THE EFFECT OF CLOMIPHENE CITRATE TREATMENT ON THE EXPRESSION OF THREE SPECIFIC GENES: ESTROGEN RECEPTOR ALPHA, 90kD HEAT SHOCK PROTEIN AND HOXA10 IN THE RAT UTERUS

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Johannesburg, 2005
DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

11th day of October 2005
Abstract

Clomiphene citrate (CC), a synthetic estrogen, is an efficient superovulator used in infertility treatment. However, pregnancy rates resulting from CC treatment are low. Research has suggested that this may be due to an aberrant effect on implantation; CC binds to estrogen receptors (ER) and may affect estrogen responsive gene expression and thus implantation. This study investigates the effect of CC on ERα, 90kDa heat shock protein (Hsp90) and Hoxa10 expression in the rat uterus. Hsp90 binds to ERα in the absence of ligand and is involved in inducing a high affinity ligand binding conformation in the ER and in transactivation of the ER. Hoxa10 has been shown to be essential for uterine receptivity to implantation. CC (0.25mg) was given to ovariectomized rats, either alone or prior to a hormonal regime known to induce uterine receptivity for implantation. Expression of ERα, Hsp90 and Hoxa10 was determined by Western blotting, fluorescence immunocytochemistry and reverse transcription polymerase chain reaction. The single dose CC treated rats were compared to the controls as well as to ovariectomized rats treated with 0.5µg 17β estradiol (E2). The CC treated pseudopregnant rats (CCPPPE treated) were compared to 5½ day pregnant and pseudopregnant rats without CC (PPPE treated), to determine CCs effect at implantation. E2 upregulated ERα and Hsp90 expression in the rat uterus compared to controls (p<0.05). The finding for ERα was unexpected as other studies have shown that E2 decreases ERα levels a few hours after administration in the uterus. The present study therefore suggests a biphasic effect of E2 on ERα expression in the rat uterus. The effect of E2 on Hsp90 and ERα also proposes a balance between the levels of these two proteins in the uterus, to keep ERα in its optimal state and suggests that too high and too low a concentration of Hsp90 may both be inhibitory to ERα functioning. No significant difference was found in ERα and Hsp90 expression between the non-receptive (vehicle treated) and the receptive (PPPE treated) rat uteri, suggesting that these two genes are not markers for receptivity. However E2 is known to induce implantation of donor blastocysts in progesterone (P4) primed uteri. Therefore it is still essential for ERα to be present at implantation. It is of interest that CC downregulated ERα levels both in
the absence of ovarian hormones and at implantation in the rat uterus. It is therefore proposed that this antiestrogenic effect would render the uterus less sensitive to the $E_2$ required to induce implantation, thus accounting for low pregnancy rates with CC use. Although CC did not alter the expression of Hsp90 in this study, the reduction in ER$\alpha$ levels in response to CC may also upset the balance in the expression of these two genes, which may affect the transcriptional activity of ER$\alpha$, and further prevent implantation. No clear results were obtained for Hoxa10 expression with the Western blots. However based on the ICC results, CC did not appear to affect Hoxa10 expression. Since $P_4$ and not $E_2$ is known to have the predominant effect on Hoxa10 expression, it is likely that $E_2$ analogs, such as CC, would also not affect Hoxa10 expression to a significant degree. Future work will aim to separate the different uterine compartments and to determine the effects of CC on the expression of other implantation specific genes in the uterus.
Dedicated to:
My mom and dad
and to Peter Le Roux
I am forever grateful for their support and encouragement
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LIST OF ABBREVIATIONS

α \hspace{1cm} \text{alpha}
α=0.05 \hspace{1cm} \text{statistics tests performed at the 5\% level of significance}
β \hspace{1cm} \text{beta}
-/- \hspace{1cm} \text{homozygous mutant}
-RT \hspace{1cm} \text{minus reverse transcriptase control}
A \hspace{1cm} \text{untreated ovariectomized control sample}
A_{260} \hspace{1cm} \text{Absorbance at 260nm}
A_{280} \hspace{1cm} \text{Absorbance at 280nm}
ACE \hspace{1cm} \text{Associated Chemical Enterprises}
AF \hspace{1cm} \text{activation function}
ANOVA \hspace{1cm} \text{one-way analysis of variance}
APS \hspace{1cm} \text{ammonium persulfate}
B \hspace{1cm} \text{saline treated (vehicle control) sample}
bp \hspace{1cm} \text{base pairs}
BSA \hspace{1cm} \text{bovine serum albumin}
C \hspace{1cm} \text{oil treated (vehicle control) sample}
C_1 \hspace{1cm} \text{initial concentration}
C_2 \hspace{1cm} \text{final concentration}
CC \hspace{1cm} \text{clomiphene citrate}
CCPPPPE \hspace{1cm} \text{treatment regime: 0.25mg clomiphene citrate on 1\textsuperscript{st} day followed by 5mg progesterone on the 2\textsuperscript{nd} and 3\textsuperscript{rd} days and then 5mg progesterone and 0.5\mu g 17\beta estradiol on the 4\textsuperscript{th} day}
cDNA \hspace{1cm} \text{complementary deoxyribonucleic acid}
conc \hspace{1cm} \text{concentration}
COX \hspace{1cm} \text{cyclooxygenase}
D \hspace{1cm} \text{0.25mg CC treated sample (single treatment)}
DAB \hspace{1cm} \text{diaminobenzidine}
DBD \hspace{1cm} \text{DNA binding domain}
DF \hspace{1cm} \text{degrees of freedom}
DF NUM \hspace{1cm} \text{degrees of freedom of the numerator}
DF DEN \hspace{1cm} \text{degrees of freedom of the denominator}
dH\textsubscript{2}O \hspace{1cm} \text{distilled water (autoclaved)}
DNase  deoxyribonuclease

dNTPs  deoxynucleotide triphosphate mix

DTT  dithiothreitol

E  0.5µg 17β estradiol treated sample

E2  17β estradiol

EDTA  ethylenediamine tetraacetate

ELISA  enzyme linked immunosorbant assay

EP3 and EP4  prostaglandin E2 receptor subtypes

ER  estrogen receptor

ERα  estrogen receptor alpha

ERβ  estrogen receptor beta

ERE  estrogen response element

F  SOOO treated (vehicle control) sample

Fig.  Figure

FITC  fluorescein isothiocyanate

FSH  follicle stimulating hormone

G  5½ day pregnant sample

GE  glandular epithelium

GIFT  gamete intra-fallopian tube transfer

gl  glands

GnRH  gonadotropin-releasing hormone

GR  glucocorticoid receptor

GST  glutathione-S-transferase

H  PPPE (pseudopregnant) sample

H&E  Haematoxylin and Eosin stain

H12  helix 12 of the estrogen receptor structure

H2O2  hydrogen peroxide

HeLa  uterine cervical adenocarcinoma

Hox  homeobox

Hsps  heat shock proteins

Hsp70  70kDa heat shock protein

Hsp90  90kDa heat shock protein

I  CCPPPE (CC treated pseudopregnant) sample
Prob  probability
RBC  red blood cells
RNase  ribonuclease
rpm  revolutions per minute
rRNA  ribosomal ribonucleic acid
RT-PCR  reverse transcription polymerase chain reaction
S  saline treatment
sc  subcutaneous
SDS  sodium dodecyl sulphate
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM  Standard Error of the Mean
SERM  selective estrogen receptor modulator
SOOO  treatment regime: saline on 1st day; oil on the following 3 days
Std Dev  standard deviation
str  stroma
TAE  Tris acetate EDTA
TBE  Tris borate EDTA
TBS  Tris buffered saline pH8
TBS-Tween  Tris buffered saline with 0.05% Tween-20®
TEMED  tetramethylethylenediamine
TGF-β  transforming growth factor beta
TM  melting temperature
UV  ultraviolet light
V₁  initial volume
V₂  final volume
VEGF  vascular endothelial growth factor