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# Effect of Maternal Ethanol Consumption during Pregnancy and Lactation on Kinetic Parameters of Folic Acid Intestinal Transport in Suckling Rats

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**Abstract** Ethanol ingestion is known to interfere with folate absorption and metabolism. A fostering/crossfostering analysis of maternal ethanol exposure effects on jejunum and ileum kinetic parameters in vivo of offspring rat folic acid absorption at 21 days postpartum was carried out. The rats were divided into four groups: CP, control pups; GP, pups exposed to ethanol only during gestation; LP, pups exposed to ethanol only during lactation; GLP, pups exposed to ethanol during gestation and lactation. Jejunal and ileal loop transport studies were performed using in vivo perfusion at a flow rate of 3 ml/min for 5 min. Folic acid concentrations of 0.25, 0.5, 1, 1.5 and 2.5 $\mu$M were used. Jejunal and ileal absorption values were determined by the difference between the initial and the final amounts of substrate in the perfusate and expressed as picomoles per square centimeter of intestinal surface every 5 min. The results indicated that ethanol consumption by the dams during gestation and/or lactation led to significant changes in $V_{max}$, with no significant changes in apparent $K_m$. These findings suggest that exposure to ethanol during gestational and suckling periods leads to a general delay in postnatal body weight and that intestinal folate absorption appears to be upregulated in suckling rats, this effect being higher in the LP group.

**Keywords** Ethanol toxicity · Folic acid transport · Gestation · Lactation · Offspring · Kinetic parameter

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## Introduction

Chronic ethanol abusers are frequently malnourished, even with adequate dietary intake, because the excessive consumption of alcohol results in various vitamin and mineral deficiencies (Lieber, 2003; Addolorato et al., 2000). Folate deficiency is the most common sign of malnutrition in chronic alcoholism (Bode & Bode, 2003).

Humans and other mammals must obtain folate from exogenous sources by intestinal absorption. For this reason, the intestine plays a central role in the control and regulation of folate homeostasis. Several groups have shown that the transport of folate across intestinal brush-border membranes is a carrier-mediated process that is dependent on a transmembrane pH gradient and is competitively inhibited by folate analogs and anion exchange inhibitors (Said & Strum, 1983; Selhub & Rosenberg, 1981; Said, Ghishan & Redba, 1987). Some experimental studies have shown that chronic ethanol causes a reduction of folate intestinal absorption (Romero, Tamura & Halsted, 1981; Villanueva, Devlin & Halsted, 2001), while other studies have shown no such effect (Reisenauer et al., 1989). Recent studies suggest that chronic ethanol exposure decreases the intestinal absorption of folic acid by altering the expresion of reduced folate carrier (RFC) and consequently its transport kinetics in jejunal brush border (Villanueva et al., 2001). Actually, Balamurugan & Said (2006) demonstrated that the RFC system is the major folate uptake system that is functional in intestinal epithelial cells.

Pregnant and lactating women are belived to be particularly vulnerable to suboptimal folates status due to their increased dietary requirement to facilitate enhanced anabolic activitiy. Therefore, suboptimal folate status has been involved in many negative maternal and fetal outcomes, including low weight at birth, *abruptio placenta*, cervical

dysplasia and neural tube defects (Achon et al., 2000). Recent studies from our laboratory have shown that chronic alcohol administration during gestation and/or lactation adversely affects pup growth at weaning as indicated by its effect on milk consumption and organ weight (Murillo-Fuentes et al., 2001).

The present study was performed to determine the effects of maternal alcohol consumption during gestation or lactation on folic acid intestinal absorption by rat pups.

## Materials and Methods

### Animals

Wistar rats weighing 150-200 g were divided into two groups: control and ethanol-treated. Alcohol-treated rats (GE) were given 5% ethanol in drinking water for 1 week, 10% ethanol during the second week, 15% ethanol during the third week and 20% ethanol during 4 additional weeks (Tavares et al., 1998). At the end of this period, males and females were mated to obtain offspring; during these 3 weeks, the animals received 20% ethanol. Pregnant GE rats were housed individually and continued drinking ethanol 20% until the end of the experimental period. Control rats (GC) received no treatment and were handled the same way as those treated with alcohol.

The day of parturition was designated as day 1 of lactation, and day 21 was designated as the final day of the lactation period. During the suckling period, the pups had free acces to the nipples.

To study the effect of chronic alcoholism during lactation or gestation separately, at the second day postpartum control mother's pups were cross-fostered to ethanol dams (GE) and the pups from ethanol-treated mothers (GE) were cross-fostered to control dams (GC). Thus, four experimental groups of pups were obtained:

CP: Control pups receiving no treatment during gestation and lactation ($n$ = 10)
GP: Pups exposed to ethanol only during gestation ($n$ = 10)
LP: Pups exposed to ethanol only during lactation ($n$ = 10)
GLP: Pups exposed to ethanol during gestation and lactation ($n$ = 10)

Animals were maintained under an automatically controlled temperature (22–23°C) and a 12-h light-dark cycle. Animal care complied with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington DC, 1996).

### Diets

Diets were prepared according to The Council of the Institute of Laboratory Animal Resources (ILAR, 1979), which details the known nutrient requirements for most of the common laboratory animals (Murillo-Fuentes et al., 2001). The diet was offered to the animals as pellets. Coprophagy was avoided by placing wire nets over the cage floor.

### Blood and Milk Collection

On day 21 of lactation and 3 h after removing the litters from the mothers, dams were killed and blood samples were taken by cutting the end of the tail. Serum was prepared using low-speed centrifugation, and folic concentrations were obtained by immunoluminometric assay (Enguix, Garcia & Hernández, 1992). The amount of milk consumed was estimated by subtracting the weight of the pups obtained just prior to returning them to the dam from the weight at the end of 30 min of suckling, as previously described (Subramanian, 2000). Milk samples were immediately obtained by gently massaging the area around each of the 12 mammary glands and then by pressing upward from the base of the gland toward the nipple. The milk samples were immediately stored at –80°C. Determination of the concentration of folic acid in milk was made as described above (Enguix et al., 1992).

### Folic Acid Absorption

Pups 21 days old were used for this study. Animals were anesthetized with subcutaneous uretane 10% (1 ml/100 g animal weight). An intestinal loop was isolated and connected by inflow and outflow cannulas to a peristaltic pump. The loop was rinsed with 0.9% NaCl solution and perfused as previously described by Ponz, Ilundain & Lluch (1979). A flow rate of 3 ml/min in a closed-circuit (multiple-pass perfusion) was used.

$^{3}$H-folic acid (0.01 μCi/ml) was prepared in a saline solution containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM morpholinoethanesulfonic acid (MES, pH 5.8) and cold folic acid at 0.25, 0.5, 1.0, 1.5, 2.5 and 5 $\mu$M concentrations. Prewarmed solutions (10 ml) were perfused for 5 min, starting with the lowest and ending with the highest folic acid concentrations. Between successive perfusions, the intestinal loop was washed with saline for 5 min at the same perfusion rate. The offspring were maintained under controlled temperature (37°C) with a heating pad.

Folic acid absorption was determined as the difference between the initial and final amounts of substrate in the perfusate and expressed as picomoles per square centimeter of intestinal surface every 5 min. Intestinal surface was measured as previously described (Winne, 1976).

### Kinetic Analysis

Total folic acid absorption from at least ten independent rats was analyzed by nonlinear regression using the Enzfitter program (Biosoft, Cambridge, UK). As errors associated with experimental intestinal absorption values were roughly proportional to their values, applying proportional weighting to the data was considered appropiate. Kinetic parameters were calculated considering a model equation comprised of one saturable Michaelis-Menten component plus a linear, nonspecific component.

### Statistical Analysis

The results are expressed as means ± standard error of the mean (SEM). The data were analyzed using the Instat software program (GraphPad, San Diego, CA) by analysis of variance (ANOVA); statistical significance was established at $p < 0.05$. When ANOVA resulted in differences, multiple comparisons between means were studied by the Tukey-Kramer test.

## Results

### Pup Body Weight throughout the Suckling Period

As shown in Figure 1, no significant differences were found among the groups of animals in body weight at birth. Body weight at 7 days of age was only lower in offspring of the GLP group. At 14 and 21 days old, the offspring body weight of the GLP and LP groups was significantly lower in comparison with the CP and GP groups. Throughout the suckling period no differences in body weight between the CP and GP groups were found.

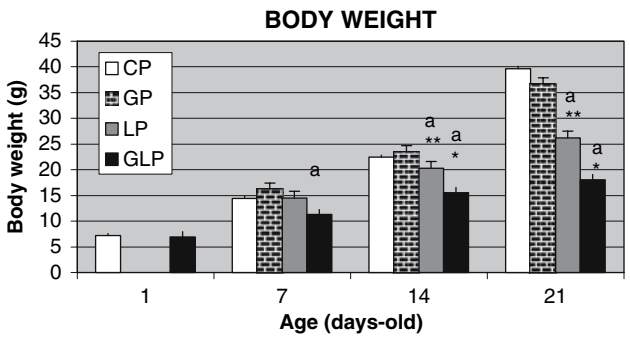


**Fig. 1** Body weight (g) in offspring of ethanol-fed dams throughout the suckling period. Values represent mean ± SEM. GLP *vs.* CP, $^{a}p < 0.001$; LP *vs.* CP, $^{a}p < 0.001$; GLP *vs.* GP, $*p < 0.001$; LP *vs.* GP, $**p < 0.01$ ($n = 10$)

### Feed Intake

The amount of milk consumed by pups during 30 min of suckling, obtained by weighing the litters before and at the end of the suckling period (Rockwood & Riley, 1986), is shown in Table 1. There were significant differences between groups. Comparisons revealed that milk consumption was lower for alcohol-administered groups compared with control (CP) ($p < 0.001$). Among the three alcohol goups, milk consumption was lower for the GLP group.

### Milk and Blood Folic Acid Levels

Figure 2 shows the concentration of folic acid in milk and blood serum obtained from ethanol-fed dams (GE) and control dams (GC) at the end of the lactation period. Decreased values in ethanol-fed dams (GE) can be observed.

### Folic Acid Intestinal Absorption

Folic acid absorption by the jejunum and ileum of rat pups in the different experimental groups was examined using isolated loops of the indicated intestinal segment at different folic acid concentrations. Different animals were used to study jejunal and ileal absorption. The results in Figure 3 show that folic acid jejunal absorption increased along with the increase of folic acid concentrations in the perfusate. The absorption in the LP group was higher than in the GP, GLP and CP groups. Figure 4 shows the results obtained with ileal loops. The first amazing finding was the absence of absorption in the CP group and its presence in the LP, GP and GLP groups. In all the groups the absorption values were significantly lower in the ileum than in the jejunum. The relationship between the folic acid concentration in the perfusate and the intestinal absorption was measured in the range 0.25-5 μmol/liter folic acid. Intestinal absorption in all groups could be fitted to a kinetic model comprised of one saturable component plus a nonsaturable one. The calculated kinetic parameters are shown in Table 2. The $K_d$ (nonsaturable component) was similar in all the groups (jejunum and ileum), and its value was 4-6 pl/cm$^2$ intestine.

These results showed that pups exposed to ethanol during the gestation and/or lactation period present an increased $V_{max}$ of folate intestinal uptake, with no significant changes in apparent $K_m$.

## Discussion

The aim of the present study was to examine the effect of maternal ethanol consumption during gestation and

**Table 1** Body weight and milk consumption by offspring rats at postpartum day 21

| Offspring at 21 days postpartum | CP ($n$ = 10) | GP ($n$ = 10) | LP ($n$ = 10) | GLP ($n$ = 10) |
|---|---|---|---|---|
| Body weight (g) | 40.95 ± 1.53 | 37.07 ± 1.38 | 27.08 ± 1.24 | 18.1 ± 0.52 |
| Milk consumption | 1.20 ± 0.09 | 0.69 ± 0.06 | 0.52 ± 0.05 | 0.45 ± 0.03 |

GP *vs.* CP, $p < 0.001$; LP *vs.* CP, $p < 0.001$; GLP *vs.* CP, $p < 0.001$; GLP *vs.* GP, $p < 0.001$; GLP *vs.* LP, $p < 0.001$

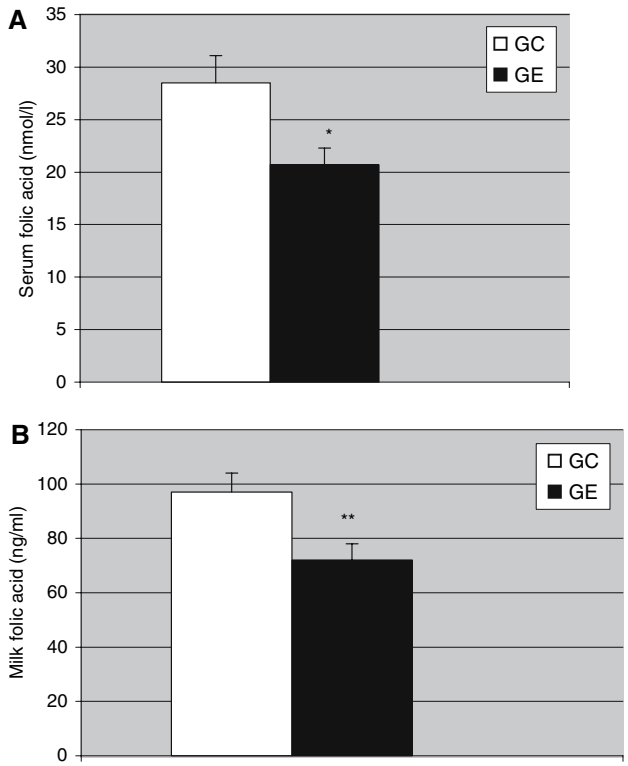


**Fig. 2** Folic acid concentrations in blood (**a**) and milk (**b**) of alcoholic (GE) and control (GC) dams. Samples were taken at the end of the lactation period. Values represent mean ± SEM. GE *vs.* GC, $*p < 0.001$, $**p < 0.005$

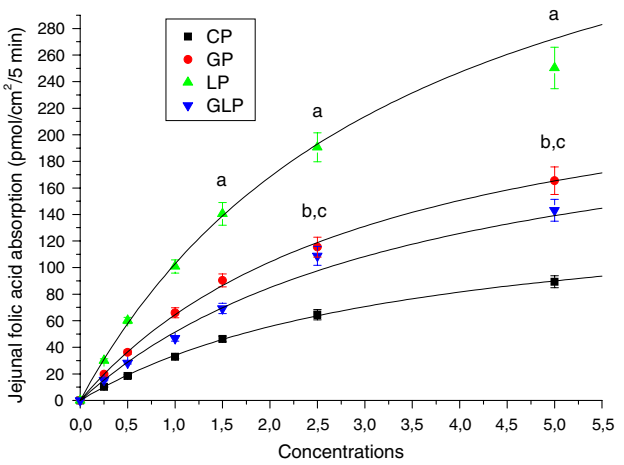


**Fig. 3** Jejunal folic acid absorption (pmol/cm$^2$/5 min) in offspring of control (CP), gestation ethanol-exposed (GP), lactation ethanol-exposed (LP) and gestation-lactation ethanol-exposed (GLP) rats. Values represent mean ± SEM. LP *vs.* CP, $^ap < 0.001$; GP *vs.* CP, $^bp < 0.01$; GLP *vs.* CP, $^cp < 0.01$ ($n$ = 10)

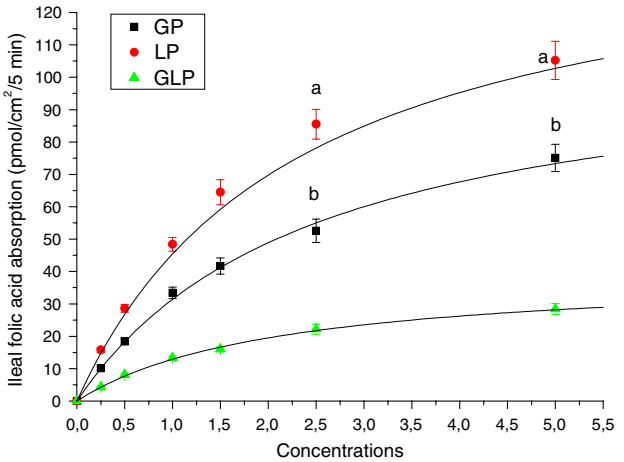


**Fig. 4** Ileal folic acid absorption (pmol/cm$^2$/5 min) in offspring of gestation ethanol-exposed (GP), lactation ethanol-exposed (LP) and gestation-lactation ethanol-exposed (GLP) rats. Values represent mean ± SEM. LP *vs.* GLP, $^ap < 0.001$; GP *vs.* GLP, $^bp < 0.01$ ($n$ = 10)

lactation on the ability of the jejunum and ileum of 21-day-old rat pups to absorb folic acid. The study was performed using the rat as the animal model because previous studies have established the suitability of this species for such investigations (Said & Strum, 1983; Selhub & Rosenberg, 1981; Said et al., 1987). Induction of alcoholism was accomplished as previously described (Tavares et al., 1999). After parturition, some pups were cross-fostered in order to discriminate between gestational and lactational alcohol effects. This type of pup manipulation has been previously used by us and other laboratories with satisfactory results (Murillo-Fuentes et al., 2001; Vorhees, 1989; Belluardo et al., 1993).

Body weight at birth did not differ among the groups of pups studied (Fig. 1). However, a significant reduction in body weight was observed at 7, 14 and 21 days in pups exposed to ethanol during the gestation and lactation periods (GLP) and at 14 and 21 days in pups exposed to ethanol only during lactation (LP). The body weight of the GP group was only slightly lower than that of the CP group, indicating a normal lactation ability to reverse the

**Table 2** Kinetic parameters of folic acid absorption in jejunum and ileum of control (CP), gestation ethanol-exposed (GP), lactation ethanol-exposed (LP) and gestation-lactation ethanol-exposed (GLP) weanling rats

| Offspring groups | Jejunum | | Ileum | |
|---|---|---|---|---|
| | $K_m$ ($\mu$M) | $V_{max}$ (pmol/cm$^2$/5 min) | $K_m$ ($\mu$M) | $V_{max}$ (pmol/cm$^2$/5 min) |
| CP ($n$ = 10) | 3.5 ± 0.6 | 153 ± 11$^{*,**,***}$ | — | — |
| GP ($n$ = 10) | 3.2 ± 0.7 | 271 ± 17$^{*,**}$ | 2.5 ± 0.5 | 110 ± 10$^{*,****}$ |
| LP ($n$ = 10) | 3.5 ± 0.5 | 463 ± 29$^{*}$ | 2.3 ± 0.6 | 150 ± 12$^{*,****}$ |
| GLP ($n$ = 10) | 3.7 ± 0.7 | 242 ± 15$^{*,***}$ | 2.1 ± 0.4 | 40 ± 4$^{*}$ |

Values represent mean ± SEM of at least ten different animals in each group. Values are significantly different: $^{*,**,***}p < 0.001$, $^{****}p < 0.05$

adverse effect of alcohol intake during the gestational period. These results, demonstrating adverse effects of maternal ethanol consumption on body weight of the offspring during lactation, are consistent with previous data (Tavares et al., 1998; Weinberg, Kwon Kim & Wayne, 1995). This lower weight should be a consequence of the reduced milk consumption previously described (Chen, Driscoll & Riley, 1982; Rockwood & Riley, 1986).

In examining the effect of maternal ethanol consumption on dietary folate supply to the fetuses (gestational period) and pups (lactational period), we first examined the effect of ethanol on dietary intake and serum levels of folate in rat dams. Dietary intake (μg/rat/day) was 14.5 ± 0.9 in control rats and 8.4 ± 0.4 in ethanol-fed rats during the gestational period and 18.2 ± 1.5 in control rats and 9.2 ± 0.8 in ethanol-fed rats during the lactational period. Serum levels of folate (nmol/liter) were determined at the end of the gestational period and were 28.5 ± 2.6 (control rats) and 20.7 ± 1.6 (ethanol-fed rats). These data are consistent with the results reported by Fernández, Murillo & Carreras (2000) in adult male rats. The decreased folate intake in ethanol-fed rats could be a consequence of the solid-diet calorie substitution for calories from liquid.

During lactation, previous results (Murillo-Fuentes et al., 2001) have demonstrated that alcohol consumption by gestating and nursing dams reduced milk intake by the suckling pups. On the other hand, determination of folate concentrations in the milk obtained from control and ethanol-fed dams revealed a lower value in the latter group (Fig. 2).

Taken together, these results indicate that during gestation and lactation the rat fetuses and pups suffer a decreased folate availability. In order to examinate this effect on the intestinal folate absorption process, we studied the in vivo absorption of folic acid in the jejunum and ileum of 21-day-old rats of the different experimental groups.

The results obtained in jejunum showed that the GP, LP and GLP groups presented an increased absorption of folate in comparison with the CP group. Recently, Said et al., (2000) indicated that dietary folate deficiency in adult rats leads to a significant upregulation in transepithelial transport, this upregulation being specific for folate. Our results are consistent with these findings, taking into account that the GP, LP and GLP groups had been exposed to a dietary folate deficiency. The upregulation in folate uptake was found to be mediated by a significant increase in the $V_{max}$ of the process, with no significant change in apparent $K_m$. These findings indicated that decreased folate intake induced a change in the number and/or turnover of the intestinal folate carriers, with no changes in their affinity. This effect probably started in the gestational step of rat development (GP group).

Comparison of the $V_{max}$ obtained in the different groups showed that the LP group presented the highest value (463 ± 29 pmol/cm$^2$ intestinal surface/5 min), followed by the GP and GLP groups (271 ± 17 and 242 ± 15, respectively) and the CP group (153 ± 11). The most striking and conflicting results were those of the LP group in comparison with the GLP group. It could be that the effect of ethanol exposure during gestation and lactation (GLP group) should be equal to or higher than the effect of ethanol exposure only during lactation (LP group), but the results indicated the opposite. An explanation of these apparently conflicting results could be that ethanol, in the context of our experimental scope, produced a double effect. One of them, as has been indicated, is the appearance of a dietary folate deficiency and, consequently, upregulation of folate absorption. The other one is the effect of ethanol per se over the expression of a folate carrier. Recently, Villanueva et al., (2001) showed that chronic ethanol intake in adult micropigs decreased intestinal absorption of folic acid by altering expression of the folate carrier. For this reason, in our experimental animals two opposite effects should be taken into account, the dietary folate deficiency that increased the intestinal absorption and the dietary ethanol intake (via placenta in the GP group and milk in the LP group) that reduced the intestinal absorption. During gestation, the ethanol intake effect should be prevalent over the dietary folate deficiency, so the $V_{max}$ in groups GP and GLP was slightly increased in comparison with the CP group. However, in the LP group, the pups had been gestated by control dams and the dietary folate deficiency effect (produced by decreased milk intake and lower milk

folate level) should be prevalent over the ethanol effect; therefore, the $V_{max}$ in the LP group was the highest.

Results obtained in the ileum showed that the folate absorption mechanism was not expressed in the control group (CP), whereas in the GP, LP and GLP groups this transport mechanism was present. In the adult rats, ileal folate transport is inefficient and occurs at a rate five- to tenfold lower than in the jejunum (Said & Strum, 1983; Said, Ghishan & Murrell, 1985; Said et al., 1987). In weaning rats the transport of folates by intestinal everted sacs (Said et al., 1985) is also similar to that of adult rats in that it is higher in the jejunum than in the ileum (approximatly tenfold). It is possible that the poor expression of this transporter in the ileum and the different experimental conditions (in vivo *vs.* in vitro) explained the absence of folate transport in our CP group.

Our results in the GP, LP and GLP groups showed that the upregulation observed in the jejunum is paralelled also in the ileum, where a folate transport system appeared that was negligible in the CP group. The affinity constant ($K_t$) of this system compared with that obtained in the jejunum was similar (2 *vs.* 3 $\mu$M, respectively), suggesting that it is the same transporter.

These results show that upregulation in folate transport also occurs in the ileum after a deficient folate intake. This shows that the ileum has developed a carrier mechanism for extracting folate from the intestinal contents. Interestingly, a similar mechanism has been described in the ileum of adult rats following extensive resection of the proximal and middle small intestine (Said et al., 1988). This upregulation in the folate uptake process has been associated with a parallel increase in the steady-state mRNA level of reduced folate carriers and their protein (Said et al., 2000).

With regard to the number or activity of transporters ($V_{max}$), differences between the GP, LP and GLP groups were observed (Table 1). The explanation of these differences should be the same as that previously indicated for jejunum.

In summary, our study demonstrates that the activity of the intestinal folate absorption process is upregulated in rat pups with decreased folate intake, this effect being higher when the decreased folate intake appeared during the suckling step.

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