DOI: 10.5137/1019-5149.JTN.14518-15.3
Received: 05.03.2015 / Accepted: 18.06.2015
Published Online: 06.03.2016

Original Investigation

Targeting Apoptosis Through FOXP1, and N-cadherin with Glatiramer Acetate in Chick Embryos During Neural Tube Development

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ABSTRACT

AIM: To demonstrate the effect of glatiramer acetate (GA) in chick embryos on neural tube (NT) development, and to explore its effects of FOXP1, apoptosis, and N-cadherin.

MATERIAL and METHODS: One hundred fertile, specific pathogen free eggs were divided into 5 groups for this study. The eggshell was windowed specifically at 24 hours of incubation. The embryos in Group 1 (n=20) were treated with 10 µl physiological saline; in Group 2 the embryos (n=20) were given 10 µl GA (equal to daily human therapeutic dose); 20 µl GA (equal to twice daily human therapeutic dose) was injected to embryos in Group 3 (n=20); in Group 4 and 5, 30 µl and 40 µl GA were administered to the embryos (n=20) (equal to x3 and x4 daily human therapeutic dose, respectively). Each egg was re-incubated for 24 hours more. Then, histological and immunohistochemical evaluation of the subjects were done.

RESULTS: The embryos with NT defect showed FOXP1 expression without N- cadherin or staining with N-cadherin in another location in our study. We interpreted this result as GA leading to an NT closure defect by increasing FOXP expression. Moreover, we also showed the reverse relation between FOXP1 and N-cadherin at the immunohistochemical level for the first time.

CONCLUSION: GA affects the spinal cord development through FOXP in the chick embryo model at high doses.

KEYWORDS: Chick embryo, Glatiramer acetate, FOXP, N-Cadherin, Spinal cord development

INTRODUCTION

Neural tube (NT) defects are uncommon malformations occurring 6 in 10000 pregnancies (60). Congenital malformation incidence is 3-5% in newborns and NT defects cause 7% of newborn deaths related to congenital malformations (13).

Multiple Sclerosis (MS), a chronic demyelinating and degenerative disease of the central nervous system (CNS), is the most common chronic neurologic disability in young adults in their childbearing ages of 20 to 45 (20, 34, 35). The issue of pregnancy planning in these patients makes treatment tricky.

Glatiramer acetate (GA), one of the first-line therapies currently approved for relapsing-remitting multiple sclerosis (RRMS) (34), was originally designed as a synthetic analogue of myelin basic protein (1). Data about exposure to GA during early pregnancy period in humans is limited but animal studies have revealed no fetal risk to date so GA is classified by

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the United States Food and Drug Administration (FDA) as a Category B drug in relation to pregnancy. The manufacturer's post-marketing surveillance about the safety of GA suggested no increased risk in terms of spontaneous abortion and other outcomes and this was also confirmed by real life data from multicentric observational studies (22). GA has been suggested to offer neuroprotection since 2001. We just wondered whether this neuroprotective role of GA might have produced the drug's safety profile during neurulation (49)

The clinical effect of GA has long been attributed to a shift in the cytokine secretion of CD4+ T helper (Th) cells. Recently, its broader immunomodulatory effect on cells of both the innate and adaptive immune system has been elucidated. The immunomodulatory processes related to GA include binding to major histocompatibility complex (MHC) molecules, shifting from a Th1 cytokine profile to a Th2-biased anti-inflammatory profile, the activation of FOXP3+ regulatory T cells, and the inactivation of inflammatory monocytes (2, 7, 17, 47).

Apoptosis, principally regulated by the Bcl2 family of proteins, participates in the morphogenesis and homeostasis of the course of central nervous system development (8). Recently, the ability to regulate apoptosis and tumorigenesis of the subfamily members of the forkhead-box (Fox) family has also been reported (30).

FOX family of transcription factors functions as both transcriptional activators and repressors in the regulation of embryonic development of various organs, including the control of cell differentiation, cell cycle regulation, and pattern formation (6, 28, 32, 57).

Among the FOXP subfamily of transcription factors within the Fox family (36, 50), FOXP proteins play critical roles in immune responses, organ development and cancer pathogenesis.

The switch between E- and N-cadherin has been found to play a key role on the effects of FOXP2 and FOXP4 upon neural differentiation in the spinal cord during early morphogenesis. Increased FOXP expression suppresses the N-cadherin expression that plays a key role in the adherens junctions of the neuroepithelial cells. Regression of N-cadherin by FOXP transcription factors disrupts apical adherens junctions (42, 46).

FOXP2 and FOXP4 are highly expressed during spinal cord neurogenesis. FOXP1 has been suggested as linking effectors of both neuronal migration and axonal projections, but there are only a few studies that assess FOXP1 expression during spinal cord neurogenesis (19). The knowledge about putative functions of FOXP resulting in a spectrum of NT defects associated through disordered neuroepithelial tissue architecture and GA's effect on FOXP led us to investigate the potential dose-dependent teratogenic effect of GA on the spinal cord development in the chick embryo model (46).

To the best of our knowledge, there is no study in the literature about the effects of GA on NT development. The aims of this study were to demonstrate the effect of GA in chick embryos on NT development and, if present, to explore its effects of FOXP1, apoptosis, and N-cadherin.

MATERIAL and METHODS

Chick Embryos

Fertile, specific pathogen free eggs of the domestic fowl (Has tavuk®, Gallus gallus, Bursa, Turkey) were used for this study. The eggs were incubated at 37.5°C and 75% relative humidity for 24 hours until the embryos reached stage six of development according to Hamburger and Hamilton (24). The eggs at that stage were divided into five equal groups. The embryos in Group 1 (n=20) were treated with 10 µl physiological saline; in Group 2 the embryos (n=20) were given 10 µl GA (equal to daily human therapeutic dose); 20 µl GA (equal to twice daily human therapeutic dose) was injected to embryos in Group 3 (n=20); in Group 4 and 5, 30 µl and 40 µl GA were administered to the embryos (n=20) (equal to x3 and x4 daily human therapeutic dose, respectively) (Table I).

Method of Injection

At the sixth stage of development, the eggs were washed

Table I: Statistical Analyses of the Groups

	NT Open (%)	NT Close (%)
Group 1(n=19)(10µl SF)	0	19 (100)
Group 2(n=20)(10µl GA)	4 (20)	16 (80)
Group 3(n=19)(20µl GA)	7 (36.80)	12 (63.20)
Group 4(n=17)(30µl GA)	7 (41.20)	10 (58.80)
Group 5(n=17)(40µl GA)	6 (35.30)	11 (64.70)
p-value	**0.003**	
Pairwise Comparisons		
Gr 1- Gr 2: p=0.106 **Gr 2- Gr 3:** p=0.417 **Gr 3- Gr 4:** p=1.000 **Gr 3- Gr 4:** p=1.000		
Gr 1- Gr 3:p=0.008Gr 2- Gr 4:p=0.297 **Gr 3- Gr 5:** p=1.000		
Gr 1- Gr 4:p=0.002Gr 2- Gr 5:p=0.460		
Gr 1- Gr 5:p=0.006		

NT: *Neural tube,* ***GA:*** *Glatiramer acetate,* ***SF:*** *Physiological saline.*

with 70% alcohol and labeled properly on the outer shell. A hole was made on the blunt pole of the eggs with a sharp and thick needle. Using a sterile Hamilton® syringe, GA or saline was injected from the blunt end under the embryonic disc at doses in accordance with the groups. The holes were sealed with paraffin, turned upside down and the eggs were then placed into an incubator for another 24 hours, reaching developmental stage 12.

Embryo Collection

At the end of the incubation for a total of 48 hours from the onset of the experiment, the eggs were cracked open and the embryos were transferred to a Petri dish after careful dissection of the allantoic stalk from other embryonic structures. All chick embryos were evaluated with the stereomicroscope and light microscope according to the Hamburger- Hamilton classification (24).

Histological Preparation and Analysis

All the embryos were fixed with 10% buffered formalin and examined under the stereomicroscope (Nikon, SZX 1000) to assess the closure of the NT and presence of NT developmental abnormalities, if present. After washing with tap water, they were dehydrated through a graded series of ethanol. The embryos were incubated in xylene after two washes and were transferred into a paraffin-embedded mixture. Then 4 µm transverse serial sections were taken and stained with Hematoxylin–Eosin (HE) according to its routine protocols. Slides were evaluated and photographed under light microscopy (Zeiss, Axio Scope A1) by blinded histologists.

Immunohistochemistry

Four µm thick tissue sections mounted on poly-lysine coated slides were incubated at 60°C overnight. The slides were deparaffinized in xylene and rehydrated through graded alcohol into water and subjected to antigen retrieval using a microwave oven. The tissues were cooled to room temperature. The limits of sections were drawn with a pap pen (Invitrogen Corporation, CA, USA) and incubated in 3% hydrogen peroxidase for 15 min to inhibit the endogenous peroxidase activity. The tissues then were given three 5-min washes in PBS and incubated in blocking solution. Then, sections were incubated for 1 h at 37°C with primary antibodies rabbit polyclonal anti-FOXP1 (1:200, Abcam- ab16645, Boston, USA) and monoclonal anti-N-Cadherin/A-CAM (1:100, Sigma C 3865, Missouri, USA). After washing with PBS, the secondary antibody (SPlink HRP Broad DAB Bulk Kit for Mouse and Rabbit Antibodies, GBI Labs, Mukilteo, WA, USA) was applied for 30 min followed by three washes in PBS. The streptavidin–peroxidase complex was added for 30 min and washed with PBS three times. Then, slides were incubated in fresh 3, 3'-diaminobenzidine (DAB) (GBI Labs, Mukilteo, WA, USA) chromogen for 1-2 min (prepared in a ratio of 1:20). The slides were then washed in water to remove the excess DAB, dehydrated, cleared, and mounted with mounting medium. The presence of a brown precipitate indicated positive findings for the primary antibody. Serial sections were stained with concurrent counter stain hematoxylin for N cadherin. The negative controls received the same treatment, with rabbit IgG or mouse IgG instead of the primary antibody, with hematoxylin solution. The scoring of immunostaining expressions were evaluated as – none, + weak, ++ moderate, +++ severe.

Apoptosis Assay

For the labeling of apoptotic cells, tissue samples, fixed in formalin, were embedded in paraffin and sectioned at 4 µm thickness. We used a standard terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate nick end labeling assay (TUNEL) technique to detect the fragmented DNA associated with apoptosis. For this purpose, the In Situ Cell Death Detection Kit Peroxidase (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. After standard deparaffinization, hydration with progressively decreasing alcohol concentrations, incubation with proteinase K, and blocking of endogenous peroxidase, tissue sections were incubated in a humidified chamber; first, with TdT and digoxigenin–deoxyuridine triphosphate (TUNEL reaction mixture) at 37°C for 60 min; and second, with alkaline phosphatase (AP) converter antifluorescein antibody at 37°C for 30 min. Color was developed with diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) and sections were counterstained with Harris hematoxylin. For negative control purpose, some slides were incubated with label solution not containing TdT. We searched for apoptotic cells showing cell shrinkage with condensed nuclei (pyknosis) and nuclear fragmentation (karyorrhexis) under light microscopy. Cells containing weakly to moderately TUNEL-positive nuclei in the absence of these additional morphological features were not assessed as apoptotic. The stained specimens were examined in a blinded fashion by experienced histologists.

Statistical Analyses

Categorical variables were represented with frequency and related percentage values and compared among groups by performing Fisher-Freeman-Halton exact test, Fisher's exact test and chi-square test with Yates correction. SPSS v.21 vas used for statistical analysis and statistical significance was set at $p<0.05$ (Table I).

■ RESULTS

In this study, we investigated the effect of GA at different dosages on NT development and closure of the neural plaque in a chick embryo model. Groups and NT developments were summarized in Table II.

Nineteen of 20 embryos in group I reached the expected developmental stage and their NTs were closed. There was immaturity in only one embryo in this group (Figure 1A, B). There was no FOXP1 immunoreactivity in the NTs of group I (Figure 1C).

N-cadherin immunoreactivity was severely expressed in epithelial cells and especially those neighboring the surface ectoderm of the NT and moderately expressed in epithelial cells neighboring the luminal side in group I (Figure 1D). There were a few apoptotic cells in the NT epithelium by TUNEL staining in group I (Figure 1E).

Stereomicroscopic evaluation of the drug treated groups, namely groups 4, 7, 7, 6 showed an open NT defect. There was 1 at group 3, 3 at each of group 4 and 5 immature embryos (Figure 2A, B). Light microscopy findings of sections from open NT embryos were in line with the stereomicroscopic evaluation (Figure 2C). Weak and moderate FOXP1 staining at the deep side of the NT epithelium and somites in embryos with open NT was detected. There were no concordance with the increasing drug dosage and FOXP1 staining (Figure 2D). However, FOXP1 immunoreactivity was not detected in the closed neural plate sites. There was no N- cadherin staining at the luminal side of the neuroepithelial cells in the open NT embryos of the drug treated groups. However, there was severe expression for N- cadherin in the middle part of the epithelial wall of some embryos (Figure 2E). N-cadherin staining in closed NT embryos was consistent within group 1. A few apoptotic cells were detected with TUNEL staining in the NT epithelium and somites of all drug treated groups (Figure 2F).

There was statistical significance according to NT malformation between control group and groups 3, 4, 5 (p=0.008, p=0.002, and p=0.006, respectively).

Table II: Stereomicroscopic Examination of the Neural Tube at Different GA Dosages

Neural tube	Group I (n=20)	Group II (n=20)	Group III (n=20)	Group IV (n=20)	Group V (n=20)
Immature	1	0	1	3	3
Open	0	4	7	7	6
Close	19	16	12	10	11

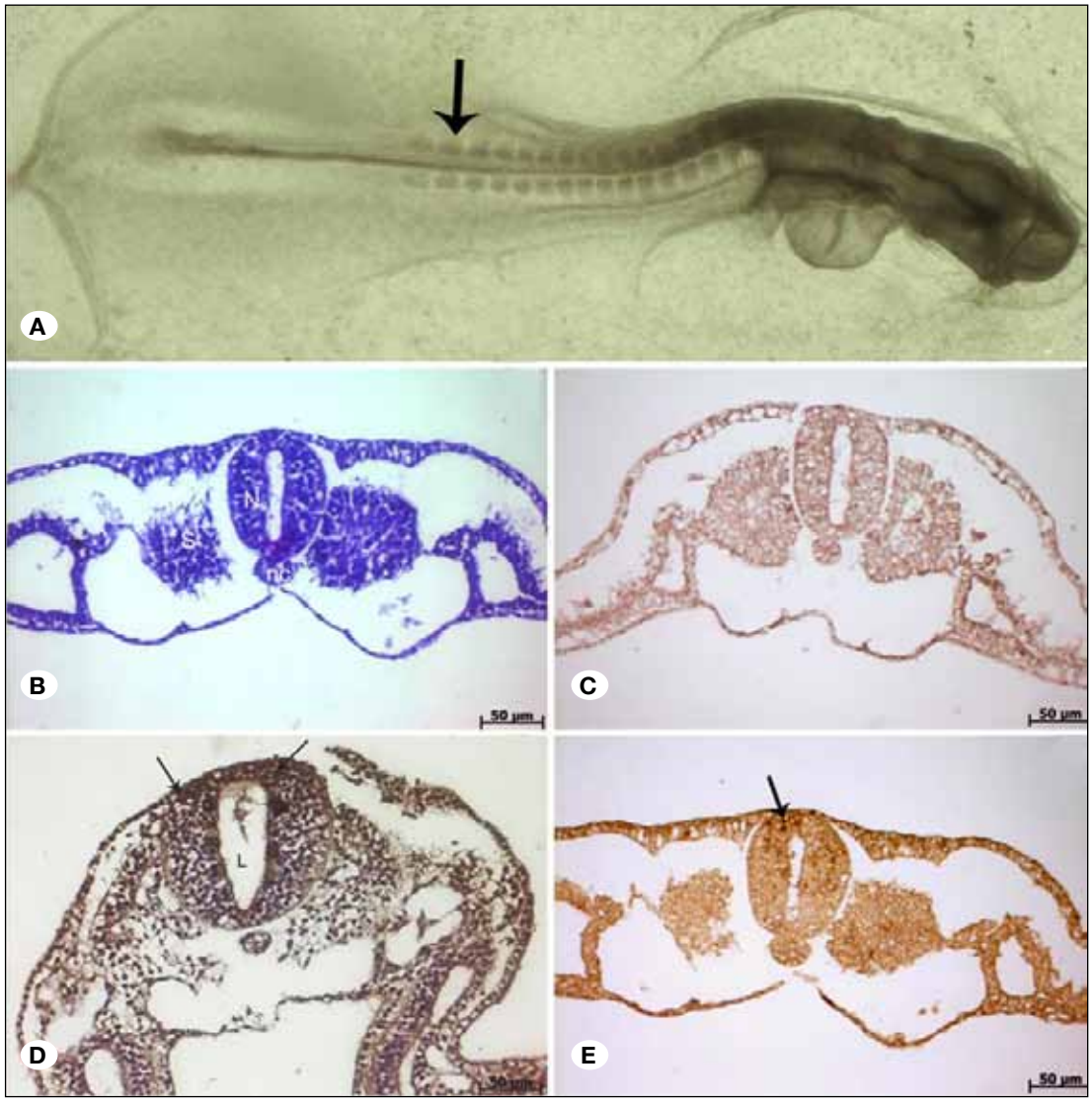

Figure 1:
A) Stereomicroscopic image of closed NT of chick embryo at group I; **B)** Cross section of closed NT at group I (stain: H-E) (N: neural tube, nc: notochord, S: Somite); **C)** No FOXP1 immunoreactivity at NT nucleus at group I; **D)** Severe N-cadherin immunoreactivity [arrow] at adjacent cells of NT surface ectoderm and moderate expression at luminal surface epithelial cells (L: lumen); **E)** Few apoptotic cells at NT epithelium with TUNEL staining at group I [arrow].

DISCUSSION

Neurulation, the process of formation of the brain and spinal cord, includes the formation of the neural plate, and its folding into lateral neural folds which then come together to complete the fusion of the NT (12). There are many potential causes of NT closure defects. Apoptosis is an important mechanism in the morphogenesis and homeostasis of the developing central nervous system, especially during the formation and fusion of the neural folds. Animal models of NT defect have shown increased apoptosis in the neuroepithelial cells (25, 43). There was no difference between the experimental and control groups in terms of apoptosis in our study. Van Boxtel et al. have recently proposed action of FOXP1 through a negative feedback loop to suppress Fox transcription factor class O (Foxo)-induced apoptosis (56). The similarity of the apoptotic cell numbers between the GA-treated group with NT defect and the control group in our study may be explained in the context of the suppression of Foxo-induced apoptosis through increased FOXP1.

NT defects affect about 1 in 1,000 neonates in the United States (53). In some chronic diseases like MS, variable concentrations of drugs are taken during the patient's whole life. GA has been used widely for the treatment of MS worldwide. The limited data on pregnancy and fetal outcomes after in-utero exposure to GA in patients with MS comes from the manufacturer's post-marketing surveillance. This suggests no increased risk in terms of spontaneous abortion and other outcomes (21). However, there is no controlled experimental study on this subject.

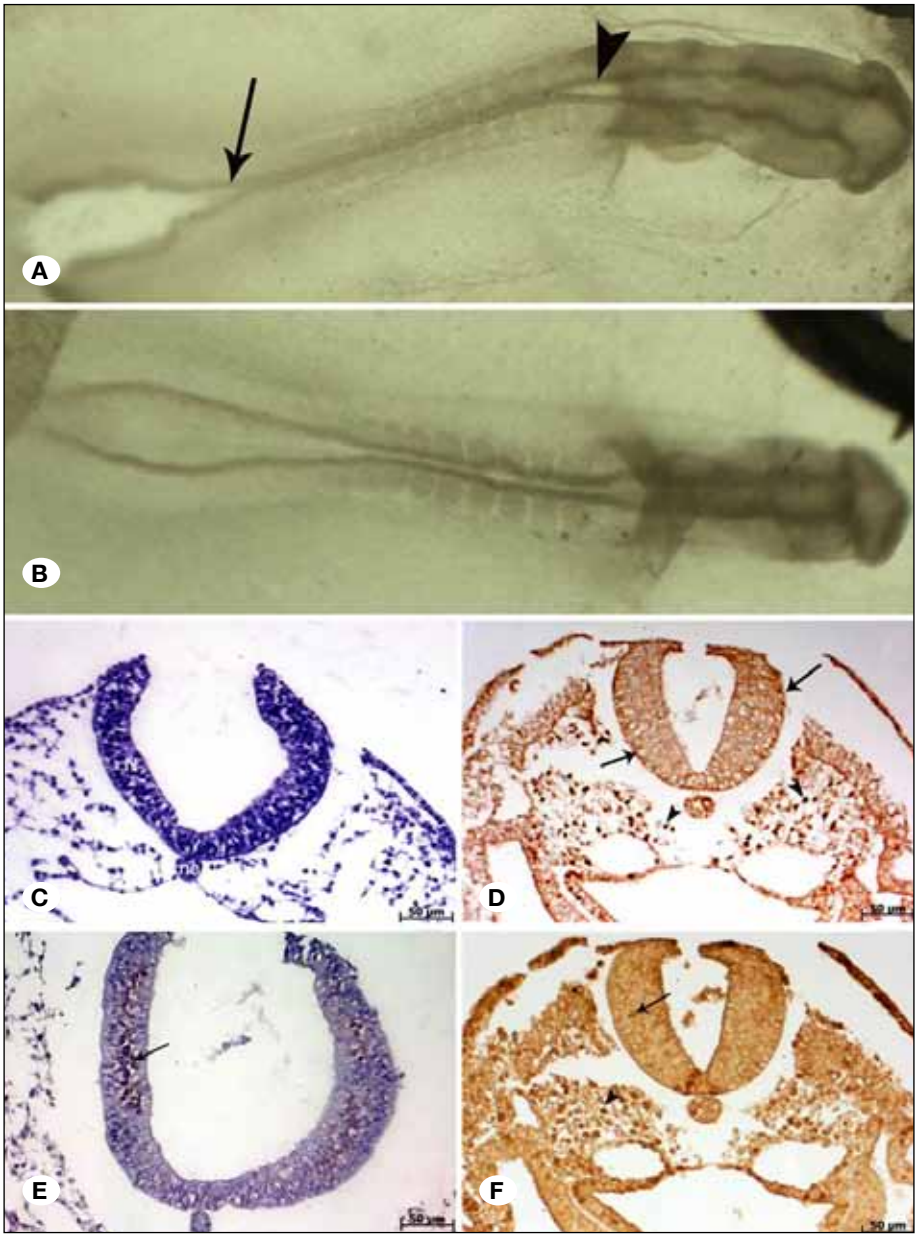

Figure 2: A) Open NT at hindbrain [arrowhead] and caudal [arrow] region at group IV; **B)** Developmental delay at group V chick embryo; **C)** Open NT at 48th hour at group IV. (Stain: H-E) (N: neural tube, nc: notochord); **D)** Mild FOXP1 expression [arrow] at NT epithelium and moderate [arrowhead] expression at somites at group III; **E)** N- cadherin expression [arrow] at group IV; **F)** Few apoptotic cells at NT epithelium [arrow] and somites [arrowhead] with TUNEL staining at group III.

We chose an old, but still commonly used method, chick embryo-model, to investigate the developmental anomalies and to show presence of toxicity or neuroprotection of the drug. Safety and application of this model to humans was well studied in the literature (37, 38, 55). To best of our knowledge, this is the first published GA effect study on the chick embryo model. Group II had 16 (80%) closed NTs while there were 10 (50%) and 11 (55%) closed NTs in groups IV and V, respectively. This shows close association of NT defect in GA with a dose dependent manner.

Of the disease-modifying drugs approved by the FDA, GA is an option for female MS patients of childbearing age. GA led to a significant increase in the FOXP3 expression of CD4+ T cells (33). In GA-treated MS patients, high levels of FOXP3 correlated with increased T-cell regulation. When mice with experimental autoimmune encephalomyelitis (EAE, animal model of MS) were treated with GA, development of type II monocytes, Th2 differentiation of T cells and expansion of Treg were reported. Monocytes isolated from GA-treated mice secreted less pro-inflammatory TNF and IL-12, but more anti-inflammatory IL-10 and transforming growth factor-β (TGFβ), a cytokine with key function for the generation of FOXP3+ Treg (48). Thus, GA is accepted to normalize the frequency and function of Treg in MS (26). Since FOXP is the key regulatory gene in the development of regulatory T cells, GA may affect spinal cord development through this mechanism (23, 27, 60).

FOXP1 and FOXP2 are expressed in various tissues, including the lung, heart, spleen, and the developing and adult CNS, such as the striatum, cerebral cortex, and spinal cord (19, 50-52). FOXP1 plays an important role in the development of the spinal cord (15, 45), and is expressed in some interneurons of the ventral spinal cord during mid- to late embryogenesis (40). FOXP1 has two opposing functions in different cell types: a tumor suppressor in some, and an oncogene in others (3-5, 31). Its function is possibly through apoptosis in tumorigenesis (15, 44).

FOXP2 has also been reported to be expressed in the developing spinal cord (14, 50). Morikawa et al suggested that FOXP1 and FOXP2 may be involved in the determination of the cell type identities during late embryogenesis in 2009 (39).

FOXP3 was initially identified by severe autoimmune diseases associated with its mutations in mouse and human (9, 59), and has emerged as a key transcriptional regulator for the development and function of regulatory T cells (Treg) (27). Because the immune response that characterizes Tregs is realized through the action of FOXP3 that can bind 700 genes and intergenically encode microRNAs, they may have opposing effects on different genes, facilitating the transcription of some genes while repressing the transcription of others. FOXP3-dependent genes mainly have functions in immune response, apoptosis, and tumorigenesis. Changes or defects in the coding sequence of the FOXP3 gene result in the development of different pathological conditions, and one of them is the alteration of Tregs functions leading to further specific autoimmune disorders (30). Tregs have roles in the control of CNS inflammation and activated T cells are predominantly regulated by favoring their commitment to apoptosis (41).

FOXP4, another member of the FOXP family highly homologous to FOXP1, has been shown to dimerize with other FOXP proteins. FOXP4 expression and function in T lymphocytes have also shown recently (Figure 3) (54, 58).

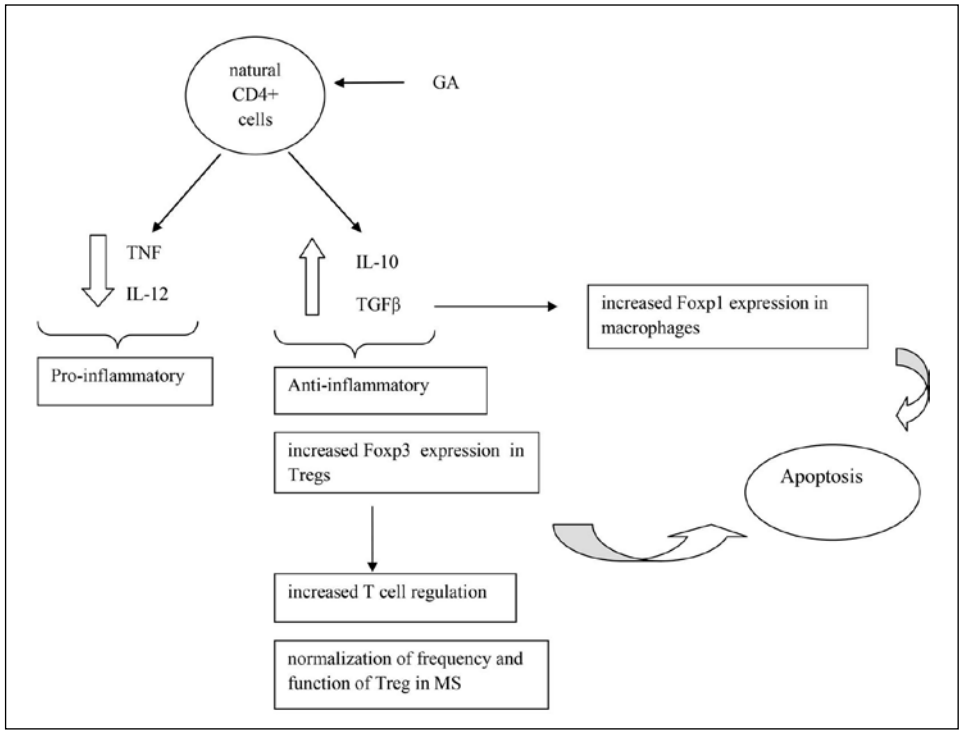

Figure 3: Diagram that shows the possible sequence mechanism of GA (56, 60).

The exciting studies about the effects of FOXP upon neural differentiation in the spinal cord during early morphogenesis, and the role of E- and N-cadherin, and apoptosis in this process necessitate new experimental studies to be designed to analyze these complicated relations.

This is the first study to show the concentration-dependent effect of GA on NT closure in the chick embryo model. At normal concentrations (10 µl), it had no adverse effect on neural development as there was no significant difference in terms of NT developmental defects between 10 µl GA treated and control groups. This amount of GA is close to its human therapeutic plasma levels. The adverse effect began at the 20 µl level. Teratogenicity on NT development increased significantly at higher levels. The investigators found out that replacement of the frequent 20 mg daily GA injections with less frequent 40 mg every other day injections had the same efficacy in RRMS patients (29). The reduction of GA dosing frequency was reasonable for MS patients tired of daily self-injections. Some issues about more injection reactions with the 40 mg every other day regimen are still present (10, 11). Moreover, accumulation of GA when accidentally used during gestation may create spinal developmental anomalies.

As far as has been shown, the mechanism of the effect in MS seems to involve FOXP3 but it may affect other members of FOXP family as well, leading to increased expression of other subgroups in the family. While anti-inflammatory process induced by FOXP3 in GA-treated subjects may be protective and warranted initially, GA, in a dose dependent manner may result in NT defects through the actions of FOXP1, 2, and 4. Although the 3 higher doses of GA differed from the control, they did not differ from each other. So, a dose-related effect needs further evaluation.

We found closure defects in chick embryos at the Hamburger Hamilton stage 12 which were administered GA. There were two openings, one cranially, and another one caudally. A cranially located opening defect in the chick embryo at Hamburger Hamilton stage 12 corresponds to the level of the midbrain-hindbrain and/or hindbrain-spinal cord boundary (16, 18). The clinical significance of this finding needs further investigation. With the knowledge of neurulation, which goes down caudally, we would like to draw attention to the presence of defects with high dose GA during neurulation, which is an ill-defined and complex process. This study also may remind the scientists of the chick embryo model, an old but still relevant one for understanding neurulation, human birth defects, and teratogenicity of drugs.

There was a relationship between FOXP1 expression at the immunohistochemical level and the presence of NT defect in GA-treated groups, while a reverse relationship was observed between FOXP1 expression and N-cadherins.

The embryos with NT defect showed FOXP1 expression without N-cadherin or staining with N-cadherin in another location in our study. We interpreted this result as GA's leading to NT closure defect by increasing FOXP expression. Moreover, we also showed the relation between FOXP1 and N-cadherin at the immunohistochemical level for the first time.

The FOXP-based transcriptional mechanism regulating the integrity and cytoarchitecture of neuroepithelial progenitors was revealed by Rousso et al. (46). They indicated that FOXP2 and FOXP4 play a crucial role in suppressing the expression of N-cadherin. We provided proof for the FOXP1 and N-cadherin relationship.

CONCLUSION

GA affects spinal cord development through FOXP in the chick embryo model at high doses. These results should be further explored in additional experimental and clinical studies.

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