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Impact of Methotrexate and Leucovorin on Hormonal Regulated Enzymes of Carbohydrate Metabolism in Accessory Reproductive Tissues of Ovariectomized Rats

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Abstract

Background: Female reproductive tract cells are fast proliferating like cancer cells. Methotrexate (MTX), an anticancer drug when used to treat non-neoplastic diseases in young women results in deleterious effects leading to infertility. Oviduct, uterus, cervix and vagina depend on ovary for their structural and functional sustenance. Ovarian ablation followed by chemotherapy in premenopausal women is a common practice in the treatment of cancer. Hence, methotrexate effect on reproductive organs in Ovariectomized (OVX) rats was investigated.

Objective: To reveal the direct influence of MTX on estradiol and progesterone in OVX rats. Also, the protective role of leucovorin (LCN) an antidote to MTX, estradiol (E219) and progesterone (P) replacement was investigated.

Methods: Animals were randomly divided into the following groups (n=6): group 1: OVX, group 2: OVX+MTX, group 3: OVX+MTX+LCN, group 4: OVX+E2, OVX+MTX+LCN+E222, group 5: OVX+P, group 6: OVX+MTX+LCN+P, treated once/day intramuscularly (im) for 4 days and sacrificed on day 5. At the end of the experiment reproductive 24 tract tissues oviduct, uterus, cervix and vagina were used for total protein, glycogen, Glucose-6-Phosphate26 Dehydrogenase (G6PD), Lactate Dehydrogenase (LDH) and Alkaline Phosphatase (ALP) enzyme activities.

Results: MTX treatment reduced protein, glycogen, G6PD, LDH and ALP activities in oviduct, uterus, cervix and vagina of OVX rats. However, LCN supplementation was partially protective on some enzymes, which was tissue dependent. Hormonal replacement improved protein and enzyme levels in all the tissues. Estradiol treatment showed a distinct enhancement over progesterone in elevating such levels. However, MTX and LCN in a combination treatment with steroids reversed the activities of enzymes in all the tissues.

Conclusion: The results suggest that MTX severely affects protein, glycogen, G6PD, LDH and ALP levels in accessory reproductive organs of OVX rats. These studies conclude that in premenopausal women ovarian ablation and chemotherapy affect the accessory reproductive tissues, consequently distressing their quality of life.

Keywords: Cervix; Enzymes; Female reproductive tract; Leucovorin; Methotrexate; Oviduct; Steroids; Uterus; Vagina

Introduction

Female reproductive tract cells are fast proliferating like cancer cells. Recent report from our group lead to hypothesize that Methotrexate (MTX), an anticancer drug when used alone or in a combination therapy to treat non-neoplastic diseases (psoriasis, arthritis, spondylitis, ectopic pregnancy, gestational trophoblastic neoplasia, or interstitial twin pregnancy) in young women results in deleterious effects leading to infertility [1]. This study lead to the potential of MTX for direct cellular contact, factors from serum and ovary to influence the functions of each tissue in female reproductive tract [1]. Results from this study reveal that MTX treatment severely damages ovarian structure and function. Accessory reproductive tissues oviduct, uterus, cervix and vagina depend on ovary for their structural and functional sustenance. Ovarian ablation followed by chemotherapy in premenopausal women has been employed for over a century in the treatment of breast cancer [2]. It is effective in delaying recurrence and increasing survival rates [3,4]. However, whether ovarian ablation suppression helps chemotherapy in premenopausal women still remains unclear. Chemotherapy induced ovarian damage, leads tomenstrual cycle changes, and menopausal symptoms, bringing about infertility in young women [5,6]. Also, endocrine disorders are common in many pediatric cancer survivors [7]. Further, the extent to which individual chemotherapy drugs impact hormonal therapy and genes in accessory reproductive tissues after ovarian ablation in premenopausal women are poorly understood. To the best our knowledge no observational studies either on patients or animals have investigated the interaction of MTX, Leucovorin (LCN) supplementation and ovarian ablation on carbohydrate metabolism enzymes in accessory reproductive tissues. The question in address is 'to what degree are the accessory reproductive tissues affected by MTX after ovarian ablation?' Therefore, the current study was undertaken to investigate the mechanism(s) of action of MTX on Ovariectomized (OVX) rats, which serves as a model to study the effect of the drug on accessory reproductive tissues (oviduct, uterus, cervix and vagina) in absence of ovaries and also a menopausal model.

Methotrexate (MTX)

Methotrexate (MTX) is a methyl-derivative of aminopterin and was first described in the year 1947 [8]. MTX is widely used in the treatment of chemotherapy as a common constituent of multi-drug regimens [9]. Additionally, MTX is used to treat non-neoplastic disorders viz; juvenile arthritis [10], rheumatoid arthritis [11], psoriasis [12] and ectopic pregnancy [13,14]. MTX action involves inhibiting the synthesis of nucleic acids, thymidylates, and proteins, which make

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it an antimetabolite, antifolate and a cytotoxic drug to treat cancer [15]. MTX inhibits Dihydrofolate Reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis [16]. MTX is a folic acid antagonist, which inhibits de novo synthesis of the nucleoside thymidine, a prerequisite for DNA synthesis [15]. In addition, MTX subdues folate, an essential participant in purine base synthesis. As a result MTX, a potent anticancer drug, acts on rapidly dividing cells suppressing the growth and proliferation in malignant [17] and some non cancerous cells [18].

Folic acid (FA)

Folic Acid (FA) is a water-soluble vitamin, which is involved in the synthesis of purine and pyrimidine, precursors to DNA. Folinic acid or Leucovorin (LCN) is the reduced form of FA that evades the inhibition of DHFR [19]. Folate supplementation during MTX therapy reduces both toxicity and side effects without compromising the efficacy [20,21]. Further, LCN supplementation reduces the common side effects of MTX in genotoxicity [22] and cytogenetic damage [23]. Hence, LCN supplementation was incorporated in the present study. Nevertheless, a relationship between MTX, LCN and steroid hormones may help establish the efficacy of therapeutic management. Thus, estradiol and progesterone hormones were used to treat OVX animals either individually or in combination with MTX and LCN in the current study.

Estrogen and progesterone

Estrogen and progesterone play a vital role in the regulation and protection of the structural, physiological, biochemical and molecular events of female accessory reproductive organs viz; oviduct, uterus, cervix and vagina. Ovarian ablation/ovariectomy is known to cause alteration in the structure and function of female accessory reproductive tissues, by changing levels of estrogen and progesterone [24,25]. Endocrine therapy in premenopausal cancer patients (< 50yrs) after ovarian ablation significantly improves their long-term survival [26,27]. Our group recently established the antiestrogenic and anti-progestational activity of methotrexate [28]. Additionally, MTX treatment to female rats revealed inconsistency of estrogen, progesterone, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) concentrations. We reported the histopathological differences in ovary, oviduct, uterus, cervix and vagina to be MTX dose dependent [1]. Recently, we also reported the anti-implantation activity of MTX and alterations in glycogen content in ovary, oviduct, uterus, cervix and vagina of rats caused by MTX [29,30]. On the other hand, sustainable benefits following chemotherapy with ovarian ablation technique during early and advanced stages of cancer remains ambiguous till date. Whether or not MTX direct action on steroids affects the accessory reproductive tissues after ovarian ablation was intriguing. Hence, in the present study the impact of ovariectomy, chemotherapy and hormonal replacement therapy was investigated on protein, glycogen, Glucose-6-Phosphate Dehydrogenase (G6PD), Lactate Dehydrogenase (LDH) and Alkaline phosphatase in accessory reproductive tissues of OVX rats.

Hormones regulate the metabolic activities by controlling RNA and protein synthesis in female reproductive tract [31]. MTX blocks the synthesis of nucleic acids and proteins, the end products of folate pathway [16]. Oviduct, uterus, cervix and vagina change under the influence of steroid hormones (estrogen and progesterone) during reproductive cycle, pregnancy, menopause and disease conditions [24]. Ovariectomy results in decrease of protein concentration and hormonal treatment causes a return to its normal levels in female reproductive tract [24]. Hence, in the present study protein concentrations were measured after MTX and LCN treatments either alone or in combination with estradiol or progesterone. Glycogen is the major constituent and an energy metabolism marker [32]. Ovariectomy and ovarian hormones are reported to have an adverse effect on glycogen levels in female reproductive tract [33-36]. Therefore, the effect of MTX, LCN and steroid hormones was also investigated on glycogen levels in the current study. The key regulatory enzyme in the Hexose Monophosphate (HMP) shunt is Glucose-6-Phosphate Dehydrogenase (G6PD). This enzyme catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone. This results in the production of NADPH to meet cellular needs for reductive biosynthesis and maintenance of the cellular redox status [37]. NADPH on the other hand is essential for the synthesis of cholesterol, an important constituent of cell membranes and steroidogenesis [38]. Exogenous steroid hormones are known to have an influence on G6PD levels in oviduct [39], uterus [40], cervix [41] and vagina [42]. A positive correlation between DNA synthesis and G6PD activity suggests that HMP shunt may limit cell proliferation by limiting NADPH availability for the synthesis of cholesterol and its metabolites, which are necessary for initiating DNA synthesis [43,44]. Earlier studies show that G6PD inhibition by steroid protects against the growth of chemically induced tumors and is also able to block cell division [45]. On the other hand the inhibition of tumor induction might be due to the failure of G6PD deficient cells to metabolize chemicals that are 136 able to induce cancer [46]. Hence, MTX and LCN effect on G6PD was examined in the present study. Lactate dehydrogenase (LDH) is an enzyme of the glycolytic pathway associated with the carbohydrate metabolism and serves as an energy source for folliculogenesis [47]. LDH catalyzes the interconversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD^+ [48]. At high concentration of lactate, the enzyme exhibit feedback inhibition and the rate of conversion of pyruvate to lactate is decreased. This is an important step in energy production in cells [49]. Ovariectomy causes alterations in LDH levels, which are also responsive to ovarian steroid hormones in female reproductive tract [50-53]. Therefore, in the present study MTX and LCN effect on LDH was explored. Alkaline Phosphatase (ALP) is involved in the differentiation, morphogenesis [54], active transport and secretion of steroid hormones [55]. ALP is an important enzyme that maintains the structure and function of female genital tract [1,56,57]. Variations in ALP reflect the secretory patterns of endogenous ovarian hormones during menstrual cycle [56-60]. Studies revealed that OVX caused a significant reduction in ALP activity [57]. Hence, in the present study MTX and LCN effect on ALP was studied. Glycogen, G6PD, LDH and ALP enzymes were reported as markers in various types of cancers in female genital tract [61-64]. Hence, the activities of these enzymes in accessory reproductive organs (oviduct, uterus, cervix and vagina) were measured in the current study.

The underlying mechanisms of ovarian ablation as adjunct to chemotherapy to clinically improve premenopausal women survival rates, quality of life have been a serious concern. As the target population includes ovarian ablation in premenopausal women, we used Ovariectomized (OVX) rats mimicking such a condition in the present study. Ovariectomized reproductive tract is known to be at its extremes in structure and functional activity. We reported earlier that MTX caused severe effects on uterus and has antiestrogenic and antiprogestational activity [1,28].

Thus, it leads to an interesting hypothesis that MTX treatment after ovarian ablation in premenopausal women may have deleterious

effects on the accessory reproductive tissues. Current investigation is devoted to quantitative aspects of changes in enzymes that accompany the functional changes in accessory female reproductive tissues. The objective of the study was designed to determine a) MTX effect on enzymes b) rescue by LCN c) estradiol and progesterone supplementation and d) hormonal therapy in combination with MTX and LCN. Enzyme assay methods used in this study were a) Total protein b) Glycogen c) G6PD d) LDH and e) ALP activities in Oviduct, Uterus, Cervix and Vagina of OVX rats.

Materials and Methods

Animals

Healthy adult female albino rats of Wistar strain (Drug testing laboratory, Bangalore, India), 3-4 months old weighing 150-180g were used in this study. Rats were bilaterally ovariectomized [65]. Briefly, the animals were anesthetized with ether and a nose cone with cotton plug ether was used during performing the surgery. A single transverse incision was made across the midline on the ventral side of the skin three centimeters above the vaginal opening. Embedded in a pad of fat ovary was visible through the abdominal wall. The tip of a pair of fine forceps was introduced grasping the pad of fat around the ovary; care was taken not to rupture the capsule of the ovary itself. A pair of artery forceps was used to crush the tip of the uterine horn and by a single cut with scalpel ovary was removed, leaving the oviduct intact with the uterine horn. Two or more interrupted sutures closed the skin incision. Animals were housed with 12h alternate light-dark cycle. Food and water were provided ad libitum. Rats were rested for fifteen days after ovariectomy and were further used for treatments. Experiments 181 were performed according to approved institutional guidelines for animal care.

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 0.5 mg/Kg body weight/day [1]. The reason for choosing LCN dose in the current study was that the use of massive doses of MTX, followed after an interval by LCN, allowed the effects of transient, complete inhibition of DNA synthesis to be studied [66]. The dose of MTX in the present study was undertaken as per our earlier report, to determine its effect on ovariectomized rat to tolerate inhibition of DNA synthesis for one estrous cycle length and to examine effects after progesterone and estrogen replacement therapy [28]. The doses and repeated injections of estradiol and progesterone increase their receptors in female reproductive tract after 4-5 days [67], hence these doses were used in the current study.

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

Group 1: Control: Vehicle saline

Group 2: MTX: 0.5 mg/Kg body weight/day

Group 3: MTX+LCN (leucovorin): 0.3 mg/Kg body weight /day (LCN)

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

Group 5: MTX+LCN+Estradiol-17 ß

Group 6: 202 Progesterone- 2 mg/100 g body weight/day

Group 7: MTX+LCN+ Progesterone

Animals were treated once per day to study the short-term MTX effects on OVX rats. Repeated injections of estradiol and progesterone increase their receptors after 4-5 days [67]. Hence, 4 days treatment schedule was chosen in the present study. LCN was injected after 4hrs [68] of MTX treatment followed by estradiol or progesterone after additional 2 hrs. Intramuscular injections were either made into the front or back of the thigh muscle of rats with volumes of 25-50 µl of the drugs or steroids. Rats were sacrificed on day 5. Animals were observed daily for general health. On the day of necropsy, all animals were anesthetized with ether. Oviduct, uterus, cervix and vagina were collected, cleaned, weighed and immediately frozen in liquid nitrogen and stored in freezer until further enzyme assays analysis.

Analysis of Protein, Glycogen, G6PD, LDH and ALP Activities

Analysis of protein, glycogen, G6PD, LDH and ALP enzyme assays in oviduct, uterus, cervix and vagina were followed as reported in our previous work [1,29].

Protein

Protein concentration was determined by Lowry method [69] and measured at 720 nm. Briefly, tissue was homogenized in 0.25 M sucrose solution and centrifuged at 3000 g for 15 min. Using Bovine Serum Albumin (BSA) as standard copper-protein complex reaction with Folin-Ciocalteau reagent was measured at 720 nm.

Glycogen

Glycogen was estimated in ovary, oviduct, uterus, cervix and vagina by the method of Hassid and Abraham [70] as modified by Morales et al. [71]. Briefly, tissues were digested with 30% Potassium Hydroxide (KOH) solution in boiling water bath for 20-30 min. The digest was cooled, saturated sodium sulfate was added and glycogen was precipitated with 95% ethanol, gently boiled, cooled and centrifuged at 3000 rpm for 10 min. The precipitate 225 was washed with water and re-precipitated with 95% ethanol. The final precipitate dissolved in water was treated with freshly prepared 0.2% anthrone reagent in 95% Sulphuric Acid (H_2SO_4) with the test tube placed in cold water to avoid excessive heating. After mixing by lateral shaking the tube was placed in boiling water for 10 mins for color development and tubes cooled with water. Glycogen was determined as glucose by the method of Karri S et al. [29]. Standard was generated using glucose. Spectrophotometrically O.D was recorded at 620 nm. The amount of glucose was converted to glycogen by dividing with the Morris factor 1.11 [30].

Glucose 6 Phosphate dehydrogenase (G6PD)

Glucose 6 Phosphate dehydrogenase (G6PD) specific activity of G6PD in the tissues was assayed by previously established methods [72,73]. Briefly, tissues homogenates made with ice cold 0.1 M Tris buffer (pH 7.6) were centrifuged at 20,000x g for 20 min at 2-4°C and the supernatants were used for enzyme assays. The total activity of G6PD was determined by following the change in absorbance at 340 nm due to NADPH production. Enzyme activity was expressed as µ/min/mg of protein.

Lactate dehydrogenase (LDH)

LDH activity in the tissue homogenates was determined by

previously described methods [72,74] and enzyme activity was measured with development of the color measured at 440 nm. Total LDH enzyme activity was measured in tissue homogenates or water in control tubes. Buffered substrate (pH 10) containing 0.1 M glycine buffer, 0.1 N NaOH and 0.135 M lithium lactate was added to the samples or control and incubated at 378°C.Further, tubes were incubated at 378°C for 15 min by the addition of NADP. 2,4 dinitrophenylhydrazine in 1N HCl was used to arrest the reaction for 15 min, while addition of NaOH (0.4 N) enhanced the maximal development of the color, which was measured at 440 nm. Standards with sodium pyruvate (0.5M) and known amounts of coenzyme NADH (10 mM) made allowance for the chromogenity of NADH2 formed. Enzyme activity was 248 expressed as µ/min/mgof protein.

Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) activity was estimated by using paranitrophenyl phosphate substrate [75,76]. Aliquots of the tissue homogenates were incubated with 0.012 M of p-nitrophenyl phosphate and 0.1 M-glycine buffer (pH 10.5) at 37°C. After incubation with enzyme, 0.2 M NaOH was added to stop the reaction and free p-nitrophenol was measured at 410 nm. By adding HCl (12 M), second reading was measured at 400 nm. Second reading was subtracted from the first reading. The enzyme activity was calculated from the standard p-nitrophenol curve run parallel with samples and expressed as µmol/h/g tissue.

Statistical Analysis

Prism software (version 4.02, Graph Pad Inc., San Diego, CA, USA) was used for graphical presentation and statistical analysis. Data are presented as Standard Error Mean (SEM) values of 'n' independent experiments with variability given as mean ± SE. Analyses of Variance (ANOVA) performed included one-way ANOVA for matched samples followed by Newman- Keuls post-hoc test of differences between all group means. P<0.05 was considered statistically significant.

Results

Effect of MTX on total protein in oviduct, uterus, cervix and vagina

Total protein levels in oviduct, uterus, cervix and vagina significantly (P<0.05) decreased in OVX+MTX, OVX+MTX+LCN groups compared to OVX group. LCN supplementation significantly (P<0.05) increased protein levels in uterus compared to MTX treated group. Hormonal therapy in OVX+E2 and OVX+P groups increased protein levels significantly (P<0.05) compared to OVX group in all the tissues. However, no such effect 270 was observed in oviduct after progesterone treatment. A combination of hormones and drugs in OVX+MTX+LCN+E2 and OVX+MTX+LCN+P groups significantly (P<0.05) caused a reduction in protein levels in all the tissues (Figure 1).

Effect of MTX on glycogen in oviduct, uterus, cervix and vagina

Glycogen levels in OVX+MTX and OVX+MTX+LCN groups decreased significantly (P<0.05) compared to OVX in all tissues. In OVX+MTX+LCN group glycogen levels significantly (P<0.05) increased in all tissues compared to OVX+MTX group except in vagina. In OVX+E2 and OVX+P group's glycogen levels were significantly (P<0.05) higher compared to OVX group. However, progesterone treatment had no significant change in uterus and cervix compared to OVX group. While, in OVX+MTX+LCN+E2 and OVX+MTX+LCN+P groups glycogen levels decreased significantly (P<0.05) compared to OVX group in all tissues (Figure 2).

Effect of MTX on glucose 6 phosphate dehydrogenase (G6PD) in oviduct, uterus, cervix and vagina

G6PD levels in OVX+MTX and OVX+MTX+LCN groups decreased significantly (P<0.05) compared to OVX in all tissues. LCN supplementation significantly increased G6PD levels in vagina compared to OVX+MTX group. In OVX+E2 and OVX+P groups G6PD levels were significantly (P<0.05) higher compared to OVX group in all tissues. While, in OVX+MTX+LCN+E2 and OVX+MTX+LCN+P groups G6PD levels in all tissues decreased significantly (P<0.05) compared to OVX group (Figure 3).

Effect of MTX on lactate dehydrogenase (LDH) in oviduct, uterus, cervix and vagina

LDH levels in OVX+MTX and OVX+MTX+LCN groups decreased significantly 292 (P<0.05) in all the tissues compared to OVX group. In OVX+MTX+LCN group LDH levels significantly (P<0.05) increased in vagina compared to OVX+MTX group. In OVX+E2 group LDH levels were significantly (P<0.05) higher in oviduct, uterus and cervix compared to OVX group. In OVX+P group LDH levels were significantly higher (P<0.05) in oviduct compared to OVX group. In OVX+MTX+LCN+E2 group LDH levels decreased significantly (P<0.05) in uterus and cervix compared to OVX group. In OVX+MTX+LCN+P groups LDH levels decreased significantly (P<0.05) in oviduct, uterus and cervix compared to OVX group (Figure 4).

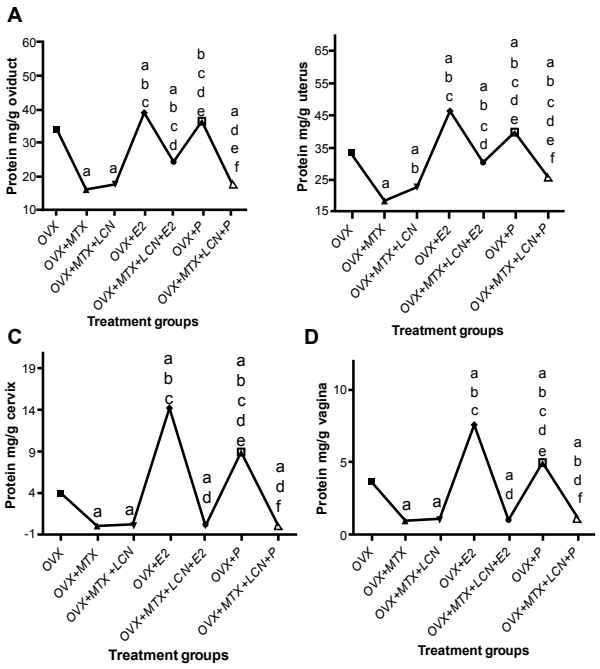

Figure 1: Effect of MTX on total protein in oviduct (A) Uterus (B) Cervix (C) Vagina (D) Ovariectomized rats.
OVX: Ovariectomized; MTX: Methotrexate, LD: Low dose, HD: High Dose, LCN: Leucovorin; E2: Estradiol; P: Progesterone P<0.05, 'a' vs Control, 'b' vs OVX+MTX, 'c' vs OVX+MTX+LCN, 'd' vs OVX+E_2, 'e' vs OVX+MTX+LCN+E_2, 'f' vs OVX+P. Values are Mean ± SE, N=6. Note marked reduction in total protein concentrations in MTX treated group and in combination with LCN and steroids. Also, note the significant differences between treatment groups and tissues.

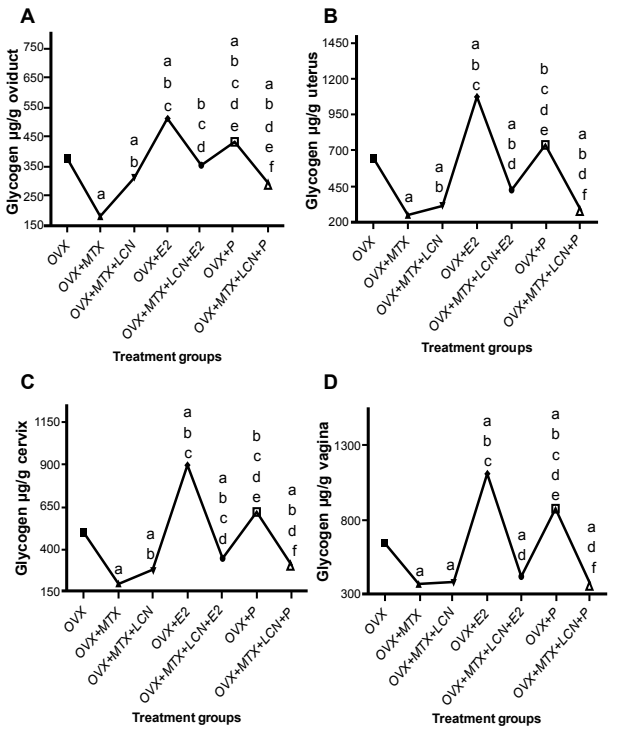

Figure 2: Effect of MTX on glycogen in oviduct (A) Uterus (B) Cervix (C) Vagina (D) Ovariectomized rats. OVX: Ovariectomized; MTX: Methotrexate; LD: Low dose; HD: High Dose; LCN: Leucovorin; E2= Estradiol; P: Progesterone P<0.05, 'a' vs Control, 'b' vs OVX+MTX, 'c' vs OVX+MTX+LCN, 'd' vs OVX+E_2, 'e' vs OVX+MTX+LCN+E_2, 'f' vs OVX+P. Values are Mean ± SE, N=6. Note marked reduction in glycogen concentrations in MTX treated group and in combination with LCN and steroids. Also, note the significant differences between treatment groups and tissues.

Effect of MTX on Alkaline phosphatase (ALP) in oviduct, uterus, cervix and vagina

Alkaline phosphatase levels in OVX+MTX and OVX+MTX+LCN groups decreased significantly (P<0.05) in all the tissues compared to OVX group. In OVX+E2 group ALP levels remained significantly (P<0.05) higher in uterus and cervix compared to OVX group. In OVX+P group, ALP levels significantly increased (P<0.05) in uterus compared to OVX group. ALP levels inOVX+MTX+LCN+E2 306 and OVX+MTX+LCN+P groups decreased significantly (P<0.05) in all tissues compared to OVX group (Figure5).

Over all, Protein, glycogen, G6PD, LDH and ALP concentrations were attenuated by MTX in a tissue dependent manner. LCN supplementation had a minimal effect on MTX-induced outcome of these enzymes. Although estrogen and progesterone replacement increased majority of the enzymes, a combination of steroids with MTX and LCN declined such intense effects.

Discussion

Methotrexate (MTX)

Methotrexate (MTX) is an anticancer drug, widely used in combination therapy to treat various types of cancers [9] and non-neoplastic diseases [11-14]. Leucovorin (LCN) a folinic acid is used as an antidote to MTX treatment [20,21]. Female accessory reproductive tissues (oviduct, uterus, cervix and vagina) are known to be dependent on ovarian steroids for their normal structure and function. Recent research from our group suggests drastic effects of MTX on female reproductive tract [1] and its anti-implantation activity [30]. Also, our previous studies show that ovariectomy, MTX and LCN treatments is known to cause variations in the structure and function of reproductive tract, a reduction in body and organ weights and antiestrogenic and antiprogestational [28] properties. However, the mechanisms following MTX and LCN treatments after ovarian ablation and their effects on enzymes of oviduct, uterus, cervix and vagina without or with hormonal replacement therapy are not well established. Thus, we hypothesize that MTX treatment after ovarian ablation in premenopausal women may have deleterious effects on the accessory reproductive tissue enzymes. In the present study, we demonstrate that MTX decreases protein, glycogen, G6PD, LDH and ALP activities in oviduct, uterus, cervix and vagina of OVX rats. Partial recovery of the enzymes by LCN was tissue dependent. However, MTX and LCN had severe effect on hormonal regulated enzymes in these tissues after ovarian ablation.

Methotrexate inhibits synthesis of nucleic acids and proteins [15], thereby restrains the fast proliferation of cells [16]. Hormones regulate metabolic activities in female reproductive tract by controlling RNA, DNA and protein synthesis [24]. Ovariectomy and chemotherapy disrupts menstrual changes, fertility potential and induces menopausal symptoms [5,6]. The interruption in signaling pathways for gonadal steroids reveals reduction in protein synthesis in regular cycling rats [1]. Previous studies have indicated that steroid hormones (estrogen and progesterone) play a major role during female reproductive tract

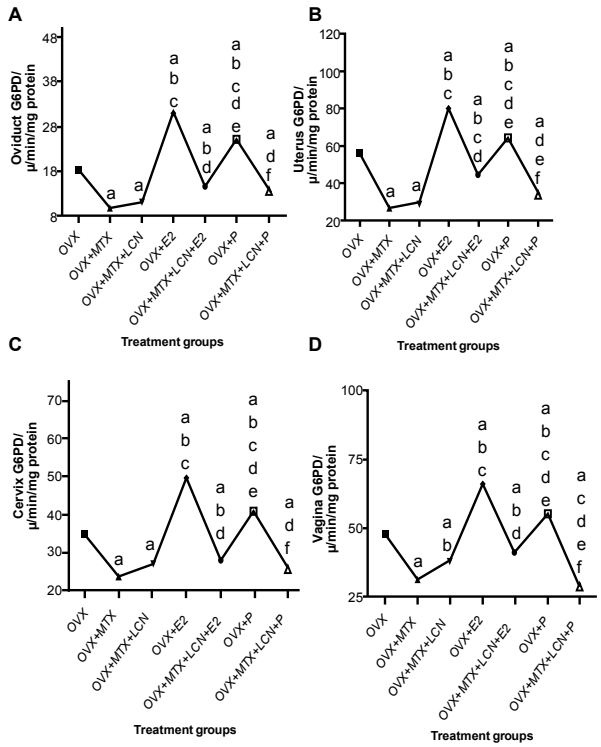

Figure 3: Effect of MTX on G6PD in oviduct (A) Uterus (B) Cervix (C) Vagina (D) Ovariectomized rats. OVX= OVX: Ovariectomized; MTX: Methotrexate; LD: Low dose; HD: High Dose; LCN: Leucovorin; E2= Estradiol; P: Progesterone;G6PD: Glucose-6-Phosphate Dehydrogenase P<0.05, 'a' vs Control, 'b' vs OVX+MTX, 'c' vs OVX+MTX+LCN, 'd' vs OVX+E_2, 'e' vs OVX+MTX+LCN+E_2, 'f' vs OVX+P. Values are Mean ± SE, N=6. Note marked reduction in G6PD activity in MTX treated group and in combination with LCN and steroids. Also, note the significant differences between treatment groups and tissues.

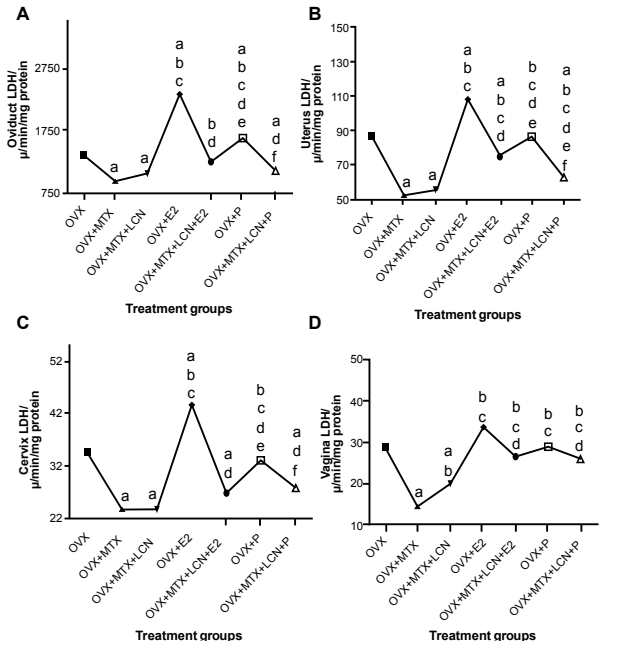

Figure 4: Effect of MTX on G6PD in oviduct (A) Uterus (B) Cervix (C) Vagina (D) Ovariectomized rats. OVX= OVX: Ovariectomized; MTX: Methotrexate; LD: Low Dose; HD: High Dose; LCN: Leucovorin; E2= Estradiol; P: Progesterone; LDH: Lactate Dehydrogenase P<0.05,'a' vs Control, 'b' vs OVX+MTX, 'c' vs OVX+MTX+LCN, 'd' vs OVX+E_2, 'e' vs OVX+MTX+LCN+E_2, 'f' vs OVX+P. Values are Mean ± SE, N=6. Note marked reduction in LDH activity in MTX treated group and in combination with LCN and steroids. Also, note the significant differences between treatment groups and tissues.

development. Decrease in protein levels in MTX treated groups in the current study relate to the MTX's inhibition of folate pathway [16]. The inhibition of protein synthesis reflects on distressed cellular growth, chromosomal damage and cytotoxicity caused by MTX [1,77]. Administration of estradiol to OVX rats increased DNA, RNA (data not shown) and protein levels in the current study. The increase in protein levels after hormonal replacement in the present study corresponds with the previous studies [24]. MTX treatment is known to cause an asynchronous population of dividing cells in accessory reproductive organs, perturbing the ability of cells to undergo mitosis, thereby altering nucleic acids and protein levels. LCN and steroid hormone supplementation may enable synchrony of dividing cells, thus increasing nucleic acids and protein content of cells. An increase in protein levels observed in uterus after LCN supplementation may be due to such a synchrony of dividing cells. While, the anti-estrogenic and antiprogestational properties of MTX [28] may be responsible for inhibiting LCN action on dividing cells and thereby lowering protein levels in oviduct, cervix and vagina in the current study.

Glycogen

Glycogen content in the female reproductive tract is important for energy-consuming events like contraction [32]. Thus, glycogen is a vital marker in female reproductive tract in normal and pathological conditions. Low levels of glycogen caused by MTX treatment in oviduct, uterus, cervix and vagina may be due to the depletion of ovarian hormones induced by ovariectomy. Decrease in tissue weights caused by MTX shown in our previous studies [28], may also be a reason for low glycogen levels. LCN supplementation and hormonal therapy to OVX rats increased the organ weights in our earlier studies [28] reflecting an increase in glycogen levels. However, MTX decreased such levels in a combination therapy with steroid hormones. Deficiency of folic acid is known to be one of the causes for glycogen storage disease [78]. In the current study the low levels of glycogen may be associated to the glycogen metabolism in these tissues. The progressive decrease in the glycogen content may also be due to decreased synthesis as a result of damage of cellular mitochondria in the female reproductive tract cells [1]. Therefore, low amount of ATP with decline of mitochondrial content of the cell reduces glycogen formation [79].

Glucose-6-phosphate to 6-phosphogluconolactone (G6PD)

G6PD catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone producing NADPH. NADPH is vital for the synthesis of cholesterol, an important constituent of steroidogenesis [37,38]. Inhibition of steroid levels [1], antiestrogenic and antiprogestational activity of MTX [28] correlates with the low levels of G6PD in the female reproductive tissues. It is certainly possible that G6PD levels may be low due to the inhibition of G6PD catalysis by MTX resulting in NADPH blockage. This may impact cholesterol synthesis thereby reduction in steroids as observed in our previous studies [1]. Results in the current study indicate that replacing the hormones further increased G6PD levels. Exogenous steroid hormones are known to have an influence on G6PD levels in oviduct [39], uterus [40], cervix [41] and vagina [42]. Previous studies showed that MTX treatment reduced DNA levels in the female reproductive tract tissues [1]. A positive correlation between DNA synthesis and G6PD activity suggests that HMP shunt may limit cell proliferation by limiting NADPH supply for the synthesis of cholesterol and its metabolites, which are necessary for initiating DNA synthesis [43,44]. In the current study DNA concentrations decreased in the accessory reproductive

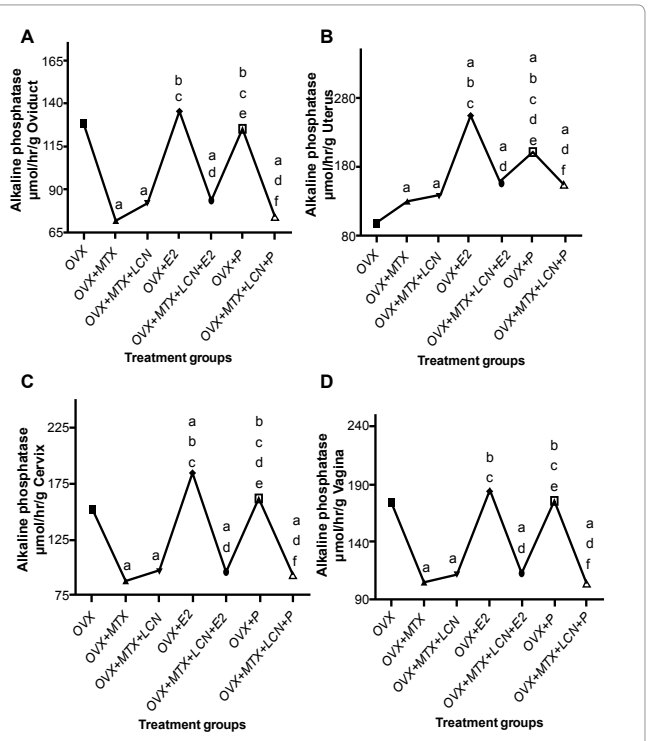

Figure 5: Effect of MTX on ALP in oviduct (A), uterus (B), cervix (C) and vagina (D) of ovariectomized rats. OVX: Ovariectomized; MTX: Methotrexate; LD: Low Dose; HD: High Dose; LCN: Leucovorin; E2= Estradiol; P: Progesterone; ALP= Alkaline Phosphatase P<0.05, 'a' vs Control, 'b' vs OVX+MTX, 'c' vs OVX+MTX+LCN, 'd' vs OVX+E_2, 'e' vs OVX+MTX+LCN+E_2, 'f' vs OVX+P. Values are Mean ± SE, N=6. Note marked reduction in ALP activity in MTX treated group and in combination with LCN and steroids. Also, note the significant differences between treatment groups and tissues.

tissues (data not shown), which correlate with the low levels of G6PD. MTX effect on the growing cells of female reproductive tract may also be a reason for the reduced levels of G6PD. As shown earlier, G6PD activity can enhance cell growth, and that decreased G6PD activity is associated with decreased cell growth [1,80,81]. Increased G6PD levels in vagina may reflect an increase in cell growth after LCN supplementation.

Lactate dehydrogenase(LDH)

Decrease in LDH activity may be due to elevated lactic acid content and depleted pyruvic acid. Such situation may arise due to the inhibition of NAD-LDH that converts lactic acid to pyruvic acid. Increase in LDH activity may be related to an increase in estrogen levels. OVX reduces steroid levels causing decrease of LDH levels in the tissues. Hormonal replacement showed an increase in LDH levels, which concurs with earlier studies that circulating steroids have an influence on LDH activity in reproductive tract [82]. However, previous studies report the inhibition of LDH by estradiol in human placenta [83]. This contradiction may be attributed to the differences in tissues and species used in the studies. Decrease in LDH activity in OVX animals reproductive tissues may be due to MTX antiestrogenic and anti progestational effects [28]. The decrease in LDH activity may also suggest decrease in anaerobic glycolysis. Anaerobic glycolysis produces ATP, an essential source of energy during estradiol synthesis. Hence, enhanced LDH activity is observed after LCN supplementation in vagina and estradiol treatment. Reduced LDH activity in some tissues treated with progesterone may indicate low glycolysis after progesterone treatment. Earlier reports confirm such tissue specific differences in estradiol and progesterone actions on LDH activity [52,53].

Alkaline phosphatase (ALP)

Alkaline Phosphatase (ALP) maintains the structure of female genital tract [57]. A decreasein ALP levels in OVX animals was tissue dependent in the current study. From the earlier studies it's known that ALP activity increases with estradiol and progesterone treatments [60]. Lower steroid levels decrease ALP activity that indicates a decrease in the cleavage of phosphate esters Phosphatases in animal tissues are known to be involved in processes such as carbohydrate metabolism, nucleotide metabolism, and calcium deposition [84]. Previous studies on alkaline

Phosphatase indicates its probable role in glycogen synthesis and the transport of secretory substances across cell membranes [85]. The pattern of the reduced ALP in tissues reflects on the lack of endogenous ovarian hormones due to ovariectomy, which is known to significantly reduce ALP activity [57]. The increase in ALP activity after estradiol treatment is in agreement with the earlier studies [58]. Antagonistic action of MTX in combination with steroids is confirmed in our studies by the decrease in ALP activity. In rats there are conflicting reports 404 claiming an increase [59], no change or a decrease [58] in ALP activity following estradiol administration. This decrease in ALP activity in the current study may be due to reduced facilitation of the transport of amino acids into the tissue cells ultimately leading to a decrease in protein synthesis. In the current study alkaline phosphatase activity in uterus indicates completely different pattern of response to MTX and LCN treatment compared to the enzyme activity in other tissues. MTX and LCN treatments caused a significant decrease in ALP activity in oviduct, cervix and vagina, while an increase in the enzyme activity was observed in uterus. The reason for this difference may be due to the sensitivity of uterus to the drugs. Our previous studies have shown that MTX causes disturbance in the cyclicity of animals leading to prolonged diestrus stage [1]. MTX and LCN treatment to ovariectomized rats may be responsible for the sensitivity of uterus, thus mimicking an immature rat uterus, hence elevated levels of ALP activity. Our results concur with the earlier reports that the total alkaline phosphatase activity of the uterus in the immature rats is significantly higher than that of the virgin animals in all stages [86]. An increase in ALP activity was observed upon estrogen and progesterone treatments in the present study, which also concurs with the previous reports [87]. MTX disrupted uterine histoarchitecture shown in our previous studies [1,28], such an impact on the uterine cells may be a reason for indicating low ALP activity. The differences in elevated ALP activity in uterus appears to be a complex relationship between MTX, LCN, steroids and uterine histoarchitecture and needs further investigation. Withdrawal of MTX treatment in the current investigation to study the reversibility of its effects revealed no return of enzyme levels to normalcy in the tissues of study (data not shown).

Limitations

Whilst the findings of the study could be applied in most instances, there were some important exceptions. A) In particular, it was found that we could not carry out long-term studies with MTX treatment, and in fact rats did not survive for longer periods after ovarian ablation and MTX treatment. B) We do not rule out the possibility of prolonged withdrawal of MTX treatment to study its reversibility of effects.

Conclusion

The increasing use of anticancer drugs for a variety of illnesses raises questions regarding its potential benefits and side effects. The current study demonstrates that MTX has a deleterious effect on protein, glycogen, G6PD, LDH and ALP levels in accessory reproductive organs of OVX rats. While these effects are due to the deprivation of steroids in response to ovariectomy, such an outcome may also be due to a) the direct effect of MTX on these enzymes b) the low potency of LCN as a rescue agent and c) MTX antagonism against estradiol and progesterone. This is the first report indicating the relative potency of ovarian ablation, chemotherapy and leucovorin supplementation with respect to carbohydrate metabolism enzymes in oviduct, uterus, cervix and vagina of OVX rat model in response to exogenous hormones. Such information will help circumvent MTX toxic effects, when used to treat young women with either neoplastic or non neoplastic diseases.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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