Original Article

Correlating Estradiol Levels with Folic Acid and Cobalamin Supplementation: Dynamics of the Rat Oestrus Cycle

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ABSTRACT

OBECTIVE: To examine the effects of supplementary doses of Folate and Cobalamin on the oestrous cycle of female *Sprague Dawley* rats concerning serum estradiol levels.
METHODOLOGY: This experimental study was conducted at the Physiology and Anatomy department's research laboratory at CMH Multan Institute of Medical Sciences, Pakistan. One hundred twenty female *Sprague Dawley* rats, aged 5-6 months and weighing 150-300g, obtained from the University of Lahore. The sampling technique was non-probability convenience sampling. The rats were divided into groups A (Control) and B1, B2, and B3 (Experimental), with thirty rats each. Group A received a daily oral gavage of 2ml/100g of pure distilled water for four weeks. For four weeks, the experimental animals B1, B2, and B3 were given 0.2 mg/kg of Folic Acid, 45µg/kg of Cobalamin, and 0.2 mg/kg of Folic Acid and 45µg/kg of Cobalamin orally via gavage. Every day, vaginal smears were taken. PAP smear slides were prepared. After completion of the study at four weeks, all the animals were euthanized by chloroform inhalation. Blood was obtained to evaluate biochemical parameters using the cardiac puncture method. Serum estradiol levels were measured in pg/ml. Data was analyzed by applying SPSS version 26.
RESULTS: The animals in experimental groups B1 and B3 exhibited an atypical oestrus cycle. Following the comparison between groups, a p-value of 0.000 was obtained, which was statistically significant.
CONCLUSION: Present research indicates that administering a prolonged supplemental dose of Folic Acid results in an extended oestrous cycle in *Sprague Dawley* rats, accompanied by elevated serum estradiol levels.

KEYWORDS: Androgen Receptors (AR), Cobalamin, Endocrine disrupting chemicals (EDC), Estradiol, Folate

INTRODUCTION

The water-soluble B vitamins work with the enzymes required for proper cellular operation as cofactors. B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B5 (Pantothenic acid), B6 (Pyridoxine), B7 (Biotin), B9 (Folate), and B12 (Cobalamin) are members of the vitamin B family[1]. Folate and Cobalamin are dietary essentials. Any cell undergoing chromosomal replication and division will have impaired DNA synthesis if it is deficient in folate compound[2]. Recommended dietary allowance (RDA) of Folic Acid and Cobalamin in the adult person is 400 and 2.4 micrograms/day, respectively[3]. Food is supplemented with this vitamin because of its importance. Because of dietary fortification, the population consumes more Folate than the body needs to function. When serum Folate levels exceed 45 nmol/L, fasting serum levels are commonly regarded as supraphysiological[4]. Folic Acid, sometimes called pteroyl glutamic acid, comprises glutamate and para-aminobenzoic acid components joined by a methylene bridge to a pteridine ring. It is not a chemical that is physiologically active. The liver transforms into the physiologically active forms of tetrahydrofolic acid and dihydrofolic acid[5]. For one-carbon metabolism, tetrahydrofolate is essential because it facilitates the synthesis of thymidylate, purines and pyrimidines crucial for the synthesis of DNA. Cobalamin and Folic acid are frequently employed in managing megaloblastic anemia and as preventative measures against neural tube defects (NTDs) .[6] Research conducted on humans revealed that Folic Acid disrupts the one-carbon metabolic pathway through its interaction with homocysteine and methionine[7]. As per a recent study, an increased intake of Folic Acid and Cobalamin is associated with changes in the DNA methylation of several genes[8].

Androgen receptors can indirectly affect DNA metabolism, redox balance, and epigenetic processes by influencing one-carbon metabolism. The female reproductive tract tissues exhibit potent androgen receptor (AR) activation[9]. Endocrine disruption is triggered by the action of (EDCs), and Folic Acid is recognized as one such chemical capable of inducing

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this type of disruption[10]. These substances interfere with androgen receptors' regular signaling and functions[11]. The hypothalamic-pituitary-gonadal axis is impacted by the disturbance of the androgen receptor signaling pathway, which increases the release of Follicle-Stimulating Hormone, Gonadotropin hormone-releasing hormone, and Luteinizing hormone. This ultimately causes the oestrous cycle to extend and serum estradiol levels to rise[12].

The rationale of this study was to investigate the effects of supplementary doses of Folic Acid and Cobalamin on the oestrous cycle of female *Sprague Dawley* rats, particularly concerning serum estradiol levels. Since no experimental studies have been conducted at the national level yet to confirm the correlation between the supplementary doses of these vitamins and serum estradiol levels.

METHODOLOGY

This experiment was conducted in the Physiology and Anatomy department's research laboratory at the CMH Multan Institute of Medical Sciences in Pakistan. One hundred twenty female *Sprague Dawley* rats were obtained from the University of Lahore. The study excluded any rats that were pregnant or exhibited any health concerns. A random sampling technique was used to select the rats. Groups A (Control) and B1, B2, and B3 (Experimental) had thirty rats. Control Group (A) received a daily oral gavage of 2ml/100g of pure distilled water for four weeks. For four weeks, the experimental animals B1, B2, and B3 were given 0.2 mg/kg of Folate, 45µg/kg of Cobalamin, and 0.2 mg/kg of Folate and 45µg/kg of Cobalamin orally via gavage in two milliliters of pure distilled water[13,14]. A pilot method was carried out in addition to reviewing a reference article to find the ideal dosage. For four weeks, a vaginal smear was taken between 11 a.m. and 12 p.m. daily. The rat's tail was lifted to expose the vaginal area. Subsequently, a pipette made up of plastic, holding 8 µL of distilled water, was cautiously introduced into the rat's vaginal canal, and gentle pressure was applied to the bulb of the pipette two or three times to extract vaginal fluid. The diameter of the pipette's tip was not more than 1.5 mm. This procedure was conducted meticulously, ensuring gentle treatment of the rats to reduce stress, and the entire collection process lasted no longer than thirty seconds[15]. After vaginal lavage, equal portions of each animal's sample were placed on distinct microscopic glass slides and allowed to air dry for three to four hours. PAP smear slides were prepared and used to study the oestrous cycle. A light microscope was used at a 40X objective lens to examine the oestrous cycle. Rats exhibiting a consistent four-stage pattern were considered to have a normal oestrus cycle. This pattern included proestrus, estrus, metestrus and diestrus lasting eleven to thirteen, twenty-four to twenty-eight, five to seven and fifty-seven to fifty-nine hours, respectively. The histological changes observed in a vaginal smear include nucleated epithelial cells during proestrus, cornified squamous epithelial cells during estrus, an equal proportion of cornified cells and leukocytes during metestrus, and predominantly leukocytes during diestrus. Typically, this cycle persisted for four to five days[16]. However, any changes in the length or order of these phases were considered to indicate an abnormal cycle[17]. Percentages were used to express the data, and the Chi-square test was employed. A P-value of 0.05 or less than 0.05 was considered statistically significant.

After completion of the study at four weeks, all the animals were euthanized by chloroform inhalation. Terminal blood (4ml) was obtained to evaluate biochemical parameters by the cardiac puncture method. Serum estradiol levels were measured in pg/ml by ELISA. Mean ± S.D. was used to express all the data. For quantitative variables, one-way ANOVA was used with the Post Hoc Tukey test to identify significant differences between the experimental and control groups.

RESULTS

In Group A, designated as the Control group, 5 out of 30 rats (17%) exhibited irregular oestrous cycles. Conversely, in Group B1, treated with Folic Acid, all 30 rats (100%) displayed abnormal oestrous cycles. Notably, in Group B2, administered with Cobalamin, none of the rats exhibited abnormal oestrous cycles. Lastly, in Group B3, receiving a combination of Folic Acid and Cobalamin, all 30 rats (100%) demonstrated abnormal oestrous cycles. After comparing all groups, a p-value of 0.000 was statistically significant. (**Figure I & Table I**)

Serum estradiol levels were normal in control groups A and B2 (Cobalamin group), and the levels were elevated in groups B1(Folic Acid group) and B3 (Folic Acid + Cobalamin group). (**Figure II**)

Tukey's posthoc analysis showed that the Estradiol levels were significantly higher in group B1- Folic Acid and B3 - F.A. + Cobalamin groups than in group A-Control and B - Cobalamin groups (p=0.000). (**Table II**)

TABLE I: PHASES OF OESTROUS CYCLE

Groups	Normal	Abnormal	p-value
A (n= 30)	83%	17%	0.000*
B1 (n= 30)	0%	100%	
B2 (n= 30)	100%	0%	
B3(n= 30)	0%	100%	

Significance was inferred for p-values less than or equal to 0.05 and indicated by Asterix (*)

FIGURE I: PHOTOMICROGRAPH SHOWS A COMPARISON BETWEEN THE PHASES OF THE OESTRUS CYCLE IN BOTH CONTROL (A) AND EXPERIMENTAL (B1, 2, 3) GROUPS AT 40X (PAPANICOLAOU STAIN)

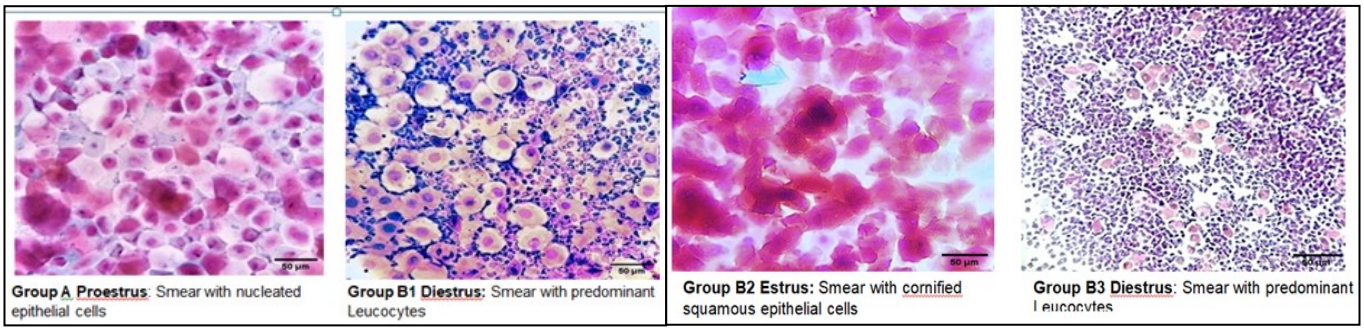
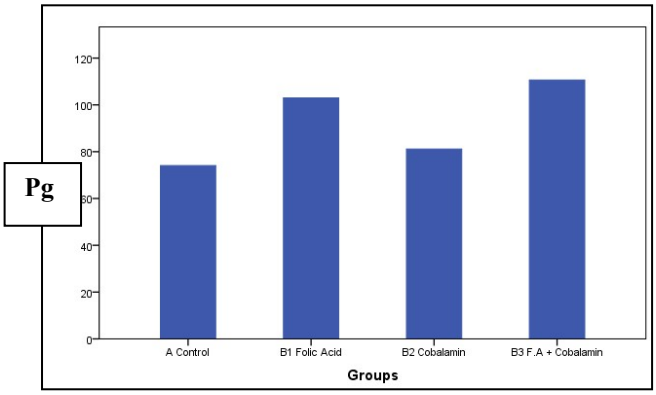


TABLE II: PAIRWISE COMPARISONS – SIGNIFICANCE VALUES FROM POSTHOC TUKEY TEST

Variable	A vs B1	A vs B2	A vs B3	B1 vs B2	B1 vs B3	B2 vs B3
Serum Estradiol Levels	.000*	.439	.000*	.000*	.366	.000*

The P-value indicates the significance level by the Post-hoc Tukey test. P-value <0.05 was considered significant & marked by Asterix (*)

FIGURE II: SERUM ESTRADIOL LEVELS



DISCUSSION

For female mammals, including rats, the oestrous cycle is vital to their reproductive health. This cycle can be affected by various factors, including some food items. Folic acid and Cobalamin are widely used to treat megaloblastic anemia and prevent neural tube defects[6]. Due to their significance, Folate and Cobalamin are routinely added to food for fortification, resulting in a widespread increase in folate and cobalamin intake within the population. In fasting samples, serum or plasma Folic Acid levels above 45 nmol/L are frequently regarded as supraphysiological[18]. Excessive concentrations of supraphysiologic Cobalamin and Folic acid can function as hormone-disrupting agents. Because normal hormone signaling and activity are known to be affected by this imbalance, increased levels of Cobalamin and Folate must be carefully considered in the context of endocrine health.

Because supraphysiological Folic Acid is present in an unmetabolized state that may be harmful, it is primarily focused on its effects on reproductive health. Experimental data suggests that elevated Folic Acid levels could disrupt the normal physiological function of sex hormones, potentially affecting menstrual cycles and fertility[10,19]. Concerns regarding the possible effects on the reproductive systems of men and women are raised by these findings.

Proestrus, estrus, metestrus, and diestrus are the four phases of the oestrous cycle that rats display. Unique hormonal shifts and behaviors distinguish every stage. Estrus denotes the period of intimate responsiveness, metestrus indicates a changing stage, diestrus denotes the luteal phase, and proestrus involves the uterus preparing for potential implantation. Any deviation from the length or order of these phases is regarded as abnormal and could have an impact on reproductive health and fertility[20,21].

This study indicates that excess Folic Acid supplementation may disturb rats' typical oestrous cycle pattern. Prolonged exposure to elevated Folic Acid levels has been noted to potentially modify the length and order of oestrous cycle stages, resulting in irregular and extended cycles. Such alterations could impact the physiological balance of hormones and the rats' conception and reproductive well-being. Nevertheless, additional research is required to elucidate the exact mechanisms driving these outcomes and to ascertain their lasting implications. One potential avenue through which Folic Acid may induce its effects of endocrine disruption involves alterations in gene expression and DNA methylation patterns. These alterations in epigenetic regulation can lead to the dysregulation of hormone synthesis, secretion, and signaling pathways. All the animals of the experimental group B1 & B3 showed an abnormal oestrous cycle. The Folic Acid in both groups may have changed the androgen signaling pathway, which is the likely explanation. The hypothalamic-pituitary-gonadal axis is impacted by modifications in the androgen receptor signaling pathway, which has been linked to elevated levels of serum estradiol, an extension of the oestrous cycle, and an increase in the secretion of sex hormones such as luteinizing hormone, gonadotrophin-releasing hormone, and

follicle-stimulating hormone[14].

This study establishes a link between folic acid exposure and endocrine disruption, resulting in abnormal estrous cycles in experimental groups B1 and B3. The disruption of hormonal pathways caused by Folic Acid-induced changes in DNA methylation and gene expression supports the association between extended oestrous cycles and higher serum estradiol levels. The findings offer crucial and novel insights into the influence of Folate on reproductive dynamics.

Understanding the effects of supraphysiological Folic Acid levels on the estrous cycle of rats is crucial for assessing the potential risks associated with excessive Folic Acid supplementation. This research may have broader implications for human reproductive health as well. Further investigations should focus on elucidating the underlying cellular and molecular mechanisms through which Folic Acid influences the oestrous cycle. Additionally, studying the impact of different dosages and durations of folic acid and cobalamin supplementation can provide valuable insights into the optimal levels required for maintaining reproductive health.

CONCLUSION

The present research indicates that administering a prolonged supplemental dose of Folic Acid results in an extended oestrous cycle in Sprague Dawley rats, accompanied by elevated serum estradiol levels.

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AUTHOR CONTRIBUTION

Awan BUK: Data collection and Manuscript writing
Irfan A:Idea conception, data collection, data analysis
Abid S: Proof Reading, technical advice
Nauman MB: Data interpretation and Literature review

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