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Use of AgNOR staining to determine the effect of metoclopramide on neural tube development in early chick embryos

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ABSTRACT

Nausea and vomiting during pregnancy are common problems and prolonged pharmacological treatment often is needed; however, the teratogenic effects of anti-emetic drugs on neural tube (NT) development are not clear. We investigated the effects of different doses of metoclopramide on NT development in 48 and 72 h chick embryos using an argyrophilic nucleolar organizing region (AgNOR) staining method. We used 150 fertile, specific pathogen-free eggs incubated for 28 h, then randomly divided into five equal groups: group A, sham control was administered 0.9% saline; groups B – E were administered 0.15 mg/egg, 0.3 mg/egg, 0.6 mg/egg and 1.2 mg/egg, respectively. Half of the eggs in each group were taken from the incubator at 48 h incubation and the other half at 72 h incubation. After incubation, eggs were opened, embryos were dissected from their membranes, fixed with 10% formalin and examined by light microscopy. The NT status, i.e., open or closed, and somite number, crown-rump length, morphological features and gross developmental abnormalities were recorded. Excised embryos were sectioned and stained using hematoxylin and eosin or the AgNOR procedure and examined for morphology and histopathology. Delayed NT closure was observed in all 48 h drug exposed embryos, but in the 72 h groups, this occurred only in high-dose groups. Somite number was reduced significantly in groups C – E compared to the control group. Crown-rump length was decreased in both 48 and 72 h embryos. We found a decreased total AgNOR area:nuclear area ratio in 48 and 72 h embryos of all experimental groups. We found that metoclopramide delayed NT closure in chick embryos in a dose-dependent manner.

KEYWORDS

AgNOR; chicken; embryo; metoclopramide; neural tube

Nausea and vomiting affect nearly 80% of pregnant women; these usually diminish by the end of the 20th week of pregnancy (Bérard et al. 2019), although in 20% of women, the problems persist (Fazliogullari et al. 2013). The etiology of this problem is not fully understood and treatment is a priority. Obstetricians generally administer anti-emetics including dimenhydrinate, trimethobenzamide HCl and metoclopramide (Arvela et al. 1983; Ruedy 1984; Briggs et al. 2005; Vlastarakos et al. 2008; Ellaithy et al. 2020).

At low doses, metoclopramide blocks dopamine D2 receptors in the central and peripheral nervous system, but at higher doses, it blocks 5-hydroxytryptamine type 3 (5-HT3) serotonin receptors. The peripheral action of metoclopramide increases motility of the stomach and intestine. During peristaltic movement, the drug strengthens contractions in the cranial portion of the gastrointestinal region and relaxes the caudal region; therefore, metoclopramide facilitates caudal movement of the intestinal contents (O'Donnell et al. 2016; Shakhatreh et al. 2019).

Although metoclopramide crosses the placenta to the fetus, it is recommended frequently by obstetricians (Arvela et al. 1983; Ruedy 1984; Briggs et al. 2005). The effects of metoclopramide on the nervous and other systems are not well characterized. Some investigators assert that use of metoclopramide during pregnancy causes serious side effects (Poortinga et al. 2001; Fazliogullari et al. 2013), while others report otherwise (Pasternak et al. 2013; Tufan et al. 2016). The possibility that the drug can cause malformations and significant side effects in newborns, therefore, requires further study.

Neural tube defects (NTD) are well known congenital anomalies. Both genetic and environmental factors contribute to the development of NTD (Battiato et al. 1996; Salih et al. 2014; Bulloch et al. 2020). Neuronal and spinal development in chick embryos is similar to

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humans (Hamburger and Hamilton 1951) (H-H). Consequently, chick embryos have been used to investigate effects of teratogenic agents and drugs on the neural tube (NT) during pregnancy (Sawitzke et al. 2005; Umur et al. 2013; Tural Emon et al. 2015; Mete et al. 2016; Taskapilioglu et al. 2016; Ertekin et al. 2019; Atay et al. 2020). Most of these investigators evaluated morphology and developmental anomalies (Tural Emon et al. 2015; Mete et al. 2016; Ertekin et al. 2019; Atay et al. 2020). Other investigators used immunohistochemical staining to evaluate apoptotic pathways (Umur et al. 2013; Taskapilioglu et al. 2016). We have found no report of investigation of cell proliferation in the developing NT of chick embryos.

Silver staining techniques have been used widely for staining different tissues. The silver proteinate technique is useful for staining nerve cells (Vargas and Hurtado 1991). Silver staining techniques are useful for evaluating NT formation (Ertekin et al. 2021; Rakip et al. 2021). Consequently, we investigated possible teratogenic effects of metoclopramide on NT development in chick embryos using AgNOR staining. In addition, we investigated possible secondary effects of metoclopramide exposure during pregnancy.

Material and methods

We used 150 pathogen-free 65 ± 5 g eggs of the domestic fowl (White Leghorn) obtained from the Manisa Research Institute of Poultry Disease and Vaccination Centre. Approval for experimental animal use was obtained from the Institutional Animal Ethics Committee, Afyon Kocatepe University, reference no. 49533702/06–14/01/2019.

Experimental design

Day 0 fertilized eggs were incubated at 37.5 ± 0.2 °C and 65 ± 5% humidity. After incubation for 28 h, H-H stage 8, the eggshells were sterilized with 70% ethanol and 10% povidone-iodine. Holes, 0.5 cm diameter, were drilled into the blunt end of the eggs to expose the embryonic discs. Eggs were randomly divided into five groups of 30, and 30 µl of various solutions, specified below, were administered via the sub-blastodermic route using a Hamilton microinjector. Group A was a sham control administered 0.9% saline. Groups B – E were administered different amounts of metoclopramide in saline (0.15 mg/egg, 0.3 mg/egg, 0.6 mg/egg and 1.2 mg/egg, respectively (Lipham et al. 1992)). After injection, the windows were closed with sterile paraffin tape. The eggs were turned 180° and incubation was continued. Half the eggs in each group were opened at 48 h (H-H stage 12); the other eggs were opened at 72 h (H-H stage 20).

After incubation for 48 or 72 h, eggs were opened and embryos were dissected from their membranes and placed in petri dishes containing 10% formalin, then examined using a light microscope (Olympus C×21; Tokyo, Japan). The NT status (open or closed), developmental stages determined by somite counts and crown-rump lengths, morphological features, and gross developmental abnormalities were recorded.

Staining

Five embryos from each group were fixed with 10% formalin for 48 h, then washed with distilled water to remove fixative. Embryos were dehydrated through a graded alcohol series, cleared with xylol and embedded in paraffin. Sections were cut at 5 µm and affixed to polylysine-coated slides. Sections were deparaffinized and rehydrated, then stained with hematoxylin and eosin (H & E) (Demir 2001). The sections were examined using a light microscope.

Ten remaining embryos from each group were stained using the argyrophilic nucleolar organizing region (AgNOR) staining method (Lindner 1993; Ertekin et al. 2016) with a slight modification. Sections 5 µm thick containing the somites and NT were affixed to polylysine-coated slides and deparaffinized through graded alcohols. Gelatin solution, 1%, was prepared by adding formic acid to 2% gelatin solution dissolved in ultrapure water. The mixture was combined with 50% silver nitrate in ultrapure water. Sections were stained for 15–30 min at room temperature in the dark. After staining, sections were washed with distilled water and placed in 5% thiosulfate solution for 10 min. The AgNOR stained cells were observed and photographed using a light microscope. We evaluated 50 randomly selected nuclei for each embryo. Both total AgNOR area (representing the nucleolus):nuclear area (TAA:NA ratio) and the mean number of AgNOR/nucleus were obtained using image processing software (ImageJ version 1.47t, National Institutes of Health, Bethesda, MD) for each specimen.

Statistical analysis

Statistical analysis was performed using the SPSS 22.0 software. The somite count, crown-rump length, TAA:NA ratio and mean AgNOR number were analyzed using nonparametric Kruskal Wallis tests. The data related to NT closure were analyzed using the chi square test. The Dunn test was used as a *post hoc* test. Values for $p \leq 0.05$ were considered statistically significant.

Results

48 h embryos

Group A eggs were administered 0.9% saline. The mean number of somites was 20.87 ± 2.33; the embryos were at H-H stage 13. The mean crown-rump length was 661.54 ± 85 µm. No developmental malformations or developmental retardation were observed (Table 1). Embryos exhibited normal histomorphology consistent with their H-H stage (Figure 1). We observed that the head was turned to the left, the anterior neuropore was closed, and the primary optic vesicles and optic stalks were well developed. The mean number of AgNOR and the TAA:NA ratio were higher in this control group than in any other group (Table 1). The AgNOR stained tissue is shown in Figure 5.

Group B eggs were administered 0.15 mg/egg metoclopramide. Four embryos exhibited delayed NT closure; the NT of 11 embryos was closed. All embryos of group B were stage 13 according to the H-H classification and their overall development was similar to group A. The mean number of somites and crown-rump length were 19.53 ± 2.33 and 625.11 ± 69.64 µm, respectively.

Group C eggs were administered 0.3 mg/egg metoclopramide. Five embryos exhibited delayed NT closure (Figure 2); the NTs of 10 embryos were closed. The mean number of somites and crown-rump length were 18.87 ± 1.96 and 578.59 ± 66.03 µm respectively. Embryonic development was determined by somite number; the H-H stage was 12 and their development was considered normal.

Group D eggs were administered 0.6 mg/egg metoclopramide. Eight embryos exhibited delayed NT closure and three were underdeveloped based on H-H classification; one embryo was classified stage 9, the others stage 10. The mean number of somites and crown-rump length were 17.93 ± 1.33 and 560.31 ± 83.01 µm, respectively.

Group E eggs were administered 1.2 mg/egg metoclopramide. Nine embryos exhibited delayed NT closure and five were underdeveloped according to H-H classification. One embryo with development retardation was classified stage 8, another was stage 9 and the remaining underdeveloped embryos were stage 10. The mean number of somites and crown-rump length were 16.33 ± 3.72 and 547.76 ± 98.64 µm, respectively.

The means of crown-rump length, number of somites, TAA:NA ratios and number of AgNOR are shown in Table 1. We found significant differences in NT closure among the groups. Mean number of somites, crown-rump length, TAA:NA ratios and number of AgNOR were decreased incrementally from the control group to group E. We observed significant differences between group A and groups C – E in number of somites ($p < 0.05$ for all comparisons), between group A and E in crown-rump length and number of AgNOR ($p < 0.001$ for both comparisons) and between control and all experimental groups in TAA:NA ratios ($p < 0.001$ for all comparisons).

Table 1. Embryonic development in control and experimental groups for 48 h embryos.

	NT open/closed	Crown-rump length (µm)	Somite count	TAA:NA ratio	AgNOR number
Group	0/15	661.54 ± 85.00	20.87 ± 2.33	0.340 ± 0.15	2.97 ± 1.40
Group B	4/11*	625.11 ± 69.64	19.53 ± 2.13	0.261 ± 0.15*	2.76 ± 1.27
Group C	5/10*	578.59 ± 66.03	18.87 ± 1.96*	0.196 ± 0.95*	2.39 ± 1.16
Group D	8/7*	560.31 ± 83.01	17.93 ± 1.33*	0.154 ± 0.06*	2.58 ± 1.31
Group E	9/6*	547.76 ± 98.64*	16.33 ± 3.72*	0.147 ± 0.06*	1.77 ± 0.93*
p	< 0.001	< 0.001	0.015	< 0.001	< 0.001

Data are means ± SD. *Statistically significant compared to group A.

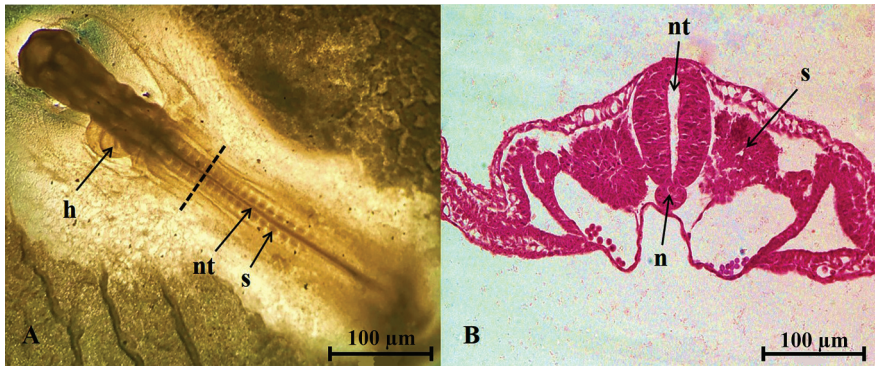

Figure 1. Chick embryo, 48 h, of control group appears normal. A) Wholemount; dashed line indicates plane of section for panel (B). B) Section through thoracic segment. H, heart; n, notochord; nt, neural tube; s, somite. H & E staining.

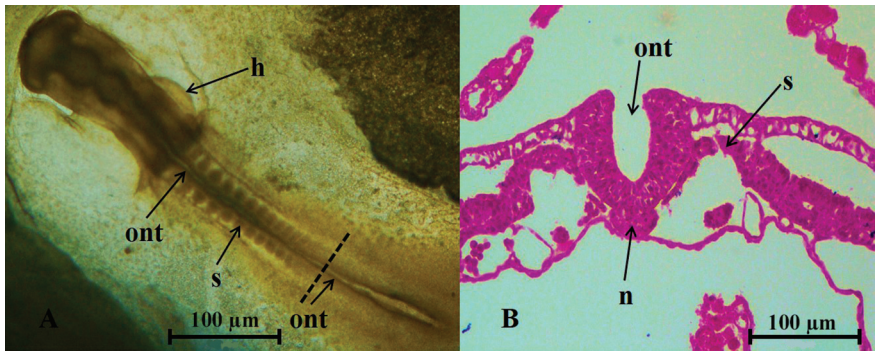

Figure 2. Chick embryo, 48 h, of experimental group C showing open NT. A) Wholemount; dashed line indicates plane of section for panel (B). B) Section through lumbar segment. h; heart; n, notochord; ont, open neural tube; s, somite. H & E staining.

72 h embryos

In groups A – C, the NTs of all embryos were closed and no malformation or developmental retardation was detected (Figure 3). The mean crown-rump length of groups A, B and C were 884.10 ± 123.84, 806.21 ± 96.81 and 775.84 ± 106.31 µm, respectively (Table 2). The mean TAA:NA ratio was greatest in group A and was significantly different from group E only (Table 2). A section of AgNOR stained tissue is shown in Figure 5. In 72 h embryos, development precludes their transparent appearance by light microscopy; therefore, it is not possible to count somites by microscopic examination. The cervical flexure was increased and second and third branchial arches were developed. We assessed H-H stages to be 19–20.

In group D, four embryos exhibited delayed NT closure in H & E stained sections and all specimens were underdeveloped (Figure 4). Macroscopic examination indicated that one of the embryos with developmental retardation was H-H stage 16, two were stage 17 and the other stage 18. The mean crown-rump length was 760.51 ± 211.84 µm.

In group E, five embryos exhibited delayed NT closure in H & E stained sections; all were underdeveloped. Macroscopic examination revealed that one of the embryos with developmental retardation was H-H stage 15, three were stage 17 and one was stage 18. The mean crown-rump length was 670.95 ± 132.01 µm.

The incidence of delayed NT closure in groups D and E was significantly greater than for groups A – C ($p < 0.001$ for all comparisons). The means of crown-rump length,

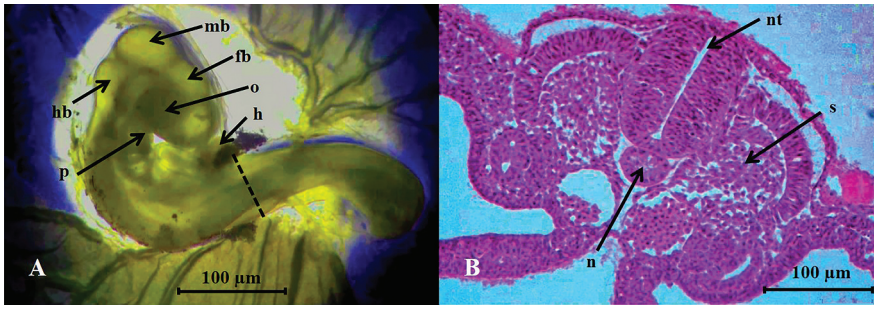

Figure 3. Chick embryo, 72 h, of control group appears normal. A) Wholemount; dashed line indicates plane of the section for panel (B). B) Section through thoracic segment with open neural tube. Fb, forebrain; mb, midbrain; h, heart; hb, hindbrain; n, notochord; nt, neural tube; o, optic vesicle; p, pharyngeal arch; s, somite. H & E staining.

Table 2. Embryonic development in control and experimental groups for 72 h embryos.

	NT open/closed	Crown-rump length (µm)	TAA:NA ratio	AgNOR number
Group A	0/15	884.10 ± 123.84	0.265 ± 0.26	2.27 ± 1.16
Group B	0/15	806.21 ± 96.81	0.214 ± 0.21	2.60 ± 1.30
Group C	0/15	775.84 ± 106.31	0.208 ± 0.17	2.01 ± 1.00
Group D	4/11*	760.51 ± 211.84	0.201 ± 0.03	2.02 ± 1.07
Group E	5/10*	670.95 ± 132.01*	0.187 ± 0.11*	2.46 ± 1.20
p	< 0.001	< 0.001	< 0.001	0.397

Data are means ± SD. *Statistically significant compared to group A.

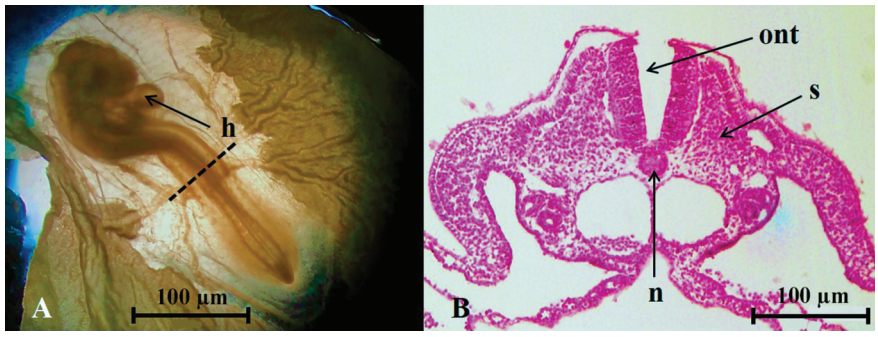

Figure 4. Chick embryo, 48 h, of experimental group D with open NT. A). Wholemount; dashed line indicates plane of section for panel (B). B) Section through thoracic segment. h, heart; n, notochord; ont, open neural tube; s, somite. H & E staining.

TAA:NA ratios and number of AgNOR are shown in Table 2. We found a significant decrease in crown-rump length and TAA:NA ratios between groups A and E ($p < 0.001$), but no significant difference in number of AgNOR among all experimental groups. Somites could not be counted in 72 h embryos due to their fusion (H-H stage 20); therefore, this parameter was excluded from the evaluation.

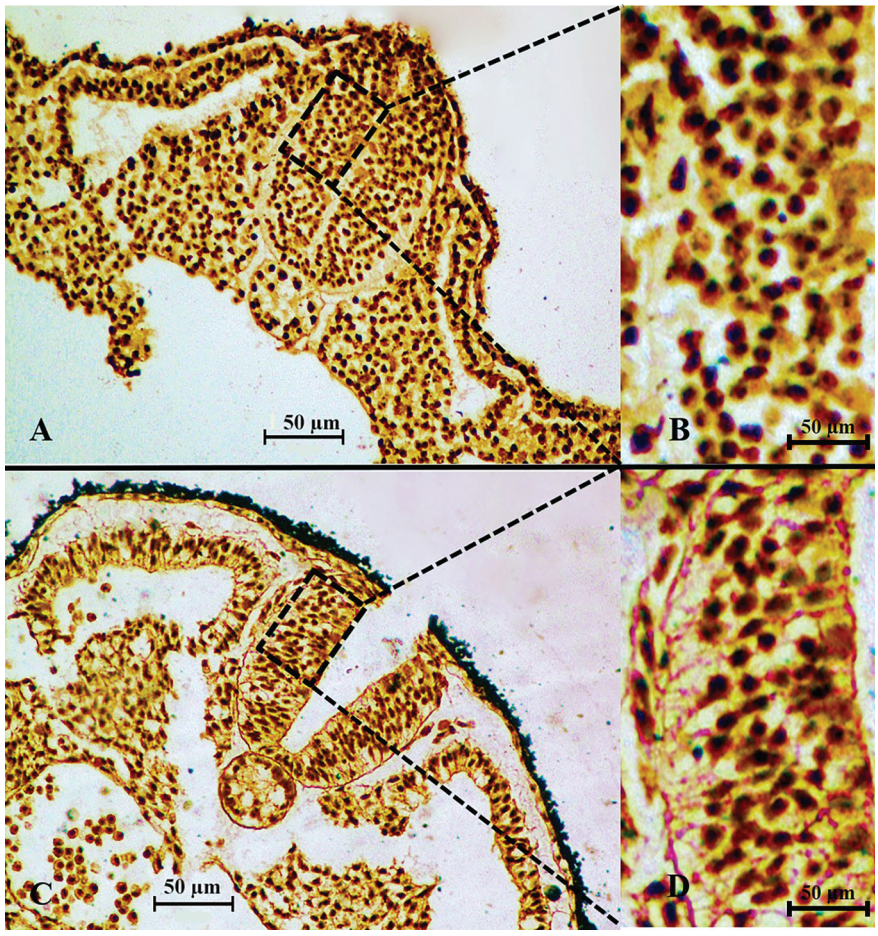

Figure 5. AgNOR stained cells in chick embryo. A) Control group: cells around the neural tube are numerous. B) Control group: AgNOR staining in the cells of the neural tube are stained intensely. C) Group E. Fewer stained cells around the neural tube than in the control group. D) Absence of AgNOR staining in the cells in the open NT of embryo of group E.

Discussion

Early neural development is a multistep process with morphologically distinct stages; however, the molecular events responsible for morphologic development are not well understood (Sahin Inan and Unver Saraydin 2019). Exposure to teratogens and toxins during this process can cause NTD due to failure of NT closure. Failure of NT closure causes brain and spine malformation; these malformations originate between days 18 and 60 of pregnancy in humans (Botto et al. 1999; Mohd-Zin et al. 2017; Mühl-Benninghaus 2018).

Many models including mammals, poultry, amphibians and computer modeling have been used to investigate NTD; advantages and disadvantages attend all of these. The early chick embryo corresponds well to the first month of embryonic development in mammals and therefore is a useful model for studying NTDs (Drake et al. 2006).

Metoclopramide is a commonly used anti-emetic that is classified as category B by the Food and Drug Administration, i.e., it has not been demonstrated to be a risk to the fetus, but there are no definitive studies in pregnant women. Metoclopramide can cross the human placental barrier (Arvela et al. 1983; Briggs et al. 2005; Vlastarakos et al. 2008) and has been reported to exhibit significant side effects and occasional malformations in newborns (Ruedy 1984; Çekmez et al. 2016). Fazliogullari et al. (2013) reported that use of metoclopramide during the perinatal period may cause teratogenic effects on some organs. In adults, adverse effects on the extrapyramidal system have been reported following exposure to high concentrations of metoclopramide (Poortinga et al. 2001).

Reports concerning the effects of metoclopramide on the central nervous system during prenatal development are contradictory. Matok et al. (2009) reported that 182 of 3458 infants (5.3%) exposed to metoclopramide during the first trimester exhibited major congenital malformations; however, metoclopramide exposure was not significantly associated with congenital malformations, low birth weight or perinatal death. Pasternak et al. (2013) reported that among 1,222,503 pregnant women, no significant association was found between malformations and metoclopramide use during the first trimester of pregnancy. Other investigators reported that metoclopramide treatment during the first trimester caused no adverse outcomes for the fetus (Matok et al. 2009; Pasternak et al. 2013). To the contrary, Çekmez et al. (2016) reported that the number of implanted human embryos decreased when high doses of metoclopramide were administered. Ruedy et al. (1984) asserted that some anti-emetics, including metoclopramide, cause fetal malformations in animals and that it could not be stated unequivocally that the drug was without risk to the fetus. Fazliogullari et al. (2013) used an embryo culture model to investigate the teratogenic and toxic effect of anti-emetics. These investigators reported that metoclopramide was the least toxic anti-emetic among the drugs tested and that it was teratogenic only at high concentrations.

We investigated the effects of metoclopramide on NT development using chick embryos. We found that the H-H stages of all embryos in both the 48 h and 72 h control groups were consistent with embryonic stages, i.e., stages 12–13 and 19–20, respectively, and that side effects of metoclopramide treatment were increased in a dose-dependent manner. High-dose metoclopramide treatment delayed closure of the NT in chick embryos. We conclude that use of metoclopramide may produce teratogenicity only at high doses. Owing to our experimental design, we cannot comment on possible adverse effects of long-term exposure.

We investigated cells during NT development using AgNOR staining. The AgNOR positive proteins are argyrophilic nucleolar proteins that are expressed strongly in rapidly dividing cells (Carbajo et al. 1993; Derenzini et al. 1995), while their expression is low in nonproliferating cells (Gos et al. 2008; Ertekin et al. 2016). We determined the mean AgNOR numbers and TAA:NA ratios (Figure 5). To our knowledge, ours is the first investigation in which AgNOR staining was used to assess the effects of metoclopramide on NT development in chick embryos. We investigated whether metoclopramide affects cell proliferation by assessing the amount of argyrophilic protein detected by AgNOR staining. Because counting AgNOR clusters using light microscopy is poorly reproducible, we also assessed the TAA:NA ratio, which increased accuracy and reliability. We found significant decreases in TAA:NA ratio in both 48 h and 72 h embryos in all experimental groups compared to control groups (Tables 1 and 2). NT development was delayed by higher doses of metoclopramide in all experimental groups. Based on number of AgNOR positive cells and TAA:NA ratio, we found a dose dependent decrease in cell division. Under normal conditions, the amount of AgNOR protein is expected to increase during cell division. We found that increasing drug doses delayed cell division and therefore neurogenesis.

We found that metoclopramide retarded NT formation in chick embryos in a dose dependent manner. We also found that metoclopramide significantly decreased the crown-rump length and number of somites in high-dose experimental groups compared to controls. Because animal

studies do not always accurately predict effects in humans, the significance of delayed NT formation due to metoclopramide is not entirely clear for humans; further studies are required to clarify the effects of metoclopramide on embryonic development. We also demonstrated that estimation of the number of AgNOR number and TAA: NA ratio are useful markers for neurogenesis and NT development.

Disclosure statement

The authors declare no conflict of interest.

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