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The effect of clomiphene citrate

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2006-10-26T08:03:27Z
http://hdl.handle.net/10539/1455

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APPENDIX

I. SOLUTIONS AND RECIPES

5.1 Vaginal Smears

5.1.1 Shorr's staining solution

500ml 100% Ethanol

2.5g Biebrichscarlet (Gurr Microscopy Materials; BDH Limited (Ltd.), Poole, England)

0.25g Orange G (Saarchem Merck Chemicals, Gauteng, South Africa (SA))

0.375g Fast Green FCF (Gurr Microscopy Materials; BDH Ltd., England)

2.5g Phosphotungstic Acid (Merck, Germany)

2.5g Phosphomolybdic Acid (Merck, Germany)

5ml Glacial Acetic Acid (Saarchem Merck Chemicals, Gauteng, SA)

Filtered overnight before used

Method of staining

Smeared cells onto glass slides.

Fixed in Hairspray.

Stained in Shorr's solution for 1 minute.

Dehydrated through graded alcohols and mounted in Entellen (Microscopy, Merck, Germany) with coverslip.

5.2 Miscellaneous

5.2.1 Phosphate Buffered Saline (PBS) pH7.5

8.5g NaCl (ACE, Southdale, SA)

1.07g Na_2HPO_4 (ACE, Southdale, SA)

0.39g $NaH_2PO_4H_2O$ (Saarchem Merck Chemicals, Gauteng, SA)

pH to 7.5 with 1N NaOH (ACE, Southdale, SA)

Made to 1 litre with dH_2O.

5.2.2 1X Tris Buffered Saline (TBS) pH8

10mM Tris-HCl pH8 (Tris: Sigma-Aldrich Co, St Louis MO, USA)

150mM NaCl

5.3 Western Blotting

5.3.1 Homogenizing buffer pH7.5

50mM Tris-HCl pH7.5

10% Glycerol (ACE, Southdale, SA)

5mM Magnesium Acetate (Sigma-Aldrich Co, St Louis MO, USA)

0.2mM ethylenediamine tetraacetate (EDTA) (UnivAR Saarchem, Muldersdrift, Krugersdorp, SA)

0.5mM dithiothreitol (DTT) (Boehringer Manheim, Manheim, Germany)

1% Triton-X-100 (Sigma-Aldrich Co, St Louis MO, USA)

To every 10ml of Homogenizing Buffer 50µl protease inhibitor cocktail stock (P2714: Sigma-Aldrich Co, St Louis MO, USA) was added just before use. Protease inhibitor cocktail stock: (was reconstituted with 10ml sterile dH_2O) 2mM AEBSF; 1mM EDTA; 130µM Bestatin; 14µM E-64 1mM Leupeptin; 0.3µ M Aprotinin.

5.3.2 30%T 2.7%C_{Bis} Acrylamide monomer stock solution

Acrylamide 29.2g/100ml (Promega, Madison, WI, USA)

Bisacrylamide 0.8g/100ml (Promega, Madison, WI, USA)

Stored at 4^0C in a dark bottle.

5.3.3 10% SDS-polyacrylamide separating gel

30% Acrylamide Monomer Stock Solution	5ml
1.5M Tris-HCl pH6.8 (4X separating gel buffer)	3.75ml
10% sodium dodecyl sulphate (SDS) (ACE, Southdale, SA)	150µl
dH_2O	6.025ml
10% Ammonium persulfate (APS) (UnivAR Saarchem, Muldersdrift, Krugersdorp, SA)	250µl
N,N,N',N'-tetramethylethylenediamine (TEMED) (Fluka Biochemika, Switzerland)	15µl

5.3.4 4% SDS-polyacrylamide stacking gel

30% Acrylamide Monomer Stock Solution	665µl
0.5M Tris-HCl pH6.8 (4X stacking gel buffer)	1.25ml
10% SDS	50µl
dH_2O	3.05ml
10% APS	100µl
TEMED	5µl

5.3.5 2X Sample buffer pH6.8

0.125M Tris-HCl pH6.8 (diluted from stacking gel buffer)
4% SDS
20% Glycerol
10% β mercaptoethanol (Sigma-Aldrich Co, St Louis MO, USA)
10µg/ml Bromophenol blue (BDH Laboratories, Poole, England)

5.3.6 Electrophoresis tank buffer

0.025M Tris pH 8.3 (Sigma-Aldrich Co, St Louis MO, USA)
0.192M Glycine (Saarchem Merck Chemicals, Gauteng, SA)
0.1% SDS
pH was tested to check if it was around 8.3.

5.3.7 Coomassie Blue staining solution

0.125% Coomassie Blue R250 (made from a 1% Coomassie stock in dH_2O, which was filtered before use) (UnivAR Saarchem, Muldersdrift, Krugersdorp, SA)
50% Methanol (Saarchem Merck Chemicals, Gauteng, SA)
10% Acetic Acid

5.3.8 Destaining solutions

Destaining Solution 1:	Destaining Solution 2:
50% Methanol	5% Methanol
10% Acetic Acid	7% Acetic Acid

5.3.9 Transfer buffer pH 8.3

0.025M Tris

0.192M Glycine

20% Methanol

pH to 8.3 and kept at 4^0C until used

5.3.10 Primary antibody solutions for Western Blotting

For ERα:

Rabbit anti-ERα (Santa Cruz Biotechnology, CA, USA; sc-542): 1:100 (100µl)

Mouse anti-Actin (Sigma-Aldrich Co, MO, USA; A-2547): 1:1600 (125µl of 1:20)

Diluted in 1X TBS-Tween (for a total of 10ml): (9.775ml)

For Hsp90

Mouse anti-Hsp90 (Santa Cruz Biotechnology, USA; sc-13119): 1:100 (100µl)

Mouse anti-Actin: 1:1600 (125µl of 1:20 stock)

1X TBS-Tween: (9.775ml)

For Hoxa10

Goat anti-Hoxa10 (Santa Cruz Biotechnology, USA; sc17158): 1:100 (100µl)
or 1:50 (200µl)

Mouse anti-Actin: 1:1600 (125µl of 1:20 stock)

1X TBS-Tween: 1:100 (9.775ml) or 1:50 (9.675ml)

5.3.11 Secondary antibody solutions for Western Blotting

For ERα

Donkey anti-Rabbit (Santa Cruz Biotechnology, USA; sc-2313): 1:500 (20µl)

Goat anti-Mouse (Santa Cruz Biotechnology, USA; sc-2005): 1:2000 (5µl)

1X TBS-Tween (to make a total of 10ml): 9.975ml

For Hsp90

Goat anti-Mouse: 1:500 (20µl)

1X TBS-Tween 9.980ml

For Hoxa10

Solution 1:

Donkey anti-Goat (Santa Cruz Biotechnology, USA; sc-2020): 1:500 (20µl)

1X TBS-Tween: 9.980ml

Incubated membrane in this solution for 45 minutes before incubating in solution 2

Solution 2:

Goat anti-Mouse: 1:2000 (5µl)

1X TBS-Tween 9.995ml

Following 1st incubation, membrane was incubated in this 2nd solution for 45 minutes.

5.3.12 Diaminobenzidine solution (made in this order)

Diaminobenzidine (DAB) 10mg (BDH Laboratories, Poole, England)

0.05M Tris-HCl pH7.6 20ml

1% H_2O_2 in cold dH_2O 200µl (Merck, Gauteng, SA)

Mixed well and immediately used.

5.4 Light Microscopy and Fluorescence Immunocytochemistry

5.4.1 2% 3-Aminopropyl-triethoxysilane in acetone treated slides

Soaked glass slides in 10% Super 10 (Detergent) overnight (Golden Neo-Life Diamite International, Spartan, SA).
Rinsed in Hot water for 2 hours and left to dry in 60°C oven.
Incubated in 2% 3-Aminopropyl-triethoxysilane (Sigma-Aldrich Co, St Louis MO, USA) in Acetone (ACE, Southdale, SA) for 30 minutes.
Dipped slides in 2 changes of 100% acetone and then in dH_2O.
Incubated in 42^0C oven overnight.

5.4.2 Haematoxylin staining solution

Haematoxylin	4g	(Saarchem Merck Chemicals, Gauteng, SA)
Sodium Iodate	3g	(BDH Laboratories, Poole, England)
Potassium Alum	50g	(Saarchem Merck Chemicals, Gauteng, SA)
Citric Acid	75g	(BDH Laboratories, Poole, England)
Chloral Hydrate	75g	(Saarchem Merck Chemicals, Gauteng, SA)
dH_2O	to 1 litre	

5.4.3 Eosin staining solution

1% Eosin	500ml	(Merck, Germany)
1% Phloxine	250ml	(Merck, Germany)
dH_2O	to 1.5 litres	

5.4.4 PBS-BSA-Triton-X

0.1% Bovine Serum Albumin (BSA) (Sigma-Aldrich Co, St Louis MO, USA)
0.25% Triton-X-100 (Sigma-Aldrich Co, St Louis MO, USA)
Made up in PBS pH7.5.

5.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

5.5.1 50X Tris Acetate EDTA (TAE) buffer pH8

Tris base	242g	(Sigma-Aldrich Co, St Louis MO, USA)
Glacial Acetic Acid	57.1ml	(Saarchem Merck Chemicals, Gauteng, SA)
0.5M EDTA	100ml	(UnivAR Saarchem, Muldersdrift, Krugersdorp, SA)

Adjusted to pH8 with acetic acid, and made up to 1 litre with dH_2O.

Diluted 1:50 with sterile dH_2O to make 1X TAE buffer

5.5.2 1.2% Agarose gel in 1X TAE buffer pH8

Agarose Molecular Grade (low EEO)	0.6g	(Whitehead Scientific, Brankenfell, SA)
1X TAE buffer pH8	50ml	

Boiled in microwave until agarose dissolved, then poured into casting unit to set.

5.5.3 10X Tris Borate EDTA (TBE) buffer pH8.3

0.89M Tris base

0.02M EDTA

0.89M Boric Acid (Saarchem Merck Chemicals, Gauteng, SA)

Checked pH is ±8.3.

Diluted 1:10 with sterile dH_2O to make 1X TBE

5.5.4 1.6% Agarose gel in 1X TBE buffer pH8.3

Agarose	0.8g
1X TBE Buffer pH8.3	50ml

Boiled in microwave until agarose dissolved then poured into casting unit to set.

II CALCULATIONS

5.6 Example of Calculating Protein Concentration and Loading Volume from Absorbance Values

Example: E3

E3 protein extract in homogenizing buffer was diluted with sterile dH_2O

20µl protein extract + 180µl sterile dH_2O = dilution factor of 10

Table 5.1 Example of BSA standard concentrations and absorbencies at 595nm to determine the standard curve for samples A3-E3

BSA Concentrations (mg/ml)	Absorbance
0 (blank) (0.1X homogenizing buffer)	0
0.25	0.32
0.5	0.55
1	1.05
1.4	1.26

Regression Line: y=0.9057x + 0.0647 (y = absorbence; x = concentration [mg/ml]).

(See Fig.2.1 for an example of such a graph)

Average Absorbance of diluted E3 sample = 0.7265 (taken from 3 readings)

∴ Concentration (conc) in mg/ml (x) = (y – 0.0647) ÷ 0.9057

= (0.7265-0.0647) ÷ 0.9057

= 0.730706mg/ml

∴ Concentration of original extract = conc of diluted sample x dilution factor

= 0.730706 x 10

= 7.30706mg/ml (µg/µl)

Concentration of E3 mixed with 2X sample buffer 1:1

= concentration of original extract ÷ 2

= 7.30706 ÷ 2

= 3.65353µg/µl

Amount of protein (µg) = Concentration of protein (µg/µl) x volume (µl)

∴ Loading Volume (to load 17.5µg protein) = 17.5µg ÷ conc of sample loaded

= 17.5µg ÷ 3.65353µg/µl

= 4.8µl (≈ 5µl)

∴ loaded 5µl of E3 protein extract mixed in 2X sample buffer onto SDS-PAGE gel

5.7 Calculating RNA Concentration and Purity from Absorbance Values

Example: F2

5µl RNA extracts was diluted with 95 µl sterile nuclease free H_2O.

∴ Dilution Factor = 20.

Absorbance at 260nm (A_{260}) of 1 = RNA Concentration of 40µg/ml

A_{260} of F2 diluted RNA extract = 0.1968

∴ Concentration of original RNA extract = A_{260} x 40µg/ml x Dilution Factor

= 0.1968 x 40 x 20

= 157.4µg/ml

Purity of RNA extract = A_{260} ÷ Absorbance at 280nm (A_{280})

A_{280} of F2 diluted RNA extract = 0.1072

∴ Purity of F2 = 0.1968 ÷ 0.1072

= 1.8

Since this is between 1.8 and 2, F2 is pure (does not contain protein contamination).

5.8 Calculation to Dilute All RNA Extract Samples to the Same Concentration for RT-PCR

Lowest Concentrated Sample between Treatment groups A-E = A1 = 20.2 µg/ml

Therefore since 2µl of RNA was to be added to the cDNA synthesis reaction, (20.2µg/ml x 0.002ml) 0.04µg RNA is added to the reaction

Example: D4

Concentration of D4 RNA extract = 97.6µg/ml

Initial Concentration (C_1) x Initial Volume (V_1) = Final Concentration (C_2) x Final Volume (V_2) (which can be any volume, 10µl was chosen)

∴ 97.6µg/ml x V_1 = 20.2µg/ml x 0.01ml

∴ V_1 = (20.2µg/ml x 0.01ml) / 97.6µg/ml

= 0.00207ml

= 2.07µl

∴ Added 2.07µl of D4 RNA extract + (10µl – 2.07µl) 7.93µl sterile nuclease free H_2O.

III STATISTICS FOR WESTERN BLOTTING

5.9 ERα Protein Expression for Single Dose Treatments (Samples A-E)

5.9.1 Raw data from scans of Western blots

Table 5.2 Raw volumes of ERα and Actin bands and the quantities of ERα protein expression in ovariectomized rat uteri treated with a single dose of CC or E_2, as obtained from Western blot scans

A represents untreated, B: saline, C: oil, D: CC and E: E_2 treated protein samples

SAMPLE	BLOT #	RAW VOLUME OF ERα	RAW VOLUME OF ACTIN	EXPRESSION OF ERα PROTEIN (ERα/Actin)
A1	**1**	2622.58	18438.29	0.142235
A2	**2**	2492.19	18693.39	0.133319
A3	**3**	5560.37	42782.29	0.129969
A1	**4**	3090.86	39677.66	0.077899
A2	**5**	3191.97	58404.30	0.054653
A3	**6**	5665.35	44653.34	0.126874
B1	**1**	2902.90	12083.98	0.240227
B2	**2**	5089.32	20206.83	0.251862
B3	**3**	5353.60	41060.67	0.130383
B1	**4**	7826.09	46088.09	0.169807
B2	**5**	2955.33	50600.01	0.058406
B3	**6**	2714.16	27814.23	0.097582
C1	**1**	2392.46	11837.00	0.202117
C2	**2**	5367.52	17121.30	0.313500
C3	**3**	3540.83	43816.39	0.080811
C1	**4**	23248.40	60148.05	0.386520
C2	**5**	2734.92	57106.00	0.047892
C3	**6**	2987.82	31752.13	0.094098
D1	**1**	0	4341.90	0
D2	**2**	0	18255.00	0
D3	**3**	0	33653.30	0
D4	**4**	0	43327.24	0
D5	**5**	0	51475.01	0
D6	**6**	0	38667.57	0
E1	**1**	2716.25	5951.80	0.456375
E2	**2**	5937.71	10541.02	0.563295
E3	**3**	11590.70	23752.53	0.487977
E4	**4**	11844.46	41684.63	0.284145
E5	**5**	3658.81	38871.96	0.094125

E6	**6**	4830.83	37949.85	0.127295

5.9.2 Summaries of statistics tests for significant differences between the mean ERα protein expression in the untreated (A) and vehicle treated (B and C) controls

Table 5.3 Means and standard deviations of control groups

Treatment Group	n (sample size)	Mean	Standard Deviation (Std Dev)	Standard Error of Mean (SEM)
Untreated (A)	6	0.110825	0.035654	0.01456
Saline treated (B)	6	0.158044	0.077515	0.03165
Oil treated (C)	6	0.187490	0.138055	0.05636

Table 5.4 Tests for homogeneity of the data (equality of variance) in the control groups for ERα protein expression using the O'Brien's and Bartlett's tests at the 5% level of significance (α=0.05)

Null Hypothesis (N.H): The variances are equal

Test	F Ratio	Degrees of Freedom (DF) of the numerator (DF NUM)	DF of the denominator (DF DEN)	Probability >F (Prob>F)
O'Brien's	4.2580	2	15	0.0343
Bartlett's	3.5279	2		0.0294

$p<0.05$ so the N.H cannot be rejected. The variances are therefore equal and the usual one-way analysis of variance (ANOVA) is used.

Table 5.5 One-way analysis of variance test for significant differences between the mean ERα protein expression in the control groups (α=0.05)

N.H: There is a significant difference between the means.

Total Number of Observations: 18

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	2	0.0179483	0.008974	1.0222
Error	15	0.1316941	0.008780	**Prob > F**
C Total	17	0.1496423	0.008802	0.3836

$p>0.05$ so the NH is rejected. Therefore there is no significant difference between the means of the control groups.

The data for the expression of ERα protein in the control (untreated [A], saline [B] and oil treated [C]) groups were therefore pooled into one group (controls).

5.9.3 Summaries of statistics tests for significant differences in the mean ERα protein expression between the single dose treatment groups (Controls, D, E)

Table 5.6 Means and standard deviations of ERα protein expression in the different groups treated with a single dose of CC or E_2 (See Fig. 3.2I)

Group	n	Mean	Std Dev	SEM
Controls (ABC)	18	0.152120	0.093822	0.02211
CC treated (D)	6	0	0	0
E_2 treated (E)	6	0.335535	0.196977	0.08042

Table 5.7 Tests for homogeneity of the data (equality of variance) in the single dose treatment groups (Controls, D, E) for ERα protein expression using the O'Brien's and Brown-Forsythe tests (α=0.05)
N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	8.9526	2	27	0.0010
Brown-Forsythe	10.3349	2	27	0.0005

$p<0.05$ so the N.H. cannot be rejected. Therefore the variances are equal and the usual ANOVA is used.

Table 5.8 One-way analysis of variance test for significant differences between the mean ERα protein expression in the single dose treatment groups
N.H: There is a significant difference between the means.

Total Number of Observations: 30

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	2	0.3395147	0.169757	13.3379
Error	27	0.3436417	0.012727	**Prob > F**
C Total	29	0.6831564	0.023557	<0.0001

$p<0.05$ so the N.H cannot be rejected. Therefore there is a significant difference in the mean expression of ERα protein between the single dose treatment groups.

Results of the Tukey-Kramer *post hoc* test (groups are arranged with the means in descending order from left to right)
E_2 treated group (E) vs Controls group (ABC): significantly different

E_2 treated group (E) vs CC treated group (D): significantly different

Controls group (ABC) vs CC treated group (D): significantly different

5.9.4 Summaries of statistics tests for significant differences in the mean raw volumes of actin bands in the ERα Western blots between the single dose treatment groups (A-E)

Table 5.9 Means and standard deviations of the raw volume of Actin bands in the ERα Western blots in the different single dose treatment groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	6	37108.2	15728.5	6421.1
Saline (B)	6	32975.6	15323.6	6255.8
Oil treated (C)	6	36963.5	20211	8251.1
CC treated (D)	6	31620	17365.5	7089.4
E_2 treated (E)	6	26458.6	15486.7	6322.4

Table 5.10 Tests for homogeneity of data of the raw volume of Actin bands in the ERα Western blots in the single dose treatment groups using the O'Brien's and Bartlett's tests (α=0.05)

N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob >F
O'Brien's	0.4003	4	25	0.8065
Bartlett's	0.1344	4		0.9697

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.11 Welch ANOVA test for significant differences between the mean raw volume of Actin bands in the ERα Western blots in the single dose treatment groups (α=0.05)

N.H.: There is a significant difference between the means.

Total Number of Observations: 30

F Ratio	DF NUM	DF DEN	Prob > F
0.3744	4	12.472	0.8226

p>0.05 so the N.H is rejected. Therefore there is no significant difference in the mean raw volume of Actin bands in the ERα Western blots between the single dose treatment groups. This suggests that actin is an appropriate internal control.

5.10 Hsp90 Protein Expression for Single Dose Treatments (Samples A-E)

5.10.1 Raw data from scans of Western blots

Table 5.12 Raw volumes of Hsp90 and Actin bands and the quantities of Hsp90 protein expression in ovariectomized rat uteri treated with a single dose of CC or E_2, as obtained from Western blot scans

A represents untreated, B: saline, C: oil, D: CC, and E: E_2 treated protein samples

SAMPLE	BLOT #	RAW VOLUME OF HSP90	RAW VOLUME OF ACTIN	EXPRESSION OF HSP90 PROTEIN (Hsp90/Actin)
A1	**1**	1974.04	55841.47	0.035351
A2	**2**	3963.05	36881.71	0.107453
A3	**3**	1293.01	29618.97	0.043655
A1	**4**	5213.07	58574.6	0.088999
A2	**5**	8471.79	81879.79	0.103466
A3	**6**	5315.4	34844.82	0.152545
B1	**1**	936.53	27627.79	0.033898
B2	**2**	6918.48	43343.15	0.159621
B3	**3**	2000.82	28994.09	0.069008
B1	**4**	5301.29	62052.21	0.085433
B2	**5**	9240.23	79626.75	0.116044
B3	**6**	9395.31	47485.95	0.197854
C1	**1**	1386.28	31703.35	0.043727
C2	**2**	5387.26	40183.47	0.134067
C3	**3**	1501.27	18751.16	0.080063
C1	**4**	8734.06	56564.36	0.154409
C2	**5**	8982.16	78674.84	0.114168
C3	**6**	5614.11	25916.43	0.216624
D1	**1**	1118.71	44783.38	0.024980
D2	**2**	3712.65	33247.37	0.111667
D3	**3**	2408.76	22915.23	0.105116
D4	**4**	10451.01	38739.21	0.269779
D5	**5**	17558.42	70081.28	0.250544
D6	**6**	6259.12	38271.32	0.163546
E1	**1**	3514.78	35549.74	0.098869
E2	**2**	11451.29	30835.64	0.371365
E3	**3**	5979	42823.57	0.139619
E4	**4**	14651.16	56182.43	0.260778
E5	**5**	22015.05	69694.34	0.315880
E6	**6**	7047.13	29900.34	0.235687

5.10.2 Summaries of statistics tests for significant differences between the mean Hsp90 protein expression in the untreated (A) and vehicle treated(B and C) controls

Table 5.13 Means and standard deviations of control groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	6	0.088578	0.043637	0.01781
Saline (B)	6	0.110310	0.060485	0.02469
Oil treated (C)	6	0.123843	0.060127	0.02455

Table 5.14 Tests for homogeneity of the data in the control groups for Hsp90 protein expression using the O'Brien's and Bartlett's tests (α=0.05)
N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	0.3936	2	15	0.6814
Bartlett's	0.2945	2		0.7449

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.15 Welch ANOVA test for significant differences between the mean Hsp90 protein expression in the control groups (α=0.05)
N.H.: There is a significant difference between the means.

Total Number of Observations: 18

F Ratio	DF NUM	DF DEN	Prob > F
0.6850	2	9.7364	0.5268

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the means of the control groups.

The data for the expression of Hsp90 protein in the control (untreated [A], saline [B] and oil treated [C]) groups were therefore pooled into one group (controls).

5.10.3 Summaries of statistics tests for significant differences between the mean Hsp90 protein expression in the single dose treatment groups (Controls, D, E)

Table 5.16 Means and standard deviations of Hsp90 protein expression in the different groups treated with a single dose of CC or E_2 (See Fig.3.2II)

Group	n	Mean	Std Dev	SEM
Controls (ABC)	18	0.107577	0.054063	0.01274
CC treated (D)	6	0.154272	0.093428	0.03814
E_2 treated (E)	6	0.237033	0.103350	0.04219

Table 5.17 Tests for homogeneity of the data in the single dose treatment groups (controls, D, E) for Hsp90 protein expression using the O'Brien's, Brown-Forsythe's and Bartlett's tests (α=0.05)
N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	3.6423	2	27	0.0398
Brown-Forsythe's	2.3905	2	27	0.1107
Bartlett's	2.2410	2		0.1063

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.18 Welch ANOVA test for significant differences between the mean Hsp90 protein expression in the single dose treatment groups (α=0.05)
N.H.: There is a significant difference between the means.

Total Number of Observations: 30

F Ratio	DF NUM	DF DEN	Prob > F
4.3484	2	7.9107	0.05

p=0.05 therefore the N.H cannot be rejected. Therefore there may be a significant difference in the mean Hsp90 protein expression between the single dose treatment groups.

Results of the Tukey-Kramer *post hoc* test (groups are arranged with the means in descending order from left to right)
E_2 treated group (E) vs CC treated group (D): not significantly different

E_2 treated group (E) vs Controls group (ABC): significantly different

CC treated group (D) vs Controls group (ABC): not significantly different

5.10.4 Summaries of statistics tests for significant differences between the mean raw volumes of actin bands in the Hsp90 Western blots in the single dose treatment groups (A-E)

Table 5.19 Means and standard deviations of the raw volume of Actin bands in the Hsp90 Western blots in the single dose treatment groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	6	49606.9	19698.7	8042
Saline (B)	6	48188.3	19978.5	8156.2
Oil treated (C)	6	41965.6	22206.6	9065.8
CC treated (D)	6	41339.6	15868.6	6478.3
E_2 treated (E)	6	44164.3	15832.5	6463.6

Table 5.20 Tests for homogeneity of the raw volume of Actin protein data in the Hsp90 Western blots in the single dose treatment groups using the O'Brien's and Bartlett's tests at the 5% level of significance

N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	0.2657	4	25	0.8972
Bartlett's	0.2066	4		0.9349

$p>0.05$ so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.21 Welch ANOVA test for a significant difference between the mean raw volume of Actin bands in the Hsp90 Western blots in the single dose treatment groups at the 5% level of significance

N.H.: There is a significant difference between the means

Total Number of Observations: 30

F Ratio	DF NUM	DF DEN	Prob > F
0.1986	4	12.4440	0.9345

$p>0.05$ so the N.H is rejected. Therefore there is no significant difference in the mean raw volume of Actin bands in the Hsp90 Western blots between the single dose treatment groups. This suggests that actin is an appropriate internal control.

5.11 ERα Protein Expression for the Pseudopregnant and Pregnant Rat Uteri at the Time of Implantation (Samples A, F-I)

5.11.1 Raw data from scans of Western blots

Table 5.22 Raw volumes of ERα and Actin bands and the quantities of ERα protein expression in pseudopregnant and pregnant rat uteri with or without CC treatment, as obtained from Western blot scans
A represents untreated, F: saline x1day oil x 3 days (SOOO), G: 5½ day pregnant rat uteri (Pregnant), H: pseudopregnant (P_4 x2 days, P_4 and E_2 on 3rd day) (PPPE) and I: CC treated pseudopregnant (CCPPPE) protein samples

SAMPLE	BLOT #	RAW VOLUME OF ERα	RAW VOLUME OF ACTIN	EXPRESSION OF ERα PROTEIN (ERα/Actin)
A1	**1**	30046.3	38100.67	0.788603
A2	**2**	25439.36	58056.52	0.438183
A3	**3**	16093.13	66377.15	0.242450
F3	**1**	7073.74	24745.89	0.285855
F4	**2**	6151.73	50153.89	0.122657
F5	**3**	11532.25	82120.9	0.140430
G3	**1**	23647.73	34245.94	0.690527
G4	**2**	15623.89	68802.15	0.227084
G5	**3**	11159.81	76221.55	0.146413
H3	**1**	17874.33	56805.07	0.314661
H4	**2**	15669.62	61981.25	0.252812
H5	**3**	20975.65	68673.3	0.305441
I3	**1**	12750.87	46638.51	0.273398
I4	**2**	8027.14	72986.21	0.109982
I5	**3**	12570.18	46803.47	0.268574

5.11.2 Summaries of statistics tests for significant differences between the mean ERα protein expression in the untreated (A) and vehicle treated (SOOO) (F) control groups

Table 5.23 Means and standard deviations of control groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	3	0.489745	0.276703	0.15975
SOOO (F)	3	0.182981	0.089534	0.05169

Table 5.24 Tests for homogeneity of the data (equality of variance) in the control groups for ERα protein expression using the O'Brien's and Bartlett's tests at the 5% level of significance

N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob >F
O'Brien's	1.4095	1	4	0.3008
Bartlett's	1.7112	1		0.1908

p>0.05 so the N.H is rejected, therefore there is an inequality of variances and the Welch ANOVA is used. (Welch ANOVA is the same as the adjusted students t-test for 2 groups with unequal variances).

Table 5.25 Welch ANOVA test for significant differences between the mean ERα protein expression in the control groups at the 5% level of significance

N.H: There is a significant difference between the means.

Total Number of Observations: 6

F Ratio	DF NUM	DF DEN	Prob > F (|t|)
3.3378	1	2.4143	0.1870
t-Test (√F Ratio)			
1.8270			

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the means of the control groups.

The data for ERα protein expression in the control (untreated [A] and SOOO [F]) groups were therefore pooled into one group (controls).

5.11.3 Summaries of statistics tests for significant differences between the mean expression of ERα protein in pregnant and pseudopregnant rat uteri, with or without CC treatment

Table 5.26 Means and standard deviations of ERα protein expression in the pregnant and pseudopregnant rat uteri with or without CC treatment (Controls, G-I) (See Fig. 3.8I)

Group	n	Mean	Std Dev	SEM
Controls (A+F)	6	0.336363	0.249126	0.10171
5½ day Pregnant (G)	3	0.354675	0.293640	0.16953
PPPE (H)	3	0.290971	0.033367	0.01926
CCPPPE (I)	3	0.217318	0.092987	0.05369

Table 5.27 Tests for homogeneity of the data (equality of variance) in the pregnant and pseudopregnant treatment groups (Controls, G-I) for ERα protein expression using the O'Brien's and Bartlett's tests at the 5% level of significance

N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	0.7676	3	11	0.5357
Bartlett's	2.2481	3		0.0805

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.28 Welch ANOVA test for significant differences between the mean ERα protein expression in the pregnant and pseudopregnant treatment groups at the 5% level of significance

N.H: There is a significant difference between the means.

Total Number of Observations: 15

F Ratio	DF NUM	DF DEN	Prob > F
0.5453	3	4.6079	0.6740

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the mean expression of ERα protein in the pregnant and pseudopregnant treatment groups at the time of implantation.

5.11.4 Summaries of statistics tests for significant differences between the mean raw volumes of actin bands in the ERα Western blots in the pregnant and pseudopregnant treatment groups (A, F-I)

Table 5.29 Means and standard deviations of the raw volume of Actin bands in the ERα Western blots in the pregnant and pseudopregnant treatment groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	3	54178.1	14531.7	8390
SOOO (F)	3	52340.2	28749.9	16599
5½ day Pregnant (G)	3	59756.5	22402.1	12934
PPPE (H)	3	62486.5	5950.2	3435
CCCPPPE (I)	3	55476.1	15164.5	8755

Table 5.30 Tests for homogeneity of the raw volume of Actin protein data in the ERα Western blots in the pregnant and pseudopregnant treatment groups using the O'Brien's and Bartlett's tests (α=0.05)
N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob >F
O'Brien's	0.8216	4	10	0.5402
Bartlett's	0.8963	4		0.4650

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.31 Welch ANOVA test for significant differences between the mean raw volume of Actin bands in the ERα Western blots in the pregnant and pseudopregnant treatment groups (α=0.05)
N.H.: There is a significant difference between the means.

Total Number of Observations: 15

F Ratio	DF NUM	DF DEN	Prob > F
0.2558	4	4.5531	0.8936

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the mean raw volume of Actin bands in the ERα Western blots in the pregnant and pseudopregnant treatment groups. This suggests that actin is an appropriate internal control.

5.12 Hsp90 Protein Expression for the Pseudopregnant and Pregnant Rat Uteri at the Time of Implantation (Samples A, F-I)

5.12.1 Raw data from scans of Western blots

Table 5.32 Raw volumes of Hsp90 and Actin bands and the quantities of Hsp90 protein expression in pseudopregnant and pregnant rat uteri with or without CC treatment, as obtained from Western blot scans
A represents untreated, F: saline x1day oil x 3 days (SOOO), G: 5½ day pregnant rat uteri (Pregnant), H: pseudopregnant (P_4 x2 days, P_4 and E_2 on 3rd day) (PPPE) and I: CC treated pseudopregnant (CCPPPE) protein samples

SAMPLE	BLOT #	RAW VOLUME OF HSP90	RAW VOLUME OF ACTIN	EXPRESSION OF HSP90 PROTEIN (Hsp90/Actin)
A1	**1**	13800.39	59099.59	0.233511
A2	**2**	12586.12	67162.34	0.187398
A3	**3**	9887.71	70549.94	0.140152
F2	**1**	14674.64	54292.53	0.270288
F4	**2**	5488.72	41178.79	0.133290
F5	**3**	5502.23	73824.2	0.074532
G2	**1**	12243.62	57667.06	0.212316
G4	**2**	6297.21	54998.54	0.114498
G5	**3**	7612.52	59074.82	0.128862
H2	**1**	11467.6	44306.69	0.258823
H4	**2**	9980.04	63284.52	0.157701
H5	**3**	16011.74	91492.02	0.175007
I2	**1**	11745.45	48431.32	0.242518
I4	**2**	5622.71	62126.27	0.090505
I6	**3**	7633.34	75559.97	0.101024

5.12.2 Summaries of the statistics tests for significant differences between the mean Hsp90 protein expression in the untreated (A) and vehicle treated (SOOO) (F) control groups

Table 5.33 Means and standard deviations of control groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	3	0.187020	0.046681	0.02695
SOOO (F)	3	0.159370	0.100450	0.05799

Table 5.34 Tests for homogeneity of the data in the control groups for Hsp90 protein expression using the O'Brien's and Bartlett's tests at the 5% level of significance

N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	1.0441	1	4	0.3646
Bartlett's	0.8599	1		0.3538

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used. (Welch ANOVA is the same as the adjusted students t-test for 2 groups with unequal variances).

Table 5.35 Welch ANOVA test for significant differences between the mean Hsp90 protein expression in the control groups at the 5% level of significance

N.H.: There is a significant difference between the means.

Total Number of Observations: 6

F Ratio	DF NUM	DF DEN	Prob > F (|t|)
0.1869	1	2.8253	0.6963
t-Test (√F Ratio)			
0.4324			

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the means of the control groups. The data for Hsp90 protein expression in the control (untreated [A] and SOOO [F]) groups were therefore pooled into one group (controls).

5.12.3 Summaries of statistics tests for significant differences between the mean Hsp90 protein expression in pregnant and pseudopregnant rat uteri, with or without CC treatment (Controls, G-I)

Table 5.36 Means and standard deviations of Hsp90 protein expression in the pregnant and pseudopregnant rat uteri with or without CC treatment (See Fig. 3.8II)

Group	n	Mean	Std Dev	SEM
Controls (A+F)	6	0.173195	0.071673	0.02926
5½ day Pregnant (G)	3	0.151892	0.052819	0.03050
PPPE (H)	3	0.197177	0.054084	0.03123
CCPPPE (I)	3	0.144682	0.084891	0.04901

Table 5.37 Tests for homogeneity of the data in the pregnant and pseudopregnant treatment groups (Controls, G-I) for Hsp90 protein expression using the O'Brien's and Bartlett's tests at the 5% level of significance
N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	0.3738	3	11	0.7736
Bartlett's	0.1854	3		0.9064

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used

Table 5.38 Welch ANOVA test for significant differences between the mean Hsp90 protein expression in the pregnant and pseudopregnant treatment groups at the 5% level of significance
N.H.: There is a significant difference between the means.

Total Number of Observations: 15

F Ratio	DF NUM	DF DEN	Prob > F
0.3655	3	5.0104	0.7816

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the mean expression of Hsp90 protein in the pregnant and pseudopregnant treatment groups at the time of implantation.

5.12.4 Summaries of statistics tests for significant differences between the mean raw volumes of actin bands in the Hsp90 Western blots in the pregnant and pseudopregnant treatment groups

Table 5.39 Means and standard deviations of the raw volume of Actin bands in the Hsp90 Western blots in the pregnant and pseudopregnant treatment groups

Group	n	Mean	Std Dev	SEM
Untreated (A)	3	65604	5882.1	3396
SOOO (F)	3	56431.8	16427.5	9484
5½ day Pregnant (G)	3	57246.8	2070.4	1195
PPPE (H)	3	66361.1	23742.6	13708
CCCPPPE (I)	3	62039.2	13564.5	7831

Table 5.40 Tests for homogeneity of the raw volume of Actin protein data in the Hsp90 Western blots in the pregnant and pseudopregnant treatment groups using the O'Brien's and Bartlett's tests at the 5% level of significance
N.H: The variances are equal.

Test	F Ratio	DF NUM	DF DEN	Prob>F
O'Brien's	1.0569	4	10	0.4264
Bartlett's	1.9248	4		0.1032

p>0.05 so the N.H is rejected. Therefore there is an inequality of variances and the Welch ANOVA is used.

Table 5.41 Welch ANOVA test for a significant difference between the mean raw volume of Actin bands in the Hsp90 Western blots in the pregnant and pseudopregnant treatment groups at the 5% level of significance
N.H.: There is a significant difference between the means.

Total Number of Observations:

F Ratio	DF NUM	DF DEN	Prob > F
1.0244	4	4.2764	0.4863

p>0.05 so the N.H is rejected. Therefore there is no significant difference between the mean raw volume of Actin bands in the Hsp90 Western blots in the pregnant and pseudopregnant treatment groups. This suggests that actin is an appropriate internal control.