde de gerileme olduğu ve beyin veziküllerinin hacimlerinin, kontrol grubuna oranla azaldığı görüldü.

Araştırma sonuçları, bir folik asit antimetaboliti olan metotreksatın, terapötik dozlarda enjeksiyonu sonucunda nöral tüp kapanma defektine neden olduğunu desteklemektedir. Folik asit sinir sistemi gelişimi için esastır, bu yüzden folat antagonistleri de santral sinir sistemi üzerinde diğer gelişen organlara oranla daha fazla zararlı etkilere sahip olabilir.

Anahtar Sözcükler: Metotreksat, erken dönem tavuk embriyosu, sinir sistemi

Introduction

Neural tube closure is accomplished by a complex morphogenetic program requiring precisely choreographed cellular proliferation, differentiation, adhesion and migration (1). Neurulation proceeds in a series of steps by which the neural plate is shaped, elongated and bent to form a tube that extends the entire length of the anterior-posterior axis (2). To form the neural tube, the neural plate undergoes a bending process by which the lateral edges, or neural folds, elevate, rotate around the actin-rich dorsal-lateral hinge points, and converge at the dorsal midline (2). After that, the edges fuse to form a seamless tube. In the cranial region, closure is initiated at defined fusion points located at the anterior point of the forebrain, the forebrain/midbrain boundary, and the hindbrain/spinal chord boundary (3). Following fusion, closure along the remainder of the neural tube proceeds in a zip-like fashion in defined directions. There are a number of intrinsic and extrinsic genetic, molecular and environmental factors that regulate neural tube morphogenesis and the lack of these factors can cause neural tube closure defects (4-5). While some factors determine neurulation, other factors allow the development of neural folds to the neural tube. Folic acid plays an important role during nucleic acid synthesis in the cell. Its biological effects contribute to the carriage of molecules which consist of a single carbon. Folic acid participates in single-carbon transfer only if it is in the tetrahydro reduced form. The dihydrofolic acid reductase enzyme plays a role in this transfer. Methotrexate (Emetopterin, Mtx, Meksat) blocks the transforation of dihydrofolic acid to tetrahydrofolic acid by inhibiting the dihydrofolic acid reductases. Therefore, the synthesis of thymidine and purine cannot proceed to DNA, RNA and thus protein synthesis. For this reason, we decided to investigate the effects of different doses of methotrexate, which has an effect on neural tube closure during chick development by affecting the folic acid pathway.

Materials and Methods

Forty white Leghorn type specific pathogen free (SPF) eggs were obtained from the State Chick Research Centre, Manisa, Turkey. The eggs were weighed measured (60 g ± 5) and they were then incubated at 37.8 ± 2 °C for 30 h to let them develop to stage 8. The eggs were divided into 2 groups: a methotrexate injected group (study group, n=30) and a serum physiological (SP) injected group (control group, n=10). The study group was sub-divided into 3 equal groups. Methotrexate was injected in the following order for each group: Group 1A: 10 mg/m^2, Group1B: 20 mg/m^2 and Group 1C: 40 mg/m^2. All injections were performed under the embryonic disc. Embryos from all groups were incubated until 48 h and 72 h of development. After that, embryos were collected and examined under dissection microscopy to detect increases in somite number, which is the proof of a growing embryo in culture media, and the presence of any abnormalities. They were then fixed with a 10% (v/v) formalin solution for 2 h and then dehydrated through a graded series of ethanols 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 µ) were then taken from each group. Sections were stained with haematoxylin solution. Slides were mounted using entellan and covered with glass coverslips prior to viewing and photography under Olympus BX-40 light microscopy.

To determine the regression of brain development, the total volume of developing brain vesicules was calculated on serial sections using the Cavalieri technique (6). The points on the calculation grid that fall on the brain tissue were detected and the volume of the brain vesicles was calculated. Student's t test was used for statistical comparisons.

Results

All embryos were incubated for 18 h and 24 h after injection and then collected. While normal development proceeded in all embryos until stage 8 at 30 h incubation, the methotrexate injected embryos from Group 1B (20 mg/m^2) and 1C (40 mg/m^2) were not alive at 48 h of development. However, the 10 mg/m^2 of methotrexate injected embryos (Group 1A) were alive at 48 and 72 h of development; therefore, this group became the study group. Within the first 18 h of incubation (48 h of development), the head-to-tail orientation of the embryos became visible macroscopically as the primitive streak in the control group (Figure 1a). After histological examination, developmental proceses for neural tube, optic vesicle and mesodermal cells were detected at that developmental stage in the control group (Figure 1b-c).
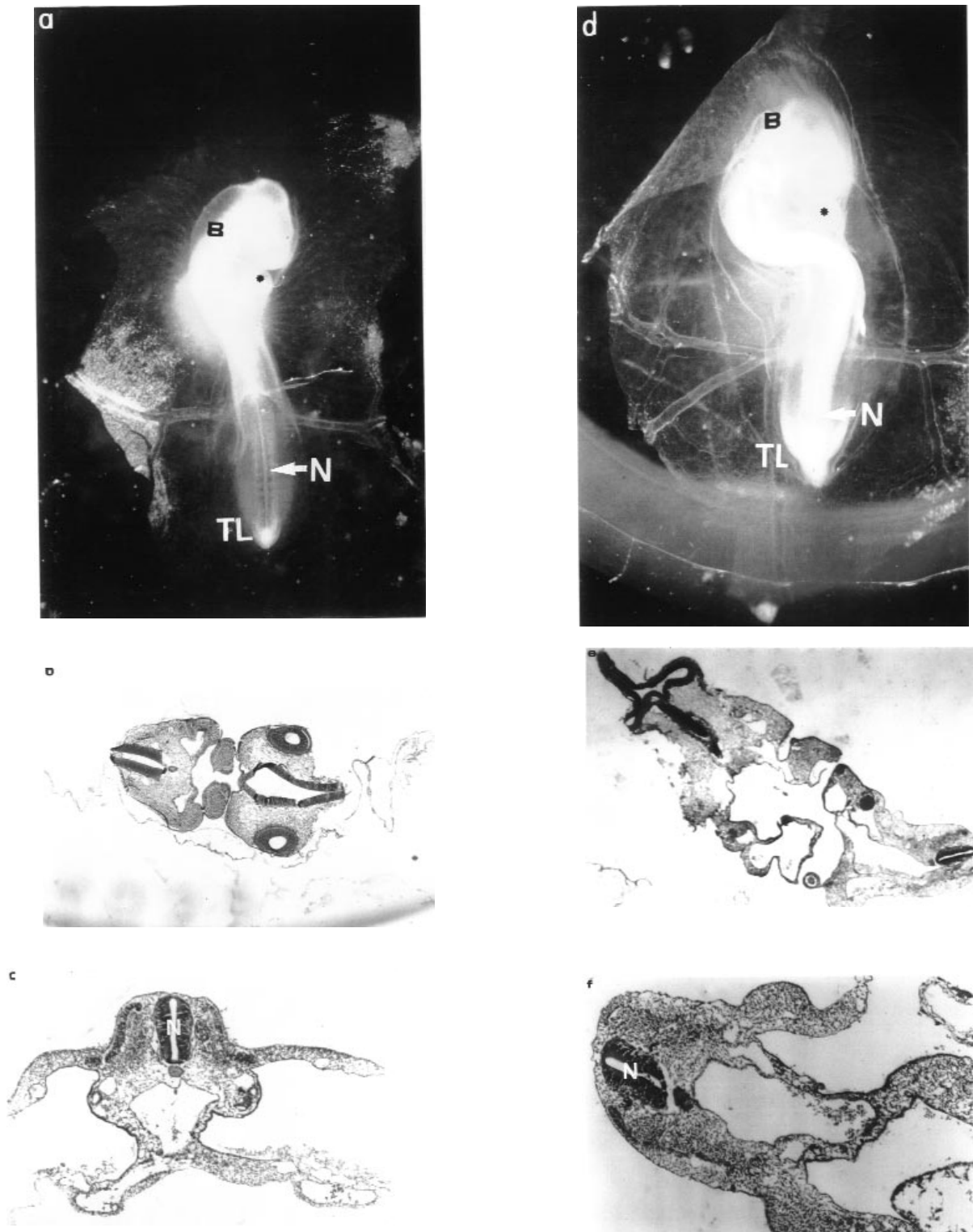
Figure 1. Macroscopic and microscopic observations of chick embryos from the control group at 48 (a-c) and 72 h (d-f) of developmental stage. Head-to-tail orientation, brain vesicles (B) cardiac process (*) and neural tube (N) were detected in 48 h (a) and 72 h (d) chick embryos under dissection microscopic observations. Neural tubes head-to-tail orientated were closed (N) and optic vesicles were detected after both 48 (b-c) and 72 h of developmental stage. X10 a,d (without staining); X40 b,e; X200 c,f, Haematoxylin.

When the embryos were incubated 48 h more (72 h of development), they developed further and the head turned and lay at right angles to the trunk (Figure 1d-f).

While head-to-tail orientation developed in the methotrexate injected embryos at the 48 h developmental stage, the embryos were smaller than those of the control group (Figure 2). Vascularization of the embryonal disc membrane was also retarded, brain vesicles were decreased, while microcephaly and growth retardation especially in the nervous system was detected in the study group (Figure 2a,b). In addition, macroscopically characteristic developmental proceses such as increased somite pairs, the head turning to the left side, the appearance of cardiac activity, primary optic vesicles and optic stalk were noted to be well established under dissection microscopy in that group. At 48 h of development the lumen and keel of the pharynx, epicardium, endocardium and lumen of the ventricle were developed in the methotrexate injected embryos (Figure 2c). The neural tube was also closed and basement membrane remained under the neurectoderm cells (Figure 2d). However, dense chromatine deposition and cytoplasmic inclusion were detected in neuroectoderm and mesoderm cells in the study group (Figure 2d).

At 72 h developmental stage, the growth retardation of methotrexate injected embryos was clearly visible, and neuroectoderm cells were disorganised, endocardial cells were destroyed, while the lumen of the ventricle
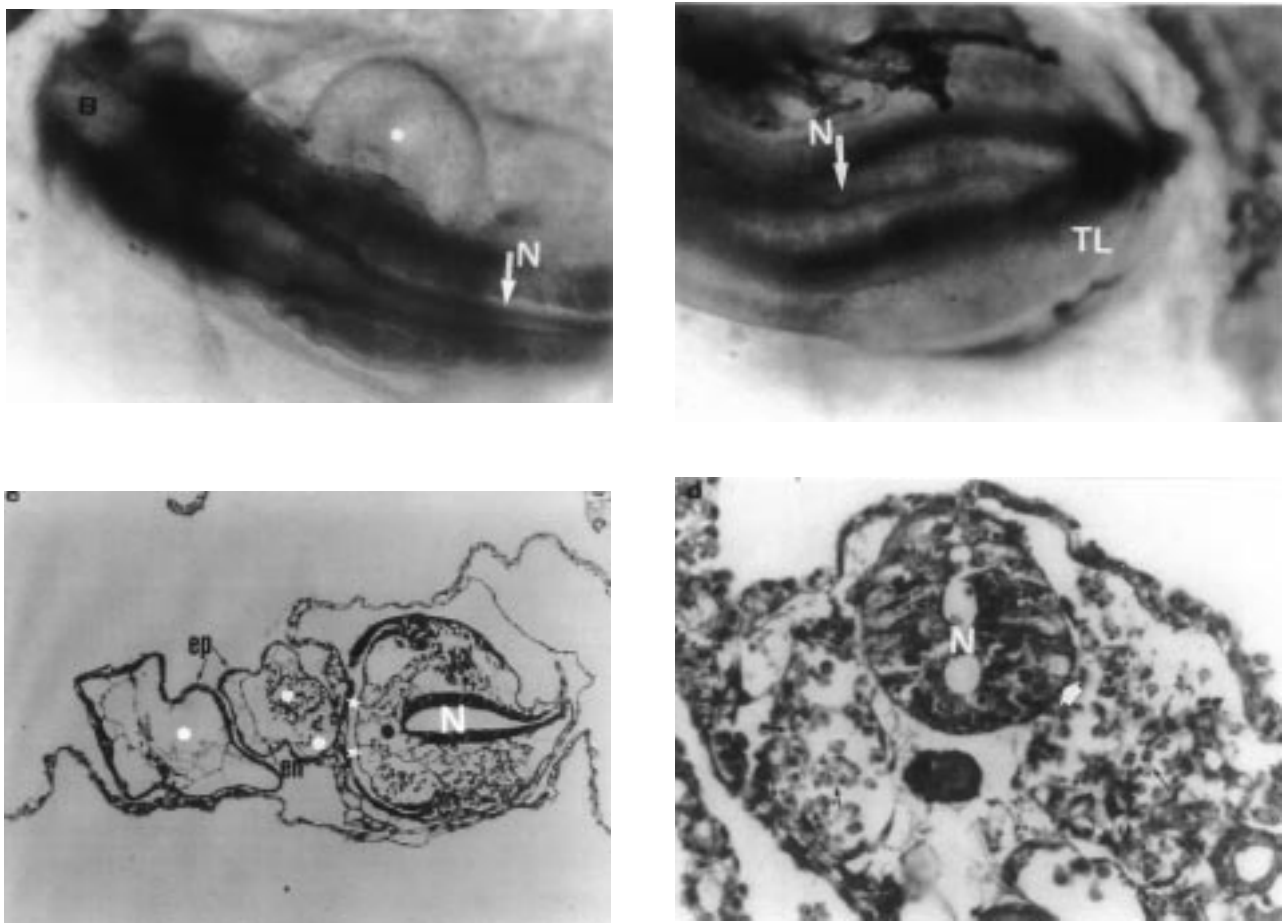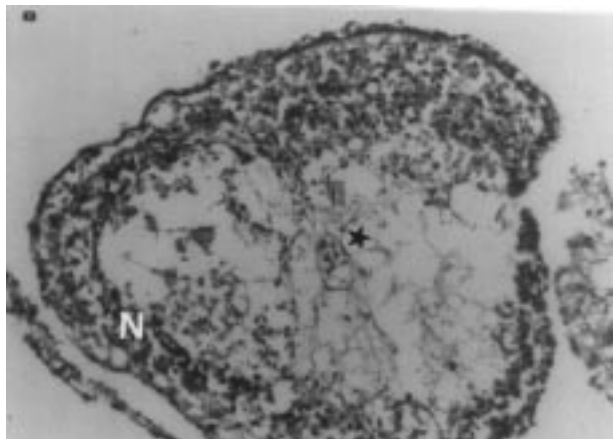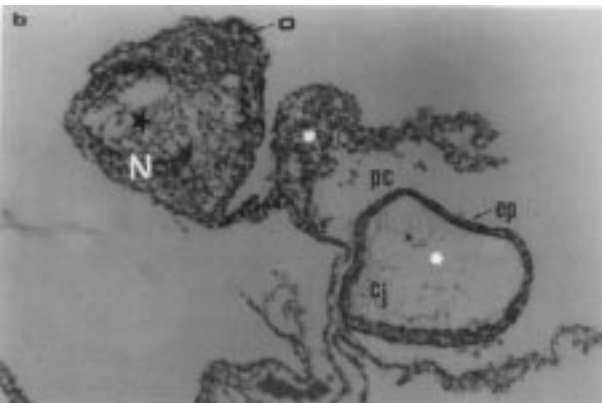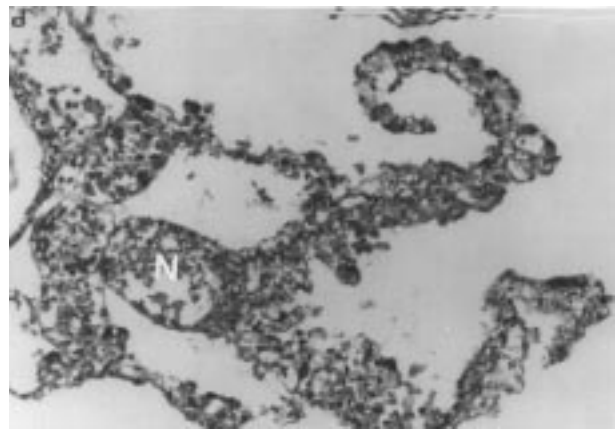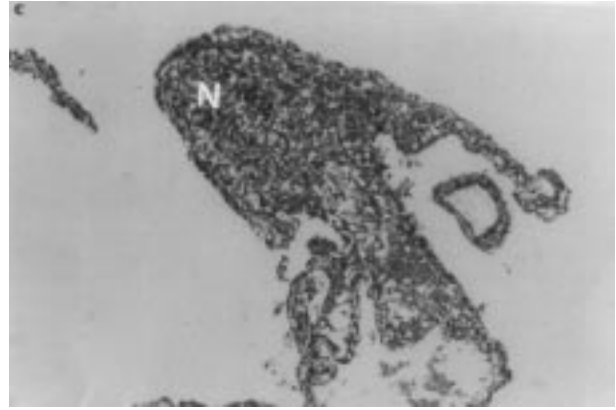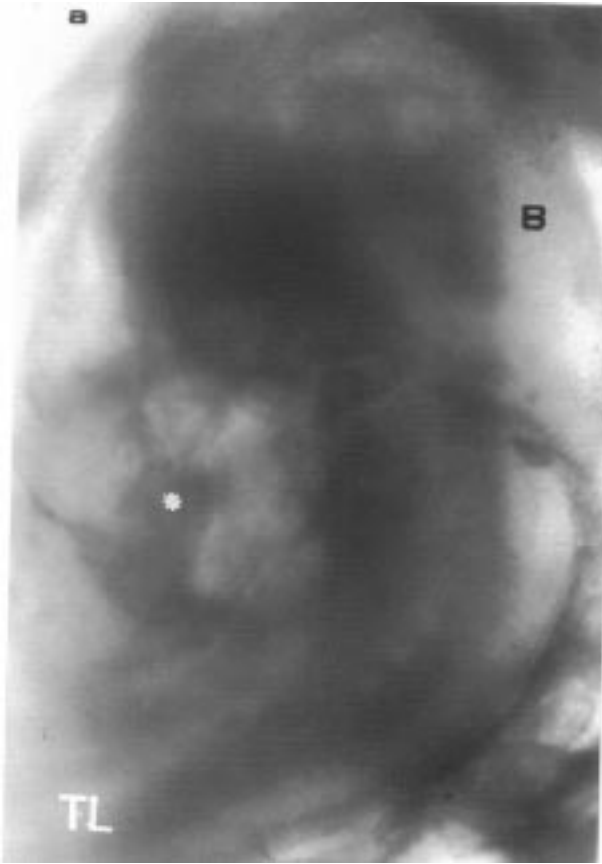

Figure 2. Dissection and light microscopic analysis of methotrexate injected chick embryos after 48 h of development.
Embryos were smaller than control embryos, and brain vesicles were decreased (B), growth retardation, especially on the nervous system, was detected under dissection microscopy (a-b). Embryos developed head-to-tail (TL) orientation, lumen and keel of pharynx (star), epicardium (ep), endocardium (en) and lumen of ventricle (*) (c). The neural tube (N) was also closed and basement membrane (thick arrow heads) remained under the neurectoderm cells. Chromatin dense cells were also detected (thin arrows). X10 a-b (without staining); X100 c; X200 d, Haematoxylin.
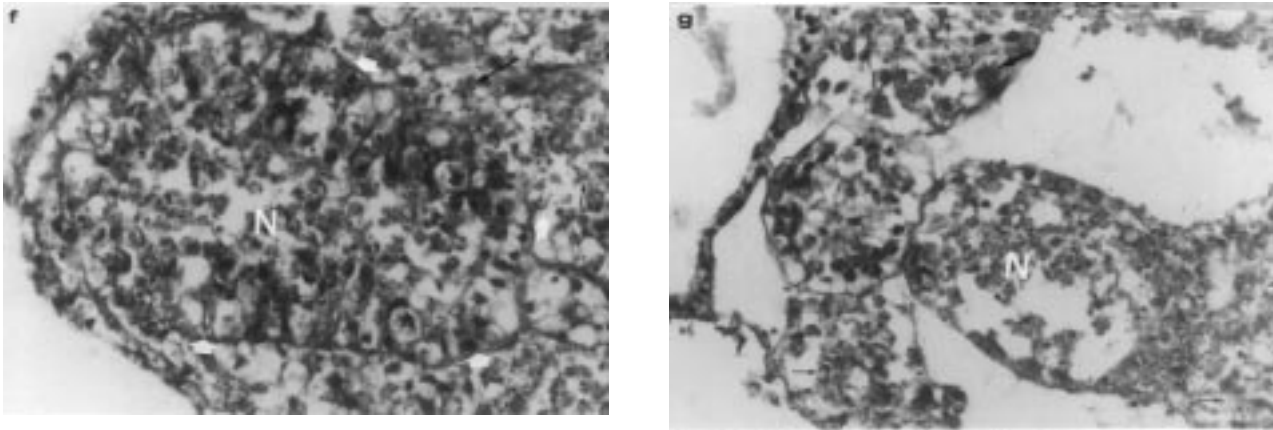
Figure 3. Dissection and light microscopic analysis of methotrexate injected chick embryos after 72 h of development. Methotrexate injected embryos were not developed properly; however, brain vesicles (B), neural tube (N) and tail (TL) orientation were still detected (a). The lumen of the ventricle (*) and epicardium (ep) were developed (a), and the optic pit (O) was only detected in these embryos (b,e). Cardiac jelly (cj) and pericardial cavity (pc) were also detected in this embryo (b).While the neural tube (N) was opened in the middle sections of embryos (c,f), the basement membrane remained in that group (arrows-c,f). The closed neural tube was also detected on sections from the tail (d,g). Increased deposition of dense chromatin and cytoplasmic inclusion was also still detected in the methotrexate injected group at this development stage (b-g). X10 a (without staining); X100 b-d; X200 e-g, Haematoxylin.

remained (Figure 3a,b,e). Cardiac jelly and the pericardial cavity were also detected in the embryos. While the neural tube was also opened, the basement membrane remained on the middle sections of the study group (Figure 3c,f). In addition, increased deposition of dense chromatin and cytoplasmic inclusion were still detected in the methotrexate injected group at this development stage.

The total volume of developing brain vesicles was measured by using the Cavalieri method. It was observed that the total volume of the brain from methotrexate injected group was lower (0.093 mm^3) than that of the control group (0.165 mm^3). This result was statistically significant (P < 0.05). This observation was confirmed macroscopically in both groups.

Discussion

The known reasons for disorders related to non-closure of the neural tube are genetic factors (Trisomi 13, 18, 21), geography, mother's age, social economic factors, zinc and folic acid metabolism disorders, diabetes mellitus, increased mother's body temperature in the first month of pregnancy, using alcohol and the toxic effects of valporic acid (7,8). In the former investigations, the effects of diazepam and verapamil on the neural tube were investigated and the effect of these agents on the neural tube was demonstrated (9,10). Later research noted that the detection of ethanol in chicken embryos could be shown histologically (11,12). Other investigations on chicken embryos revealed that folic acid metabolism disorder causes defects in the development of embryos (13). To our knowledge, there are no reports on the relation of methotrexate and developing chick embryos as far as the development of the central nervous system is concerned.

The results of our investigation disclosed that the application of methotrexate in therapeutic doses causes retardation in general embryonic progress, and further retardation in the development of the neural system by impairing the folate metabolism at the cellular level, which ends in an open neural tube.

Folic acid and congenital malformations have been intertwined for 5 decades (7,8). The connection began with animal studies of aminopterin, an antimetabolite of pteroyl-glutamic acid, or folic acid as it is usually called. A number of recent studies have demonstrated that the incidence of congenital defects related to the interruption of both neurulation and conotruncal septation could be reduced by the use of supplementary folate during pregnancy (14,15). However, the dramatic effects of folate deficency cause congenital defects especially in

neural ectodermal tissue, which is affected more than other tissues. Furthermore, it appears that the severity and duration of folate deficiency necessary to inhibit DNA synthesis are greater than the conditions necessary to produce congenitally disordered organ systems (16). Thus folate deficiency may not by itself be sufficient to explain neural tube defects in mice by the mechanism of deficient DNA synthesis. In addition, folate deficiency may not induce congenital neural tube and heart defects by generally limiting the availability of nucleic acids but by producing some more specific effect upon a narrow region of the neural ectoderm (17). Therefore, an unknown mechanism must be at work during the development of septal formation and neural tube closure,

which in turn causes defects associated with folate insufficiency.

Our results suggested that folate antagonists caused more severe embryological defects in nervous tissue than in other developing tissues. While cardiac septation and cavity were demonstrated in the study group, more dramatic retardations were detected in neural tube closure and in optic vesicle development. The folate metabolism seems to have a more important role in the function and development of the nervous system, and thus folic acid antagonists may harm the developing central nervous system more than any other part of the embryo.

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