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Decreased TGF-β1 and IGF-1 protein expression in rat embryo skull bone in folic acid-restricted diet

Mochammad Istiadjid Eddy Santoso[a], Mohammad Saifur Rohman[b,*]

[a]*Division of Neurosurgery, Department of Surgery, Brawijaya University, Malang, 65145 Indonesia*
[b]*Department of Biochemistry, Faculty of Medicine, Brawijaya University, Malang, 65145 Indonesia*

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Abstract

Folic acid deficiency during conception up to the end of the third month of gestation is believed to play the most important factor in neural tube defects (NTDs). However, the exact molecular mechanism remains to be elucidated. It has been suggested that transforming growth factor-β (TGF-β1) and insulin-like growth factor-1 (IGF-1) play a critical role in supporting bone formation. Therefore, folic acid deficiency may contribute to NTD occurrence via decreased TGF-β1 and IGF-1 expression. This study aimed to determine the correlation between folic acid deficiency and the expression of TGF-β1 and IGF-1 in rat skull bone. Thirty female Sprague–Dawley rats were divided into three groups. Purified diet containing 5 (restricted), 15 (low) and 30 μg (normal) of folic acid was given to the first, second and third groups, respectively. At 16 weeks of a given diet, blood samples were taken to examine folic acid (folate immunoassay method), TGF-β1 and IGF-1 (enzyme-linked immunosorbent assay method) levels. After forced mating, on the 18th–19th day of gestation (E18–19), the pregnant rats were subjected to hysterectomy. The skull bone samples of E18–19 rats were taken to examine the TGF-β1 and IGF-1 protein expression by immunohistochemistry. The folic acid-restricted diet (5 μg) resulted in decreased serum TGF-β1 and IGF-1 levels. Furthermore, protein expression of TGF-β1 and IGF-1 in E18–19 rat skull bones was also significantly lower in the folic acid-restricted diet than in the normal diet. Folic acid deficiency could result in reduction of TGF-β1 and IGF-1 protein levels and might contribute to formation of defects in the skull bone as observed in mengingocele patients.

Keywords: Folic acid; TGF-β1; IGF-1; Skull bone; Meningocele; Neural tube defects

1. Introduction

Meningocele is defined as a congenital defect with herniation of the meninx layer and cerebral fluid through a defect in the skull bone. In cases where the brain tissue herniated, the term *meningoencephalocele* or *encephalocele* is used [1–3]. These abnormalities are called neural tube defects (NTDs) [4–6].

Previous reports have suggested that folic acid deficiency during conception up to the end of the third month of gestation is the most important factor responsible for NTD. However, its molecular mechanism remains unknown [3,7,8].

In embryologic development, many speculations were raised to explain the cause of skull bone defect formation. One hypothesis follows the neurulation disorder theory, which states that the neuralgic ectoderm stays attached to the epidermis ectoderm in the midline occipital bone during organogenesis early in the pregnancy. Thus, there is a hindrance to the bone-forming mesoderm cells' migration to the attachment site of the two ectoderm layers. This condition causes no bone formation in the respective site; hence, a defect is formed.

Another nonseparation theory has suggested that there is no explanation for the nonseparation of the two ectoderm layers [3]. It is assumed that mediator substance, such as growth factors, plays an important role, especially the growth factors that synthesize bone tissues, such as transforming growth factor-β (TGF-β), in particular, TGF-β1 and insulin-like growth factor-1 (IGF-1) [9–11]. Previous studies have suggested that TGF-β1 and IGF-1 supported the function of bone cells [12–14].

In contrast to bone defect, for instance, in craniosinostosis cases (cranium suture closes before its proper time) and acromegaly, there have been numerous research that

* Corresponding author. Tel.: +62 341 560491; fax: +62 341 564755.
E-mail address: ippoenk@yahoo.com (M.S. Rohman).

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revealed the increase in activity of those two growth factors in supporting bone overgrowth [11,14]. Accordingly, TGF-β1 and IGF-1 expression levels seem to be a critical factor in bone formation.

An increasing body of evidence has raised an interesting issue, which claims that administering folic acid supplement during the periconception period might prevent the occurrence and reoccurrence of approximately 70% of NTD cases [15,16]. Therefore, folic acid may be involved in bone growth and formation during pregnancy.

However, the mechanism by which folic acid deficiency could prevent meningocele remains to be elucidated. One possible explanation is that folic acid deficiency may affect TGF-β1 and IGF-1 levels, which may disturb these growth factor functions in bone formation. However, there is a lack of study that investigates the correlation between folic acid deficiency and TGF-β1 and IGF-1 levels.

The present study was conducted to determine the correlation between folic acid deficiency and TGF-β1 and IGF-1 levels in pulp skull bone. We observed that folic acid-restricted diet (5 μg) results in a decrease in serum TGF-β1 and IGF-1 levels. Furthermore, protein expression of TGF-β1 and IGF-1 in pulp skull bones was significantly lower in the folic acid-restricted diet than in the normal diet (30 μg of folic acid). We also observed to a similar extent a decrease in TGF-β1 and IGF-1 expression in meningocele patients.

2. Materials and methods

Anti-rat TGF-β1 and anti-rat IGF-1 antibodies were purchased from Santa Cruz Biotechnology. Monoclonal antibody for folate binding protein/folate immunoassay was obtained from R&D Systems.

2.1. Animal care and handling

Thirty 6- to 8-week-old female Sprague–Dawley rats were randomly divided into three groups; each group consisted of 10 female rats, with an average weight of 250 g. Group A was given a diet containing very low folic acid, 5 μg (restricted); Group B, 15 μg (low); and Group C, 30 μg (normal). The process of preparing diets was based on standard books about nutrient and micronutrient contents [17,18], with a composition of 350 g of glucose, 100 g of casein, 70 g of cellulose, 5 g of succinyl-sulphatiazol, 5 g of choline-Cl, 15 ml of sunflower oil, 100 ml of mineral, 20 ml of trace element and 0.6 ml of mixed vitamin (without folic acid).

The diets were administered for 16 weeks. On the 16th week, blood was taken to measure the serum and erythrocyte folic acid levels as well as serum TGF-β1 and IGF-1 levels. All female rats were then mated with male Sprague–Dawley rats to induce pregnancy. On the 18th–19th day of gestation, the pregnant rats were subjected to hysterectomy. The care and use of the animals strictly followed the guidelines of the Animal Research Committee of Brawijaya University Medical faculty.

2.2. Measurement of serum and erythrocyte folic acid levels

One milliliter of whole blood was collected from the tail vein of female rats (total 30 rats) at 2:00 p.m. The folic acid level was measured by folate immunoassay method (FIM) as previously described [8,19].

2.3. Measurement of serum TGF-β1 and IGF-1 levels

One milliliter of serum was collected from the tail vein of female rats at 2:00 p.m. The TGF-β1 and IGF-1 levels were measured by enzyme-linked immunosorbent assay (ELISA). The wavelength absorbance at 450 and 620 nm was used after incubation and addition of stop solution.

2.4. Measurement of skull bone TGF-β1 and IGF-1 levels

Pregnant rats were subjected to hysterectomy on the 18th–19th day of gestation (E18–19), and embryos were operated on to remove the base frontal bone and midline occipital bone. The skull bones were then fixed in formalin and subjected to immunohistochemistry using anti-rat TGF-β1 and anti-rat IGF-1 antibodies [11]. The stained cells were randomly counted in five different places of the counting area. These semiquantitative data were further analyzed by a statistical test.

2.5. Statistical analysis

The significance of differences between groups was determined by paired-sample *t* test and one-way analysis of variance. Values were considered significantly different if P was $<.05$. A test was performed using Statview/SPSS software.

3. Results

3.1. Erythrocyte and serum folic acid level in maternal rats

As shown in Fig. 1, there was a significant difference in erythrocyte folic acid level between Groups A (very low folic acid) and B (low folic acid). This significant difference was further increased when the erythrocyte folic acid level of Group A was compared with that of Group C (normal folic acid). The observation suggested that the folic acid-restricted diet resulted in a significant decrease in erythrocyte folic acid levels. However, we could not detect any significant difference in erythrocyte folic acid level between Groups B and C.

There was a significant difference in serum folic acid level between Groups A and B (see Fig. 2). We also observed a significant difference in serum folic acid level among all three groups. Therefore, the folic acid-restricted diet successfully reduced the erythrocyte and serum folic acid level in this study. Accordingly, this model could be useful in evaluating the effect of folic acid deficiency on the measurement of skull bone TGF-β1 and IGF-1 expression levels.

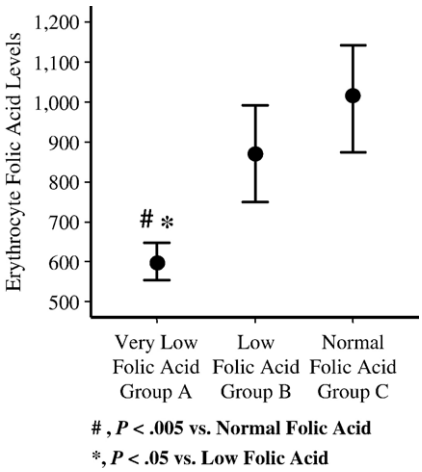

Fig. 1. Decreased erythrocyte folic acid level of maternal rats in response to a very-low-folic acid diet. The folic acid-restricted diet resulted in a significant decrease in erythrocyte folic acid levels. There was no significant difference in erythrocyte folic acid level between Groups B and C. The diets were administered for 16 weeks. On the 16th week, blood was taken to measure the erythrocyte folic acid levels. The folic acid level was measured by FIM.

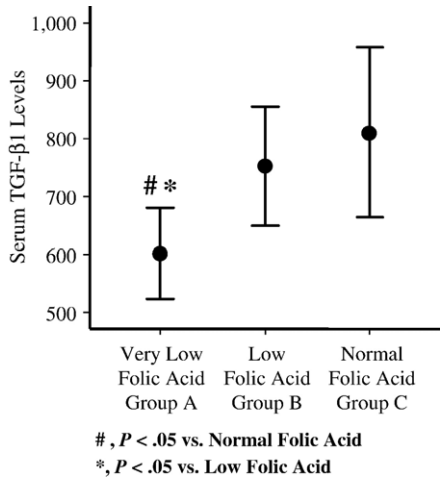

Fig. 3. A very-low-folic acid diet resulted in decreased TGF-β1 protein expression. Only by administration of very-low-folic acid diet did a significant decrease in serum TGF-β1 level result. Low-folic acid diet did not change serum TGF-β1 protein level significantly. The TGF-β1 protein level was measured by ELISA as described in the Materials and Methods.

3.2. TGF-β1 and IGF-1 levels in maternal serum

In the presence of a decreased serum folic acid level, we wanted to determine whether the decrease in folic acid level may coincide with a down-regulation of serum TGF-β1 and IGF-1 levels in these rats. As shown in Fig. 3, there was a significant difference in serum TGF-β1 level either between normal- and very-low-folic acid diet or between low- and very-low-folic acid diet. However, comparing the serum TGF-β1 level of low-folic acid diet and normal-folic acid diet, we could not find any significant difference. Therefore, only by administration of very-low-folic acid diet did a significant decrease in serum TGF-β1 level result. Fig. 4 shows a significant difference in serum IGF-1 level between Groups A (very low folic acid) and C (normal folic acid), even if Group B (low folic acid) showed a significant difference in serum IGF-1 level compared with the other two groups. These data suggested that a folic acid-restricted diet (5 μg) was necessary to induce a significant down-regulation of serum IGF-1 and TGF-β1 levels, whereas a low-folic acid diet (15 μg) did not significantly affect either the serum TGF-β1 or the IGF-1 level.

3.3. TGF-β1 and IGF-1 level in the skull bone of E18–19 rats

We then examined the TGF-β1 and IGF-1 protein levels in the skull bone of rat embryos, to determine whether the folic acid-restricted diet resulted in decreased TGF-β1 and IGF-1

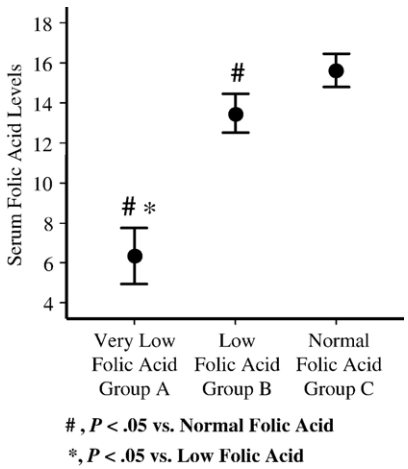

Fig. 2. Decreased serum folic acid level of maternal rats in response to very-low- and low-folic acid diets. The folic acid-restricted diet resulted in a significant decrease in serum folic acid levels. The diets were administered for 16 weeks as described in the Materials and Methods. On the 16th week, blood was taken to measure the erythrocyte folic acid levels. The folic acid level was measured by FIM.

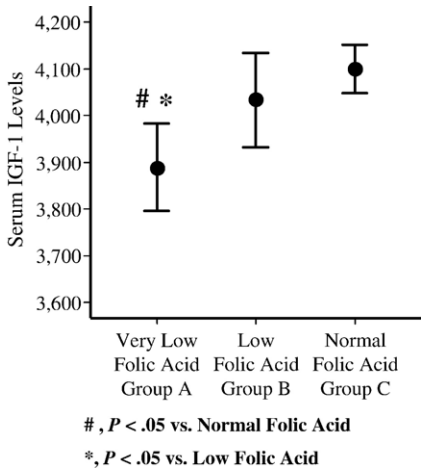

Fig. 4. Decreased IGF-1 protein expression in a very-low-folic acid diet. Low-folic acid diet did not change serum IGF-1 protein level significantly. Only by administration of a very-low-folic acid diet did a significant decrease in serum TGF-β1 level result. The IGF-1 protein level was measured by ELISA as described in the Materials and Methods.

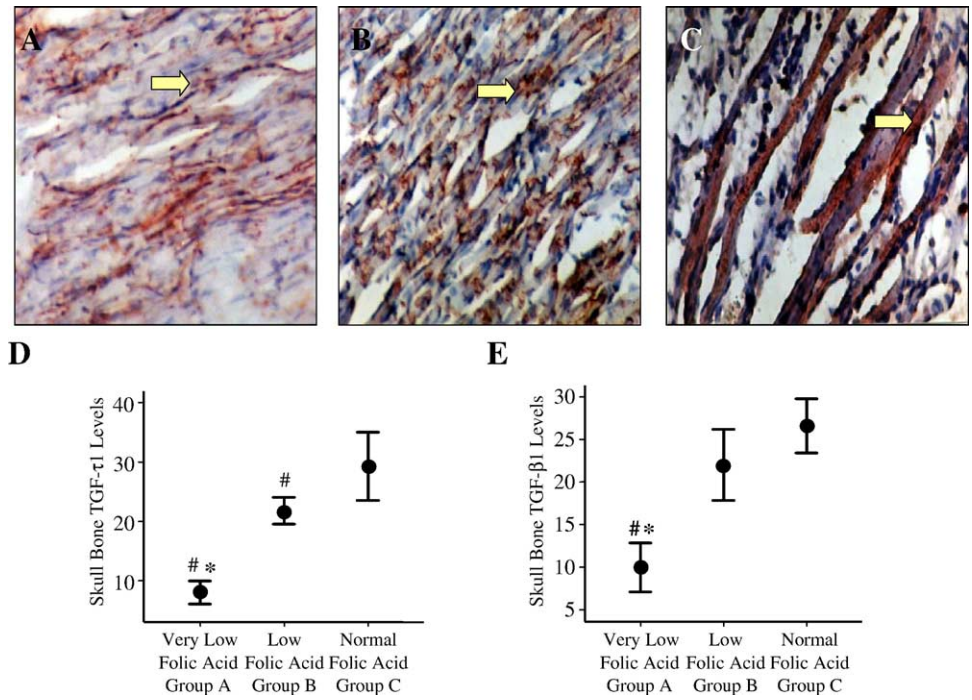

Fig. 5. TGF-β1 expression in the skull bone of E18–19 rat embryos. Immunohistochemistry with peroxide method followed by Mayer–Harris hematoxylin counterstaining of monoclonal anti-TGF-β1 antibody was used to detect TGF-β1 expression. A red-brownish appearance (arrow) is a typical sign of the TGF-β1 expression, which differs in each group. The more intense and wider the red-brownish appearance, the higher the TGF-β1 level in the base frontal skull bone. (A) Very-low-folic acid diet. (B) Low-folic acid diet. (C) Normal-folic acid diet. The stained cell specific for TGF-β1 protein was calculated as mentioned in the Materials and Methods. (D) Statistical analysis of TGF-β1 in the base frontal bone of E18–19 rat embryos. (E) Statistical analysis of TGF-β1 in the midline occipital bone of E18–19 rat embryos.

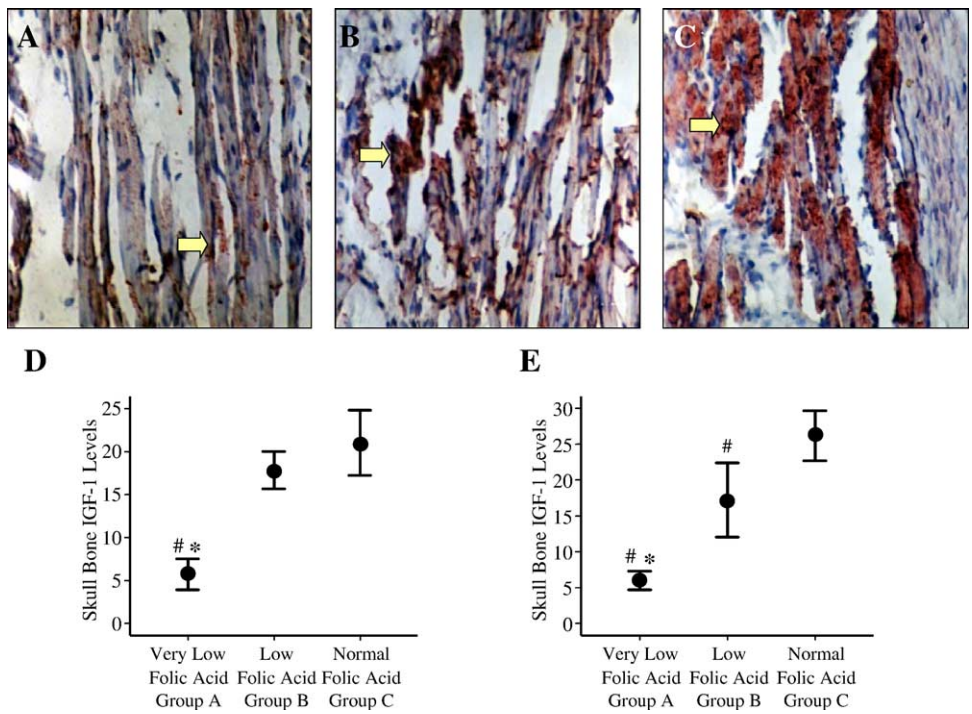

Fig. 6. IGF-1 expression in the skull bone of E18–19 rat embryos. Immunohistochemistry with peroxide method followed by Mayer–Harris hematoxylin counterstaining of monoclonal anti-IGF-1 antibody was used to detect IGF-1 expression. A red-brownish appearance (arrow) is a typical sign of the IGF-1 expression, which differs in each group. The more intense and wider the red-brownish appearance, the higher the IGF-1 level in the midline skull bone. (A) Very-low-folic acid diet. (B) Low-folic acid diet. (C) Normal-folic acid diet. The stained cell specific for TGF-β1 protein was calculated as mentioned in the Materials and Methods. (D) Statistical analysis of TGF-β1 in the base frontal bone of E18-19 rat embryos. (E) Statistical analysis of TGF-β1 in the midline occipital bone of E18–19 rat embryos.

expressions. Immunohistochemistry method was used to detect the specific expression of TGF-β1 and IGF-1 using anti-rat TGF-β1 and anti-rat IGF-1 antibodies, respectively, as described in the Materials and Methods. Immunohistochemistry with peroxide method followed by Mayer–Harris hematoxylin counterstaining of monoclonal anti-rat TGF-β1 and anti-rat IGF-1 antibodies was used to detect TGF-β1 and IGF-1 expressions. A red-brownish appearance is typical of the expression of the two growth factors, which differs in each group. The more intense and wider the red-brownish appearance, the higher the TGF-β1 and IGF-1 levels qualitatively. The stained cells specific for TGF-β1 and IGF-1 protein were calculated as mentioned in the Materials and Methods (Fig. 5A–C). In the base frontal bone, we observed a significant difference in TGF-β1 protein expressions between Groups A (very low folic acid) and C (normal folic acid). To a lesser extent, TGF-β1 protein expression in Group B (low folic acid) was significantly higher than in Group A. A significant difference was also observed in skull bone TGF-β1 level between Groups B and C (Fig. 5D). We observed in a similar fashion a decrease in skull bone TGF-β level in the midline occipital bone, even if there was no significant difference in skull bone TGF-β1 level between Groups B and C (Fig. 5C).

In the base frontal bone, we observed a significant difference in IGF-1 protein expressions between Groups A and C. We also observed that IGF-1 protein expression in Group B was significantly higher than in Group A. However, we could not detect a significant difference between Groups B and C in IGF-1 protein expression. In the midline occipital bone, we found that IGF-1 protein expression significantly decreased in both Groups A and B compared with Group C (Fig. 6).

These result suggest that a very low concentration of folic acid caused a significant decrease in TGF-β1 and IGF-1 protein expression in both the base frontal and midline occipital bone of E18–19 rat embryos. Fifteen micrograms of folic acid significantly decreased TGF-β1 level in the base frontal bone but not in the midline occipital bone. IGF-1 protein expression decreased in the midline occipital bone but not in the base frontal bone of E18–19 rat embryos when low-folic acid diet was administered to the pregnant maternal rats.

4. Discussion

We have observed that 16 weeks after dietary treatment of folic acid, the serum folic acid concentration was significantly decreased in both very-low- and low-folic acid diet treatment. It is suggested that 5 and 15 μg of folic acid supplement resulted in decreased serum folic acid level. However, low-folic acid diet did not significantly decrease erythrocyte folic acid concentration, so a smaller concentration of folic acid is necessary to reduce erythrocyte folic acid level. It has been reported that erythrocyte acts as a more stable deposit for folic acid than serum. Therefore, a small reduction of folic acid in the diet still has no effect on erythrocyte folic acid level. The level of folic acid in erythrocyte is also a hundred times higher than that in serum, because in the serum, it fluctuates according to activities, last food intake, rats' health condition, etc. Accordingly, our result supports those of previous studies, which showed that decreased erythrocyte folic acid level is induced by a very low folic acid concentration in the diet or a longer dietary treatment [17,20,21].

We observed a significant reduction in serum TGF-β1 level in Group A as compared with Group C. However, low-folic acid diet did not significantly change serum TGF-β1 concentration. These data suggested that a very small amount is necessary to reduce serum TGF-β1 level.

A similar fashion of IGF-1 down-regulation happens when a very-low-folic acid diet was given to Group A; however, in Group B, there was no significant reduction in IGF-1 level compared with Group C.

These data suggested that the serum protein level of TGF-β1 and IGF-1 decreased only in response to a very-low-folic acid diet, whereas a low-folic acid diet did not significantly reduce these growth factors.

Interestingly, TGF-β1 and IGF-1 protein expressions were significantly decreased in both the base frontal bone and occipital midline skull bone of E18–19 rat embryos given a very-low-folic acid diet. We also observed a discrepancy between TGF-β1 and IGF-1 protein expression in response to a low-folic acid diet treatment. TGF-β1 protein expression was significantly reduced in the base frontal bone but not in the midline occipital bone. In contrast, IGF-1 protein expression was significantly reduced in the midline occipital bone but not in the base frontal bone. These data suggest that there is a different pattern of decreased TGF-β1 and IGF-1 protein expression. Regarding the distinguished function of TGF-β1 and IGF-1 in bone formation, a low-folic acid diet may affect differently the base frontal bone and midline occipital bone. The normal diet revealed no significant difference in TGF-β1 and IGF-1 levels.

In agreement with these results, our previous study observed a decreased TGF-β1 and IGF-1 protein level in the edge of skull bone defect of meningocele patients. The width of the defect correlated with the degree of decreased TGF-β1 and IGF-1 protein expression (manuscript in preparation). We, therefore, suppose that the decreased TGF-β1 and IGF-1 protein expression found in meningocele patients may be due to a very low concentration of folic acid in the diet during the critical period of organ formation in the first trimester of pregnancy.

Previous studies have revealed strong evidence that folic acid supplementation during the critical period of organ formation (the first trimester of pregnancy) will decrease by 70% the incidence and prevalence of some congenital diseases, such as NTD, congenital heart defect and labio- and palatoschizis [6,16,20,22–25]. A possible mechanism of how folic acid prevents NTD might be due to the ability of folic acid to recover TGF-β1 and IGF-1 expressions, which,

in turn, may induce normal functioning of TGF-β1 and IGF-1 as growth factors.

The weight of E18–19 rats in Group A was significantly lower than that of E18–19 rats in Groups B and C. Moreover, seven E18–19 rats in Group A grew without tails, while the E18–19 rats of other groups were morphologically normal. This finding showed a macroscopic alteration in the growth and development process of E18–19 rats in Group A (data not shown).

We also found that, compared with the other two groups, it was difficult for the rats in Group A to get pregnant and breed. There are at least two mechanisms responsible for this finding. First, folic acid deficiency made the rats subfertile, resulting in conception difficulty. Second, if conception and pregnancy occur, but supporting factors are lacking causing failure of embryogenesis and implantation, then abortion or embryo resorption takes place. Theoretically, this was related to folic acid deficiency and TGF-β1/IGF-1 deficiency, which all play a role in embryogenesis and organogenesis as suggested by previous studies [26–28]. Folic acid deficiency, hyperhomocysteinemia in particular, may damage embryogenesis and even induce abortion through its impact on migration and implantation failure, preterm birth, placental abruption and low-birth-weight fetus [26–28]. Our study showed the effects of folic acid-restricted diet treatment, which is similar to folic acid deficiency, mainly on reducing TGF-β1 and IGF-1 protein expression, growth and difficulty in conception as well.

Taken together, our study showed a significant decreased effect of very-low-folic acid diet on TGF-β1 and IGF-1 protein level in skull bone of E18–19 rat embryos and a different pattern of decreased TGF-β1 and IGF-1 protein level between base frontal bone and midline occipital bone. This result may explain the mechanism of decreased TGF-β1 and IGF-1 expression found in meningocele patients with folic acid deficiency.

References

[1] Charoonsmith T, Suwanwela C. Frontoethmoidal encephalomeningocele with special reference to plastic reconstruction. Clin Plast Surg 1984;1:27–47.
[2] Davids DJ, Sheffield F, Simpson D, White J. Frontoethmoidal meningoencephaloceles; morphology and treatment. Br J Plast Surg 1984;37:271–81.
[3] Hoving EW. Frontoethmoidal encephaloceles, a study of their pathogenesis. Groningen: Rijk Universiteit; 1993.
[4] Acuna J, Yoo P, Erickson D. The prevention of neural tube defects with folic acid. CDC Pan Am Health Org 2000:1–17.
[5] Boonvisut S, Ladpli S, Sujatanond M, Tandha Vadana C, Tisavipad N. Morphology study of 120 skull base defects in frontoethmoidal encephalomeningocele. J Plast Reconst Surg 1998;101:1784–95.
[6] Martinez-Lage JF, Poza M, Sola M, Soler CL. The child with cephalocel: etiology, neuroimaging and outcome. Childs Nerv Syst 1996;12:540–50.
[7] Agthong S, Wiwanitkit V. Encephalomeningocele cases over 10 years in Thailand: a case series. BMC Neurol 2002;2:3–10.
[8] Brouwer DAJ, Welten HTM, Reijngoud DL, Van Doormal JJ, Muiskiet FAJ. Plasma folic acid cut off value, derived from its relationship with homocyst(e)ine. Clin Chem 1998;44:1545–50.
[9] Canalis E, McCarthy T, Centrella M. Isolation and characterization of IGF-1 (somatomedin-C) from cultures of fetal rat calvariae. Endocrinology 1988;122:22–9.
[10] Cohick WS, Clemmons DR. The insulin-like growth factors. Annu Rev Physiol 1995;55:131–7.
[11] Roth DA, Gold LI, Han VKM, McCarthy JG. Immunolocalization of transforming growth factor β1, β2 and β3 and insulin-like growth factor-I in premature cranial suture fusion. Plast Reconstr Surg 1997;2:300–9.
[12] Joyce ME, Roberts AB, Sporn NB, Bolander ME. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in rat femur. J Cell Biol 1990;110:2195.
[13] Massague J. The transforming growth factor-β family. Ann Rev Cell Biol 1998;6:597–641.
[14] Steed DL. The role of growth factors in wound healing. Surg Clin North Am 1997;77:576–86.
[15] Eskes TK. Neural tube defects, vitamins and homocysteine. Eur J Pediatr 1998;2:139–41.
[16] Hall DJ, Solehdin AM. Folic acid deficiency as a cause of neural tube defects. Biochem Biophys Res Commun 1998;246: 404–8.
[17] Arrington LR. Introductory laboratory animal science: the breeding, care and management of experimental animals. Danville (Ill): Interstate Printing & Publishing; 1981. p. 59–88.
[18] Van Wouwe JP. Zinc deficiency: its clinical and laboratory assessment. Bleiswijk-Nederland: NKB Offset; 1991.
[19] Brouwer IA. Folic acid, folate and homocysteine: human intervention studies. Nijmegen: Katholieke Universiteit; 1999.
[20] Fleming A, Copp AJ. Embryonic folate metabolism and mouse neural tube defects. Science 1988;280:2107–9.
[21] Fernstrom JD. Can nutrient supplements modify brain function? Am J Clin Nutr 2000;71:1669(s)–73(s).
[22] Botto LD, Mastroiacovo P. Exploring gene–gene interactions in the etiology of neural tube defects. Clin Genet 1998;53:456–9.
[23] Copp AJ. Prevention of neural tube defects: vitamin, enzymes, and genes. Curr Opin Neurol 1998;11:97–102.
[24] Corcoran J. What are the molecular mechanisms of neural tube defects? Bioessay 1998;20:6–8.
[25] Martinasevic MK, Rios GR, Miller MW, Tephly TR. Folate and folate-dependent enzymes associated with rat CNS development. Dev Neurosci 1999;21:29–35.
[26] Gardner RL, Squire S, Zaina S, Hills S, Ham CF. Insulin-like growth factor regulation conceptus composition: effects of the trophoectoderm and inner cell mass genotypes in the mouse. Biol Reprod 1999;60:190–5.
[27] Margawati ET. The effectivity of growth factors on in vitro embryo development. Media Vet 1999;6:27–34.
[28] Scholl TO, Johnson WG. Folic acid: influence on the outcome of pregnancy. Am J Clin Nutr 2000;71:1295S–303S.