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Effect of Thiocolchicoside on Midline Closure in Early Chicken Embryos

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Abstract

BACKGROUND/AIMS: Thiocolchicoside (TCC) is a semi-synthetic derivative of colchicine analog, a muscle relaxant with analgesic and anti-inflammatory activity. Studies have shown that colchicine inhibits microtubule polymerization and stops mitotic activity, and that TCC is a competitive antagonist of g-aminobutyric acid A and glycine receptors. It is known that the use of TCC during pregnancy may have teratogenic effects. We aimed to examine the effects of drugs with TCC as their active ingredient, which are frequently used in clinical practice, on the development of chicken embryos and midline closure.

MATERIALS AND METHODS: A total of 80 eggs were incubated in an incubator for 24 hours. At the 24th hour of incubation, the eggs were divided into 4 groups. Increasing doses of TCC (8 mcg, 16 mcg, 32 mcg) in 0.1 cc solutions were applied to these groups and half of the control group was administered physiological saline, corresponding to the air sac. All eggs were then closed with sterile tapes and replaced in the incubator. On the 10th day of incubation, the eggs were hatched and the embryos were evaluated morphologically and histopathologically.

RESULTS: TCC caused a high rate of early embryo death (EED) in all groups in which it was applied. Although midline closure defects were detected in some of the developing embryos, it was not statistically significant.

CONCLUSION: EED and midline closure defects were observed in some developing embryos in the TCC applied groups. It will be possible to understand the mechanism of embryonic damage, to reveal teratogenic effects and to minimize the formation of congenital defects with more comprehensive studies.

Keywords: Thiocolchicoside, neural tube defect, chicken embryo

INTRODUCTION

Neural tube defects (NTDs) are severe birth defects of the central nervous system caused by a failure of the morphogenetic process of neural tube closure during embryogenesis.[1] NTDs are malformations caused by abnormal neural tube closure at between 3 and 4 weeks of gestation,[2] with a global incidence of 1.8 per 1,000 live births and 3 per 1,000 live births in Türkiye. Spina bifida and anencephaly, the two most common types of NTDs, affect approximately 300,000 newborns worldwide annually.[3]

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Although NTDs can be found in all geographies areas, they are a major public health problem which is especially common in low-income countries and they cause psychological, economic, and sociological problems. Although the causal mechanism is not fully understood, genetic, nutritional, and environmental factors or a combination of these factors play a definite role in the development of NTDs.[4]

Most NTD cases have a genetic etiological component and involve the interaction of several environmental risk factors. Although more than 200 NTD-causing gene expressions have been identified in various studies, the folate-dependent enzymes methylenetetrahydrofolate reductase (MTHFR), MTHFR C677T, and two polymorphisms of MTHFR A1298C are among the best known risk factors. For this reason, 400 mg of folic acid supplementation has been recommended before pregnancy as a prophylaxis.[5-8]

This anomaly has been the subject of extensive research because there are preventable conditions which can cause NTDs. Even though natal/prenatal diagnosis and treatment methods have improved, being aware of the etiological factors which may cause NTDs and keeping them under control is still the unchanged fundamental approach.[9,10]

Thiocolchicoside (TCC) is a semi-synthetic derivative of colchicine, a natural anti-inflammatory glycoside obtained from the seeds of the flower of *Superba gloriosa*.[11] TCC has a selective and strong affinity for g-aminobutyric acid A (GABA-A) receptors and it acts on muscle contractures by activating GABA inhibitory pathways, thus acting as a potent muscle relaxant. GABA is the main inhibitory neurotransmitter in the human cortex. GABAergic neurons are involved in the effective mechanisms of myorelaxants, anxiolytics, sedatives, and anesthetics. GABA can also modulate heart rate and blood pressure[12] and it has an affinity for inhibitory glycine receptors (i.e., it has glycomimetic and GABA mimetic activities). Glycine is an inhibitory neurotransmitter and functions as an allosteric regulator of N-methyl-D-aspartate receptors.[13]

Studies have shown that TCC is a competitive antagonist of GABA-A and glycine receptors.[12] A maximum daily dose of 8 mg is recommended when given intramuscularly. The use of TCC in pregnancy may have teratogenic effects.[14-16]

The aim of the present study was to investigate the effects of TCC, a commonly used muscle relaxant in clinical practice, on the development of chicken embryos and midline closure.

MATERIALS AND METHODS

This study was carried out in the Animal Laboratory of Kahramanmaraş Sütçü İmam University, Ziraat Faculty of Medicine, Department of Animal Husbandry. Ethical approval was obtained from Kahramanmaraş Sütçü İmam University, Ziraat Faculty of Medicine (approval number: 2022/03, date: 27.05.2022). Patient approval was not obtained as it was performed on animals. All protocols were in accordance with the National Laboratory Animals Care Guidelines.

Pathogen-free, fertile, day zero white broiler eggs were obtained from the CIVKUR facility in Adıyaman. A total of 80 eggs were weighed (mean weight 65±5 gr) and incubated for 24 hours at 37.8±0.2 ºC and 50-75% humidity in an incubator with automatic rotation every 2 hours.

The air sac injection technique was chosen because of the low infection risk, homogeneous distribution of the injected agent, and minimal mechanical damage which may occur in the embryo due to an increase in intra-ovarian pressure compared to other methods.[17] Since there is literature evidence that other techniques themselves may cause NTD, it was decided to conduct this study using the air sac injection technique.[18]

At the 24th hour of incubation, all eggs were sterilized with alcohol, and 0.5 mm windows were opened onto the air sacs. The eggs were divided into four basic groups (n=20 per group). TCC was dissolved in saline under sterile conditions, and 0.1 cc stock solutions were prepared at predetermined concentrations. This 0.1 cc solution was injected into the eggs using an insulin injector.

Group 1a: (Sham group). The eggs were punctured and closed without injection (n=10). This group was set up to examine the possible effects of changing the pressure inside the egg by piercing the shell.

Group 1b: (Solvent) The eggs were injected with 0.1 cc of saline (n=10). This group was set up to study the possible side effects of the solvent and any volume-occupying substance injected into the egg.

Group 2: 8 mg/day/individual (embryo dose ≥8 micrograms/0.1 cc) (n=20).

Group 3: 16 mg/day/individual (embryo dose ≥16 micrograms/0.1 cc) (n=20).

Group 4: 32 mg/day/individual (embryo dose ≥32 micrograms/0.1 cc) (n=20).

After the punctures were sealed with sterile tape, the eggs were transferred to an incubator (Cimuka, Ankara, Türkiye) with 180° automatic rotation. Temperature and humidity were checked twice a day. The eggs were opened on day 10 of the incubation period to evaluate embryological development.

Macroscopic Examination

Embryo development was evaluated macroscopically. Non-fertilized embryos were separated and excluded from this study. Head circumference, head-tail length, and embryo weight were measured in those embryos with adequate development. Light microscopy was used to classify the embryos based on whether the neural tube was closed or open along the craniospinal axis. The measurement values obtained were analyzed statistically.

Histopathological Examination

For histopathological examination of the embryos, four 2-3 mm-thick transverse sections, one from the cranial and three from the spinal regions, were taken from each embryo using a microtome knife. The samples were fixed in 10% buffered formalin for 48 hours and dried. The samples were cleaned in xylene and embedded in paraffin. These tissue sections were stained with hematoxylin-eosin stain for morphological examination. All sections were evaluated by a pathologist under a light microscope (Olympus BX51, Tokyo, Japan) at 20X and 40X magnification based on the Hamburger-Hamilton Chicken Embryo Classification System.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 21.0 of the statistical package program. The crosstabs chi-square test was used to evaluate whether there was

a statistically significant difference between the groups in terms of fertilization, [early embryo death (EED)], and NTD parameters. A p-value <0.189 was considered statistically significant. In addition, the Kruskall-Wallis test was used to compare head circumference, head-tail length, and weight parameters between the groups.

RESULTS

In group 1 (control group), 17 embryos had closed neural tubes (85%), 2 had no development (EED) (10%), and 1 had a significant developmental delay (5%). NTD was not observed in any of the embryos in this group. The embryo with a developmental delay could not be sectioned. In group 2, after the administration of 8 micrograms/0.1 cc of TCC injection, 8 embryos had closed neural tubes (40%), 10 had no development (EED) (50%), 1 had a significant developmental delay (5%), and 2 had open neural tubes (10%). The embryos with developmental delays were also sectioned and examined (Table 1).

In group 3, after the administration of 16 microgram/0.1 cc of TCC injection, 5 embryos had closed neural tubes (25%), 14 had no development (EED) (70%), and 1 had an open neural tube (5%). In group 4, after the administration of 32 micrograms/0.1 cc of TCC injection, 5 embryos had closed neural tubes (25%), 14 had no development (EED) (70%), and 1 had an open neural tube (5%) (Table 1).

Viability was 90% in the control group, and statistical analysis showed no significant difference in the experimental groups injected with TCC in terms of fertilization (p>0.001) (Table 2).

Furthermore, no significant difference was found between the experimental groups in terms of NTD (p>0.001) (Table 3). The developing embryos were quantitatively measured and evaluated using the Kruskall-Wallis test (Table 4). There were no significant differences between the control group and the TCC-treated groups in terms of weight and head circumference, but the head-tail length was significantly longer in the TCC-treated groups than in the control group.

Table 1. Results of the experimental groups injected with varying thiocolchicoside doses

Embryo observation	8 mcg	16 mcg	32 mcg	Control
No development (early embryo death)	10	14	14	2
Developmental delay	1	0	0	1
Neural tube open	2	1	1	0
Neural tube closed	8	5	5	17

Table 2. Statistical analysis of the groups in terms of fertilization (p=0.189)

	Value	df	Asymp. sig. (p-value) <0.189 was considered statistically significant. sig. (two-sided)
Pearson's chi-square likelihood	3.604[a]	3	0.308
Ratio	4.769	3	0.189
Linear-by-linear association	0.142	1	0.706
(n) of valid cases	80		

[a]Chi-square tests, Asymp.: Asymptotic, sig.: Significance.

DISCUSSION

TCC, alone or in combination with other substances, is a commonly used agent in orthopedics, physical therapy, rheumatology, and neurosurgery outpatient clinics due to its beneficial muscle relaxant activity. Based on this information and previous studies, our research hypothesis was that TCC, a colchicine derivative commonly used in clinical practice, causes developmental delays and NTDs in embryos.

Since the chemical structure of TCC is similar to that of colchicine, it is expected to have a similar side effect profile.[14] Colchicine functions primarily by inhibiting microtubules (MT) polymerization. MT polymerization affects many cellular processes, including shape maintenance, signaling, division, migration, and cellular transport. MTs are structures involved in cell shaping, the transportation of intracellular substances, the release of cytokines and chemokines, cell migration, the regulation of ion channel activity, and cell division. Colchicine stops mitosis in metaphase by inhibiting MTs and it has a limiting effect on mitochondrial activity.[18-20]

In 2013, the European Medical Association (EMA) mandated that the use of TCC-containing medicines by mouth or injection should be restricted across the European Union. These medicines are now only recommended as an adjunctive treatment for painful muscle contractures caused by spinal conditions in adults and adolescents aged 16 years and older. The EMA also stated that the dose of TCC administered orally or intramuscularly should be limited. This is based on experimental evidence showing that TCC has a tendency to damage dividing cells, resulting in aneuploidy (an abnormal number or arrangement of chromosomes).[21]

Fernandez et al.[22] tested three different colchicine doses ($5x10^{-5}$ M, $5x10^{-6}$ M, and $5x10^{-7}$ M) with two experimental treatments (*in ovo* and *in vitro*) and concluded that *in vitro* colchicine treatment was always effective in depolymerizing the MTs of neuroepithelial cells.

O'Shea conducted a study with mouse embryos in 1982 and reported that colchicine inhibited the assembly and elongation of MTs, therefore, neural folds could not form.[23] This effect supports the idea that impaired

Table 3. Statistical analysis of the groups in terms of neural tube defects (p=0.174)

	Value	df	Asymp. sig. (two-sided)
Pearson's chi-square likelihood ratio	0.510[a]	3	319
Linear-by-linear association	0.972	3	174
(n) of valid cases	0.892 39	1	169

[a]Chi-square tests, Asymp.: Asymptotic, sig.: Significance.

Table 4. Statistical comparison of weight, head circumference, and head-tail length between groups (p<0.0125)

	Weight	Head-tail length	Head circumference
Chi-square	1.253	12.617	1.696
df	3	3	3
Asymp. sig. (p-value) <0.189 was considered statistically significant. sig.	0.740	0.006	0.638

Asymp.: Asymptotic, sig.: Significance.

MT function may also cause NTDs, which is consistent with the findings of our study.

It has been demonstrated that MTs undergo major changes in organization and stability during neurulation and are essential for the timely completion of neural convergence by promoting cell elongation and polarity.[24]

MTs align along the apical-basal axis of the mammalian neuroepithelium early in neural tube closure. They functionally participate in interkinetic nuclear migration, which indirectly affects cell shape. MTs are pioneers in defining the neural rod midline prior to cavitation, both by localizing apical proteins to the tissue midline and directing cell division through a symmetric MT apparatus which helps to further define the medial localization of apical polarity.[25]

In a study conducted by Briner[26] on pregnant rats in 2001, it was reported that the GABA antagonist bicuculline may cause NTDs by causing enlargement in the vertebral arch. In the present study, the fact that TCC had an effect by binding to GABA receptors supports our hypothesis.

Lee et al.[27] injected varying doses of caffeine into early chicken embryos and found that increased doses caused NTDs. The authors reported that this effect was caused by a decrease in the number and contractility of actin microfilaments.[27]

Considering that TCC also has anti-inflammatory activity, an increase in the incidence of NTDs was observed in experimental studies and case studies previously conducted with non-steroidal anti-inflammatory drugs.[28-31]

According to the severity of possible teratogenic effects, drugs are categorized by the Food and Drug Administration into five categories: A, B, C, D, and X.[32] The pregnancy category of TCC is X, and its use is contraindicated in women with the potential to become pregnant. For drugs in this category, studies on experimental animals and pregnant women have shown that the drug is harmful to the fetus. The benefits of using these drugs are insignificant compared to the harm they may cause to the fetus. Pregnant women and women with the potential to become pregnant should not use drugs in category X under any circumstances.[32]

Various agents causing NTDs have been previously investigated by different methods. However, this is the first study conducted with chicken embryos to investigate the effects of TCC, a muscle relaxant and painkiller which we frequently use in our daily lives, on neural tube development.

The absence of embryonic development observed at high rates with increasing doses of TCC injection (70% in group 2, 50% in group 3, and 50% in group 4) suggests that TCC causes infertility because it stops mitotic activity in the early embryonic stage, causes genetic anomalies, or affects MT functions and causes cytoskeleton damage.

Therefore, TCC should be used with extreme caution during the fertile period, awareness should be raised in the community, and infertile individuals should be questioned about TCC use. Health services should be organized to prevent NTDs, and all necessary training should be provided to health personnel.[33]

Study Limitations

The results obtained from animal studies do not fully reflect the conditions in humans, and the teratogenic mechanism of TCC has not been elucidated to date. Therefore, more studies are needed to demonstrate the teratogenic effects of TCC on embryonic development and to minimize its congenital defects.

CONCLUSION

In the present study, NTD was not observed in group 1, whereas two embryos in group 2, one in group 3, and one in group 4 showed NTD in histopathological examinations. Although there was no statistically significant difference in the incidence of NTDs between the groups, among the few embryos which were able to develop, 20% in group 2, 16% in group 3, and 16% in group 4 had NTDs. Considering these high rates, we believe that the statistical analysis of our study, which examined the effects of TCC on midline closure in chicken embryos for the first time in the literature, may reveal a significant difference in terms of NTD incidence if conducted with a larger number of chicken embryos.

MAIN POINTS

- Neural tube defects (NTDs) are severe birth defects of the central nervous system caused by a failure of the morphogenetic process of neural tube closure during embryogenesis. Although NTDs can be found in all geographic areas, they are a major public health problem which is especially common in low-income countries and they cause psychological, economic, and sociological problems.
- Thiocolchicoside (TCC), alone or in combination with other substances, is a commonly used agent in orthopedics, physical therapy, rheumatology, and neurosurgery outpatient clinics due to its beneficial muscle relaxant activity.
- Our study, which examined the effects of TCC on midline closure in chicken embryos for the first time in the literature, may reveal a significant difference in terms of NTD incidence if conducted with a larger number of chicken embryos.

ETHICS

Ethics Committee Approval: Ethical approval was obtained from Kahramanmaraş Sütçü İmam University, Ziraat Faculty of Medicine Animal Experiments Local Ethics Committee (approval number: 2022/03, date: 27.05.2022).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Authorship Contributions

Surgical and Medical Practices: R.E., E.C.K., Concept: R.E., E.C.K., K.D.S., Z.Y., Design: R.E., K.D.S., Data Collection and/or Processing: R.E., E.C.K., Z.K.A., İ.E.S., Z.Y., Analysis and/or Interpretation: R.E., E.C.K., Z.K.A., Literature Search: R.E., E.C.K., H.T., Writing: R.E., H.T.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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