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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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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

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 (E_2). 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. E_2 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 E_2 decreases ERα levels a few hours after administration in the uterus. The present study therefore suggests a biphasic effect of E_2 on ERα expression in the rat uterus. The effect of E_2 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 E_2 is known to induce implantation of donor blastocysts in progesterone (P_4) 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 ERα levels in response to CC may also upset the balance in the expression of these two genes, which may affect the transcriptional activity of ERα, 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	alpha
α=0.05	statistics tests performed at the 5% level of significance
β	beta
-/-	homozygous mutant
-RT	minus reverse transcriptase control
A	untreated ovariectomized control sample
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
ACE	Associated Chemical Enterprises
AF	activation function
ANOVA	one-way analysis of variance
APS	ammonium persulfate
B	saline treated (vehicle control) sample
bp	base pairs
BSA	bovine serum albumin
C	oil treated (vehicle control) sample
C_1	initial concentration
C_2	final concentration
CC	clomiphene citrate
CCPPPE	treatment regime: 0.25mg clomiphene citrate on 1st day followed by 5mg progesterone on the 2nd and 3rd days and then 5mg progesterone and 0.5µg 17β estradiol on the 4th day
cDNA	complementary deoxyribonucleic acid
conc	concentration
COX	cyclooxygenase
D	0.25mg CC treated sample (single treatment)
DAB	diaminobenzidine
DBD	DNA binding domain
DF	degrees of freedom
DF NUM	degrees of freedom of the numerator
DF DEN	degrees of freedom of the denominator
dH_2O	distilled water (autoclaved)

DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphate mix
DTT	dithiothreitol
E	0.5μg 17β estradiol treated sample
E_2	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	fluoroscein 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
H_2O_2	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

ICC	immunocytochemistry
IGF	insulin-like growth factor
ip	intraperitoneal
IVF	*in vitro* fertilization
kDa	kilo Daltons
LBD	ligand binding domain
LE	luminal epithelium
lu	lumen
M	molar
MCF7	breast carcinoma cell line
µg	microgram
mg	milligram
ml	millilitre
mRNA	messenger ribonucleic acid
MW	molecular weight marker
myo	myometrium
n	sample size (number of observations)
NegI	negative control in which the primary antibody is omitted
NegII	negative control in which the secondary antibody is omitted
ng	nanogram
N.H	Null Hypothesis
NTC	no template control
O	oil treatment
$p<0.05$	significantly different at the 5% level of significance
$p>0.05$	not significantly different at the 5% level of significance
P_4	progesterone
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	progesterone and 17β estradiol treatment
PPPE	treatment regime to induce pseudopregnancy: 5mg progesterone on 1st 2 days followed by 5mg progesterone and 0.5µg 17β estradiol on the 3rd day
PR	progesterone receptor

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
V_1	initial volume
V_2	final volume
VEGF	vascular endothelial growth factor