

Effects of Subacute Administration of Co-Trimoxazole and Folic Acid on Ovarian Tissue in Adult Female Rats

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What's Known

- Some antibiotics such as sulfasalazine and co-trimoxazole can affect sexual function and fertility capacity, but the exact underlying mechanism(s) of action is not known.
- Folate is important for female reproduction *vis-à-vis* oocyte quality and maturation, implantation, placentation, fetal growth, and organ development.

What's New

- High dose of co-trimoxazole treatment caused significant structural abnormalities and decreases in ovarian quality, including the number of germinal vesicles and metaphase I and metaphase II of rat oocytes.
- Adverse effects of co-trimoxazole on the ovarian tissue and ovarian mature status were reversed partially by folic acid co-administration in rats.

Abstract

Background: Previous studies have reported the antifertility activities of sulfonamides. This study was designed to evaluate the effects of co-trimoxazole and its co-administration with folic acid on ovarian tissue in female rats.

Methods: A total of 54 rats were randomly divided into 9 groups (n=6). Group I served as the control and group II (vehicle) received saline. Other groups, III to IX, received co-trimoxazole (30, 60, and 120 mg/kg; i.p.), folic acid (1 mg/kg; i.p.) or their combination for 14 days, respectively. The oocytes were obtained from each group at the end of the 14th days and scored for maturational status as germinal vesicle (GV), metaphase I (MI), or metaphase II (MII). The number of primordial follicle (PrF), primary follicle (PF), and secondary follicle in formalin-fixed ovaries were counted under light microscopy. The data were analyzed by one-way ANOVA followed by post-hoc Dunnett test using SPSS statistical software (version 17.0). Results were considered statistically significant at P<0.05.

Results: Co-trimoxazole (60 and 120 mg/kg) treatment for 14 days caused a significant decrease in the number of GV (P=0.02, P<0.001), MI and MII (P=0.03, P<0.001), a significant increase in structural abnormalities, including PrF, PF and secondary follicle (P<0.001) as well as congestion, inflammation and necrosis of ovarian tissue compared to the vehicle group. Folic acid co-administration with co-trimoxazole reversed partially all these parameters compared to the co-trimoxazole group (P<0.001).

Conclusion: The data showed the adverse effects of co-trimoxazole on the ovarian maturational status and tissue structure which was reversed partially by folic acid co-administration in rats.

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Keywords • Cotrimoxazole • Folic acid • Ovarian maturation status • Ovarian histopathology • Rats

Introduction

Infertility is a worldwide problem, affecting 13–18% of couples in the globe and the rate varies among different regions and countries,¹⁻³ Infertility is defined by the World Health Organization as the pregnancy failure after 12 months or more of regular unprotected sexual intercourse.³

Ovulation disorders, tubal damage, sexually transmitted diseases (STDs) are among the main causes of female infertility. Moreover, occupational, environmental, drug-induced, emotional, and particularly genetic factors are among the main causes of male infertility, represented as the abnormal sperm quality and/or structural abnormalities.^{1,4-7}

Drug-induced infertility causes primary infertility through the alteration of the hypothalamic-pituitary-gonadal axis or a direct toxic effect on the gonads. Antineoplastic agents,⁸⁻¹⁰ psychotherapeutic agents,⁷ exogenous estrogens,¹¹ anabolic steroids,^{12,13} sulfasalazine and co-trimoxazole,¹⁴⁻¹⁶ tetrahydrocannabinol (the main psychoactive component of marijuana),¹⁷ alcohol and abused drugs,¹⁸ and pesticides¹⁹ are the main drugs affecting sexual function and fertility capacity.

Co-trimoxazole, the combination of sulfamethoxazole (SMX) and trimethoprim (TMP), which act by inhibition of a metabolic pathway for folic acid synthesis, is routinely used by urologists and fertility specialists to treat urinary tract bacterial infections occurring prior to in-vitro fertilization (IVF) treatment.^{15,20} Also, it is frequently used for the treatment of critically ill patients with infections caused by sensitive pathogens, such as *Pneumocystis jiroveci*.²¹ Sulfonamides can cause significant reductions in fertility, corresponding to significant impairments of sperm quality.^{16,22}

Folic acid is important for female reproduction, including oocyte quality and maturation, implantation, placentation, fetal growth, and organ development.^{23,24} Previous studies have shown that folate deficiency may significantly impair the female fecundity and compromise implantation and decrease the chance of a live birth.²³ Folic acid supplementation decreases homocysteine concentration and a better quality and higher degree of oocytes maturity.²⁵ Also, folate has antioxidant properties that counteract reactive oxygen species (ROS) which affect oocyte maturation, ovulation, luteolysis, and follicle atresia.²⁶

Despite the possible adverse effects of co-trimoxazole on male and female fertility, the drug is commonly used for the treatment of various infective conditions. However, there is no report on the effect of its co-administration with various antioxidants on ovarian quality. Hence, the present study was designed to evaluate the effects of co-trimoxazole and its combination with folic acid on ovarian quality in female rats.

Materials and Methods

Animals

Healthy female Wistar rats (weighing 200-250g, 10-11 weeks of age) were housed in an air-conditioned animal house at 23±2 °C on a 12h light/dark cycle with free access to standard pellet and tap water. Before experimental protocol, rats were allowed to accommodate to the laboratory environment for 1h. The experiments were conducted according to the guidelines on ethical standards for investigation of animals, which were approved by the Animal Experimentation Ethic Committee of Kerman Neuroscience Research Center (EC/KNRC/94/6).

Experimental Groups

To investigate the effects of subacute administration²⁷ of co-trimoxazole and its combination with folic acid on ovarian tissue, a total of 54 female Wistar rats were randomly allocated to 9 groups containing 6 animals each. Group I served as the normal control group and did not receive any treatment throughout the 14 days of the study period. Group II served as the vehicle control group and received daily i.p. injection of normal saline for 14 days. Groups III to V served as co-trimoxazole group and received daily i.p. injection of co-trimoxazole (Sobhan Daru, Iran) (30, 60, and 120 mg/kg) for 14 days. Group VI served as the folic acid group and rats received daily i.p. injection of folic acid (1 mg/kg) for 14 days. Groups VII to IX served as co-treatment groups and rats received daily i.p. injection of co-trimoxazole (30, 60, and 120 mg/kg) and folic acid (1 mg/kg) for 14 days.

Collection of Oocytes

At the end of the treatment period, female rats were superovulated by injecting 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma, USA) i.p. followed by an injection of 5 IU of human chorionic gonadotropin (HCG, Sigma, USA) 48 hours later. Both fallopian tubes were isolated 14-16 hours after HCG administration and maintained in 1 mL of human tubal fluid (HTF) medium (Intervet, Denmark), which was previously equilibrated in a humidified atmosphere of 5% CO₂ in air at 37 °C until use. Next, cumulus oocyte complexes (COCs) were collected from the removed oviducts and washed in 10% fetal bovine serum (FBS, Gibco, UK) supplemented with HTF medium droplet 6 times.²⁸⁻³⁰

At the end of the experiment (day 14), the treated and control females were sacrificed

by cervical dislocation and the ovaries were removed under light thiopental anesthesia and were fixed in 10% buffered formalin solution and embedded in paraffin. The paraffin ovary sections were prepared by routine 5 μm section from the paraffin blocks and stained with Hematoxylin and Eosin (H&E). Then, the H&E stained ovary samples were examined for histopathological evaluation using light microscopy, followed by counting primordial (PrF), primary (PF), and secondary follicle in each ovarian tissue.^{31,32}

Ovarian Histopathological Study

The maturational status of oocytes were scored as germinal vesicle (GV), metaphase I (MI), or metaphase II (MII) as described by Donahue (1968), using an optical microscope.³³ Assessment of nuclear status IVM oocytes were denuded from cumulus cells after treatment with 1 mg/ml hyaluronidase for 1 min by gentle repeated pipetting. The nuclear status of oocytes was determined by chromatin dye (Hoechst 33342: 10 μg/ml) under an inverted fluorescence microscope. Oocytes were classified as germinal vesicle stage (GV, containing an intact GV), germinal vesicle breakdown stage and metaphase I (GVBD and MI, containing a broken vesicle with the chromatin starting to condense and oocytes with a metaphase plate, but without a polar body, respectively), metaphase II stage (MII, containing a metaphysical plate and polar body), and degenerated oocyte (oocyte with membrane rupture or without visible chromosomes).³⁰

Statistical Analysis

All data were expressed as mean±SEM (standard error of the mean) of 6 rats in each group. Data were analyzed using one-way analysis of variance (ANOVA), followed by post-hoc Dunnet test. In addition, 95% level of significance (P<0.05) was considered statistically significant. Statistical analysis was performed using SPSS version 17.0 statistical software.

Results

Effects of Co-Trimoxazole, Folic Acid, and their Co-Administration on Ovarian Maturational Status

Figure 1 shows the ovarian maturational status parameters (germinal vesicle, metaphase I and/or metaphase II) in rats following 14 days of treatment with co-trimoxazole, folic acid, and their combination. Our results showed that no significant changes in maturational status among the vehicle group compared to the control group (P=0.83-0.99) (figure 1).

Co-trimoxazole administration (30, 60, and 120 mg/kg) for 14 days was associated with a dose-dependent significant decrease in the number of GV (P=0.04, P=0.02, and P<0.001, respectively), MI (P=0.04, P=0.03, and P<0.001, respectively) and MII (P=0.02, P=0.03, and P<0.001, respectively). Also, co-trimoxazole administration (60 and 120 mg/kg) caused a significant increase in structural abnormalities

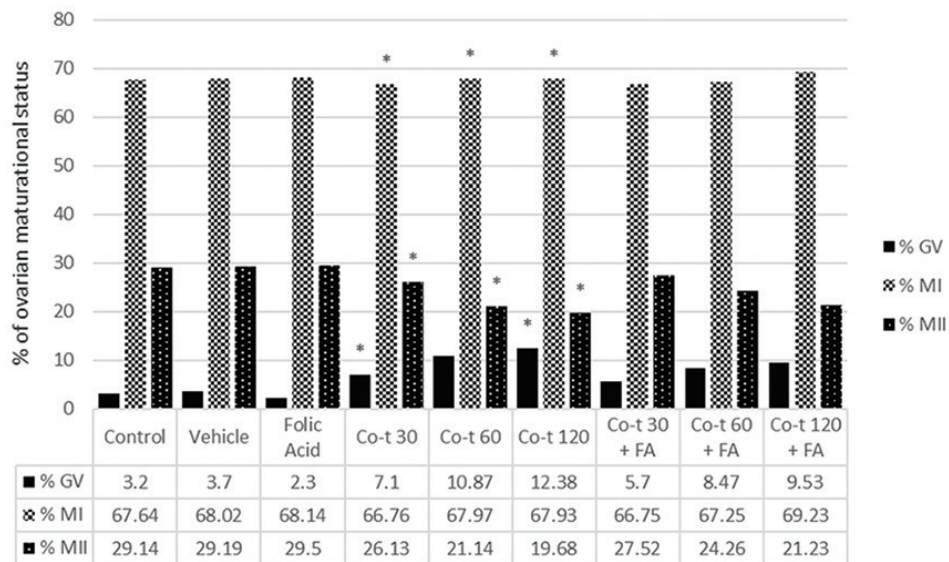


Figure 1: Effects of subacute administration of co-trimoxazole, folic acid, and their combination on ovarian maturational status in rats. Rats received co-trimoxazole (30, 60, and 120 mg/kg), folic acid (1 mg/kg) or their combination intraperitoneally for 14 days. The vehicle group received saline. Control rats received no treatment. Data are the mean±SEM (%) of 6 rats in each group. GV: Germinal vesicle; MI: Metaphase I; MII: Metaphase II; Co-t: Co-trimoxazole; FA: Folic acid; *P value compared to the control and vehicle groups.

indicated as a significant decrease in PrF (P=0.04 and P<0.001, respectively), PF (P=0.02 and P<0.001, respectively), and secondary follicle (P=0.01 and P<0.001, respectively). In addition, histological examination showed congestion, inflammation, and necrosis of ovarian tissue compared to the vehicle and control groups (figure 1).

Folic acid treatment (1 mg/kg; i.p.; 14 days) did not cause any significant change in the ovarian maturational status as compared to the vehicle and control groups (P=0.9). However, folic acid co-administration with co-trimoxazole treatment reversed partially, but not completely, the co-trimoxazole (60 and 120 mg/kg) induced decrease in the ovarian maturational status of GV (P=0.01), MI (P=0.01), and MII (P=0.05). In addition, our results showed that ovarian maturational status in folic acid co-administration with co-trimoxazole group was significantly different from cotrimoxazole (120 mg/kg) treated group, as indicated by a significant increase in GV, GV (P=0.01), MI (P=0.01), and MII (P=0.05). However, maturational status after the co-administration of folic acid and co-trimoxazole was significantly lower than both the control and vehicle groups (P=0.05) (figure 1).

Effects of Co-Trimoxazole, Folic Acid, and their Co-Administration on Ovarian Histopathology

Table 1 shows the effects of subacute administration of co-trimoxazole, folic acid, and their combination on ovarian histopathology in rats. The mean ovary weights in the experimental and control groups showed no significant difference (P=0.92). Histological examination of the ovaries showed remarkable structural changes in the experimental groups receiving high doses of co-trimoxazole (60 and 120 mg/kg) compared to the control group. The main pathological changes included a significant

decrease in the number of primordial follicle (P=0.04 and P<0.001, respectively), primary follicle (P=0.02 and P<0.001, respectively), and secondary follicle (P=0.01 and P<0.001, respectively) compared to the control group (table 1). Co- trimoxazole (30, 60, and 120 mg/kg) caused a dose-dependent change in ovarian histopathology in a dose-dependent manner, i.e. lower doses caused ovarian congestion, but the high dose of co-trimoxazole (120 mg/kg) caused follicular necrosis and atresia (figure 2). No histological changes were seen in the control specimens (figure 2). Folic acid alone did not show any significant effects on follicular structure (figure 2). Folic acid co-administration with co-trimoxazole reversed partially, but not completely, the adverse effects of co-trimoxazole (60 and 120 mg/kg) on ovarian structure abnormalities as compared to the co-trimoxazole groups (60 and 120 mg/kg) (table 1 and figure 1).

Discussion

The subacute or long-term administration of antibacterial co-trimoxazole is commonly used for the treatment of critically ill patients, such as Pneumocystis jiroveci infection in HIV patients. In the present study, the reproductive toxicity of co-trimoxazole was evaluated regarding its effect on ovarian maturational status in female rats. In our study, a period of 14 days treatment with co-trimoxazole was chosen as subacute period, which is in agreement with Noda et al (1995) study that used the same period of 14 days treatment with phencyclidine as subacute treatment.²⁷ Our results showed that subacute co-trimoxazole administration (60 and 120 mg/kg; 14 days) was associated with a significant decrease in all stages of ovarian maturational status (GV, MI, and MII). In addition, it caused remarkable

Table 1: Effects of subacute administration of co-trimoxazole, folic acid, and their combination on ovarian maturational status in female rats

Groups	Primordial follicle (n)	P value	Primary follicle (n)	P value	Secondary follicle (n)	P value	Weight of ovary (g)	P value
Control	7.83±0.31	-	5.80±0.37	-	6.03±0.41	-	0.033±0.006	-
Vehicle	7.17±0.31	0.93	5.40±0.24	0.97	6.25±0.23	0.95	0.032±0.004	0.84
FA	9.17±0.31	0.60	6.60±0.24	0.83	7.35±0.28*	0.71	0.033±0.003	0.91
Co-t 30	6.50±0.22	0.49	4.20±0.37*	0.06	5.23±0.27	0.71	0.032±0.005	0.86
Co-t 60	5.83±0.31*	0.04	4.00±0.32*	0.02	4.33±0.22*	0.01	0.035±0.004	0.81
Co-t 120	4.50±0.43*	0.001	3.00±0.32*	0.001	3.57±0.19*	0.001	0.036±0.005	0.73
Co-t 30+FA	7.17±0.31	0.97	4.60±0.24	0.34	5.73±0.33	0.66	0.031±0.006	0.86
Co-t 60+FA	6.00±0.37*	0.07	4.40±0.24	0.16	5.28±0.22	0.26	0.032±0.004	0.91
Co-t 120+FA	4.50±0.22*	0.001	3.60±0.40*	0.00	4.33±0.21*	0.01	0.034±0.003	0.89

Rats received co-trimoxazole (30, 60, and 120 mg/kg), folic acid (1 mg/kg) or their combination intraperitoneally for 14 days, the vehicle group received saline, control rats received no treatment. Data are the mean±SEM (%) of 6 rats in each group, Co-t: Co-trimoxazole; FA: Folic acid; *P value compared to the control and vehicle groups

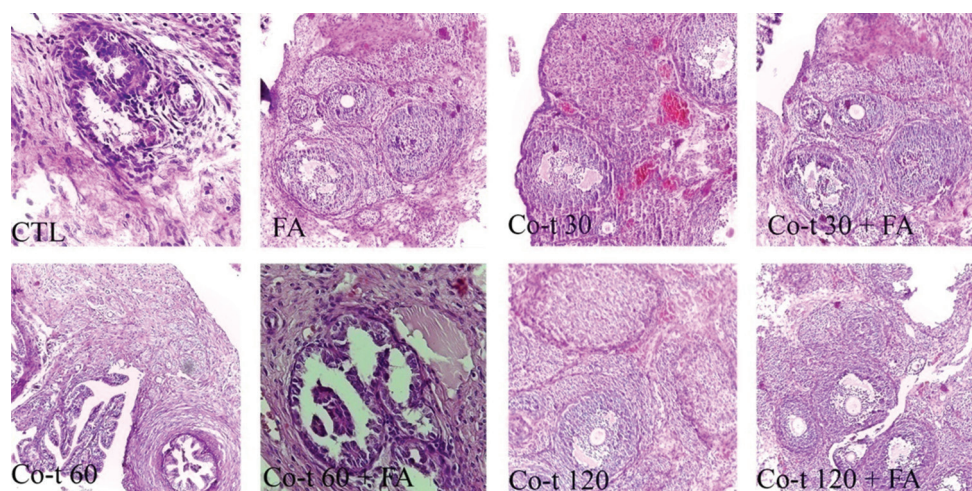


Figure 2: The effects of co-trimoxazole, folic acid, and their combination on ovarian histopathology in rats. Rats received co-trimoxazole (30, 60, and 120 mg/kg), folic acid (1 mg/kg), or their combination intraperitoneally for 14 days. The vehicle group received saline. Control rats received no treatment. CTL: Control; Co-t: Co-trimoxazole; FA: Folic acid.

structural abnormalities as indicated by a significant decrease in primordial, primary, and secondary follicles. Lower doses (120 mg/kg) caused follicular necrosis and atresia, which was partially reversed by co-administration of folic acid.

Our results are in complete agreement with previous reports indicating the antifertility activities of sulfonamides in both animal and human studies.^{14,16,22} Sulfasalazine, commonly used for the treatment of inflammatory bowel disease, causes reversible infertility in men.¹⁶ Significant decrease in the reproductive activity and reduced male fertility parameters such as sperm count, sperm motility, fertility ratio, serum testosterone level, glycogen, and protein content in sexual organs was observed in sulfasalazine or salicylazosulfapyridine-treated rats. All these parameters were restored following withdrawing the treatment.³⁴ Sulfasalazine-induced male infertility on semen quality could be mediated via a metabolite and possibly sulfapyridine.³⁵ Alonso et al. (2009) suggest that sulfasalazine induces oxidative stress as a possible mechanism of male-induced infertility through a significant decrease of superoxide dismutase (SOD) and glutathione reductase (GR). Thus, its antifertility mechanism is different from its antibacterial mechanism.¹⁴ Others have reported that sulfasalazine causes morphological abnormalities in spermatozoa as well as increasing the congenital abnormality amongst men with IBD.^{16,36} Previous reports have shown that *CD59* and decay accelerating factor (*DAF*) genes are secreted from the epididymis and play a role in sperm maturation and sulfasalazine decreases the epididymal expression of *CD59* and *DAF* genes, which causes reduced sperm motility, acrosome reactions, and sperm maturation.³⁷

It is reported that the co-administration of pyrimethamine and sulfanilamide caused reversible infertility in male Wistar rats.²² Previous reports have indicated that the antifertility effect of sulfasalazine is not mediated through the folic acid synthesis inhibition pathway.³⁸ This hypothesis is confirmed by other investigators stating that mesalazine, an active metabolite of sulfasalazine which is widely used for the treatment of inflammatory bowel disease, possess antifertility activities.^{36,39} Mesalamine caused a significant reduction in the mean values of sperm concentration, sperm motility, percentage of normal formed sperm, semen volume, and total motile sperm count. Sperm motility and total motile sperm count were significantly improved ($P < 0.05$) after mesalamine discontinuation.³⁶

Our results showed that folic acid supplementation partially reversed the co-trimoxazole induced antifertility effects by a significant increase in all stages of ovarian maturational status (GV, MI, and MII) and a remarkable increase in primordial, primary, and secondary follicles. In addition, folic acid co-administration with folic acid partially reversed the cotrimoxazole-induced inflammation, congestion, and follicular necrosis. The underlying mechanism(s) is not determined yet, but folic acid supplementation possesses free radical scavenging properties of reactive oxygen species (ROS) and antioxidant activities which antagonize the sulfonamide-induced reactive oxygen species (ROS) production and induction of oxidative stress which result in improvement of oocyte maturation, ovulation, and luteolysis.^{26,40} Others have reported that an increase in homocysteine production is associated with the induction of oxidative stress, increase in

inflammatory cytokines production, decrease in nitric oxide bioavailability, and cell apoptosis.⁴¹ Previous studies have reported that folic acid supplementation resulted in a significant decrease in homocysteine concentration and improvement of oocyte quality and higher degree of oocytes maturation.²⁵ Also, periconception folic acid supplementation (0.4-0.5 mg/d) showed beneficial effects on fetus such as increased fetal growth, higher placental and birth weight, and decreased risks of low birth weight and small for gestational age (SGA) parameter.⁴² It is reported that severe maternal folate deficiency before conception and during gestation has been associated with impairment in female fertility, folliculogenesis, and fetal development.⁴¹ Folate absorption is influenced by folate gene variation in 5,10-methylenetetrahydrofolate reductase (MTHFR) gene polymorphism 677C/T, which is necessary for the folate methylation cycle, where homocysteine is converted to methionine. Thus, folate gene polymorphism may alter the beneficial effect of folates that play a role in the metabolism of methyl groups and impair the conversion of homocysteine to methionine.⁴³ Therefore, folate genes polymorphism could be associated with unexplained female infertility which is responsible for more than 10% of couples infertility.^{24,44} In agreement with our results, Wong et al. (2010) showed the importance of maternal dietary folate/B-vitamin status during the periconceptional period in the bovine oocyte, somatic cells of the ovarian follicle, and pre-implantation embryo.⁴⁵

There are some limitations in our study. The most important limitation was the measurement of serum concentration of female sex hormone, including gonadotropins (LH and FSH), 17- β estradiol, progesterone, and prolactin level in the control and experimental groups. Furthermore, the treatment duration could be a limitation since we evaluated the treatment effects after 14 days. However, it is necessary to investigate the effects of acute and chronic administration of co-trimoxazole on ovarian structure as well as the effects of periconception folic acid supplementation on co-trimoxazole-induced ovarian functions.

Conclusion

The data show the adverse effects of co-trimoxazole on ovarian maturation status (GV, MI, and MII) and ovarian morphology as indicated by a significant decrease in primordial, primary, and secondary follicle as well as ovarian congestion and follicular necrosis in adult female rats. Folic acid co-administration

with co-trimoxazole reversed partially, but not completely, co-trimoxazole induced decrease in ovarian tissue quality and ovarian structural abnormalities in female rats.

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Conflict of Interest: None declared.

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