Original Research Article

Rat Uterine Protein Profile In Response To Tamoxifen During Estrous Cycle In Presence And Absence Of Ovary In-situ

Hiranmaya Sharma[1] and Hirendra N Sarma[2*]

[1]Department of Zoology, Dera Natung Govt. College, Itanagar-791113, Arunachal Pradesh, India

[2]Molecular Endocrinology & Reproductive Biology Laboratory, Department of Zoology (Center with Potential for Excellence in Biodiversity), Rajiv Gandhi University, Rono Hills, Itanagar- 791112, India

*Corresponding author: hnsarma@hotmail.com

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Abstract: The present study was designed to study the effect of tamoxifen in the uterus of female albino rat in presence and absence of ovary-in situ. The experiments were carried out in both the cyclic and ovariectomised (OVX) female albino rats. Tamoxifen (TAM) has been administered subcutaneously and the uterine horns were collected following the respective treatment in order to study the protein profile by SDS-PAGE in 15% gel. Estradiol-17β (E2) was used to ascertain involvement of certain other factors and TAM in modulating protein profile of the uterus. Tamoxifen induced production of new proteins in the uterus, both in presence and absence of ovary. In ovary intact cyclic female rats, new proteins were synthesized during the follicular and luteal phases along with disappearance of some other proteins in response to TAM. Ovariectomy leads to loss or decreased expression of many proteins in the uterus. Administration of E2, TAM and E2 + TAM induced restoration of many protein found during estrous and diestrous phases. In addition to the original proteins restored, few new proteins were also synthesized in response to the administration of these compounds. The study reveals that tamoxifen alters the protein profile of the ovary intact and ovariectomised female rats which may alter the normal physiology of the reproductive system of female rats.

Keywords: Tamoxifen, SDS-PAGE, Protein profile, Rat uterus

Introduction

The uterus is a versatile component of the female reproductive system. The estrous cycle, which is under the control of ovarian hormones, induces cyclic changes in the uterus, mediated through various proteins and growth factors. A healthy uterus is essential for the overall reproductive health of cyclic female. Various growth factors produced by the uterus, in coordination with the ovarian hormones and the hypothalamo-pituitary axis, maintain the dynamic state of uterus (Ciarmela *et al.,* 2011). The uterus shows a continuous cyclicity of events, which are under the control of the above mentioned factors. The estrous cycle of mouse has been described in terms of vaginal cell types (Allen, 1922). Both visual and vaginal cytology methods can be used to ascertain the stage of estrous cycle in a more accurate manner (Byers *et al.*, 2012). The mean duration of estrous cycle in rats is 4 days which includes proestrous, estrous, metestrous and diestrous phases (Marcondes *et al.,* 2002). The hormonal basis of estrous cycle in mice has been established in various studies (Michael, 1976; DeLeon *et al.,* 1990). A clear understanding of the processes involved during estrous cycle may lead to understanding of functioning of the uterus. Undesirable changes in the normal physiology of the uterus, induced by external or internal factors, may lead to endometrial cancer. Next to breast cancer, endometrial cancer is the most widely occurring cancer in

women affecting female reproductive health. Tamoxifen is a triphenylamine derivative, extensively used for treatment of breast cancer throughout the world because of its antiestrogenic effects in the breast tissue (Davies *et al.,* 2013). Though tamoxifen is widely used for prevention of breast cancer, it is seen to induce endometrial cancer in few cases (Bergman *et al.,* 2000; Jones *et al.,* 2012).

In spite of having side effects like endometrial cancer, tamoxifen is being used for treatment of breast cancer, because of its high efficiency against estrogen receptor (ER)- positive breast tumors. It acts as an estrogen antagonist in breast cells, preventing proliferation of ER positive tumor cells (Cuzick *et al.,* 2003). However, in the uterus, tamoxifen acts as an estrogen agonist causing cell proliferation (Goldstein, 2001; Pole *et al.,* 2005). This study was done in order to understand the mechanism of effect of tamoxifen in the uterus in terms of changes in protein expression, as studied by Sodium dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE). It has been speculated that tamoxifen modulates protein expression, both in presence and absence of ovary in-situ. Endometrial protein during follicular (estrus) and luteal (diestrus) phases of estrous cycle were studied to see, how tamoxifen affects protein profile in presence of native gonadal steroids, estrogen and progesterone respectively. Effect of TAM in ovariectomized (OVX) mice was studied to see the expression of uterine proteins in presence of exogenous estradiol-17β and the possible role of other endogenous factors in controlling protein expression of the uterus.

Materials and methods

Test animal

Sprague-Dawley female albino rats weighing 150 ± 10 g body weight were taken for the experiments. The animals were maintained in the central animal facility of Rajiv Gandhi University, Itanagar, Arunachal Pradesh, India. The animals were acclimatized prior to treatment with natural dark light periods and room temperature. All animal experimentation was done in accordance with institutional ethical guidelines. Rats were fed Bengal gram and water *ad libitum*. The rats were observed for regular estrous cyclicity and animals with at least three regular cycles were selected for the experiments. The estrous cycle study was done for ascertaining the stage of estrous cycle, by observing the vaginal smear taken by flushing the vagina with distilled water. Experiments were performed in the cyclic ovary intact females, both in the estrus (follicular) and diestrus (luteal) phases and adult ovariectomised (OVX) rats. Effects of tamoxifen on estrous cycle were studied by observation of cell types present in the vaginal smear.

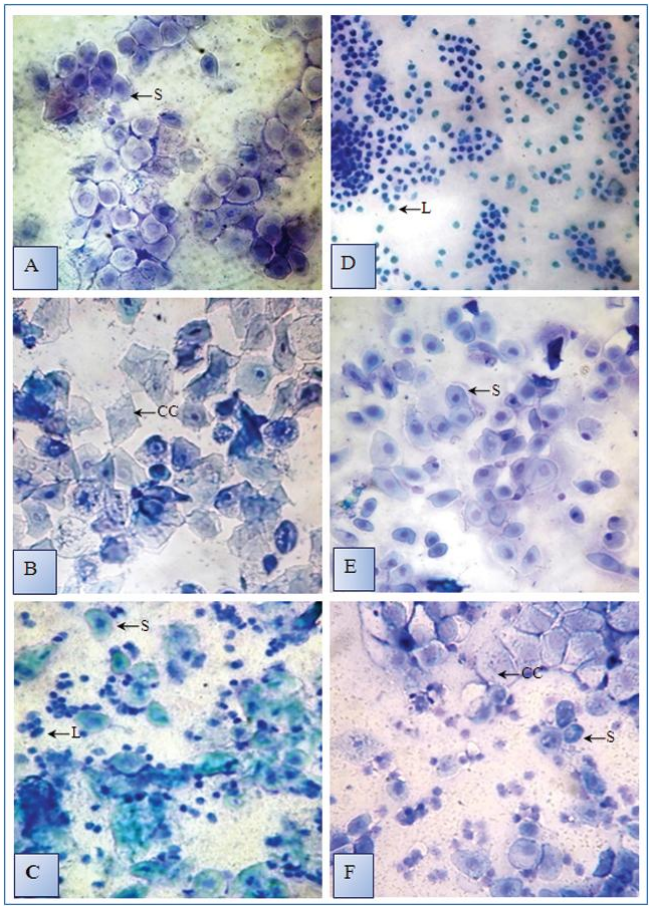

Fig. 1. Photomicrographs of vaginal cytology during estrous cycle of control (A-D), Tamoxifen (TAM) treated during follicular(estrous) phase (E) and TAM treated during luteal (diestrous) phase (F) of females. Control females showed superficial epithelial (S) cells during proestrus (A). The estrus phase (B) is characterized by cornified cells (CC). Metestrus (C) showed presence of both the cornified cells and leucocytes (L). Diestrus (D) showed large numbers of leucocytes in the vaginal smear. Administration (s.c) of tamoxifen causes extension of follicular phase for longer duration showing presence of epithelial cells undergoing cornification (E). TAM treated females during luteal phase showed presence of cornified cells induced by TAM (Fig F). Original magnification A-F x 40.

Ovariectomy of the adult female rats

The ovariectomy of adult cyclic female rats was carried out following standard laboratory method (Hogan *et al.,* 1986). Briefly, adult cyclic females were anaesthetized using diethyl ether anesthesia and two dorsolateral incisions below the last rib of vertebral column, approximately 1 cm long were made on both sides to expose the ovaries. Ovaries were removed along with small part of uterine horn and the wound closed by single cat gut suture. Neosporin powder is applied topically over the wound to prevent any infection. The OVX females were allowed to recover for 3-week period before starting the experiment.

Experimental design: administration of Tamoxifen and Estradiol-17β

Female rats either ovary intact or ovariectomized were divided in different groups as mentioned below; each group containing six numbers of rats for administration of tamoxifen and/or estradiol-17β. Control group of females received vehicle only for the respective compound in similar dose and time of administration. Tamoxifen (Sigma, USA) and estradiol-17b (Alfa aesar, USA, purchased from Himedia) were administered subcutaneously (sc) to the experimental females. Both tamoxifen and estradiol were dissolved in sesame oil and were administered in the nape of neck region of the females at an interval of 24 hours between 8.00 – 9.00 AM. Tamoxifen in a dose of 25 μ g/0.5 ml/day was injected (sc) to cyclic females for 2 days beginning with morning of proestrus and sacrificed 12 hours later of the last injection (Group A1). The dose was selected on the basis of previous studies suggesting control of N-Nitrosomethyl urea (NMU)-induced breast tumors on administration of this minimal dose in rat mammary carcinoma model (Gottardis *et al.,* 1987). A vehicle treated control group of animals in the follicular (estrus) phase was taken (Group A2). Another group of cyclic females in late metestrus were injected tamoxifen for 3 days to study the effect during the luteal phase (diestrus) (Group B1). The control group of animal was treated with vehicle during the luteal (diestrus) phase (Group B2). TAM treatment was for 2 days and 3 days for follicular and luteal phases respectively, as per the duration of the phases in the normal cyclic rats. E2 was administered to another group of OVX females in the dose of 0.1 μg/0.5 ml/day sesame oil for three consecutive days (Group C1). A control group of OVX female (Group C2) received the vehicle only in similar mode of administration. A group of OVX female was administered tamoxifen in the similar dose as mentioned earlier for three days and sacrificed 24 hours after the last injection (Group D1). A group of OVX females were administered a combined dose of E2 and tamoxifen at the similar doses (Group E1) as mentioned above.

Separation of uterine protein by SDS-PAGE

Uterine protein samples were collected from the females of above mentioned groups and extraction of protein was done following standard method used earlier in our laboratory. Briefly, the uterus was cleaned, minced on ice and homogenized in SDS sample buffer. The ground tissue was kept in a boiling water bath for 10 minutes and quenched on ice for 10 minutes. The contents were centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant containing denatured proteins were separated and centrifuged again at 10000 rpm for 5 minutes. The supernatant was collected finally and stored at -20°C until use for SDS-PAGE. The protein was extracted in 2% SDS sample buffer. The estimation of the extracted protein was done following Bradford method (Bradford, 1976). All the uterine protein samples were separated by single dimensional SDS-PAGE following standard method (Ausubel *et al.,* 2002). 80 -100 mg of protein sample was loaded in each lane. Separation of proteins was done in 15% separating gel topped with 3.9% stacking gel. Standard molecular weight marker (Novagene, Perfect protein marker, 10-225 kDa, cat # 69079-3) was used for calibration of the experimental protein samples. Electrophoresis was carried out at an environment of 4°C. The gels were fixed and stained overnight with 0.5 % Coomassie brilliant Blue (CBB) G-250 solution and then destained with changes of destaining solution. Digitized image of gels was taken using Gel Doc system (Perkin Elmer, Geliance 200 Imaging system), bands were quantified using Gene tools software from Perkin Elmer and molecular weights determined (Nevile *et al.,* 1971).

Results

To begin with the experiments on uterine protein profile, estrous cycle of adult cyclic females was studied by observation of vaginal cytology in smear. The uterine protein profile changes during the different phases of the estrous cycle. The estrous cycle in rats is divided into four phases - proestrus, estrus, metestrus and diestrus, of which proestrus and estrus were considered as the follicular phases while, metestrus and diestrus as the luteal phase. Tamoxifen administration to adult cyclic females altered the pattern of cell types that appeared in vaginal cytology (Fig. 1). The ovariectomised (OVX) females lose their cyclicity due to absence of gonad; however, capable of uterine cellular proliferation and subsequent cornification following exogenous E2 and TAM effects (Fig. 2).

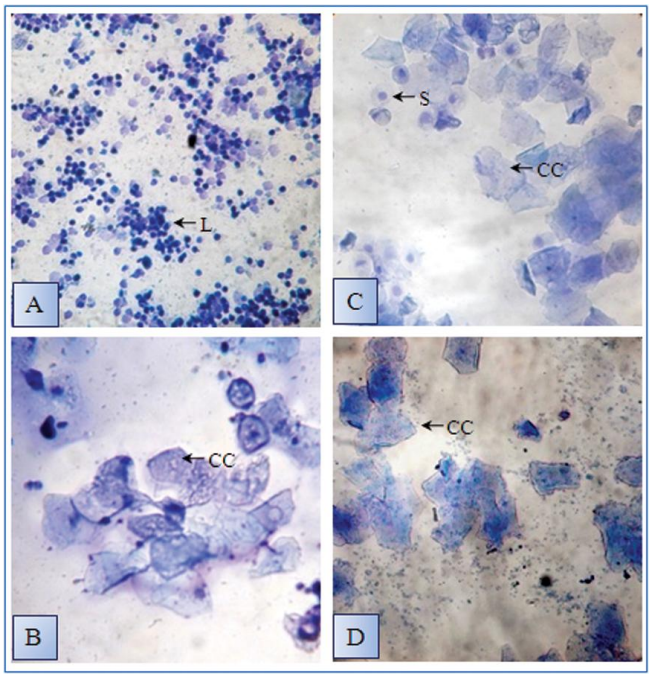

Fig. 2. Photomicrographs of vaginal cytology of OVX females treated with TAM (B), estradiol-17β (C) and vehicle treated control (A). Administration of TAM and E2 induced vaginal epithelial cell proliferation and maturation producing superficial (S) cells (B-C) and cornified (CC) cells (B-D). Original magnification A-D ×40.

Protein profile during the follicular (estrus) and luteal (diestrus) phase

A study of the protein profile during follicular (estrus) and luteal (diestrus) phases showed 26 and 24 protein bands respectively, ranging from molecular weight of 295 kDa to 20 kDa (Fig. 3). Out of the total bands, 23 bands were common to both phases. Three protein bands having molecular weight ≈250 (band B), ≈42 (band U) and ≈36 (band W) kDa respectively were expressed during diestrous only which were barely detectable during the estrus phase. One protein band of molecular weight ≈97 kDa (band I) was expressed during estrus (group A2) which was not visible during the diestrus phase (group B2).

Effect of tamoxifen on uterine protein during follicular (estrus) phase

Administration of tamoxifen to ovary intact cyclic females in follicular (estrus) phase altered the expression of uterine protein profile compared to that of the control females (Fig. 3 and 4). Four protein bands of molecular weight ≈127 (band E), ≈56 (band P), ≈34 (band Y) and ≈28 (band Z1) kDa respectively disappeared from control females in estrus phase upon administration of TAM. Three bands of molecular weight ≈117 (band G), ≈84 (band M) and ≈63 (band O) kDa appeared in the ovary intact cyclic females in response to TAM administration.

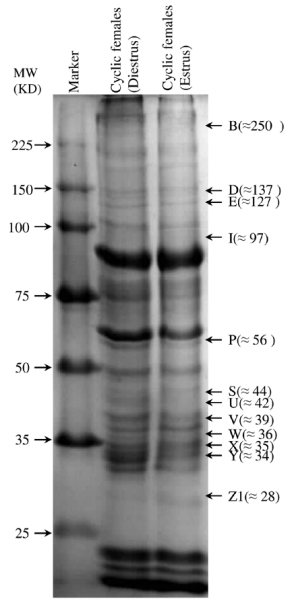

Fig. 3. Uterine protein profile of vehicle treated control ovary intact females in follicular (estrus/ group-A2) and luteal (diestrus/ groupB2) phase separated by SDS-PAGE (15% gel). Only the differentially expressed proteins have been marked in the figure. Three proteins bands (B, U and W) were expressed in the ovary intact females in diestrus phase whereas one protein (band I) was expressed in the estrus phase. Four proteins present in estrous phase (E, P, Y and Z1) were absent in TAM treated rats in estrus phase (group A1, Fig.4). Four protein bands were seen in diestrus phase (band D, S, V and X) which were absent in TAM treated rats in same phase (group B1).

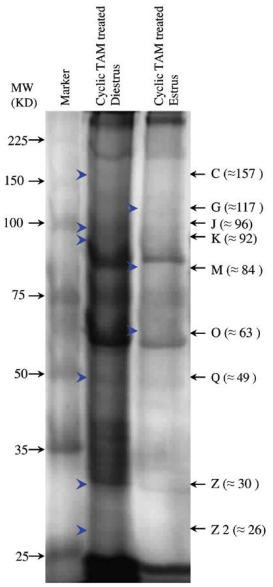

Fig. 4. Uterine protein profile of Tamoxifen (TAM) treated ovary intact females in follicular (estrus/ group A1) and luteal (diestrus/ group B1) phase. The follicular stage treated with TAM (group A1) showed expression of three proteins (band G, M and O) not present during estrous (group A2, Fig 3). The luteal phase treated with TAM (group B1) showed expression of six protein bands (band C, J, K, Q, Z and Z2) not present during diestrus of control group (group B2, Fig 3). Proteins were compared with the standard molecular weight (MW) marker proteins expressed in Kilo Dalton (kDa). Proteins were separated by SDS-PAGE (15% gel).

Effect of tamoxifen on uterine protein during luteal (diestrus) phase

Administration of TAM to the ovary intact cyclic females in luteal (diestrus) phase also altered the expression of uterine protein profile compared to that of the control females (Fig. 3 and 4). A total of four proteins of molecular weight ≈137 (band D), ≈44 (band S), ≈39 (band V) and ≈34 (band X) kDa were observed only during the diestrus phase of females that could not be visualized following TAM treatment in females during same phase (diestrus) of estrous cycle. Six protein bands of molecular weight ≈157 (band C), ≈96 (band J), ≈92 (band K), ≈49 (band Q), ≈30 (band Z) and ≈25 (band Z2) were newly synthesized on administration of TAM during diestrus which was not present in the control females. The rest 19 proteins were common in both the control and treated group.

Study on ovariectomised (OVX) females

The OVX females were studied to observe the effects of TAM in absence of ovarian estrogen, as well as for comparative analysis of effects of exogenous E2 and TAM on expression of uterine protein in absence of ovary *in situ*. The OVX females treated with exogenous E2, TAM and E2 & TAM together showed further changes (Fig. 5). The ovariectomized females showed disappearance of many protein bands in absence of ovary in situ. The OVX control females showed minimal number of bands in all the samples studied.

Effect of Estradiol-17β on uterine protein of OVX females

Exogenous estradiol-17β stimulates the OVX rat uterus to synthesize many proteins which were absent following removal of ovary in situ. Eight additional protein bands were present in the E2 treated OVX rats which were absent in the OVX control rats (Fig. 5). The proteins with molecular weight ≈267 (band A), ≈119 (band F), ≈113 (band H), ≈97 (band I), ≈69 (band N), ≈45 (band R), ≈43 (band T) and ≈28 (band Z1) kDa respectively appeared in the uterus of OVX rats in response to treatment with exogenous estradiol-17β. Out of these proteins, band I, R, T and Z1 were also present in the ovary intact cyclic rats during follicular (group A2) and luteal (group B2) phase.

Effect of TAM on uterine protein of OVX rats

TAM induced synthesis of four proteins which were not there in the OVX control females. Protein bands with molecular weight ≈113 (band H), ≈97 (band I), ≈69 (band N) and ≈45 (band R) kDa respectively were synthesized in response to TAM administration which were absent in OVX rats (Fig. 5). The above mentioned proteins which were speculated to be specific for TAM response, and two other protein bands, (band I and R) were present in ovary intact cyclic rats during follicular (group A2) and luteal (group B2) phase.

Effect of TAM on uterine protein of OVX rats in presence of estradiol-17β

TAM, when administered along with estradiol-17β, resulted in 5 new protein bands, as compared with OVX control. New proteins with molecular weight ≈86 (band L), ≈69 (band

N), ≈45 (band R), ≈43 (band T) and ≈28 (band Z1) kDa was synthesized in response to combined effect of E2 and TAM on the OVX rats (Fig. 5). Administration of TAM and E2 together in the OVX rats restores the protein bands of molecular weight ≈294, ≈180, ≈145, ≈126, ≈45, ≈33, ≈32 and ≈28 kDa respectively, which were also observed in the ovary intact cyclic females in follicular and luteal phase.

Discussion

The purpose of this investigation is to study the effect of tamoxifen (TAM) on the uterine protein profile in albino rat. The protein profile has been studied during follicular and luteal phases of the estrous cycle to understand the underlying role of TAM in presence and absence of various ovarian hormones. During the present study, the protein profile of TAM treated animals were seen to ascertain whether any new protein appeared, any previous protein disappeared, or there is some other change in the protein profile, in response to TAM administration. It has been speculated that TAM alters some of the basic metabolic pathways in the uterus of albino rat, which may alter the protein synthesized within the uterus, as reflected in the protein profile. The altered protein profile is responsible for carrying out restructural changes in response to TAM. The effect of TAM has been studied for its effect on uterine protein profiles either in presence or absence of ovary in situ. The study was carried out using E2 for comparing its effect with TAM in OVX rat models. Earlier studies from this laboratory showed effects of certain compounds on the uterine protein profile in OVX rats (Hazarika *et al.,* 2006; Goswami *et al.,* 2009).

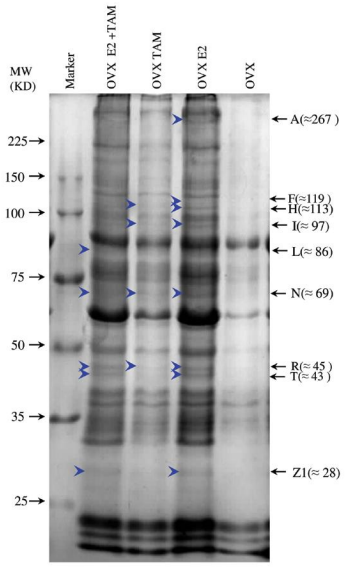

Fig. 5. Uterine protein Profile of ovariectomised control (OVX, group C2), ovariectomised E2 treated (OVX E2, group C1), ovariectomised TAM treated (OVX TAM, group D1) and ovariectomized TAM +E2 treated (OVX E2+TAM, group E1) females. E2 induces production of eight new bands (A, F, H, I, N, R, T and Z1, group C1). Four new protein bands (H, I, N, R) were observed in response to TAM (group D1) as compared to OVX vehicle treated control females. Five additional protein bands (L, N, R, T and Z1) were newly synthesized in OVX E2+TAM rats (group E1) as compared to OVX females. Uterine proteins were compared with the standard molecular weight (MW) marker proteins expressed in Kilo Dalton (kDa). Proteins were separated by SDS-PAGE (15% gel).

In order to study the effect of TAM in both follicular (estrus) and luteal (diestrous) phases, the uterine proteins of the experimental animals were separated by SDS-PAGE, using 15% gel. Experiments were performed in ovary intact cyclic rats to see the effect of TAM in presence of ovarian estrogen, while OVX model treated with E2 was used to study the effect of factors other than ovarian estrogen, modulating the protein profile. The present study showed differences in banding pattern of uterine proteins in follicular and luteal phases, as determined by estrous cycle study and SDS-PAGE. A total of twenty six protein bands were detected during diestrus whereas twenty four proteins were detected during estrus. A difference of four protein bands was observed in between these two phases, of which three proteins with molecular weight ≈250, ≈42 and ≈36 kDa respectively were expressed only during the diestrus, but not in estrus phase. These proteins could be induced by progesterone, as progesterone is the dominant hormone of the diestrus phase required for uterine receptivity. One protein of molecular weight ≈97 kDa was expressed during estrus which was not seen during diestrus, suggesting its expression in response to elevated levels of estrogen during estrus. Studies on mice also reveals eight bands of proteins, of which five were restricted to estrus and three other in diestrus, out of 80 proteins detected in their uterine protein profile (Horvat, 1991).

The present study showed loss of certain proteins in uterus in response to TAM treatment. Four proteins of molecular weight ≈12, ≈56, ≈34 and ≈28 kDa respectively were absent in TAM treated rats during estrus. During diestrus also, four proteins of molecular weight ≈137, ≈44, ≈39 and ≈35 kDa were lost in response to TAM. TAM may bind competitively with the estrogen receptors or induces structural changes in the receptors, which might result in loss of these proteins. TAM treatment during estrus induces production of three new proteins of molecular weight ≈117, ≈84 and ≈63 kDa respectively which were found to be absent during estrus. TAM treatment during diestrus also results in synthesis of six proteins of molecular weight ≈157, ≈96, ≈92, ≈49, ≈30 and ≈26 kDa respectively, absent in cyclic ovary intact rats during diestrus.

Ovariectomy, which causes removal of natural estrogen source, led to loss of several uterine proteins, resulting in least number of bands amongst all the samples studied. A total of twenty bands were expressed in the OVX rats' uteri. Various literatures showed alteration of uterine protein profile of OVX rats in response to steroid hormone (E2) treatment. Exogenous E2 treatment to OVX rats results in increased synthesis of many proteins of different molecular ranges (Komm *et al.,* 1985). In our study, administration of E2 to OVX rats resulted in restoration of four protein bands present in the normal cyclic rats. In addition, four new protein bands of molecular weight ≈267, ≈119, ≈113 and ≈69 kDa were also synthesized in the E2 treated OVX females which were not detected during estrus or diestrus phase. TAM induced synthesis of four new proteins in the OVX rats. Of these, two protein bands (≈97 and ≈45 kDa) were originally present in the cyclic rats, and also induced by E2, showing resemblance of TAM with E2, though it induced synthesis of two more protein bands (≈113, ≈69 kDa) which were not there either during estrus or diestrus phase of cyclic rats. Earlier reports also suggest induction of a wave of DNA synthesis in epithelial cells of OVX mice by TAM with kinetics similar to estradiol-17β treatment. However, TAM is much less potent than E2 and never attains full estrogenicity (Zhang *et al.,* 2005). In addition to its analogous effects with E2, TAM may activate other pathways in the uterus, which may lead to synthesis of new proteins in OVX rats. Also, removal of the ovary may lead to removal of certain other factors, leading to loss of repression, inducing synthesis of new proteins by TAM in the OVX uterus.

In the present study five new proteins have been detected in OVX rats' uteri following treatment of TAM and E2 (TAM + E2) together. Of these, three proteins were present in normal cyclic rats during estrus or diestrus phase. Two more proteins with molecular weight ≈86 and ≈69 kDa respectively were also synthesized which were not present either in estrus or diestrus of cyclic females. Proteins of molecular weight ≈250, ≈137 and ≈35 kDa, present in normal cyclic rats during estrus and diestrus, were not found in any of the treated animals. The synthesis of these proteins may be controlled by some factors other than estradiol-17β, which got removed along with the ovary during ovariectomy. Haem oxygenase (HO-1) expression was seen to maximal during the estrus in mice. Proteins expressed during estrus may be because of similar attributes.

A protein of molecular weight ≈69 kDa was expressed in uteri following administration of exogenous E2, TAM and TAM + E2 treated rats but not in ovary intact cyclic rats, either during follicular or luteal phases. This protein could be expressed because of loss or repression of certain factors as a result of removal of the ovary. Two proteins of molecular weight ≈267 kDa and ≈119 kDa were observed only in OVX+E2 treated rats. These proteins are believed to be induced by estrogen, but in the normal rats, some other factors from the ovary might stop them from getting expressed in the uterus. One protein of molecular weight ≈86 kDa was expressed only in OVX females treated with TAM and E2 together. Another protein of molecular weight ≈45 kDa was absent only in OVX females. Presence of this molecule was detected in ovary intact cyclic rats during both estrous and diestrous and also in the OVX females following treatement of E2, TAM and TAM + E2 seperately. This protein could be

controlled exclusively by endogenous E2 in ovary intact rats and by exogenous E2 and TAM in treated OVX rats.

Thus, TAM exerts its effect in both ovary intact cyclic females and the OVX rats, which were expressed as changes in the uterine protein profile. Elevated levels of different hormones, estrogen and progesterone, during different stages of estrous cycle in ovary intact rats, affects the mechanism of action of TAM on the uterus, as reflected in the disappearance of some proteins and expression of certain new proteins in the uterine protein profile. In OVX rats, treatment of E2, TAM and TAM + E2 induced production of new proteins. All these compounds restore some proteins present in the ovary intact cyclic rats; in addition to some new proteins not found earlier. The differential protein profile is believed to be due to E2 and TAM, whose effects could depend on certain other factors released from the ovary.

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