BIORESEARCH BULLETIN
Sapience Research Labs

ORIGINAL PAPER OPEN ACCESS

Antiestrogenic and antiprogestational activity of methotrexate and its effect on uterine histoarchitecture of ovariectomized albino rats

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Received: 01 November 2010 / **Accepted:** 12 November 2010 / **Published online:** 23 November 2010

Abstract

Background: Methotrexate (MTX) is used as an antineoplastic drug in the treatment of various cancers and non-cancerous diseases. Ovarian ablation is considered to be effective in delaying recurrence of cancer and increasing survival in young women. When added to chemotherapy, it is less clear that this technique is effective. Uniqueness of the present study is that we used ovariectomized (OVX) rats as an experimental model to investigate the action of MTX and leucovorin (LCN) a universal antidote on uterus without or with estrogen and progesterone replacement therapy.

Results: The present study describes the drug effects on body and organ weights, histopathology of uterus and steroidogenic acute regulatory protein (StAR) expression in the uterus. MTX treatment decreased body and organ (oviduct, uterus, cervix and vagina) weights in OVX rats. Histopathological studies revealed that MTX treatment regressed uterine epithelium and stroma revealing its anti-uterotrophic property. Considerable increase in body and uterus weights and improvement in histology of uterus were observed after estradiol and progesterone replacement. However, such changes were significantly decreased when MTX was treated in combination with LCN, estradiol and progesterone.

Conclusion: Our results unambiguously revealed that steroidogenic marker StAR protein expression in OVX uterus was enhanced by steroids supplementation. Additionally, MTX treatment alone or in combination with LCN, E2 and P treatment inhibited StAR protein expression in uterus thereby affecting steroid hormones replacement. LCN supplementation did not bring about a rescue of uterus and the side effects were profound. These results for the first time indicate antiestrogenic and antiprogestational action of MTX.

Keywords: Estradiol, Histology, Leucovorin, Methotrexate, Ovariectomy, Progesterone, StAR protein and Uterus.

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http://bioresonline.org/archives/A120.pdf

Article citation:
Sritulasi K*, Gopalakrishnan CR and Vanithakumari G 2010. Antiestrogenic and Antiprogestational Activity of Methotrexate and its effect on Uterine Histoarchitecture of Ovariectomized Albino Rats. Bioresearch Bulletin 3: 127-136.

INTRODUCTION

Women with chemotherapy-induced ovarian damage experience menstrual changes, menopausal symptoms, changes in fertility potential, and infertility (Averette et al, 1990). Young women (≤ 40 years old) who receive adjuvant chemotherapy, particularly when they experience drug-induced menopause, are at a greater risk for negative changes in sexuality and poorer sexual-functioning outcomes (Ganz et al, 1998, Lindley et al, 1998). Endocrine therapy remains pivotal in the adjuvant therapy of premenopausal women with cancer (Pritchard, 1998). Adjuvant ovarian ablation is believed to be helpful in premenopausal women with cancer (Taylor et al, 1998, Davidson et al, 2005). It is less clear whether ovarian ablation suppression adds significantly to the role of chemotherapy in premenopausal women.

Methotrexate (MTX) is a common constituent of multi-drug regimens widely used in the treatment of chemotherapy. MTX is used in non -neoplastic disorders, rheumatoid arthritis (Black et al, 1964) and psoriasis (De Moragas, 1965). The synthesis of nucleic acids, thymidylates, and proteins is inhibited by MTX. It is an antimetabolite, antifolate and a cytotoxic drug, used extensively to treat cancer (Bertino, 1963). MTX therapeutic efficacy is the inhibition of dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis (Goldman, 1974). MTX is a folic acid antagonist, which inhibits *de novo* synthesis of the nucleoside thymidine, a pre-requisite for DNA synthesis (Bertino, 1963). In addition, MTX subdues folate, an essential participant in purine base synthesis. Thus, MTX causes toxic effect on rapidly dividing cells, suppressing the growth and proliferation in malignant, some non-cancerous cells (Skipper et al, 1967) and on reproductive system (Chapman, 1983, Averette et al, 1990, Badri et al, 2000, Padmanabhan et al, 2009). Folic acid (FA) is a water-soluble vitamin, which is involved in the synthesis of purine and pyrimidine, the essential precursors of DNA. Folinic acid or leucovorin (LCN) is the reduced form of FA that circumvents the inhibition of DHFR (Albert, 1981). Folate supplementation during MTX therapy reduces both toxicity and side effects without compromising the efficacy (Zeisel, 1990). Further, LCN supplementation reduces the common side effects of MTX in the treatment of arthritis and genotoxicity (Stover and Schirch, 1993).

The response of the uterine tissue to steroids is complex, involving many biochemical as well as morphological events resulting in uterine growth and differentiation (Clarke and Sutherland, 1990). Although the most profound mitogenic effect of estrogen appears to be in the endometrial compartment, myometrium is also sensitive to the hypertrophic actions of steroids (Soto and Sonnenschein, 1987). The mammalian uterus has a unique potential for proliferative growth and differentiation, which is cyclic (Barker et al, 1991). To elicit complete maturational and functional responses of the endometrium ovarian steroids, estrogen and progesterone are vital (Hisaw and Hisaw, 1961). Estrogens are responsible for the proliferative and progesterone for the secretory phase (Brenner and Slayden, 1994). Estrogen stimulates an initial trigger even, resulting in a cascade of sequential processes which result in true uterine growth (Walters, 1985). Although several reports exist as mentioned above on the toxic effects of MTX, there is a lacuna on the interrelationship of MTX and steroid hormones. Recently, we reported that MTX attenuated steroidogenic genes, which in turn reduced steroid hormone levels thereby causing damage to the ovary (Karri and Vanithakumari, 2010).

The induction of steroidogenesis is mediated by steroidogenic acute regulatory (StAR) protein (Clark et al, 1995). StAR protein mediates the rate-limiting step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane, where it is cleaved to pregnenolone by the inner membrane-bound $P450_{SCC}$ enzyme (Payne and Hales, 2004). Mutations in the human StAR gene cause congenial lipoid adrenal hyperplasia, a lethal disease that is characterized by marked impairment of steroidogenesis (Bose et al, 1996). Recently, StAR has been described in the uterus of rodent pregnancy (Arensburg et al, 1999, Ben-Zimra et al, 2002) and in endometriotic stromal cells (Tsai et al, 2001, Attar et al, 2009). However, to the best of our knowledge, limited studies are available on the effect of MTX on StAR expression in ovariectomized rat uterus.

We hypothesized that MTX has antiestrogenic and antiprogestational activity and is an endocrine disruptor based on our previous study (Karri and Vanithakumari, 2010). Ovariectomized (OVX) rat is a widely used model to study estrogen withdrawal and replacement because many

phenomena in rat model are similar to those occurring in postmenopausal women. To eliminate any interference from the ovarian hormones produced by intact animals, ovariectomy was performed on a group of rats, which were subsequently used to study an open question whether MTX has antisteroidogenic properties and StAR protein responsible for de novo synthesis of steroids is also a target to MTX in OVX uterus. As MTX is used in a multi-regimen chemotherapy, it is important to investigate the individual drug effect in target tissues. Furthermore, little is known about the biological effects of MTX after ovariectomy. Therefore, the focus of the present study was to investigate MTX induced effects on OVX uterus. The present study was designed to determine 1) MTX treatment alone on OVX uterus 2) MTX treatment rescue by LCN 3) estrogenic/ antiestrogenic properties of MTX 4) progestational /antiprogestational properties of MTX 5) expression of StAR protein in OVX uterus without or with MTX, LCN, E2 and P treatments.

MATERIALS AND METHODS

Animals

Healthy adult female albino rats of Wistar strain (Drug testing laboratory, Bangalore, India) 3-4 months old were used in this study. Rats were bilaterally ovariectomized (Zarrow et al, 1964, Lasota and Danowska-Klonowska, 2004). They were housed with 12h alternate light-dark cycle. Food and water were provided *ad libitum.* Fifteen days after rest period, animals were used for further treatments.

Study designs

Methotrexate (MTX) sodium salt and leucovorin (LCN) calcium salt were obtained from M/S Cynamide India Ltd., (Lederle division), India. MTX dose response studies were conducted in our laboratory and the maximum dose required to study the short-term effect on ovariectomized rat for one estrous cycle (4-5 days) was 0.5mg/Kg body weight/day (Karri and Vanithakumari, 2010). The use of massive doses of MTX, followed after an interval by folinic acid, allowed the effects of transient, complete inhibition of DNA synthesis to be studied (Berenbaum and Brown, 1965). The dose of MTX in the present study was undertaken to determine its effect on ovariectomized uterus to tolerate inhibition of DNA synthesis for one estrous cycle length, which reflects on uterus histology and to examine effects after progesterone and estrogen replacement therapy.

Ovariectomized rats were randomly divided into the following groups (n=6) and treated intramuscularly for 4 days.

Group 1: Control: Vehicle saline
Group 2: MTX: 0.5mg/Kg body weight/day
Group 3: MTX+LCN (leucovorin): 0.3mg/Kg body weight /day

Evaluation of estrogenic/antiestrogenic activity:

Group 4: Estradiol-17 ß 5µg/100g body weight/day
Group 5: MTX+LCN+Estradiol-17 ß

Evaluation of Progestational/antiprogestational activity:

Group 6: Progesterone- 2mg/100g body weight/day
Group 7: MTX+LCN+ progesterone 2mg/100g body weight/day

Animals were treated once per day to study the short-term MTX effects on uterus. Repeated injections of estradiol and progesterone increase their receptors in uteri after 4-5 days (Brenner and West, 1975). Hence, 5 days treatment schedule was chosen in the present study. LCN was injected after 4hrs (Badri et al, 2000) of MTX treatment followed by estradiol or progesterone. Rats were sacrificed on day 5. Animals were observed daily for general health. On the day of necropsy, all animals were anesthetized with ether. Body and uterus weights were recorded. Uteri were fixed in Bouin's fluid for histological analysis.

Body and organ weights

Body and oviducts, uteri, cervix and vagina weights were recorded on the day of sacrifice and are represented in grams (g) and mg/100g body weight respectively.

Histoarchitecture

Uterus was fixed in Bouin's fluid (Bancroft and Stevens, 1977) for histological analysis. Paraffin sections of tissue were cut (5mm), stained with hematoxylin and eosin and processed as reported previously (Karri and Vanithakumari, 2010).

StAR immunoblotting

StAR protein and ß-actin western blot analysis was performed as reported previously (Karri and Vanithakumari, 2010). Briefly, Equal amounts of uteri protein (25 mg), as determined by BCA method (Smith et al, 1985), were separated by

one-dimensional electrophoresis on 12% polyacrylamide gels electro transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with either StAR or ß-actin antibodies followed by respective secondary antibodies and exposed to X-ray films. Results are presented as % integrated density values.

RESULTS AND DISCUSSION

Effects of MTX on body and organ weights: (Fig.1. A-E)

Fig.1 depicts the effect of MTX on the body and organ weights (oviduct, uterus, cervix and vagina) of ovariectomized rats. Compared to OVX group, MTX and MTX+LCN treatment significantly (P< 0.05) decreased body and organ weights. Estradiol and progesterone replacement to OVX animals significantly increased the body and organ weights compared to non supplemented OVX group. While, MTX+LCN combined with either estradiol or progesteron supplementation revealed a significant (P<0.05) decrease in the body and organ weights. Additionally, significant (P<0.05) differences between the treatments groups in both body and organ weights were observed. LCN supplementation improved the body and organ weights significantly (P<0.05) compared to MTX treatment alone or in combination with estradiol and progesterone treatments. Body and organ weights significantly (P<0.05) reduced in OVX+MTX+LCN+E2 and OVX+MTX+LCN+P groups compared to OVX+E2 and OVX+P. LCN

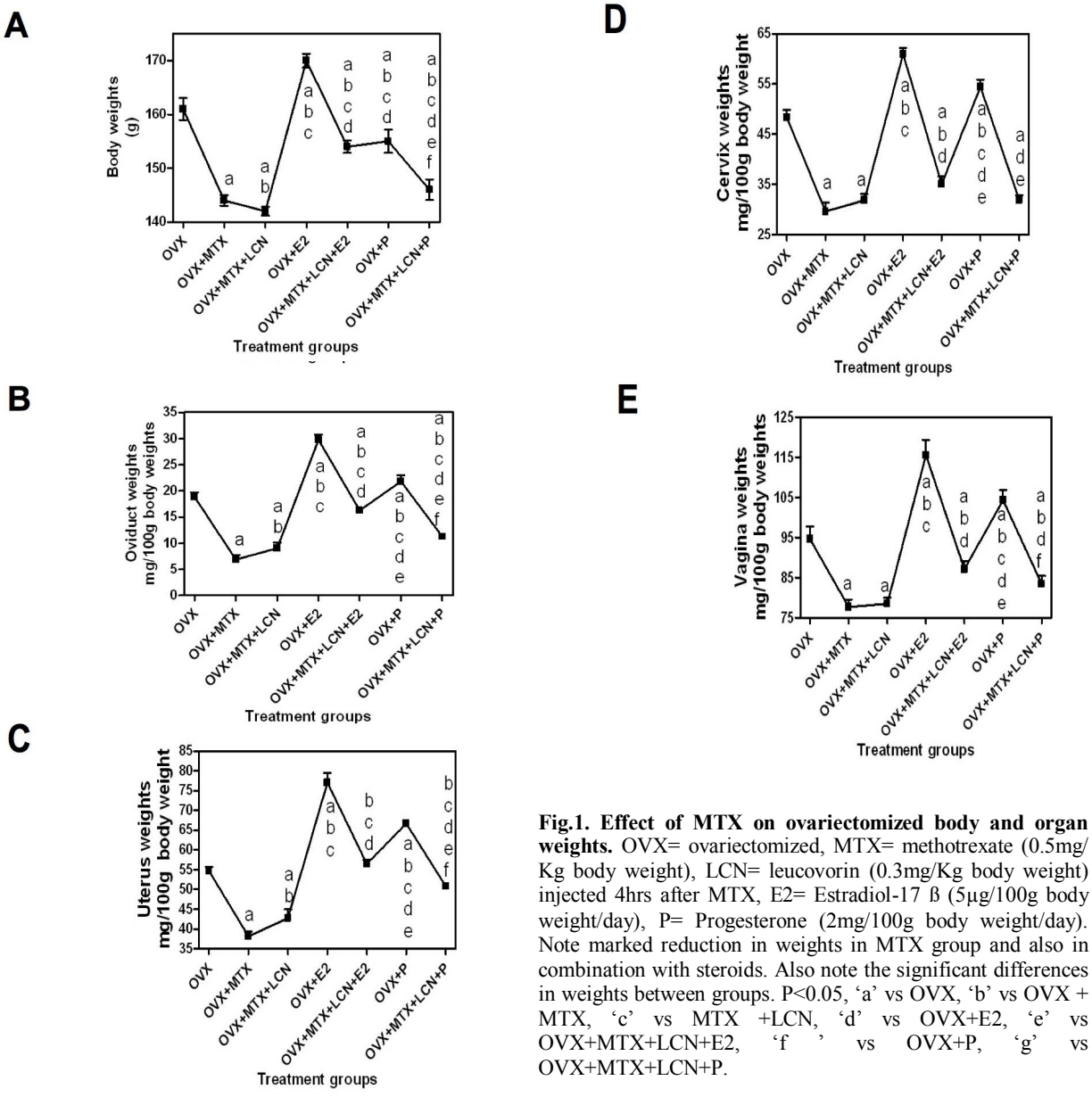

Fig.1. Effect of MTX on ovariectomized body and organ weights. OVX= ovariectomized, MTX= methotrexate (0.5mg/ Kg body weight), LCN= leucovorin (0.3mg/Kg body weight) injected 4hrs after MTX, E2= Estradiol-17 ß (5µg/100g body weight/day), P= Progesterone (2mg/100g body weight/day). Note marked reduction in weights in MTX group and also in combination with steroids. Also note the significant differences in weights between groups. P<0.05, 'a' vs OVX, 'b' vs OVX + MTX, 'c' vs MTX +LCN, 'd' vs OVX+E2, 'e' vs OVX+MTX+LCN+E2, 'f ' vs OVX+P, 'g' vs OVX+MTX+LCN+P.

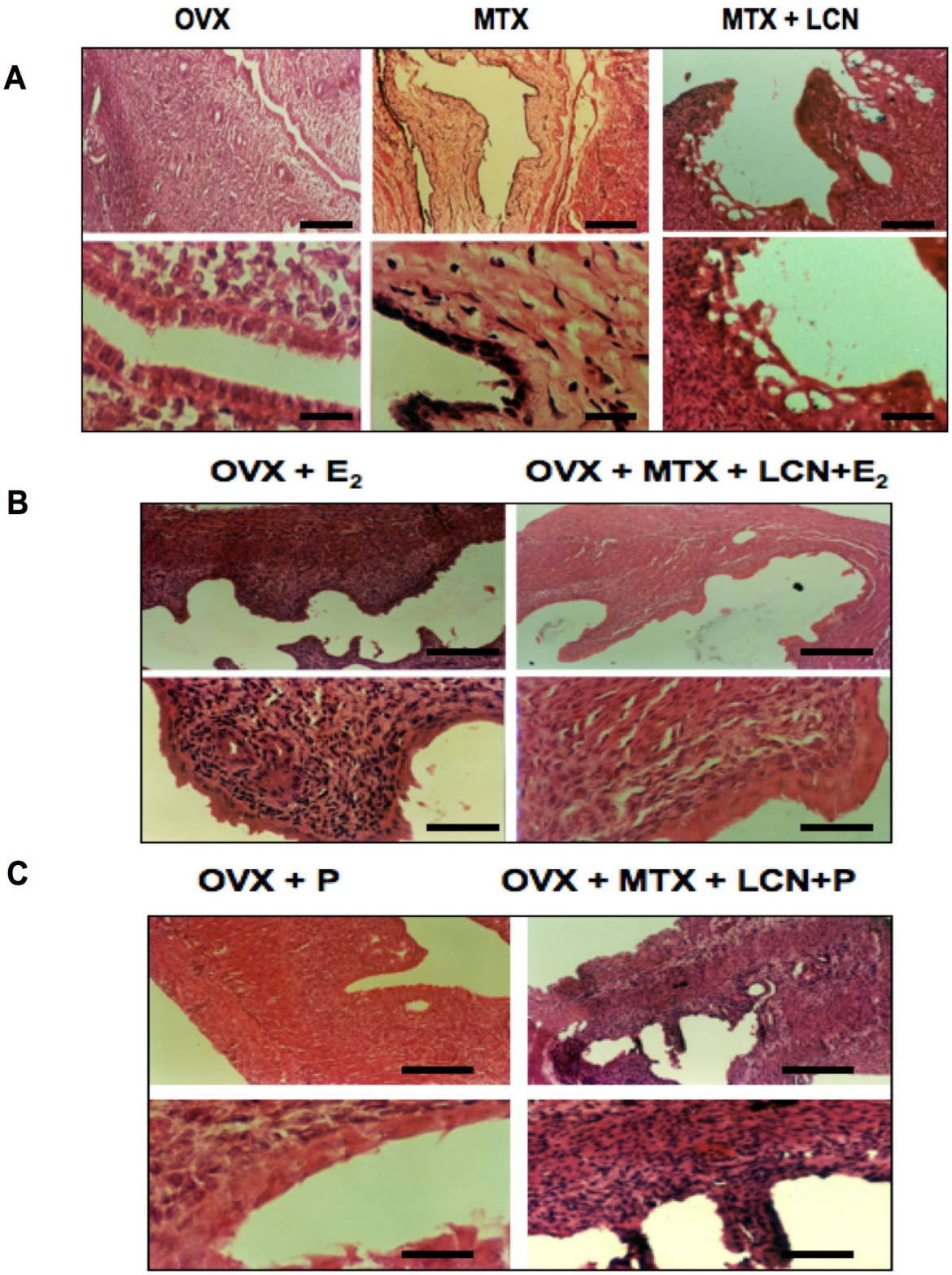

Fig.2. Phomicrographs of ovariectomized uterus showing changes in epithelium and stroma. Bar = 25µm (H&E). MTX= methotrexate, LCN= leucovorin, E2= Estradiol, P= Progesterone. Note the severe changes in the luminal epithelium and stroma of the uterus caused by MTX, improvement in the histoarchitecture after E2 and P treatments and disruption of stroma and changes in lumen by MTX in combination with E2 and P. LCN supplementation did not improve the histoarchitecture of uterus.

supplementation partially improved the weights. Increase in uterine weights by hormonal replacement depicts estrogenic and progestational activity. While, inhibition of uterine weights by MTX +LCN in combination with hormones indicate antiestrogenic and antiprogestational activity of MTX.

MTX toxicity shows signs of nausea, vomiting, ulceration of oral mucosa and diarrhea, which may alter feeding behavior (Weinblatt *et al*, 1988, Morgan et al, 1990) and that periods of under nutrition even as short as one day lead to a wide variety of metabolic responses in the body or organs which acts to decrease energy utilization and mobilize energy senses. LCN supplementation had partial significant change in MTX caused reduction of body and organ weights in OVX rats. The possible reason may be that the reduction in folate content and alteration of folate coenzyme pattern may alter cell differentiation and tissue growth. Factors affecting both protein synthesis and degradation determine cytoplasmic growth. On the other hand both progesterone and estrogen are important in consumption of food (Hervey and Hervey, 1967, Nunez *et al*, 1980, Ashby et al, 1982), hence hormonal replacement showed an increase in weights. While, MTX treatment in combination with steroids brought about a reduction in body and organ weights, revealing its antisteroidogenic property.

Effect of MTX on histopathology of the uterus in ovariectomized rats (Fig.2. A-C)

OVX: Uterus of OVX rats exhibited small luminal and atrophied glandular epithelium of simple

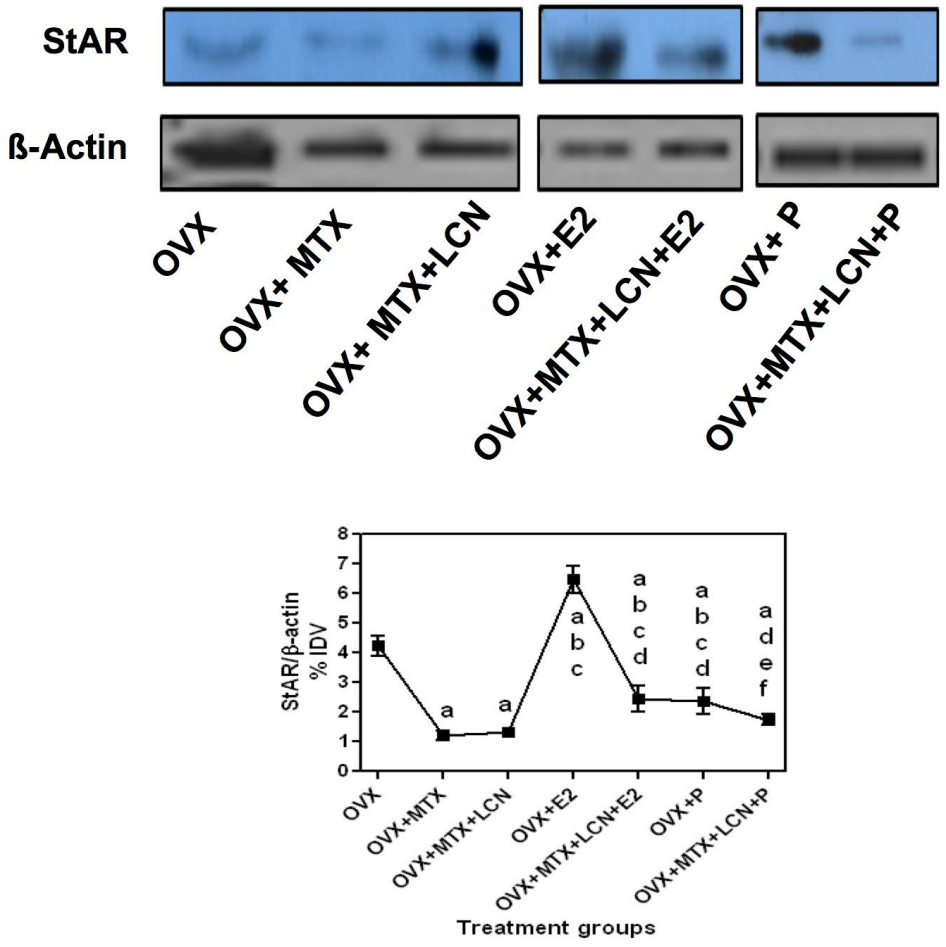

Fig.3. Immunoblot analysis of Steroidogenic acute regulatory (StAR) protein in OVX uterus. MTX= methotrexate, LCN= leucovorin, E2= Estradiol, P= Progesterone. In the representative autoradiograph, note the decrease of StAR protein levels caused by MTX and in combination with steroids. Line graph shows % IDV normalized to ß-actin. Data are mean± SE (n=3). P<0.05, 'a' vs OVX, 'b' vs OVX + MTX, 'c' vs MTX +LCN, 'd' vs OVX+E2, 'e' vs OVX+MTX+LCN+E2, 'f ' vs OVX+P.

cuboidal cells with a smooth rich eosinophilic surface. A few vacuoles containing dense granules or leukocytes were seen in the uterine epithelium. The nuclei were small and were aligned regularly in between the supra and sub nuclear cytoplasm forming a prominent band. The underlying stroma appeared undifferentiated.

OVX+MTX: OVX animals treated with MTX showed drastic changes in the luminal epithelium. The epithelial cells were thin and low with pycnotic nuclei. Absence of compactness and edema in stroma was exhibited.

OVX+MTX+LCN: LCN supplementation to MTX treated animals caused hypertrophy of glandular and luminal epithelium. Numerous shallow glands formed in the dense stroma due to extension of the columnar surface epithelium. The stroma was compact.

OVX+E2: OVX animals treated with estradiol caused hypertrophy, hyperplasia and pseudo stratification of luminal epithelium. Epithelial cells were narrow, tall and appeared pseudo stratified. Nuclei were enlarged and not regularly aligned. Lumen was filled with uterine fluid leading to a stretch of the uterine wall in addition to the differentiation of uterine cell types, the endometrial and myometrial cells. The stroma and myometrial cells were also highly stimulated.

OVX+MTX+LCN+E2: Estradiol injections in combination with MTX and LCN altered histology of uterus. Epithelium and nuclei were not well differentiated. Stroma epithelia revealed edema with few uterine glands and widened lumen.

OVX+P: Treatment of estrogen primed rats with progesterone caused a thickening of the uterine epithelium. The cells were of cuboidal type with nuclei arranged regularly. Light stained cytoplasm with sub nuclear vacuolation was prominent. Wave like projections in the surface of the epithelium was outstanding. There was a considerable hyperplasia in the stromal tissue.

OVX+MTX+LCN+P: There was a severe atrophy of the luminal epithelial cells observed. Darkly stained polygonal cells with pycnotic nuclei in stroma were observed. Atrophy of uterine glands was also seen.

The present study is the first to investigate the effect of MTX on histopathology of ovariectomized uterus. The massive hypertrophy of the luminal epithelium observed in OVX animals under estradiol treatment is consistent with the previous reports (Landau, 1976). Ballooning of the lumen was observed when MTX was treated in combination with E2 group compared to the other groups. This reflects on the accumulation of fluid in the lumen. There was not much improvement in the epithelium of this group compared to E2 treated group, which may be due to the antiestrogenic property of MTX. Such similar effect of MTX was also observed when treated in combination with progesterone. Stromal-epithelial interactions have been shown to be critical in the regulation of epithelial cells by estradiol and progesterone (Cunha et al, 2004). The uterine histology of OVX rats treated with MTX showed myometrial atrophy, decreased numbers of uterine glands and endometrial atrophy, suggesting a lack of estrogenic activity of MTX on this tissue. Furthermore, supplementation with LCN caused only marginal intrinsic uterotrophic activity, and we have confirmed these studies using MTX and LCN in the intact rat (Karri and Vanithakumari, 2010). Taken together, these data suggest that MTX has no ER agonist activity in the uterus, and in fact, is able to block natural estrogen action in the uterus. We do not rule out the possibility of long term steroid replacement studies to further establish the effect of MTX on OVX uterus.

Effects of MTX on StAR Protein in ovariectomized uterus (Fig.3)

Western-blot analysis of uterine tissue was used to examine possible alterations of key steroidogenic protein caused by MTX, LCN, estradiol and progesterone treatments. StAR protein the marker of steroidogenesis, decreased significantly ($P<0.05$) in OVX+MTX, MTX+LCN, compared to OVX group. A significant ($P<0.05$) increase in uterine StAR protein was observed in OVX+E2 and OVX+P groups compared to OVX, OVX+MTX, OVX+MTX+LCN. However, combination of OVX+MTX+LCN with either E2 and or P reduced StAR expression significantly ($P<0.05$). LCN supplementation had no significant difference in StAR protein expression compared to either MTX or steroids injected groups. The bar graph depicts the %integrated density value (IDV) normalized to ß-actin as loading control.

Our previous studies revealed that MTX caused inhibition of steroid synthesis in intact rats, associated with the expression of reduced StAR protein in the ovary (Karri and Vanithakumari, 2010). MTX treatment to OVX rats also decreased StAR expression in the uterus, which was

upregulated by estradiol and progesterone replacement. Indicating that an alteration affected by the microenvironment in the uterus, *i.e.* lack of uterine steroids is the cause for such a decrease in StAR protein. Further, MTX blocks the uterotrophic activity of steroids by reducing StAR protein in the uterus. Although, StAR protein has been shown to be expressed in uterus during pregnancy (Ben-Zimra et al, 2002), this is the first report to demonstrate the expression of StAR protein in the OVX rat uterus, which is expressed in response to steroids. Collectively, these observations propose a role for StAR in the mediation of the uterotrophic activity in MTX treated OVX rats. In addition, antiestrogenic and antiprogestational property of MTX is mediated *via* StAR protein in OVX uterus.

CONCLUSION

In conclusion, both estrogen and progesterone appears to be important target of MTX in OVX rats. This is further supported by our previous (Karri and Vanithakumari, 2010) and current study demonstrating a direct *in vivo* effect of MTX on steroid hormones. The most salient results from this study include the antiestrogenic and antiprogestational activity of MTX, reflecting on the body, organ weights and uterus histology. Also, StAR protein suppression after hormonal replacement by MTX unambiguously confirms its antisteroidogenic activity. LCN supplementation did not help rescue MTX caused antiestrogenic/ antiprogestational activity in the present study. This data may represent diminished reproductive success and it would be unwise to ignore potential risks to young women exposed to MTX after ovarian ablation and also in non-malignant treatments. Further, studies are warranted in OVX rats to reveal the genes involved in antiestrogenic and antiprogestational activity of MTX.

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