treated rat, (ii) the reduced levels of c-Jun, (iii) modulation in ERα and ERβ transformation by centchroman. This can be understood by seeing the transcriptional activity of this complex. ERα and ERβ show the differential transcriptional activity through AP-1 pathway (Peach et al., 1997). SERMs bind with ERβ and promote its transcriptional activity as shown by raloxifene and tamoxifen. Here centchroman reduced ERs expression and also AP-1 transcription factor binding to AP-1 element. Here, the effect of centchroman on transcriptional activity of AP-1 complex is not known, but reduced binding of AP-1 transcription factors to AP-1 element in the presence of centchroman might be one of the reasons for its anti-uterotrophic activity. Further, by seeing the transcriptional activity of AP-1 complex in the presence of centchroman will depict the actual scene whether centchroman has any (positive or negative) effect on AP-1 complex mediated transcription.
SUMMARY

Estrogens influence a wide range of physiological processes, including growth, differentiation and functioning of many reproductive and non-reproductive target tissues. Actions of estrogen in target tissue are mediated through its binding to the estrogen receptor, a member of ligand activated transcription factor superfamily. Consequently, there is a conformational change causing the estrogen receptor to dimerize and interact with an ERE located in the promoter region of an estrogen responsive gene. Transcriptional activation occurs, through a transcriptional complex that binds to E2-ER complex, resulting in a phenotypic response, which is specific for a particular target cell. A number of coregulator proteins interact with the DNA bound-estrogen occupied estrogen receptor to produce multiprotein cooperative coactivator complex capable of synergistically activating estrogen driven transcription. Apart from its pathway mediated via EREs, the second pathway occurs via AP-1 enhancer elements that require ligand and the AP-1 transcription factors Fos and Jun for transcriptional activation and for the recruitment of coactivators. Also ERs induce transcription in a ligand independent manner through the mediation of growth factor receptors. In addition, increasing evidence for ‘nongenomic’ mechanism has been accumulated which is mediated via membrane estrogen receptor.

The use of selective estrogen receptor modulators (SERMs) and progesterone receptor modulators (PRMs) has been the most promising approach for the development of new contraceptives for women in the recent past. A number of synthetic compounds have been developed having mixed ER agonist / antagonist profile including triphenylethylene (tamoxifen and it’s derivative), benzothiofene (raloxifene), and naphthalenes (CP336,156). In such an approach to find a postcoital drug suitable for repeated use, the efforts of CDRI have led to emergence of “Centchroman” as a nonsteroidal once-a-week oral contraceptive.

Several groups have reported that ER-antiestrogen complexes differ from ER-estrogen complexes in receptor conformation, DNA binding and recruitment of transcription factors necessary to effectively activate gene transcription. In rat, anti-implantation action of centchroman is mediated via inhibition of endometrial sensitization to the blastocyst stimulus and decidualization by its antagonism at receptor level. The asynchrony caused by centchroman in embryonic development
Summary

vis-a-vis uterine development is suggested to contribute to its contraceptive action. But the information is still lacking regarding the molecular mechanisms vis-à-vis genetic control of centchroman action in uterus which is most characterized target tissue for an estrogen agonist / antagonist. The objectives of the present study therefore, were to explore the molecular mechanism of action of centchroman as an antiestrogen and as an anti-implantation agent by studying the-

i) regulation of expression of uterine estrogen dependent genes by centchroman at uterine level under various physiological conditions.

ii) antagonism of E₂ action by centchroman mediated via ERE and AP-1 pathways,

Anti-implantation and antideciduogenic mechanism

Implantation of embryo requires a precise synchrony between the stage of ovum development and that of uterus. The sequential exposure of the uterus to preovulatory estrogen (secreted during the previous proestrus), to luteal progesterone and finally to a surge of nidatory estrogen is necessary for the proper response of the endometrial stromal cells towards decidualizing stimuli during pregnancy and pseudopregnancy. The balance of progesterone and estrogen may be distorted by disturbing either of the hormones or either of two uterine receptor systems.

In this study, the regulation of ER and PR was examined using Western blot and RT-PCR techniques in rat uterus during pre-implantation period and during the process of endometrial sensitization and decidual induction with a view to illustrate whether differential expression of ER isoforms and PR isoforms under the influence of centchroman (an anti-implantation agent showing antiestrogenic activity) is responsible for inhibition of implantation.

In ovariectomized (ovx) hormonally treated rats, uterine ERα mRNA as well as protein expression uterus decreased from 24 h to 72 h after receiving intra-luminal oil injection (decidual stimulus) as repression by high level of progesterone. ERβ mRNA continuously increased whereas protein remained unchanged throughout. In rats administered with centchroman, uterine ERα protein as well as mRNA expression was inhibited significantly at 24 h post-decidual stimulus whereas ERβ was suppressed at 48 h and 72 h. Centchroman probably inhibited sensitizing (mimicking nidatory E₂) E₂-induced increase in ERα expression. Also centchroman
modulated ERα and ERβ expression differentially changing the ratio of ER isoforms during the initial phase of decidualization induction.

During pre-implantation period, ERα level was highest on Day 4 p.c. and was regulated post-transcriptionally thereafter. While ERβ remained at higher level on Days 3, 4 and 5 evening except on Day 5 morning. In centchroman fed rat the uterine ERα level was increased on Day 3 and decreased on Day 5 morning and this regulation was also post-transcriptional as mRNA level remained unaltered. ERβ expression was increased on Day 3 and Day 5 morning and decreased on Day 4. Thus centchroman affects ERα and ERβ expression differentially on different days during pre-implantation at both transcriptional as well as post-transcriptional level. In this study, centchroman caused alteration in uterine expression levels of ERα and more profoundly in that of ERβ. This could alter the transcriptional efficacy and potency of ERα by altering the relative ratio of α–α homodimers and α–β heterodimers. Also the ability of ERβ to function as a transcriptional inhibitor or activator, depending on the agonist concentration, suggests that completely different pattern of gene expression may be observed at different hormone levels.

It is the temporal ratio of ER isoforms, which was altered in the presence of centchroman. By this, it might modulate the overall transcriptional activity of the two isoforms and ultimately gene expression carried by it. This could be one of the possible mechanisms of the antideciduogenic effect of centchroman. Also the overall changes in ER isoforms expression during pre-implantation period resulted into alteration in temporal ratio of ERα and ERβ. There must be very strict ratio of ERα / ERβ specific to each stage of implantation as any alteration in this by antiestrogen may cause inhibition in implantation.

PR isoforms expression was studied in ovx E and P treated rats receiving intraluminal oil injection and it was found PR-B protein level continued to decrease from 24 h to 72 h post decidual stimulus, whereas PR-A protein level was first increased and then decreased, changing PR-A / PR-B ratio. In rats administered with centchroman, it did not cause reduction in PR-A and PR-B protein expression as a whole but it caused differential effect at different hours, changing the relative level of two isoform. At 72 h after decidual stimulus, it caused great change in PR A / B ratio i.e. from 1.4 to ~3.5.
During pre-implantation period both uterine PR isoforms PR-A and PR-B protein changed continuously and differentially causing change in PR-A / PR-B ratio that remained between 2 to 2.5 on Day 3, Day 5 morning and evening except at Day 4, where the ratio was ~5. The mRNA expression pattern was also found to be similar to that of protein except on Day 5 (showing high level) suggesting PR regulation at both transcriptional as well as post-transcriptional level. In centchroman treated rats the expression of two isoforms was regulated differentially. Here, PR-B protein as well as mRNA levels was enhanced except at Day 4, where PR-B mRNA decreased. Thus we could observe, that PR-A / PR-B ratio changed during different stages of pre-implantation phase. Centchroman caused change in PR isoform ratio. This PR isoform ratio and hence the composition of PR might presumably alter the progesterone action by modulating interaction with nuclear factors and thereby promoted different transactivational processes.

Progesterone receptor gene is induced by estrogen and repressed by progesterone in the whole uterus. The effect of progesterone on decidualization may be modulated by centchroman indirectly i.e. by modulating the effect of ER to induce PR gene expression and ultimately by altering progesterone responsiveness. Centchroman affects decidualization and thereby implantation not only by merely changing uterine progesterone receptor concentration, but also by changing the relative levels of two isoforms, hence changing balance of two PR isoform system. As two isoforms are functionally different yet each has specific role on the decidualization specific gene expression, this altered PR-A / PR-B ratio could be responsible for the modulation in progesterone responsiveness and hence for the inhibition of implantation.

Antiestrogenic mechanism

In studies on uterine RNA polymerase activity, it was found that E2 administration in ovx rats caused uterine RNA polymerase I and II to show peak activity at 1 h and 6 h. Whereas RNA polymerase III activity was maintained at higher level till 6 h after E2 administration. It caused RNA polymerase II activity to decrease sharply at 1 h, 6 h and also at 24 h but the magnitude of inhibition was less in the later. In general, centchroman lowered E2-induced peak activity of RNA polymerase at 1 h, 6 h and 24-h hence has antagonistic effect on E2-induced
transcription. RNA polymerase activity in the presence of centchroman was found to be higher than that observed in ovx rat uteri, suggesting its partial agonistic effects.

Estrogen mediated gene expression stimulation involves two of either DNA protein interaction; (i) by binding E2-ER complex to ERE and (ii) where E2-ER does not bind directly to DNA, as interaction of ER with AP-1 element occurs through the mediation of Fos/Jun proteins called transcription factors. Antiestrogens are likely to play role in alteration in ER mediated gene expression through both mechanisms. It is well established that following binding to LBD of ER, ligand causes certain conformational changes within the H 12 helix of LBD. Nevertheless, the ligand induced activation ultimately results into the interaction of ER with specific EREs on DNA. The present studies on ER-ERE interaction have shown that centchroman interaction with ER resulted into the promotion of ER interaction with ERE when incubated alone under transformed conditions i.e. 30°C. In the presence of estradiol, vitellogenin A2 ERE binding was not induced by centchroman as was induced by estradiol alone. Pure antiestrogen CDRI-85/287 showed somewhat different behaviour as it caused inhibition of ER-ERE binding at higher concentrations. This may be the reason for lack of agonism at uterine level by this molecule. In uterus, centchroman and its active metabolite 7-hydroxy centchroman show partial agonistic and potent antagonistic activity. The interaction of ER caused by centchroman possibly leads to the formation of ER-ERE complexes that may be rather unstable and dissociate faster than those formed by estradiol and other potent agonists. The stoichiometric studies with estradiol have suggested the each monomer interacts with half of the palindromic ERE and agreed with the expected stoichiometry of one ER dimer per ERE based on NMR and crystallographic studies of the ER-DNA binding domain. Nevertheless, the effect of centchroman on distribution of ER molecules per ERE site remains to be studied and the influence of CN-ER interaction on the recruitment of co-activators and co-repressors needs to be explored further.

Effect of centchroman was examined on the AP-1 pathway mediated ER-action. For this, the effects of centchroman on the expression of ER isoforms and AP-1 transcription factors and on the formation of AP-1 complex were studied. The effect of centchroman was observed 24 h after E2 and/ or centchroman injection because it has been reported that in adult rat, E2 at 24 h after administration caused significant increase in ERα transcripts. It was found that centchroman when given in the
presence of E2, it suppresses E2-induced ERα expression. However, it caused inhibition in ERβ expression irrespective of condition whether given alone or with E2. ERα and ERβ form homodimer and heterodimer depending upon their ratio within the cell. The transcriptional activity of these dimers differs and hence the E2 responsiveness in the target cells. Centchroman altered this ERα/ERβ ratio when given alone as well as in the presence of E2. It caused a significant decrease in ERβ than that in ERα and hence it increased ERα/ERβ ratio. ERα and ERβ bind with EREs and ultimate response in the uterus depends upon ERα/ERβ ratio. Also ERα and ERβ were shown to signal in opposite ways when complexed with the natural hormone estradiol from an AP-1 site.

Further, to know whether centchroman has any effect on AP-1 protein-DNA interaction, EMSA was performed with rat uterine nuclear extract using 32P-labeled AP-1 oligonucleotide. ERs interact with AP-1 element indirectly through protein-protein contacts with AP-1 transcription factors. The level of these transcription factors (Fos and Jun family proteins) in uterus was altered by E2. Centchroman showed same effect as E2 on c-Fos expression, but when given with E2, it caused E2-induced c-Fos expression to decrease. In the case of c-Jun expression, it was increased by centchroman by ~2 fold, independent of administered alone or with E2. Centchroman caused increase in both c-Fos and c-Jun lesser than that caused by E2. But when uterine weight was observed, gain in uterine weight in presence of centchroman was much lower than that in E2-treated rat uterus. Thus centchroman itself has moderate uterotrophic activity but it lowers the E2-driven uterotrophic activity. However, the expression levels of c-Fos and c-Jun were not altered significantly. In rats treated with centchroman alone or with E2, the binding of transcription factors to AP-1 element was found to be reduced by ~70% than that observed in E2-treated rats. It might be due to (i) low levels of ERβ in uteri of centchroman and E2 + centchroman treated rats, (ii) reduced level of c-Jun, and / or (iii) modulation in ERα and ERβ transformation by centchroman.

The reduced binding of AP-1 transcription factors to the AP-1 element in centchroman treated animals might be one of the reasons for the reduced uterotrophic activity. Further examination of transcriptional activity of this AP-1 complex in the presence of centchroman would depict the actual scene whether centchroman has any (positive or negative) effect on transcription induced by AP-1 complex.
In conclusion, centchroman alters uterine estrogen receptor isoforms α and β ratio thereby altering the transcriptional activity of ERs. The altered transcriptional activity appears to be responsible for alteration in the relative levels of PR isoforms and thus the uterine responsiveness to progesterone. The estrogen–antagonistic activities of centchroman are manifested via both ERE and non-ERE mediated (AP-1 pathway) mechanisms.