



Original Article

Role of folic acid supplementation and/ or its absence during pregnancy on implantation of embryos – An experimental study of Wistar rats



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ABSTRACT

Introduction: The present study was undertaken to know the effect of folic acid (FA) supplementation and FA absence diet during pregnancy on the number of implantation as well as their endometrial changes. **Methods:** Eighteen Wistar strain Albino rats were randomly divided into three groups and given different diets: Control group with normal diet, group with FA supplementation diet, and group with FA absent diet (with added Succinyl Sulfathiazole) for 5 weeks. The number of coloured thickenings along the uterine horns were identified by injecting 1% Evan's blue dye solution. The sites of embryo implantation were fixed in 10% buffered formalin for 8 hours and embedded.

Result: Average number of implantation observed in pregnant dams fed with FA supplementation diet was 10 in contrast it was 7 in diet with FA absent diet and 8 in control groups. Histologically, implantation site in FA supplemented group showed favorable endometrial environment than the other groups.

Discussion: FA is essential in successful implantation by providing favorable receptive environment to receive the implantation-competent blastocyst for a successful pregnancy.

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1. Introduction

FA has gained a considerable attention in recent era because of its promising role in reducing the incidence of defects in neural tube, craniofacial, and cardiovascular systems, and low birth weight but the consequences of long-term supplementation remain unsettled.^{1,2}

There has been a much debate on the role of pre-/periconceptional and prenatal folic acid (FA) supplements for the long-term health of their offspring. Though we are unaware of the harmful levels of concentrations, such high concentrations may cause epigenetic changes in many tissues.

The growing field of epigenetic research has highlighted the role of 1-carbon metabolites like FA and Vitamin B12 on the developmental programming of chronic diseases in offspring. However, much less is known about the effect of one-carbon metabolism on the gametogenesis and on implantation.^{3,4}

The physical contact of blastocyst with the uterine epithelium triggers the implantation events by localized increase in the endometrial permeability. Injecting Evans blue dye solution

confirmed this response intravenously the colorant leaves the vessels at such hyperpermeable regions and gets deposited to produce blue colored spots at the implantation sites.⁵

Lucock and Bates quoted that the “FA, a genetic time bomb” as the maternal deficiency or excess of FA would likely to affect the developing fetus at critical stages of development to cause long lasting health consequences in their offspring.⁶

There is no consensus about the safe upper limit of FA intake, but is usually considered to be 1 mg/d for adults. Limited evidences suggested that serum concentrations of FA < 45–59 nmol/L are often considered to be supraphysiologic.⁷

The present study was undertaken to determine the effects of FA supplementation and FA absent diets during the pregnancy on the number of implantation and the endometrial changes at the site of implantation.

2. Material and methods

Eighteen Wistar strained Albino female rats, with their weight approximately 250 g were approved for experimental use by the Institutional Animal Ethical Committee (Ref no: IAEC/KMC/06/2017), Manipal University. Rats were randomly divided into three groups with 6 rats in each group. The First group was give normal diet (with normal amount of folic acid as suggested by animal

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research committee, 2 mg/kg body weight/day), second group was fed with a diet containing no FA. 1% Succinyl sulfathiazole was added to the diet with no FA content to reduce/inhibit the gut flora responsible for synthesis of FA in their body and to avoid coprophagy during pregnancy.¹ Third group was given diet with excess of FA (40 mg/kg body weight/day). All rats were maintained in separate cages in a specific pathogen-free animal room under controlled environment (12 h light and 12 h of darkness, 25 °C, and 35% humidity) with free access to food and water. Body weights of female rats were procured from the lab and in their pregnancy, were recorded weekly thereafter till the confirmation of their pregnancy. All rats were fed in the Central Animal Research Facility, Manipal College of Life Sciences (Ref no: 94/1999/CPCSEA, 28/04/1999), Manipal University, 5 weeks prior to mating and continued with pregnancy for another 3 weeks. Later all female rats were paired with males of their own strain overnight at room temperature (25 °C). To check whether they mated or not, vaginal smear was taken in the early morning and stained with methylene blue and confirmed the presence of sperm plug under microscopy. On confirmation of sperm plug, the gestational day was termed as day 1 (GD1). Rats were remained in their cage and diets until they were sacrificed during gestation from day GD6–GD9.

Fifteen minutes before each rat was sacrificed on GD 7 and GD8, 0.25 ml of 1% solution of Evans blue dye (T-1824, Sigma-Aldrich Co., India) in 0.9% saline was injected intravenously through the tail vein and housed in closed container with added ether. After this deep inhalation anaesthesia, rat abdomen was opened and identified the uterine horns. Two uterine horns with intact ovaries were separated meticulously and taken out to determine the number and site of implantation. Because of the leakage of Evans blue dye into the interstitial space at points of increased vascular permeability from the vessels, blue coloured nodular thickenings were visible along the site of implantation and growing embryo. The number of coloured thickenings were counted and photographed. Each horn was cut transversely containing the sites of embryo implantation were fixed in 10% buffered formalin for 8 h and embedded. Two blocks for embryo implantation sites were sectioned for each rat.

3. Results

Bodyweight of Wistar rats obtained at the time of experiment (from 0 week - 5 weeks) were analyzed using two-way ANOVA. Ordinal variables were assessed using non-parametric tests: Kruskal-Wallis and Mann-Whitney. Significant differences obtained in the two-way ANOVA were followed by Tukey's Honestly Significant Differences (HSD) *post hoc* analysis. All significance tests were considered significant at P value < 0.05.

Weight gain among the three groups (control, diet with increased FA, Diets with decreased FA) were compared after feeding with their respective diets as prescribed in the protocol from the day of procurement of Wistar rats for experiment to

5thweek. With multivariate test application, weight gained by Wistar rats were significantly (p value of 0.001) increased when compared with other two groups. Tests of within-group (factor1 = 0.001, and factor 1* = 0.58) showed no significant association between the weight gain observed in FA supplementation of FA absent diets. Tukey test (Table 1) among the multiple comparison showed significant differences among the three groups.

Kruskal-Wallis test applied to the number of implantation observed among the three groups showed statistically significant values (0.029) but fail to see the significance when applied among the individual groups, which could be due to small number of sample size. Average number of implantation observed in pregnant dams fed with FA supplementation diet was 10 in contrast it was 7 in diet with FA absent diet and 8 in control groups.

Hematoxylin and Eosin staining of the implanted site in pregnant rats fed with normal diet showed obliterated uterine lumen, increase in the number of cells and reduction in the interstitial space in the endometrium. Endometrial site in rats fed with FA absent diet showed very less number of irregular cells as well as the connective tissue cells with not marked endometrial tissue. Implantation site in FA supplemented group showed more intense acidophilic area with large number of larger, rounded, oval and polygonal shaped cells. The early invasion cells (trophoblasts) can be detected with more acidophilic area nearby. Stroma showed larger coiled arteries and thicker stromal component.

4. Discussion

The term folate is a generic name for the group of chemically related compounds based on the folic acid (FA) structure. Folate (Vitamin B9), an essential vitamin that must be administered either in diet (green leafy vegetables, legumes, egg yolk, liver and citrus fruit) or through supplementation.⁸ FA used in food fortification (artificially enriched foods and pharmaceutical vitamins) is synthetic and differ from naturally occurring FA in our diet because it is in the oxidized state, has only one conjugated glutamate molecule, higher bioavailability, and rapidly absorbed in the intestine.⁷

National Institutes of Health (NIH) have recommended 600 µg of FA be taken daily by pregnant women throughout their pregnancy to have adequate FA stores in pregnancy, and that this supplementation be continued in lactation with reduced amount to 500 µg.⁹

Studies reported that consumption of 400 µg/day of naturally available FA in food and FA supplementation resulted in supra-physiological levels with greatest increase in pregnant women.¹⁰ Concern has been raised if such over FA exposure in pregnancy will have any detrimental effects if not benefitted. In our study, the level of FA supplementation chosen was 40 mg FA/kg diet, i.e., twenty times the normal level considered for pregnant rats (2 mg FA/kg).¹¹

Table 1

Pair-wise comparison of the significance of weight gain in rats fed with food with folic acid excess, folic acid absent, and normal folic acid contents (Tusky HSD test).

Group (A)	Versus Group (B)	Mean Difference (A-B)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Increased folic acid	Absence of folic acid	15.2051	6.32048	.056	-.3508
	Control	29.0940 ^a	6.84637	.001	12.2438
Absence of folic acid	Increased folic acid	-15.2051	6.32048	.056	-30.7610
	Control	13.8889	6.96209	.130	-3.2461
Control	Increased folic acid	-29.0940 ^a	6.84637	.001	-45.9442
	Absence of folic acid	-13.8889	6.96209	.130	-31.0239

^a Statistically significance.

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