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The effect of clomiphene citrate

Thomson, Karren Judith

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CHAPTER 2: MATERIALS AND METHODS

(See Appendix I for solutions recipes)

2.1 ANIMALS

Forty five 12-14 week sexually mature female in-bred Hooded Wistar rats, weighing between 200-210g, were obtained from the Central Animal Unit at the University of Witwatersrand. Two to three rats were housed per cage and were kept at a constant 23°C. The rats were fed *ad libitum* on rat cubes and water. At sacrifice, the animals weighed between 250 and 300g. Animal Ethics Clearance was approved (AEC No: 2002/80/4)

2.1.1 Pregnant Animals

A vaginal smear was performed on six animals every afternoon and this was stained with Shorr's solution (Appendix I). Those animals identified as being in proestrus, were placed in a cage overnight with a male of proven fertility. If sperm was present in a vaginal smear the following morning then the female was deemed to be pregnant (day ½). Animals were sacrificed on day 5½ of pregnancy, corresponding to the time of implantation (Psychoyos, 1973).

2.1.2 Ovariectomy

12-14 week old animals, weighing a minimum of 200g, were bilaterally ovariectomized. Surgery was performed by the veterinarian of the Animal Service University of Witwatersrand. Following surgery the animals were allowed to recover for four to six weeks before experimentation, to clear any residual ovarian hormones in the blood (Ljungkvist, 1971a).

2.1.3 Pseudopregnant Animals

Pseudopregnancy was induced in ovariectomized rats with a sequence of daily hormone injections (2 days of 5mg of progesterone (P_4) (Sigma-Aldrich Co, St Louis MO, USA) followed by an injection of 5mg P_4 and 0.5μg 17β estradiol (E_2) (Sigma-Aldrich Co, St Louis MO, USA) on the 3rd day) (PPPE samples) (Psychoyos, 1966; 1967; 1973).

2.2 DRUG TREATMENT REGIMES:

2.2.1 Clomiphene Citrate Treatment

Ovariectomized rats were injected with a single 0.25mg dose of CC, consisting of 40% *cis* and 60% *trans* isomers (Sigma-Aldrich Co., St Louis MO, USA). The dose and protocol used were based on studies performed by Bowman *et al*., (1981) and Hosie and Murphy, (1992), and was calculated to be approximately 1mg/kg. This dose falls well within the physiological tolerance for rats (LD50 = 530mg/kg, Newberne *et al.*, 1966).

In women, using CC as a superovulator for infertility treatment, a 50mg daily dose is given over 5 days starting on day 5 of the menstrual cycle (Clark and Markaverich, 1982). It has been suggested that a single (one day) dose of 0.25mg CC would be equivalent to this in a rat that normally has a cycle of only 4-5 days (Hosie, 1997).

Clomiphene Citrate was administered as a 0.1ml intraperitoneal (ip) injection in saline. CC (1mg) (Sigma-Aldrich Co., USA) was dissolved in 0.1ml 100% ethanol. Once dissolved 0.3ml of 0.9% normal saline (Associated Chemical Enterprises (ACE), Southdale, South Africa (S.A)) was added giving a final concentration of 0.25mg/0.1ml. Placebo treated animals were injected with carrier alone. This method was based on studies by Bowman *et al.*, (1981) and Hosie and Murphy, (1992).

2.2.2 17β Estradiol Treatment

Estrogen was administered as a single 0.5μg 17β estradiol (E_2) (Sigma-Aldrich Co., USA) dose. Although other investigators have used both lower (0.1μg; Psychoyos, 1961) and higher doses (1μg; Hammer *et al*., 1978) to induce implantation in rats, 0.5μg has also been shown to elicit a physiological response in the rat uterus and has been shown to induce implantation of donor embryos in ovariectomized P_4 primed rats (Psychoyos, 1961; 1973; Ljungkvist, 1971b; Murphy and Rogers, 1981).

Estradiol 17β (1mg) was dissolved in 5ml benzyl alcohol (BDH Chemicals, Poole, England). An aliquot of this (0.5ml) was added to 3.5ml benzyl alcohol and then diluted to a concentration of 5μg/ml (0.5μg/0.1ml) with 16ml cold pressed peanut oil (Aromatic Essential Oils, Killarney, SA) (1:4 mixture of benzyl alcohol and peanut oil). Animals were then given a 0.1ml subcutaneous (sc) injection of this solution in the scruff of the neck. This method was based on studies by Brown-Grant *et al.*, (1972); Murphy and Rogers, (1981) and Hosie and Murphy, (1992).

2.2.3 Progesterone Treatment

Progesterone (P_4) (Sigma-Aldrich Co., USA) was given as a 5mg dose per rat. This dose has been shown by Ljungkvist (1971c); Psychoyos (1973) and Huet and Dey, (1987) to elicit a normal physiological response in rat uterine epithelium. In addition a minimum of 2 days of P_4 treatment is required to prime the uterus for implantation prior to a small amount of E_2 (Psychoyos, 1973).

Progesterone (50mg) (Sigma-Aldrich Co., USA) was dissolved in 0.2ml benzyl alcohol (BDH, England). Once dissolved, 0.8ml peanut oil (Aromatic Essential Oils, S.A) was added and mixed to give a final concentration of 50mg/ml. Each rat was injected with 0.1ml (sc) of this solution in the scruff of the neck to give a final dose of 5mg/0.1ml. This dose appears to be the standard P_4 dose used amongst reproductive biologists and is taken from studies by Psychoyos (1973).

2.2.4 Treatment Regimes

Two types of treatment regimes were employed in this study, the first, summarized in Table 2.1, tested the effect of a single dose of CC or E_2 on the expression of ERα, Hsp90 and Hoxa10 in the ovariectomized rat uterus.
The second, summarized in Table 2.2, tested the effect of CC on the expression of ERα, Hsp90 and Hoxa10 in the pseudopregnant rat uterus at the time of implantation.

Table 2.1: Treatment regime for the single dose treated samples

Ovariectomized animals were given a single 0.1ml (sc) injection of 0.5µg E_2 in oil (**Group E**) or a single 0.1ml (ip) injection of 0.25mg CC in saline (**Group D**). Control animals were given carrier (saline [**Group B**] or oil [**Group C**]) alone or no treatment (**Group A**). Animals were sacrificed 24 hours after the last treatment.

Group	Sample size (n)	Day 1	Day 2	Vehicle	Administration
A	3	-	Kill	-	-
B	3	Saline	Kill	Saline	(ip)
C	3	Oil	Kill	Oil	(sc)
D	6	0.25mg CC	Kill	Saline	(ip)
E	6	0.5µg E_2	Kill	Oil	(sc)

Table 2.2: Treatment regime for the pseudopregnant and pregnant samples

To induce pseudopregnancy, ovariectomized animals were treated with a series of hormonal injections over 3 days, which is known to induce implantation (Psychoyos, 1973) and was taken from studies by Hosie *et al.,* (2003). The hormonal series consisted of: day 1: 5mg P_4; day 2: 5mg P_4; day 3: 5mg P_4 and 0.5µg E_2 (PPPE).

The experimental group consisted of animals that were treated with 0.1ml of 0.25mg CC (ip) in saline on day 1, followed by the PPPE hormonal series (sc) in oil on days 2-4 (CCPPPE; **Group I**).

The first control group consisted of animals treated with only carrier over the 4 days: day 1: saline (ip); day 2-4: oil (sc) (SOOO; **Group F**).

The second control group consisted of pseudopregnant rats without CC treatment: day 1: saline (ip); day 2-3: P_4 in oil (sc); day 4: P_4 and E_2 in oil (sc) (PPPE; **Group H**). The animals were sacrificed 24 hours after the last injection.

A Pregnant control (**Group G**) was used whereby intact females were mated with fertile males and then sacrificed on day 5½ of pregnancy, corresponding to the time of implantation (Psychoyos, 1973).

Group	n	Day 1	Day 2	Day 3	Day 4	Day 5	
F	6	Saline (ip)	Oil (sc)	Oil (sc)	Oil (sc)	Kill	**SOOO**
G	6	Pregnant	Pregnant	Pregnant	Pregnant	Kill (on day 5½)	**Pregnant**
H	6	Saline (ip)	5mg P_4 (sc)	5mg P_4 (sc)	5mg P_4 + 0.5µg E_2 (sc)	Kill	**PPPE**
I	6	0.25mg CC (ip)	5mg P_4 (sc)	5mg P_4 (sc)	5mg P_4 + 0.5µg E_2 (sc)	Kill	**CCPPPE**

2.3 SACRIFICE OF ANIMALS

Animals were euthanased with ±3.5ml (1-2ml/kg body weight) (ip) of S6 Euthanaze (sodium pentabarbatone 200mg/ml) (Centaur Labs Bayer Pty. Ltd., Isando, SA). When no response to a tail pinch was observed, a central ventral incision was made to expose the uterus. The uterus was dissected away from the attached mesentery, cut just above the cervix and then removed. The uterine horns were pinned out on foil covered dental wax and cut into 3 pieces for Western blotting; Light Microscopy and Fluorescence Immunocytochemistry (Fluorescence ICC); and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The rat was then killed by opening the thorax to expose the heart.

2.4 WESTERN BLOTTING

This technique was used to measure the expression of ERα, Hsp90 and Hoxa10 protein, in order to determine the effect of CC on the expression of these genes.

2.4.1 Protein Extraction

The pieces of rat uteri were placed in a petri dish containing cold sterile phosphate buffered saline (PBS) pH 7.4 (Appendix I) to remove all blood. Using sterile scissors the tissue was minced and placed into a clean glass Dounce homogenizor. For each gram of tissue, 5mls of homogenizing buffer pH7.5, containing a protease inhibitor cocktail (P2714 Sigma-Aldrich Co, USA), (Appendix I) was added. The tissue was homogenized and placed on ice until all samples collected that day were at the same point. The samples were centrifuged at 4°C at 12000rpm for 20 minutes, the supernatant was removed and aliquoted into ± 0.2ml and then stored at -70°C until needed.

2.4.2 Protein Concentration (Bradford Assay)

The concentration of protein extract was determined using a Bradford Reagent Assay (Sigma-Aldrich Co., USA). Briefly, the protein samples were diluted to 0.1X with distilled water (dH_2O). Concentration standards were made using Bovine Serum Albumin (BSA) (Sigma-Aldrich Co., USA) in 0.1X homogenizing buffer at concentrations of 1.4mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml and 0mg/ml. Each protein sample or BSA standard (100µl) was mixed with 3ml of Bradford Reagent (Sigma-Aldrich Co., USA). These samples were allowed to stand for 5 minutes, after which the absorbance was read at 595nm on an Ultrospec 2000 Ultraviolet (UV) Spectrophotometer (Pharmacia Biotechnology, Buckinghamshire, UK). The samples were blanked against the 0mg/ml BSA standard with Bradford Reagent, and all readings were done within 60 minutes of the first reading to ensure accuracy. A standard curve of the absorbance values of the BSA standards was plotted against their concentration (Fig 2.1 represents an example of this graph). Concentrations for the diluted protein samples were read off the graph and multiplied by the dilution factor to obtain the concentration of the original protein extract. Table 2.3 represents the concentration of the protein extracts and the volume of protein added to the gel.

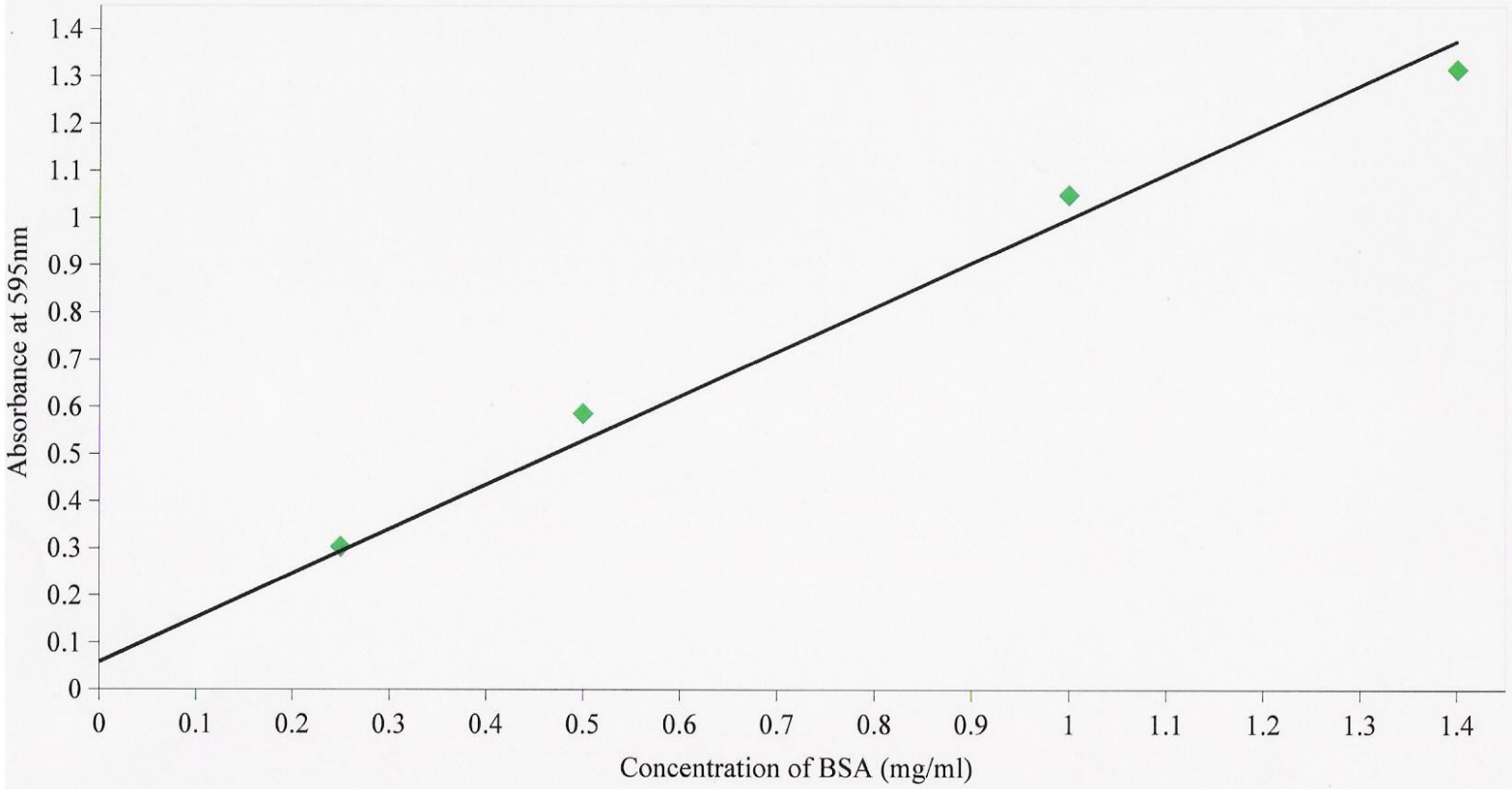

Fig. 2.1 Standard curve for the absorbance at 595nm of Bovine Serum Albumin (BSA) at known concentrations in mg/ml (regression line: y=0.9412x+0.0588) for samples A2-E2

Table 2.3: Absorbance readings, final concentration in μg/μl and Loading Volume of protein extracts. See Appendix II (5.6) for example of calculations

Blot Number	Sample	Absorbance (average of 3 readings)	Final Concentration (taken from graph x dilution factor = 10) (μg/μl)	Loading Volume (to load 17.5μg) (μl)
1	A1	0.4225	4.06364	9
	B1	0.4495	4.38065	8
	C1	0.4415	4.28672	8
	D1	0.4	3.79946	9
	E1	0.1855	1.28097	27
2	A2	0.366	3.26392	11
	B2	0.394	3.56141	10
	C2	0.4395	4.04484	9
	D2	0.2275	1.79239	20
	E2	0.6935	6.74352	5
3	A3	0.4415	4.16032	8
	B3	0.455	4.30937	8
	C3	0.383	3.51441	10
	D3	0.6945	6.95374	5
	E3	0.7265	7.30706	5
4	A1	0.4315	4.04991	9
	B1	0.4665	4.43635	8
	C1	0.4325	4.06095	9
	D4	0.438	4.12167	8
	E4	0.5445	5.29756	7
5	A2	0.2985	2.98203	12
	B2	0.3355	3.48196	10
	C2	0.358	3.78597	9
	D5	0.5825	6.81935	5
	E5	0.5955	6.995	5
6	A3	0.342	3.56979	10
	B3	0.3675	3.91434	9
	C3	0.286	2.81313	12
	D6	0.7085	8.52182	4
	E6	0.576	6.73152	5

7	A1	0.467	4.57235	8
	F1	0.4543	4.41556	8
	G1	1.0527	11.8216	3
	H1	0.774	8.37232	4
	I1	0.391	3.63164	10
8	A1	0.467	4.57235	8
	F2	0.292	2.40624	15
	G2	0.9857	10.99228	3
	H2	0.6537	6.88287	5
	I2	0.5153	5.17061	7
9	A2	0.2947	3.23302	11
	F3	0.397	4.61404	8
	G3	0.894	11.32119	3
	H3	0.7473	9.34188	4
	I3	0.6257	7.69996	5
10	A2	0.2947	3.23302	11
	F4	0.3723	4.28115	8
	G4	0.919	11.65857	3
	H4	0.6543	8.08682	4
	I4	0.623	7.66397	5
11	A3	0.3367	3.83387	9
	F5	0.2363	2.48021	14
	G5	0.8857	11.24078	3
	H5	0.587	7.21128	5
	I5	0.6597	8.19167	4
12	A3	0.3367	3.83387	9
	F6	0.3247	3.67197	10
	G6	0.855	10.82704	3
	H6	0.598	7.35969	5
	I6	0.557	6.80653	5

2.4.3 Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.4.3.1 Gel preparation

A 10% SDS-polyacrylamide separating gel with a 4% stacking gel was made in the Hoeffer Mighty Small Electrophoresis Unit (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Appendix I), and allowed to set for about 30 minutes before loading of samples and electrophoresis run.

2.4.3.2 Sample preparation

The samples loaded onto every gel consisted of the following:

1. A Sigma Marker (molecular weight marker (MW)) (Sigma-Aldrich Co., USA), which was reconstituted with 100µl sterile dH_2O.
2. A positive control (20µl) consisting of a whole cell lysate from either the MCF7 breast carcinoma cell line (sc-2206; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or the HeLa uterine cervical adenocarcinoma cell line (sc-2220; Santa Cruz Biotechnology, USA). The MCF7 whole cell lysate was used for ERα identification and the HeLa whole cell lysate was used for Hsp90 identification.
3. Protein extracts from each treatment group (A-E) or (A,F-I). Each extract was mixed in a 1:1 ratio with 2X sample buffer pH 6.8 (Appendix I) containing SDS, β-mercaptoethanol and bromophenol blue.

Both the positive control sample and protein (extract + buffer) samples were boiled for 2 minutes prior to loading.

2.4.3.3 Electrophoresis (gel running)

Electrophoresis tank buffer (Appendix I) was poured into the lower and upper buffer chambers of the electrophoresis unit and the wells were rinsed of any unpolymerized gel solution. Using a Hamilton Syringe (Hamilton Company Incorporated, Whittier, California, USA), 5µl of Sigma Marker (Sigma-Aldrich Co., USA), 20µl of one of the positive control whole cell lysates (depending on the protein to be identified) and 17.5µg of each protein sample were loaded into the wells of the gel, (See Table 2.3 for volumes of each protein sample loaded).

The Hamilton syringe was rinsed out in tank buffer between each sample to prevent contamination. The lid of the electrophoresis unit was attached and the electrodes connected to a power pack (Consort E452; Separation Scientific, Honeydew, South Africa). The proteins were separated at a constant current of 25mA for 3.7 hours for ERα identification or until the bromophenol blue dye reached the bottom of the gel for Hsp90.

2.4.3.4 Staining of molecular weight markers

Once the gels had finished running, they were removed from the electrophoresis plates. The stacking gel and unused lanes were cut off and discarded. The molecular weight lane was also removed and placed into the Coomassie Blue Stain (Appendix I) overnight on a shaker. The rest of the gel, containing the separated proteins was placed into cold transfer buffer, and used in the transfer onto nitrocellulose.

Figure 2.2. is representative of gels that were used for SDS-PAGE electrophoresis and were not transferred onto nitrocellulose but rather stained with the Coomassie Blue Stain for 3 hours on a shaker. This was followed by 1 hour in Destaining Solution 1 (Appendix I) and then the gels were placed into Destaining solution 2 (Appendix I) and stored.

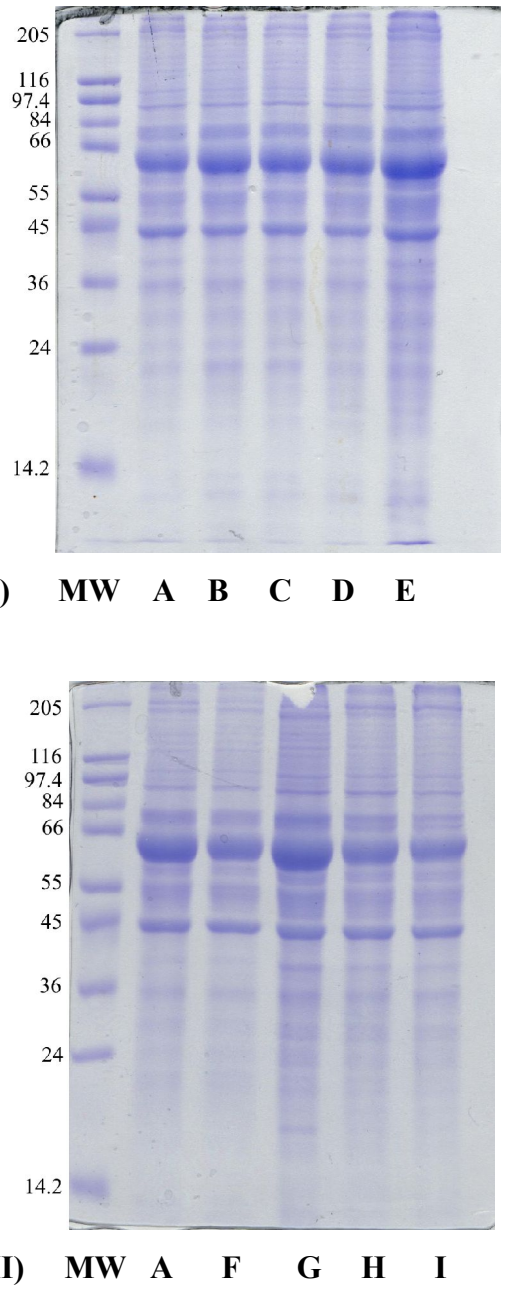

Fig.2.2 Gels representing protein present in the different treatment samples A-I. Total uterine protein (17.5µg) from the different treatment groups were loaded onto a 10% SDS-polyacrylamide gel and separated at 25mA until the bromophenol blue dye reached the bottom of the gel. In (I) the protein from the single dose treatments is shown where MW represents the molecular weight marker; A: an untreated protein sample; B: a saline treated sample; C: an oil treated sample; D: a single dose of 0.25mgCC treated sample; and E: a single dose of 0.5µg E_2 treated sample. In (II) the protein from the pseudopregnant and pregnant samples is shown with or without CC treatment. F represents the vehicle (SOOO) treated sample; G represents a 5½ day pregnant sample; H represents a pseudopregnant (PPPE) sample; and I represents a CC treated pseudopregnant sample (CCPPPE).

2.4.4 Transfer onto Nitrocellulose Membrane

Ten sheets of filter paper, 2 foam pads, and the gel containing the separated proteins were soaked in cold transfer buffer pH8.3 (Appendix I) for at least 10 minutes. A nitrocellulose sheet (Hybond C-Extra, Amersham Pharmacia Biotechnology, UK) was cut to the size of the gel and soaked briefly in dH_2O before soaking in cold transfer buffer for 10 minutes.

Whilst keeping moist with cold transfer buffer, a sandwich was assembled consisting of: the anode (positive) tray of the Hoeffer Western blotting transfer equipment (Hoeffer Scientific Instruments, San Francisco, USA); a foam pad, 5 sheets of filter paper; a nitrocellulose sheet, the gel containing separated proteins, another 5 sheets of filter paper, a second foam pad and the cathode (negative) tray of the transfer equipment, making sure that no air bubbles were trapped between the layers.

Two elastic bands were wrapped securely around the sandwich, which was placed into the transfer chamber (Hoeffer Scientific Instruments, USA), filled with cold transfer buffer. The lid of the chamber was attached and the current set at 400mA and left to transfer for 3½ hours.

Following transfer, the sandwich was removed from the chamber, the gel was placed in Coomassie Blue stain overnight to ensure that the transfer was effective, and the nitrocellulose membrane was placed into 5% fat-free milk (Clover Elite, Roodepoort, South Africa) in 1X Tris Buffered Saline (TBS) pH 8 (Appendix I) to block overnight at 4°C on a shaker.

The molecular weight marker lane and gel from the transfer were placed into the 1st Destaining Solution for an hour, followed by the 2nd Destaining Solution, in which it was stored. The nitrocellulose membrane was placed into the primary antibody solution which was diluted in 1X TBS pH8 with 0.05% Tween-20® (Merck, Germany) (TBS-Tween).

For ERα protein identification, polyclonal rabbit anti-ERα 1:100 (sc-542 Santa Cruz Biotechnology, USA) was used.

For Hsp90, monoclonal mouse anti-Hsp90 1:100 (sc-13119; Santa Cruz Biotechnology, USA) was used.

For Hoxa10, polyclonal goat anti-Hoxa10 (sc-17158; Santa Cruz Biotechnology, USA) at 1:100 and 1:50 concentrations was used.
Monoclonal mouse anti-actin 1:1600 (A-2547 Sigma-Aldrich Co, USA) was also used in each primary antibody solution. Actin was used as an internal control to account for any sample to sample variability during the loading of the proteins and to compare the expression of ERα, Hsp90 and Hoxa10 with. Appendix I contains recipes for each of these solutions.
The membranes were incubated in the primary antibody solution for 3 hours on a shaker.
The primary antibody negative control consisted of membranes that were incubated in TBS-Tween without primary antibodies.

Following the primary antibody incubation, the membranes were washed in 3 changes of TBS-Tween for 2x 10 minutes, and 1x 5 minutes, and then incubated in secondary antibodies conjugated to horse radish peroxidase and diluted in 1xTBS-Tween for 45 minutes on a shaker.
The secondary antibody used for ERα was donkey anti-rabbit (1:500) (sc-2313 Santa Cruz Biotechnology, USA). This solution also contained goat anti-mouse (1:2000) (sc-2005 Santa Cruz Biotechnology, USA) for identification of the actin internal control.
For Hsp90 goat anti-mouse (1:500) was used, which also recognized the primary antibody raised against the actin internal control.
For Hoxa10 the membrane was first incubated in donkey anti-goat (1:500) (sc-2020 Santa Cruz Biotechnology, USA) for 45 minutes and then the membrane was incubated in goat anti-mouse (1:2000) (Santa Cruz Biotechnology, USA) for 45 minutes, to identify the actin internal control. Appendix I contains the recipe for these solutions.

Following the secondary antibody incubation the membranes were washed in 3 changes of 1X TBS-Tween pH 8 for 1x 10 minutes and 2x 5 minutes, followed by a wash in 1X TBS pH 8 for 5 minutes. During this time 10mg of diaminobenzidine (DAB) (BDH Laboratories, UK) was mixed with 20ml of 0.05M Tris-HCl pH 7.6.

Once dissolved 200µl of cold 1% hydrogen peroxide (H_2O_2) (Merck, Gauteng, South Africa) was added, and used immediately. The membranes were then incubated in this DAB solution (Appendix I) for 5 minutes, then rinsed briefly in dH_2O and allowed to dry.

The molecular weight marker and blot were scanned and aligned next to each other.

Bands that aligned to 67kDa represented ERα protein, those at 90kDa represented Hsp90, those at 55kDa represented Hoxa10 and those at 42kDa represented actin, which was used as an internal control. Figure 2.3. diagrammatically represents the process of Western blotting.

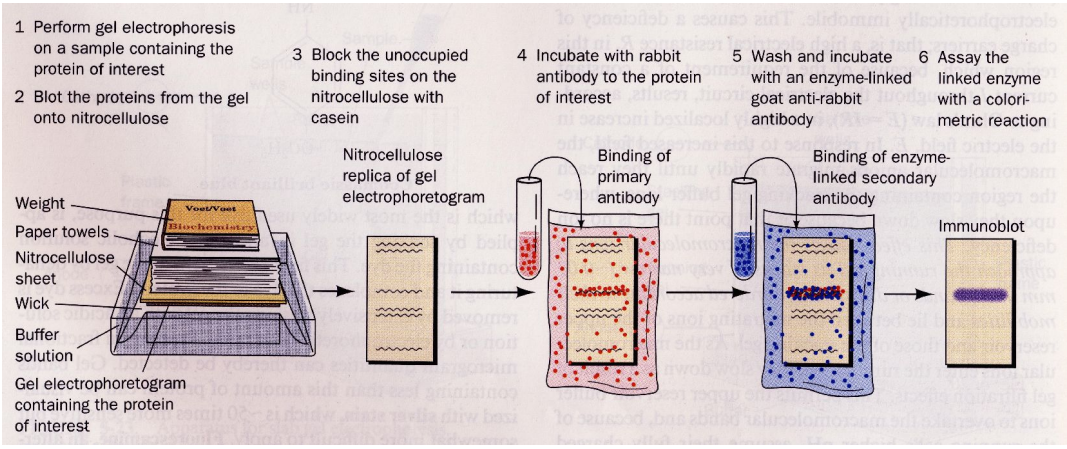

Fig.2.3 Diagram representing the Western blot process (Voet and Voet, 1995)

2.4.5 Quantifying Western Blot Data and Statistics

Raw volumes for each band in each treatment lane were obtained from the SynGene Gene Tools gel-doc system. The expression of ERα, Hsp90 or Hoxa10 with each treatment was then quantified as a ratio of the raw volume for the ERα, Hsp90 or Hoxa10 band over the raw volume for the actin band in the same treatment lane in arbitrary units.

The following statistics tests were performed to determine whether there was any significant difference between the means of the different treatment groups for the expression of each protein. Summaries of these statistic tests can be found in Appendix III.

The O'Brien's and Bartlett's (or Brown Forsythe's) tests tested whether the variances in the different treatment groups for ERα, Hsp90 or Hoxa10 expression were the same.

If the variances for a particular protein expression were found to be equal ($p<0.05$) then this test was followed by a one-way Analysis of Variance (ANOVA), to test for a significant difference between the means of the different treatment groups for the expression of that protein. If there was a significant difference between the means ($p<0.05$) then a Tukey-Kramer *post hoc* test was performed to determine which treatment groups were significantly different from each other for the expression of that protein.

However if the variances in the different treatment groups, for the expression of a particular protein, were not equal ($p>0.05$), then a Welch ANOVA was performed, which tests whether there is a significant difference between means, whilst allowing the variances to be unequal. The Welch ANOVA, based on the ANOVA F test, uses weighted means and does not require the variances to be equal (JMP statistics program version 3.1.6.2). The Welch ANOVA was also followed by a Tukey-Kramer *post hoc* test, if a significant difference between means was found, to determine which groups were significantly different to each other.

All statistics tests were performed at the 5% level of significance.

2.5 LIGHT MICROSCOPY

2.5.1 Preparation of Tissue

Once the tissue had been dissected, it was covered in Shandon Cryomatrix (Shandon Scientific, Cheswick, England), and placed on this discs of cork. The tissue was frozen by immersing the tissue into supercooled isopentane (Saarchem Merck Chemicals, Gauteng, South Africa) for 30 seconds. The frozen tissue was then immersed in liquid nitrogen for another 30 seconds, then covered in foil and stored at -70°C.

2.5.2 Cryosectioning of Tissue

The tissue was orientated so that crossections of the uterine horn could be cut on a Leica Cryostat (Microm Heidelberg, South Africa). Sections were cut at a thickness of 10μm at -20°C and immediately placed on slides pretreated with 2% 3-Aminopropyl-triethoxysilane (Sigma-Aldrich Co., USA) in acetone (Appendix I). The slides were air dried, covered in foil and stored at -70°C until needed for Haematoxylin and Eosin (H&E) staining or fluorescence ICC.

2.6 HAEMATOXYLIN AND EOSIN (H&E) STAIN

This technique was used to observe the general histology of the rat uterus and how it may change with CC.

Sections were stained with Haematoxylin staining solution (Appendix I) for 5 minutes and then blued in tap water and acid alcohol (Saarchem, Merck Chemicals, Gauteng, South Africa) (20 seconds each). The sections were then counterstained in Eosin staining solution (Appendix I) for 1 minute, washed in tap water and dehydrated in ethanol, cleared in Xylene (Saarchem, Merck Chemicals, South Africa) and mounted in Entellen (Microscopy, Merck, Germany) with a coverslip.

2.7 FLUORESCENCE IMMUNOCYTOCHEMISTRY

This technique was used to determine the location of ERα; Hsp90 and Hoxa10 protein within the uterus, and to determine whether this changes with CC treatment. Sections cut from the cryostat were fixed in acetone (ACE, Southdale, SA) for 10 minutes. The slides were washed in 3 changes of PBS for 5 minutes each. Excess PBS was removed from the slides and sections were circled with a DAKO pen (DAKO, Denmark) to prevent solutions from running off the section. The sections were covered in PBS-BSA-Triton-X (Appendix I) for 20 minutes to block against non-specific staining and permeabilize the cell membranes. Excess PBS-BSA-Triton-X was removed with a tissue and replaced with 50µl of primary antibody which was diluted in PBS with 0.1% BSA (Sigma-Aldrich Co., USA) (PBS-BSA) and incubated overnight at 4°C. The primary antibodies consisted of rabbit polyclonal anti-ERα 1:200 (sc-542; Santa Cruz Biotechnology, USA); mouse monoclonal anti-Hsp90 1:100 (sc-13119; Santa Cruz Biotechnology), or goat polyclonal anti-Hoxa10 1:50 (sc-17158; Santa Cruz Biotechnology). Negative controls were included in every run without the primary antibody, which was replaced with PBS-BSA.
The following morning the slides were washed in PBS with 0.1% BSA for 1x 10 minutes, and 2x 5 minutes. The excess PBS was removed and slides were incubated in a dark chamber with 50µl of secondary antibody conjugated to a fluorescent dye and diluted in PBS for 2 hours at room temperature. The antibodies consisted of donkey anti-rabbit conjugated to Texas Red 1:200 (sc-2784; Santa Cruz Biotechnology), goat anti-mouse conjugated to fluoroscein isothiocyanate (FITC) 1:200 (sc-2010; Santa Cruz Biotechnology) or donkey anti-goat conjugated to FITC 1:200 (sc-2024; Santa Cruz Biotechnology). Negative controls were included in every run without the secondary antibody, which was replaced with PBS. The slides were washed in a dark chamber with PBS for 2x 10 minutes and 1x 5 minutes. Slides were then mounted in antifade (MØ1; Biomeda Foster City, CA, USA) with a coverslip and sealed with clear nail varnish. The slides were kept in a black plastic bag at 4°C until the next day where they were viewed on a Zeiss Laser Confocal Microscope at the University of the Witwatersrand and the images photographed. Figure 2.4 illustrates a flow chart for the procedure of florescence ICC.

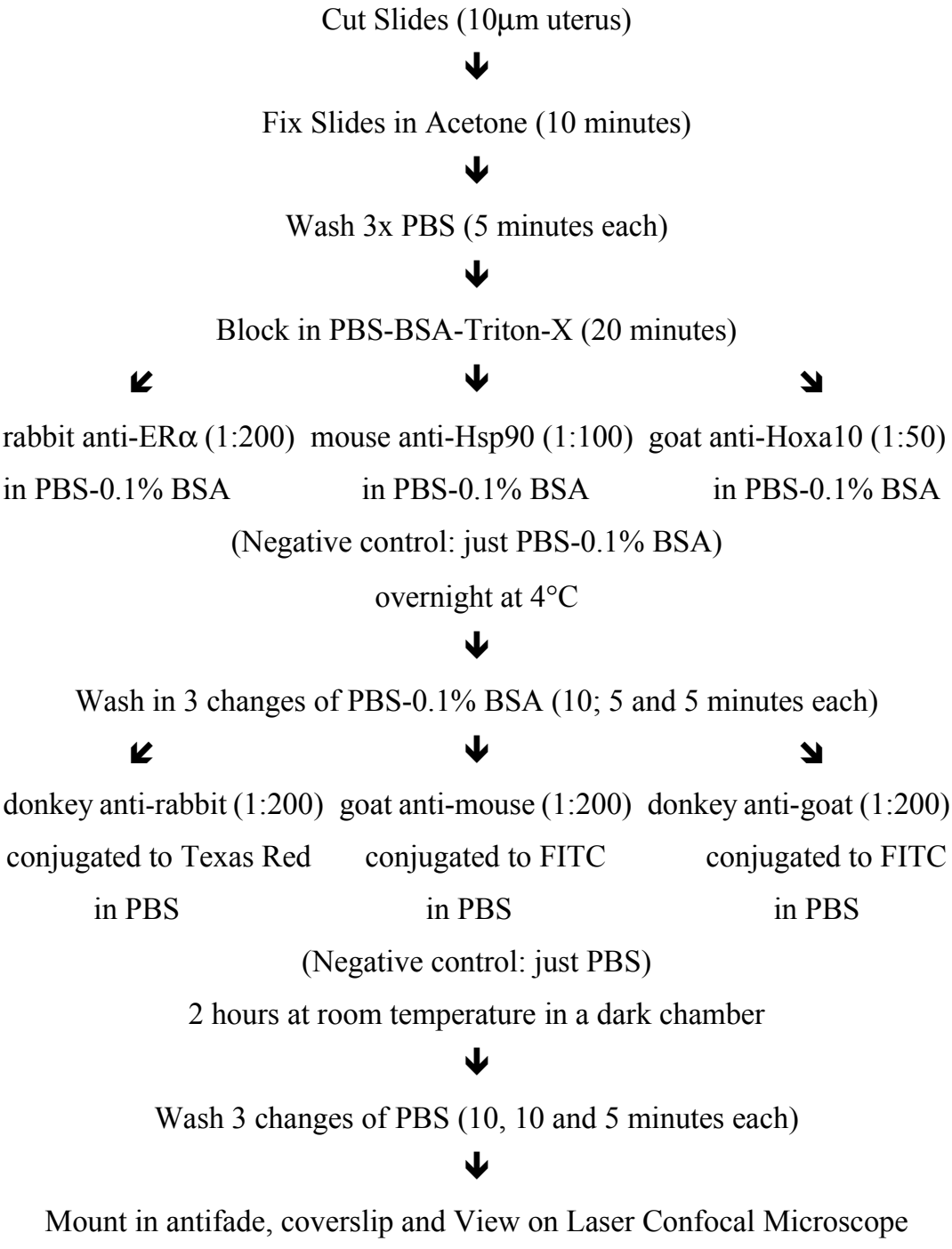

Fig.2.4 Flow diagram showing procedure for fluorescence immunocytochemistry

2.8 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

This technique was used to determine if there was any change in the expression of ERα mRNA with CC, to support the results obtained with the Western blots on this gene, and to determine at what level CC may be acting on this gene.

2.8.1 RNA Extraction

The pieces of uteri dissected from each rat were placed into a clean sterile petri-dish and weighed. The tissue had to be less than 30mg in order to use the RNeasy Mini Kit for total RNA extraction (74104; Qiagen, Maryland, USA).
Using sterile autoclaved scissors the tissue was minced and placed into a clean autoclaved eppendorf. For every 1ml of Buffer RLT from the kit to be used, 10µl of β-mercaptoethanol (Sigma-Aldrich Co., USA) was added. This Buffer RLT-β-mercaptoethanol solution (600µl) was added to the minced tissue. The tissue was then homogenized by passing the tissue several times through a 1000µl pipette tip. The tissue was vortexed and placed into a Qiashredder column (Qiagen, Maryland, USA) and centrifuged at maximum speed for 2 minutes in a HERMLE Z229 micro-centrifuge (Germany) to further homogenize the tissue. The samples were then placed on ice until all samples extracted that day were at the same stage. Once all the samples were ready, they were centrifuged for 3 minutes at maximum speed and the supernatant transferred to a new eppendorf, and the pellet discarded. Ethanol (70%) (600µl) was added to each sample and gently mixed by sucking the sample a few times through the pipette tip. An aliquot (700µl) of this sample was added to an RNeasy mini column, which was placed in a 2 ml collection tube. The tube was closed and centrifuged for 15 seconds at maximum speed and then the flow through discarded. If the original sample exceeded 700µl then the procedure was repeated by adding another 700µl of the sample to the same RNeasy mini column and centrifuging for a further15 seconds. The flow through was discarded each time until the entire sample had been used. Buffer RW1 (700µl) was then added to the RNeasy mini column and the sample centrifuged for 15 seconds at maximum speed to wash the column. The flow through and collection tube were discarded and the column transferred to a new 2ml collection tube.

Buffer RPE (500µl), to which 4 volumes of 100% ethanol had been added, was added to the column, centrifuged for 15 seconds at maximum speed and then the flow through discarded. Another 500µl of Buffer RPE was added and the column centrifuged for 2 minutes to dry the RNeasy silica gel membrane. To ensure that the membrane was dry, the column was left slightly open on the bench top for a few seconds to allow excess ethanol to evaporate and the column was transferred to a new 1.5ml collection tube. Ribonuclease (RNase)-free water (50µl) was added to the RNeasy silica gel membrane and the column centrifuged for 1 minute. The flow through was not discarded but a second volume of 20µl of RNase-free water was added and the column centrifuged for another minute. The total elution volume was therefore 70µl. The RNeasy column was then discarded and the 1.5ml collection tube stored at -70°C. No deoxyribonuclease (DNase) step was included as the columns are capable of removing most if not all of the DNA in the original sample.

2.8.2 RNA Integrity and Concentration Determination

To check the integrity of the RNA extracted a 1.2% agarose gel was made in 1X Tris Acetate EDTA (TAE) Buffer pH8 (Appendix I) (EDTA = ethylenediamine tetraacetate), which was poured and allowed to set in the horizontal mini electrophoresis unit (233,879-6; Sigma-Aldrich Co., USA). Once set the gel was covered in 250ml of 1X TAE with 25µl of Ethidium Bromide solution (E-1385; Sigma Chemical Co., St Louis, MO, USA). 6X loading buffer (G190A; Promega, Madison, WI, USA) (3µl) was mixed with 7µl (10% of original sample) of RNA extract and this was loaded into the wells of the gel. The electrodes were attached to a power pack (Consort E452; Separation Scientific, Honeydew, SA) and the gel allowed to run at a constant voltage of 80V until the blue dye in the loading buffer reached half way through the gel. The gel was removed from the unit and viewed under UV light with a UVP dual-intensity transilluminator (TM-20; UVP, CA, USA) and photographed with a DS34 Polaroid Camera (Polaroid, UK) with film (Sigma-Aldrich Co., USA).Only samples showing two clear bands of the 18S and 28S ribosomal RNA (rRNA) with a faint smear representing mRNA in the background were included in the study. Figure 2.5 shows an example of an agarose gel containing RNA extracted with the RNeasy Mini Kit.

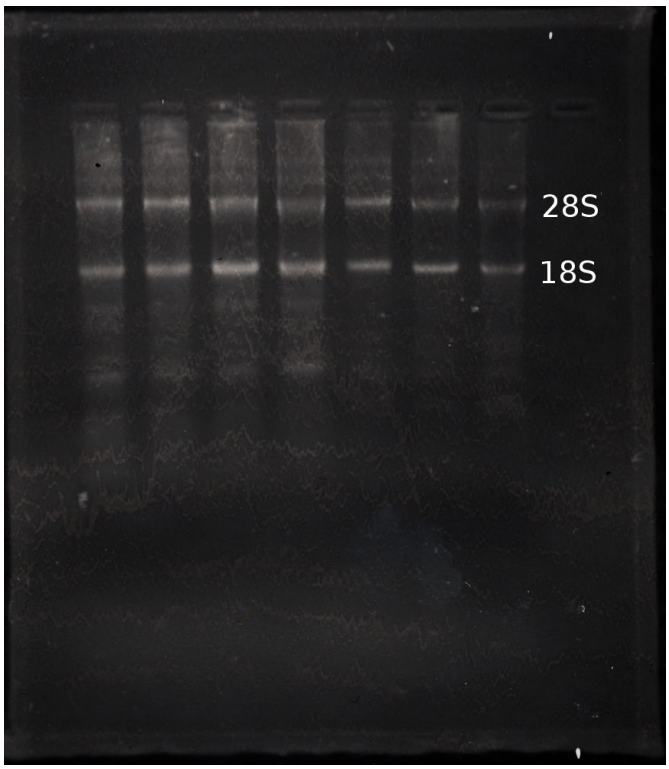

Fig.2.5 1.2% Agarose gel showing RNA samples extracted using the Qiagen RNeasy Mini Kit
Note two bands representing 18S and 28S rRNA and smear in the background representing mRNA

To determine the concentration and purity of the RNA samples, 5µl of RNA sample was mixed with 95µl of sterile nuclease free H_2O in a 200µl eppendorf. The absorbance of the samples were read at 260nm (A_{260}) and at 280nm (A_{280}), using the Ultrospec 2000 UV spectrophotometer (Pharmacia Biotechnology, UK) and blanking on the nuclease free H_2O. The samples were read 3 times and an average was taken. The concentration was determined based on the fact that an A_{260} of 1 is equivalent to 40µg/ml. Therefore the concentration of the original RNA extract is determined by the A_{260} multiplied by 40 and multiplied by the dilution factor (20) in µg/ml (Appendix II [5.7]). The Purity of the sample was determined by the ratio of the A_{260} over the A_{280}, and only those samples with a purity of between 1.8 and 2 were used in the RT-PCR process (Appendix II [5.7] contains an example of these calculations). Samples for single dose treatments were all read on the same day, and samples for the pseudopregnant and pregnant treatments were also all read in one day. Table 2.4 represents the A_{260}, A_{280}, concentration and purity of the samples.

Table 2.4: RNA absorbance values, concentration and purity (See Appendix II [5.7] for examples of these calculations)

Gel number	Sample	A_{260}	A_{280}	Concentration (µg/ml)	Purity
1	A1	0.0253	0.0125	20.2	2
	B2	0.0336	0.0153	26.9	2
	C1	0.0523	0.0247	41.8	2
	D7	0.0984	0.0457	78.7	2
	E7	0.2490	0.1211	199.2	2
2	A4	0.0658	0.0331	52.6	2
	B1	0.0418	0.0191	33.4	2
	C4	0.0485	0.0224	38.8	2
	D8	0.0613	0.0281	49.0	2
	E8	0.0962	0.0441	77.0	2
3	A5	0.0466	0.0218	37.3	2
	B4	0.0615	0.0283	49.2	2
	C5	0.0568	0.0270	45.4	2
	D2	0.1013	0.0477	81.1	2
	E9	0.1533	0.0729	122.7	2

4	A4	0.0658	0.0331	52.6	2
	B1	0.0418	0.0191	33.4	2
	C4	0.0485	0.0224	38.8	2
	D3	0.1639	0.0768	131.2	2
	E1	0.1672	0.0788	133.8	2
5	A1	0.0253	0.0125	20.2	2
	B2	0.0336	0.0153	26.9	2
	C1	0.0523	0.0247	41.8	2
	D4	0.1220	0.0656	97.6	2
	E10	0.1509	0.0708	120.7	2
6	A5	0.0466	0.0218	37.3	2
	B4	0.0615	0.0283	49.2	2
	C5	0.0568	0.0270	45.4	2
	D9	0.1658	0.0768	132.6	2
	E11	0.2247	0.1057	179.8	2
7	F2	0.2	0.1072	157.4	1.8
	G3	0.3196	0.2010	255.7	1.6
	H3	0.3279	0.1605	262.3	2
	I2	0.2190	0.1084	175.2	2
8	F4	0.0721	0.0355	57.7	2
	G1	0.2068	0.1057	165.4	2
	H7	0.1508	0.0790	120.6	1.9
	I7	0.0848	0.0450	67.9	1.9
9	F5	0.1445	0.0780	115.6	1.9
	G6	0.3448	0.1887	275.9	1.8
	H2	0.2733	0.1358	218.7	2
	I8	0.2172	0.1116	173.7	1.9
10	F6	0.2407	0.1463	192.6	1.6
	G2	0.3438	0.2220	275.0	1.5
	H4	0.2484	0.1194	198.7	2
	I9	0.2157	0.1095	172.6	2
11	F3	0.1111	0.0600	88.9	1.9
	G4	0.2513	0.1278	201.1	2
	H8	0.2144	0.1185	171.5	1.8
	I10	0.0944	0.0501	75.5	1.9

12	F7	0.0758	0.0349	60.6	2
	G5	0.1865	0.0814	149.2	2
	H9	0.1613	0.0900	129.0	1.8
	I11	0.0967	0.05	77.3	1.8

2.8.3 Complementary DNA (cDNA) Synthesis (Reverse Transcription Step)

Reverse Transcription-Polymerase Chain Reaction was performed as a two step method. In the reverse transcription step, the mRNA was converted to cDNA using the Roche 1st strand cDNA synthesis Kit for RT-PCR (AMV) (1483188; Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. Briefly the total RNA samples were diluted to the lowest concentrated sample (20.2µg/ml for samples A-E; and 57.7µg/ml for samples F-I), with SABAX water (See Appendix II (5.8) for example of this calculation). All components of the kit were thawed on ice, briefly vortexed and centrifuged before beginning. A master mix was made in a sterile 1.5ml eppendorf on ice as per Table 2.5. The volumes added were multiplied by the number of samples +1 to allow for pipetting error.

Table 2.5: Reagents and volumes used per sample in Master Mix for cDNA synthesis

REAGENT	VOLUME per 1 sample	FINAL CONCENTRATION
10X Reaction Buffer – 100mM Tris, 500mM KCl; pH 8.3	2µl	1X
25mM $MgCl_2$	4µl	5mM
Deoxynucleotide Mix (dNTPs) – 10mM each dATP, dCTP, dTTP, dGTP	2µl	1mM
Oligo-p$(dT)_{15}$ Primer – 0.02 A_{260} units/µl (0.8µg/µl)	2µl	0.04 A_{260} units/µl (1.6µg)
RNase Inhibitor – 50 units/µl	1µl	50 units
AMV Reverse Transcriptase	0.8µl	≥20 units
Sterile Nuclease Free Water	6.2µl	
TOTAL	**18µl**	

The primer is specific for the poly-A tail of mRNA, hence only the mRNA is converted to cDNA. The Master Mix was briefly vortexed and centrifuged, to ensure the components were properly mixed and to collect the sample at the bottom of the eppendorf. The Master mix (18µl) was added to 2µl of each RNA sample (0.04µg for samples A-E; and 0.12µg for samples F-I) in a 200µl eppendorf. Included in every run was a "minus reverse transcriptase control" (-RT control), in which the same amount of RNA was added to 18µl of a Master mix, in which the reverse transcriptase had been replaced with sterile nuclease free water. This control accounts for any genomic DNA in the RNA extract, and should therefore not contain any bands after it has been amplified. The samples were again vortexed and centrifuged and allowed to stand at room temperature for 10 minutes to allow for primer annealing, and then placed in a dry block heater (Hägar designs HB2, Germany) at 42°C for 1 hour, during which the mRNA is converted to cDNA by the AMV reverse transcriptase. The remainder of the RNA samples were returned to -70°C and stored. After the 42°C incubation, the samples were placed in a dry block heater (Hägar, Germany) at 99°C for 5 minutes and then cooled at 4°C for 5 minutes to denature the reverse transcriptase and stop the cDNA synthesis reaction. At this point the samples were either kept at 4°C and then proceeded to the PCR reaction step or they were stored at -20°C until needed.

2.8.4 Polymerase Chain Reaction (PCR)

In the PCR step, the cDNA encoding ERα and β-actin was amplified using the Roche PCR Core Kit (1578553; Roche Applied Science, Germany) according to the manufacturer's instructions. Briefly all reagents of the kit were thawed on ice and then briefly vortexed and centrifuged. A PCR Master Mix was made in a 1.5ml sterile eppendorf on ice as per Table 2.6. The volumes added were multiplied by the number of samples including the -RT control sample + 1 as for the reverse transcription step.

Table 2.6 Reagents and volumes used per sample in PCR Master Mix

REAGENT	VOLUME per 1 sample	FINAL CONCENTRATION
10X PCR Buffer with $MgCl_2$ - 100mM Tris/HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3	5µl	1X
dNTP Mix - 10mM dATP, dCTP, dGTP, dTTP, in sterile double distilled water, pH 7	1µl	200µM each
ERα Upstream Primer [a] - 100µM stock	0.5µl	50pmol
ERα Downstream Primer [b] - 100µM stock	0.5µl	50pmol
β-Actin Upstream Primer [c] - 100µM stock	0.5µl	50pmol
β-Actin Downstream Primer [d] - 100µM stock	0.5µl	50pmol
Taq DNA Polymerase – 250U (5U/µl) in 20mM Tris/HCl, 100mM dithiothreitol (DTT), 0.1mM ethylenediamine tetraacetate (EDTA), Nonident P40™ 0.5%, Tween 20™ 0.5%, glycerol 50%, pH8	0.25µl	1.25U/reaction
Nuclease Free Water	31.75µl	
TOTAL	**40µl**	

a – The ERα upstream primer (Inqaba Biotechnical Industries (Pty) Ltd., Hatfield, South Africa) sequence 5'-AAT TCT GAC AAT CGA AAT CGA CGC CAG-3', has a TM (melting temperature) of 60.61.

b – The ERα downstream primer (Inqaba Biotech., South Africa) sequence 5'-GTG CTT CAA CAT TCT CCC TCC TC-3', has a TM of 64.55. (Sequences for ERα obtained from Tessier *et al.,* 2000).

c – The β-actin upstream primer (Inqaba Biotech., South Africa) sequence 5'-TAC AAC CTC CTT GCA GCT CC-3', has a TM of 62.45.

d – The β-actin downstream primer (Inqaba Biotech., South Africa) sequence 5'-ACA ATG CCG TGT TCA ATG G-3' has a TM of 58.01. (Sequences for β-Actin obtained from Schwendt and Jezova, 2001).

All primers were resuspended to a concentration of 100µM with SABAX water.

The PCR master mix was then briefly vortexed and centrifuged and 40µl of it was added to 10µl of each cDNA sample including the -RT control sample, in a 200µl sterile eppendorf. In addition an extra 40µl of PCR master mix was added to 10µl of nuclease free water as a "no template control" (NTC) for every run, which accounts for any contamination during the amplification procedure, and therefore should not contain any bands after amplification. The samples were again vortexed and centrifuged and placed into the GeneAmp PCR System 2400 thermocycler (Applied

Biosystems, Pharmacia Biotechnology, Buckinghamshire, UK). No mineral oil overlay was required as the thermocycler had a top heater which prevents evaporation. The reaction volume was set at 50µl.

The cycling parameters for the PCR amplification were as follows:

1 cycle of 94°C for 5 minutes: to remove secondary structure from cDNA

30 cycles of: 94°C for 30 seconds: to denature or separate strands

55°C for 1 minute: to anneal strands to primers (based on melting temperature (TM) of primers)

72°C for 1 minute: to extend strands by Taq polymerase

1 x cycle of 72°C for 7 minutes: to finish off

Store at 4°C.

2.8.5 1.6% Agarose Gel

The results of the PCR amplification were separated and analyzed on a 1.6% agarose gel in 1X Tris Borate EDTA (TBE) buffer pH 8.3 (Appendix I). The gel was poured and allowed to set in the horizontal mini electrophoresis unit (233,879-6; Sigma-Aldrich Co., USA). Once set the gel was covered in 250ml of 1X TBE with 25µl of Ethidium Bromide solution (E-1385; Sigma Chemical Co., USA). 2µl of 6X loading buffer (G190A; Promega, Madison, WI, USA) was mixed with 5µl of each PCR product sample, including one -RT control sample and one NTC sample. 2µl of the loading buffer was also mixed with 3µl of a 100bp DNA ladder (G210A; Promega, USA). These were loaded into the wells of the gel. The electrodes were attached to a power pack (Consort E452; Separation Scientific, South Africa) and the gel allowed to run at a constant voltage of 80V until the yellow dye in the loading buffer reached the end of the gel. The gel was removed from the unit and viewed under UV with a UVP dual-intensity transilluminator (TM-20; UVP, CA, USA) and photographed with a DS34 Polaroid Camera (Polaroid, UK) with film (Sigma-Aldrich Co., USA). The size of the ERα amplification product is 344bp, according to Tessier *et al.,* (2000), and for the β-Actin amplification product it is 280bp, according to Schwendt and Jezova, (2001).