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The effect of clomiphene citrate

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CHAPTER 3: RESULTS

3.1 EFFECT OF CC ON ERα AND HSP90 EXPRESSION IN THE RAT UTERUS

3.1.1 Effect of a Single Dose of CC or E_2 on the Expression of ERα and Hsp90 in the Ovariectomized Rat Uterus 24hours after its Administration

Currently little is known about the effects of CC on the uterus at the molecular level, particularly with regard to its mode of action. Therefore this section documents the effect of a single dose of CC in the absence of ovarian hormones on the expression of two estrogen sensitive genes: ERα and Hsp90, in the rat uterus, 24 hours after administration of the drug. This was determined by Western blotting and Fluorescence Immunocytochemistry (ICC) and the effect of CC on ERα expression was also further analysed by RT-PCR. The results obtained were compared to 0.5μg E_2 treated rat uteri, to determine whether CC has an estrogenic or antiestrogenic effect on the expression of ERα and Hsp90. Both treatment groups were also compared to untreated and vehicle treated (control) rat uteri.

3.1.1.1 Western blotting analysis of ERα and Hsp90 protein expression in the ovariectomized rat uterus 24 hours after a single dose of CC or E_2

In order to determine the effects of CC and E_2 on the protein expression of ERα and Hsp90, Western blotting was performed on protein extracted from ovariectomized rat uteri, 24 hours after they had been injected with a single dose of either 0.25mg CC in saline (ip) or 0.5μg E_2 in oil (sc). Six blots for each protein were performed with primary antibodies raised against either ERα or Hsp90. Therefore the number of observations (n) is six. In addition the blots were incubated with a primary antibody raised against actin as an internal control, whose expression is unlikely to be affected by the various treatments and is therefore used to correct for sample to sample variation. Actin was also used so that the expression of ERα or Hsp90 protein in each treatment group could be compared in relation to actin's expression in the same treatment group.

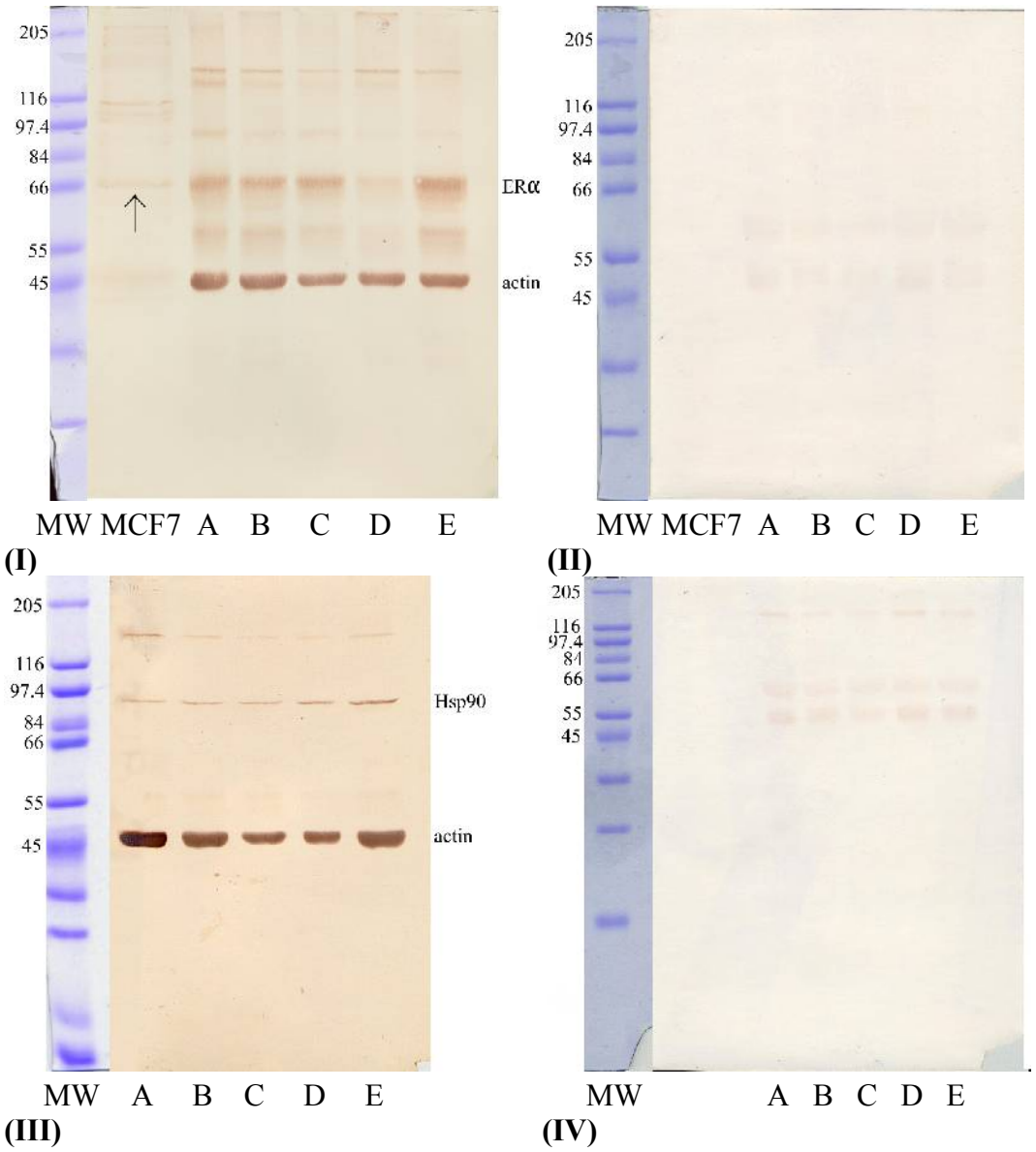


Fig.3.1 Western blots representing expression of ERα (I) and Hsp90 (III) protein, in the ovariectomized rat uterus, 24 hours after a single treatment.
Ovariectomized rats were either untreated, vehicle treated, or treated with 0.25mg clomiphene (CC), or 0.5µg 17β estradiol (E_2) and then sacrificed 24hours later. 17.5µg of uterine protein was loaded onto an SDS polyacrylamide gel, and Western blotting was performed according to materials and methods. Lanes represent molecular weight marker in kDa (MW), untreated (A), saline treated (B), oil treated (C), CC treated (D) and E_2 treated (E). Bands present at 67kDa represent ERα, at 90kDa they represent Hsp90 and at 42kDa they represent actin, which served as an internal control. Arrow indicates positive control ERα band from MCF7 breast carcinoma whole cell lysate (sc-2206 Santa Cruz Biotech). Negative controls, for ERα (II) and Hsp90 (IV), represent blots in which the primary antibody was replaced with TBS-Tween.

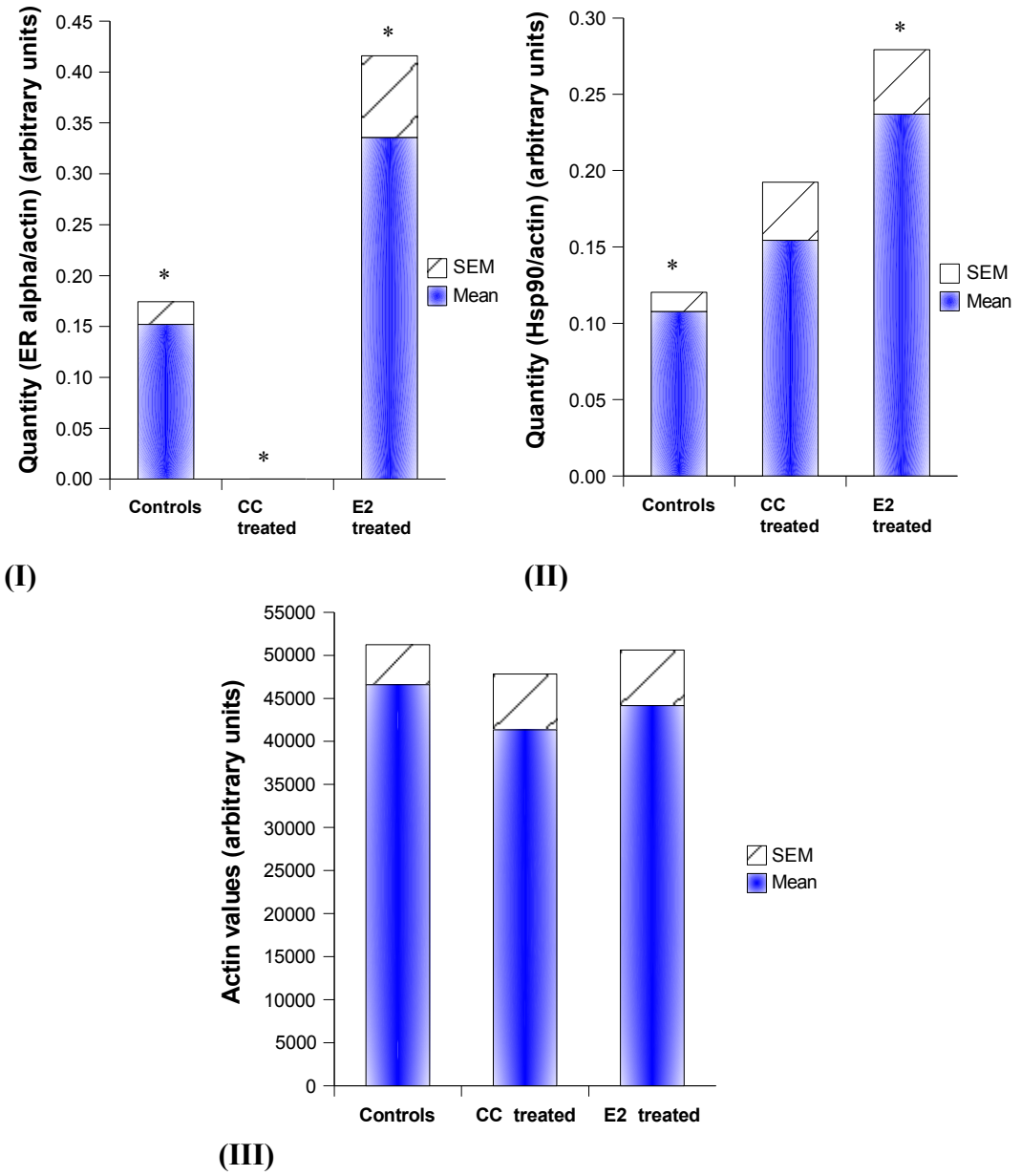


Fig.3.2 Graphs representing Mean ± SEM (Standard Error of the Mean) of ERα (I); Hsp90 (II) and actin (III) protein expression, in ovariectomized rat uteri, 24hours after a single treatment, as obtained from scans of the Western blots.
Ovariectomized rats were treated with a single (ip) injection of 0.25mg CC in saline, or a single (sc) injection of 0.5µg E_2 in oil and sacrificed 24 hours later. The controls group consists of ovariectomized rats that were untreated or vehicle treated and are taken together as one group as their values were not significantly different to each other ($p>0.05$ Appendix III [5.9.2 & 5.10.2]). Values for (I) and (II) are expressed as the quantity of ERα or Hsp90 expression over the quantity of actin expression (used as an internal control) in arbitrary units in the rat uterus.
* = significantly different ($p<0.05$) using the Tukey-Kramer *post hoc* statistics test.
In (II), the CC treated group was neither significantly different to the controls group nor to the E_2 treated group. In (III), the protein expression of actin from the blots, in arbitrary units, is shown, where none of the treatments had a significant effect on its expression ($p>0.05$), therefore showing that actin was an appropriate internal control.

Figure 3.1 represents Western blots showing the protein expression of ERα (I) and Hsp90 (III) with the different treatments A-E. In Fig.3.1(I) bands at 67kDa and 42kDa are detected and represent ERα and actin respectively, as these proteins are known to have these molecular weights. A positive control was also added in the form of a whole cell lysate of the MCF7 breast carcinoma cell line, which is known to express ERα. The band at 67kDa, in the MCF7 sample lane (arrow), aligns with the bands at 67kDa in the lanes A-E, where A represents untreated; B: saline; C: oil; D: CC; and E: E_2 treated samples. Therefore these bands do represent ERα protein. A negative control blot, where the primary antibody to ERα was replaced with TBS-Tween, is also shown in Fig.3.1(II). Although some very light bands are visible in the negative and positive blots, none of these align to 67kDa or 42kDa, therefore indicating that the primary antibodies were specific to the ERα or actin protein respectively in (I).

In Fig.3.1(III) bands at 90kDa and 42kDa are detected representing Hsp90 and actin respectively, as these proteins are known to have these molecular weights. A negative control blot is also shown in Fig.3.1(IV) and although there are some light bands in the negative blot, they do not align to 90kDa or 42kDa, and hence indicate primary antibody specificity in (III).

The presence of the non-specific bands between 205 and 116kDa in both the ERα (I) and Hsp90 (III) blots, indicates that these bands originate from the antibody used to identify actin. They, most likely, arise from the primary antibody, due to their absence in the negative blots. It must however be emphasized that the non-specific bands did not align with the known molecular weights of ERα or Hsp90, and therefore do not interfere with the results.

In Fig.3.2 the protein expression of ERα (I) and Hsp90 (II) are represented in graphical form as the mean ± standard error of the mean (SEM) in arbitrary units. The observations for the control groups (untreated, saline and oil treated [A-C]) were pooled into one group, as their means were not significantly different to each other ($p>0.05$ Appendix III [5.9.2 & 5.10.2]). The number of observations (n) for the controls was therefore 18 (6 per control treatment) and for both the CC (D) and E_2 (E) treatment groups it was six.

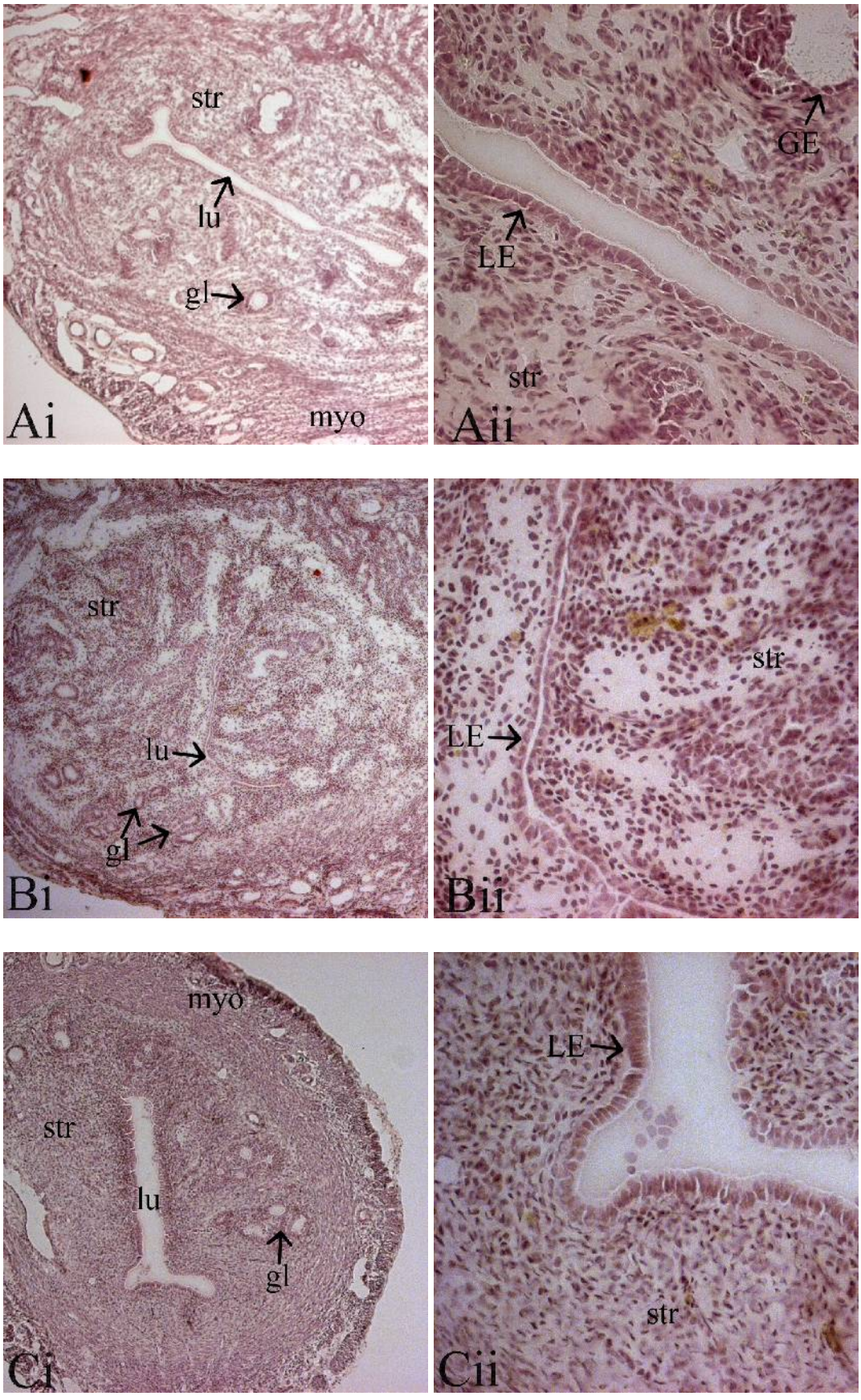
Western blot analysis (Fig.3.1(I) and Fig.3.2(I)) shows that a single dose of E_2 significantly upregulated the expression of ERα protein in the rat uterus (0.34 ± 0.08; $p<0.05$), compared to controls (0.15 ± 0.02), 24 hours after treatment (Appendix III [5.9.3]). However, CC downregulated ERα protein to undetectable levels in the rat uterus (0.00 ± 0.00) 24 hours after treatment. This decrease was both significantly different to the controls and to the E_2 treated groups ($p<0.05$; Appendix III [5.9.3]).

In Figs.3.1(III) and 3.2(II), the expression of Hsp90 protein is shown. A single dose of E_2 significantly upregulated Hsp90 expression (0.24 ± 0.04; $p<0.05$), 24 hours after treatment, compared to the control rat uteri (0.11 ± 0.01), (Appendix III [5.10.3]). In the CC treated samples (0.15 ± 0.04), although there was a decrease in expression of Hsp90 protein compared to the E_2 treated samples, and an increase in expression compared to the controls, this difference was neither significantly different to the E_2 treated nor to the control samples ($p>0.05$) (Appendix III [5.10.3]).

Both CC and E_2 have been shown to affect cell division, histology and ultrastructure of the uterus (Murphy *et al.,* 1979; 1981; Luxford and Murphy, 1989; Hosie and Murphy, 1992; 1995; Knobil and Neill, 1994; Murphy, 1995). Therefore it was thought that E_2 and/or CC treatment might affect the level of actin expression in the rat uterus and thus indicate that actin might not be an adequate internal control for Western blotting (and RT-PCR). However, no significant difference was found between the raw volume values (Fig.3.2(III)) obtained for the actin bands alone in the different treatment groups ($p>0.05$; Appendix III [5.9.4 & 5.10.4]). Therefore the expression of actin was not affected by E_2 or CC treatment, at the doses used, and was an appropriate internal control. However, this does not disregard the possibility that CC and E_2 can redistribute actin within the rat uterus (Luxford and Murphy, 1989).

3.1.1.2 Histology of ovariectomized rat uteri 24 hours after a single dose of E_2 or CC

Before the spatial expression of ERα and Hsp90 proteins could be determined, the histological effects of a single dose of E_2 or CC, on the rat uterus, was observed by Light Microscopy and Haematoxylin and Eosin (H&E) staining and compared to previous studies.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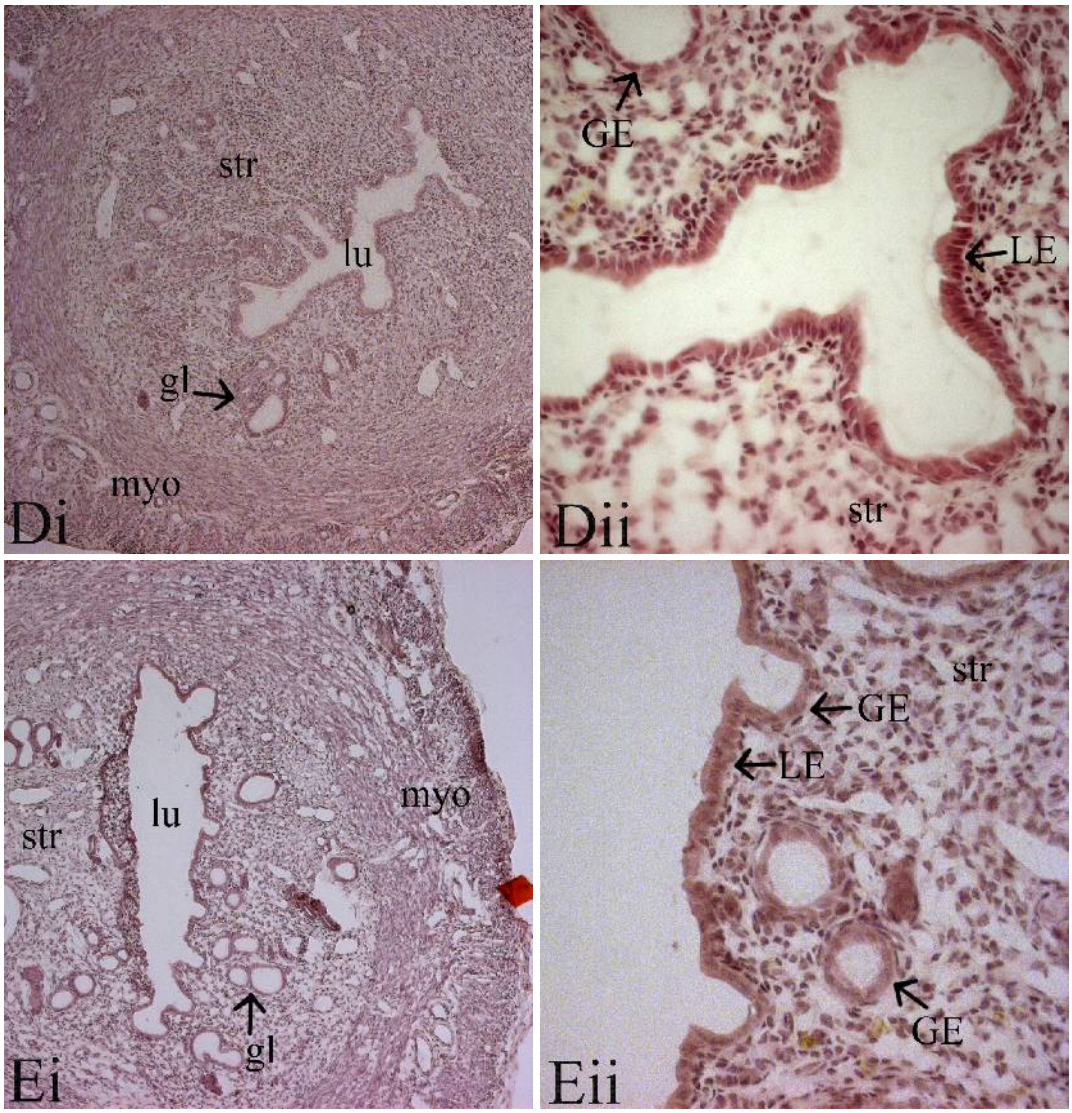


Fig.3.3 Light microscopy images representing crossections of ovariectomized rat uteri 24 hours after a single treatment and stained with Haematoxylin and Eosin. Ovariectomized rats were either untreated, vehicle treated or treated with 0.25mg CC or 0.5µg E_2, and sacrificed 24 hours later. The uteri were frozen in liquid nitrogen and 10µm crossections cut and stained with Haematoxylin and Eosin (H&E) as per the Materials and Methods. Sections represent untreated (A), and saline (B), oil (C), CC (D) and E_2 (E) treated rat uteri. LE = luminal epithelium, GE = glandular epithelium, str = stroma, lu = lumen, gl = glands, myo = myometrium. (i) = 100X; (ii) = 400X

A (untreated), **B** (saline treated), **C** (oil treated) **(controls)**: Small diameter with narrow slit-like lumen. The luminal epithelium is straight (not folded) and its cells are cuboidal in shape although, in the oil control, the cells are more columnar with basal nuclei.

D (CC treated)**:** Larger diameter than control and E_2 treated sections with a more convoluted or notched lumen than the controls. The luminal epithelium is uneven (folded) with very tall highly packed columnar cells and basal nuclei.

E (E_2 treated)**:** Slightly larger diameter than controls. Lumen is more convoluted and there are more glands in the stroma than CC and control sections. Luminal epithelium is columnar with basal nuclei, but cells are not as tall and uneven as CC treated sections.

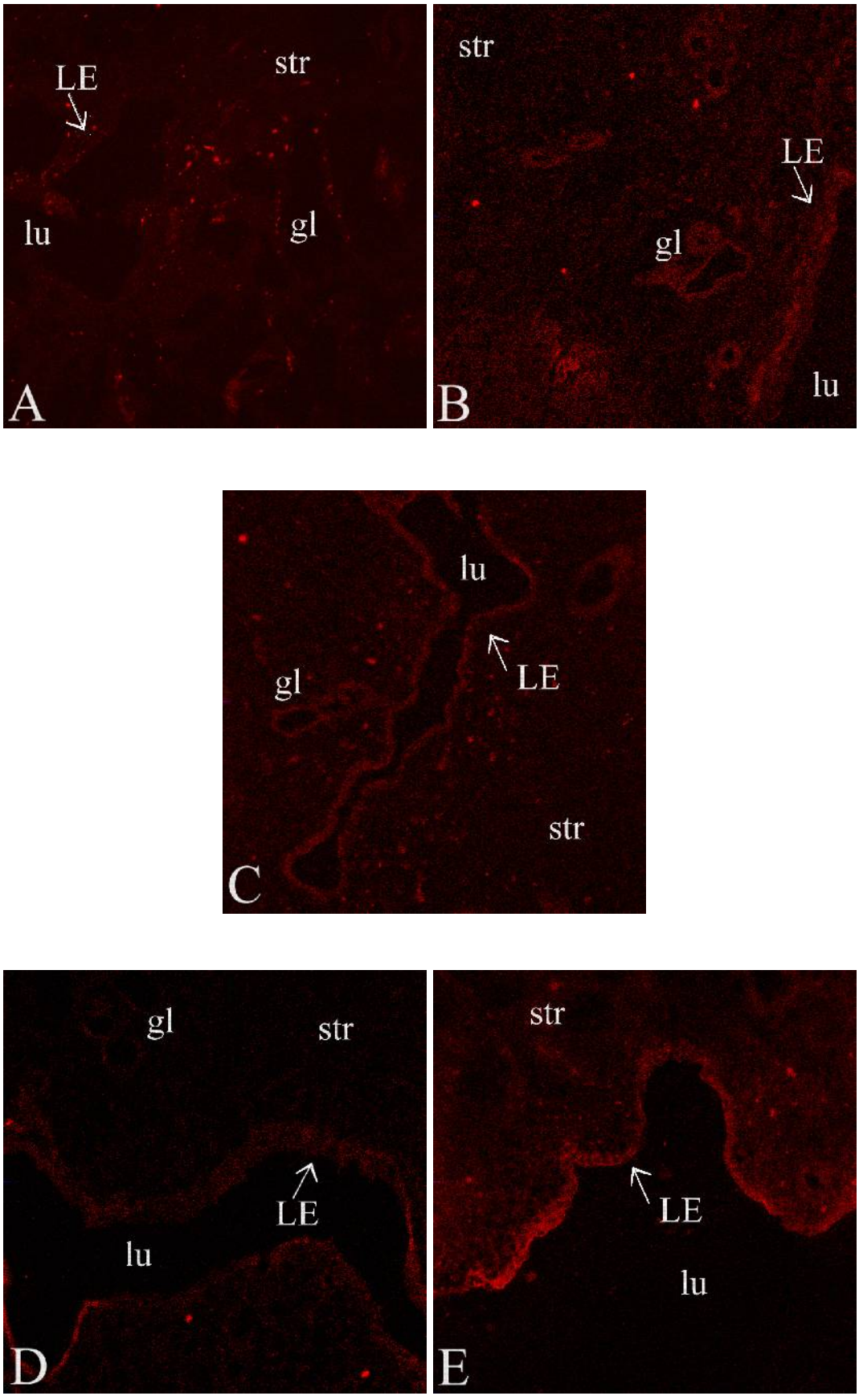
In Fig.3.3, low power (i) and high power (ii) light microscopy images of crossections of ovariectomized rat uterus, which are untreated (A), saline treated (B), oil treated (C), CC treated (D) or E_2 treated (E), are shown.
In Fig.3.3A (untreated control uteri), a typical cross section of rat uterus is shown. It has a small diameter and is round to oval in shape. All cell layers of the uterus are relatively thin. The lumen (lu) is long and narrow (slit-like), with only two indentations towards the mesometrial end, such that the surface profile of the epithelium is straight. The lumen is lined with simple, evenly spaced, cuboidal epithelium (LE), with central nuclei and well defined cell borders. The stroma (str) is approximately twice as thick as the myometrium (myo), is very cellular and contains blood vessels and glands (gl), which invaginate from the lumen. The glandular epithelium (GE) is similar to the luminal epithelium (LE) and is simple with cuboidal cells. The myometrium (myo) consists of two smooth muscle layers: an inner circular and an outer longitudinal layer. Large blood vessels are seen between the two muscle layers.
Figures 3.3B (saline treated) and C (oil treated) are similar in morphology to A (untreated uteri), except that in the oil treated section (Cii) the luminal epithelium (LE) appears to be more columnar rather than cuboidal. Overall the results of the control sections confirm and extend those of Ljungkvist, (1971a), in that they represent rat uteri that are understimulated by ovarian hormones.

In Fig.3.3D (CC treated), the most notable change that occurs is that the diameter is much larger than the control (A and B) or E_2 treated (E) sections, with an increase in the size of all compartments. The lumen shape is also more irregular (lu) and is lined by a simple tightly packed columnar epithelium (LE), the surface of which is irregular in places. These features have been observed in previous studies to be due to CC's effect on hypertrophy (increase in cell size), which has been suggested to have an adverse effect on implantation (Hosie and Murphy, 1992; 1995). It is also observed that the nuclei of the luminal epithelium, in the CC treated rat uteri, are basally situated. In the glandular epithelium there is no histological change, in comparison to the controls (A, B), and therefore it has a simple cuboidal epithelium. Overall the results confirm those of Clark and Markaverich, (1982); and Hosie and Murphy, (1992; 1995).

In Fig.3.3E (E_2 treated), there is a slight increase in the diameter of the uterus compared to the control sections (A and C), although it is not as large as in the CC treated sections (D). The lumen (lu) is more irregular than the control sections and is more open than in the control (A and C) and CC treated (D) sections. It is lined by a columnar epithelium, which is evenly spaced, and there is an increase in the height of the cells compared to the controls (A and C), although the cells are not as high as in the CC treated (D) sections. The surface is also smooth (straight) and not irregular as in the CC treated sections (D) and the nuclei are basally situated. There appears to be more glands (gl) within the stroma than in the control (A and C) and CC treated (D) sections, and the gland profiles are clustered suggesting coiling. The glandular epithelium also appears slightly taller than in the control and CC treated sections, but is still cuboidal with central nuclei. In general the results of the E_2 treated sections confirm those of Ljungkvist, (1971c); Psychoyos, (1973); Hosie and Murphy, (1992; 1995); and Knobil and Neill, (1994).

3.1.1.3 Fluorescence Immunocytochemistry of ERα and Hsp90 protein expression in the ovariectomized rat uterus 24 hours after a single dose of E_2 or CC

Although Western blotting shows the expression of proteins in a tissue, it does not locate these proteins within the tissue or cell. It is known that substances like CC and E_2 can have varying effects within a tissue, for instance between the luminal epithelium, glandular epithelium and stroma of the uterus (Clark and Markaverich, 1982). Fluorescence ICC was therefore performed to show the spatial expression of ERα and Hsp90 proteins, within the uterus and within the cells of the various compartments of the uterus, and how this expression changes with a single dose of E_2 or CC.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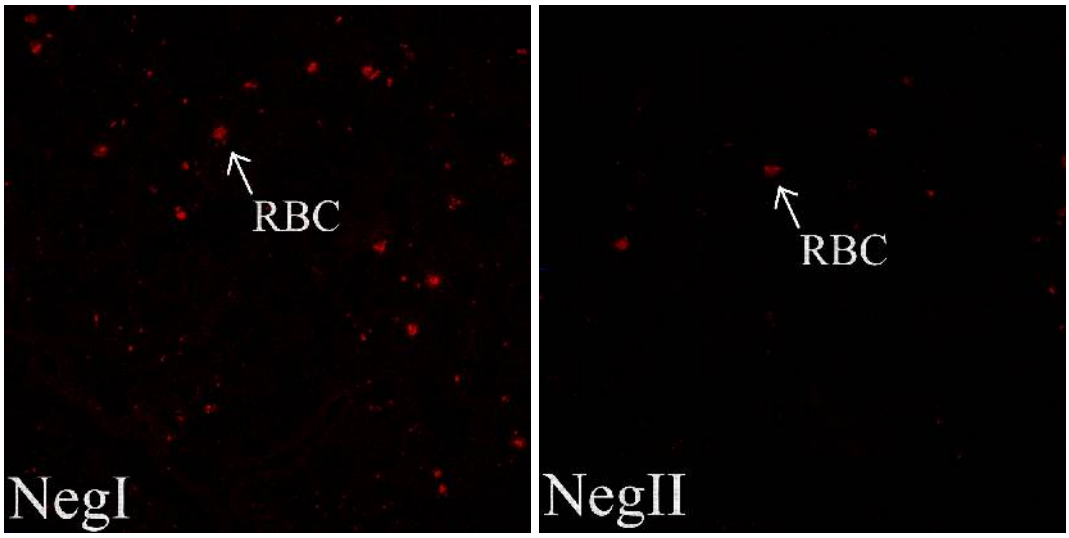


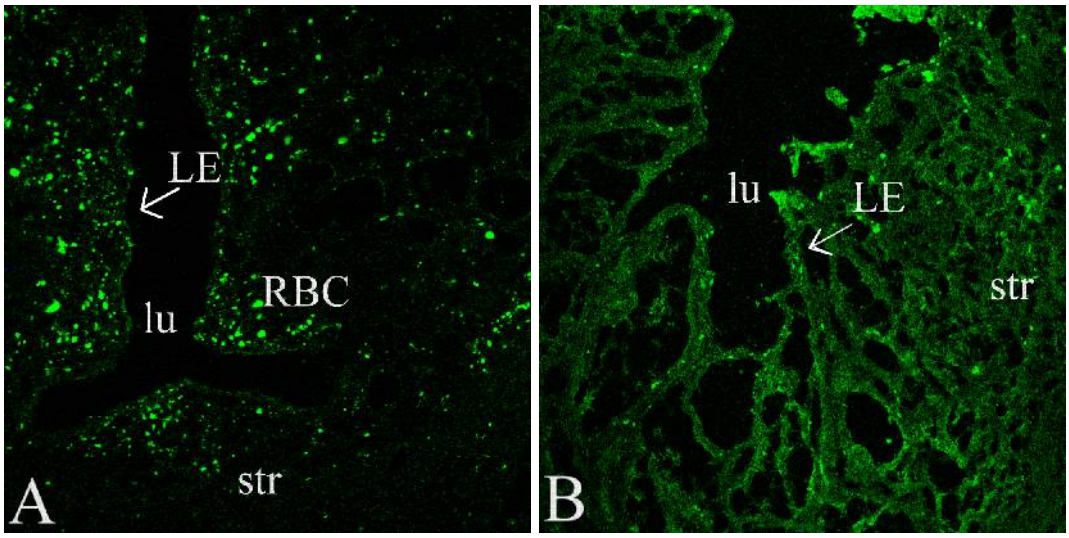
Fig.3.4 A-E, NegI and II. Fluorescence Immunocytochemistry (ICC) confocal images representing ERα protein expression, in the ovariectomized rat uterus, 24 hours after a single treatment. Crossections (10 μm) of frozen rat uteri were subjected to fluorescence ICC, as per materials and methods, using primary antibodies against ERα. Fluorescence was observed using secondary antibodies conjugated to Texas Red. Sections represent untreated (A), saline treated (B), oil treated (C), CC treated (D) and E_2 treated (E) rat uteri. Negative controls are represented by NegI (primary antibody replaced with PBS + 0.1% BSA) and NegII (secondary antibody replaced with PBS). LE = luminal epithelium, lu = lumen, str = stroma, gl = glands, RBC = red blood cells. Magnification = 250x.

B (saline treated) **and C** (oil treated)**:** Staining appears slightly more pronounced than in untreated control (A).

D (CC treated)**:** Staining reduced in stroma compared to controls (A and B) with some staining evident in luminal epithelium, which appears slightly more pronounced than in saline (B) and untreated (A) control sections.

E (E_2 treated)**:** Staining more pronounced than in control (A and C) and CC treated (D) sections, particularly in the luminal epithelium, which appears to be cytoplasmic.

NegI and II: Only autofluorescing red blood cells visible.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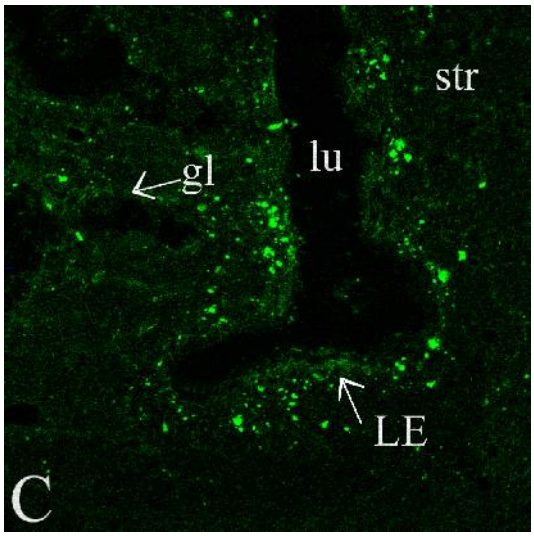
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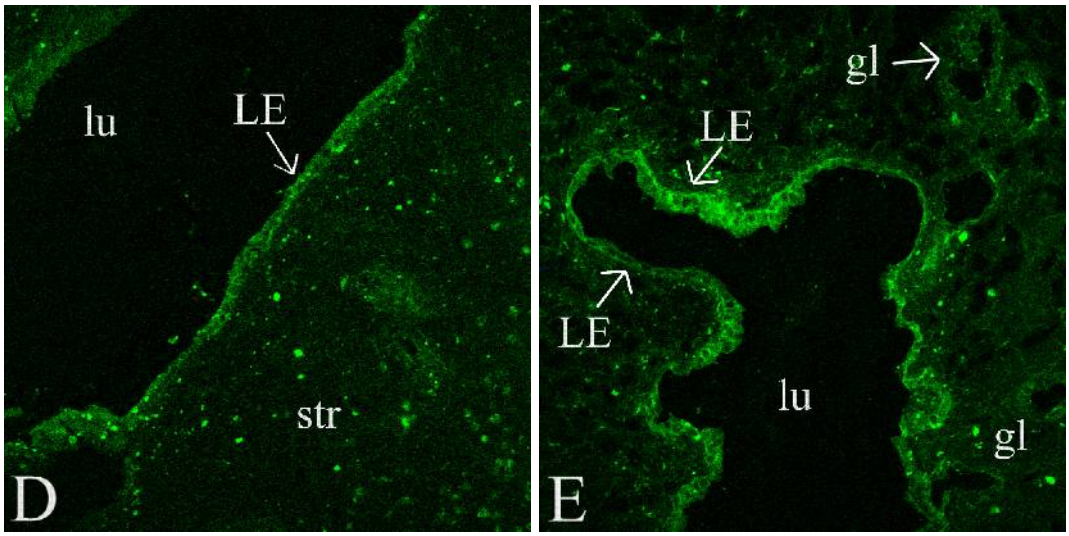
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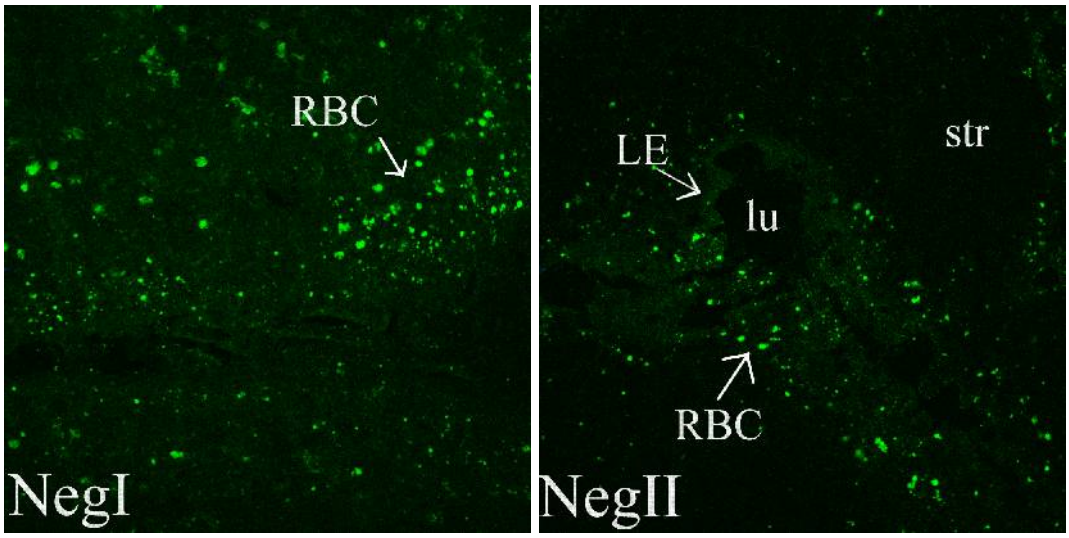


Fig.3.5 A-E, NegI and II. Fluorescence ICC confocal images representing Hsp90 protein expression, in the ovariectomized rat uterus, 24 hours after a single treatment. Crossections (10 μm) of frozen rat uteri were subjected to fluorescence ICC, as per materials and methods, using primary antibodies against Hsp90. Fluorescence was observed using secondary antibodies conjugated to FITC. Sections represent untreated (A), saline treated (B), oil treated (C), CC treated (D) and E_2 treated (E) rat uteri. Negative controls are represented by NegI (primary antibody replaced with PBS + 0.1% BSA) and NegII (secondary antibody replaced with PBS). LE = luminal epithelium, lu = lumen, str = stroma, gl = glands, RBC = red blood cells. Magnification = 250x.

A (untreated), **B** (saline), **C** (oil) **(controls)**: Controls not obviously different to each other, perhaps more staining in stroma of saline and oil treated sections.

D (CC treated)**:** Staining present mostly in the luminal epithelium, which is absent in control sections (A and B), but not as prominent as in E_2 treated (E) section. Very little staining in glandular epithelium.

E (E_2 treated)**:** Staining present in luminal epithelium, which is more pronounced than both CC treated (D) and control (A and C) sections, and appears to be cytoplasmic. Very little staining is present in the glandular epithelium (expression same as in stroma).

NegI and II: Some background staining in stroma in section without primary antibody (NegI). Autofluorescing red blood cells also present in both NegI and II.

Figure 3.4 A-E shows the expression of ERα protein in ovariectomized rat uteri that were treated with a single dose of CC or E_2 using fluorescence ICC.

In the negative controls (Fig.3.4 NegI and NegII), only autofluorescing red blood cells (RBCs), which are usually situated in small vessels, are visible indicating that the fluorescence observed in the treated sections is specific to ERα protein.

No obvious staining is observed in the control sections (Fig.3.4A and B), although some expression may be present in the stroma. In the oil treated control sections (Fig.3.4C), expression of ERα protein is seen in the luminal and glandular epithelium, whereas there was no expression in the untreated (Fig.3.4A) and saline treated (Fig.3.4B) controls. This should be taken into account when interpreting the E_2 treated samples, particularly since the H&E sections (Fig.3.3) showed an increase in luminal epithelial cell height in the oil treated sections (Fig.3.3C) in comparison to the other controls (Fig.3.3A and B). This may suggest that the oil used as a vehicle may have a slight effect on the expression of ERα as well as on epithelial cell height. It must however be emphasized that in the Western blots (Fig.3.2), no significant difference was found in ERα expression between the untreated and oil treated rat uteri. The multiple dose oil (SOOO) treated control in Fig.3.9F, also showed a similar morphology to the untreated section in Fig.3.4A, suggesting that oil does not have a significant effect on these two parameters.

In the E_2 treated sections (Fig.3.4E), expression of ERα protein is observed in the stroma and luminal and glandular epithelium (although the latter is not visible in this section). However the highest expression is found in the luminal epithelium. Although the results were not quantified, it appears as if E_2 increases expression of ERα protein in the uterus, particularly in the luminal epithelium, and this increase correlates with results obtained with the Western blots, which examined ERα expression in the whole uterus. The expression of ERα protein appears to be cytoplasmic, which is in contrast to most histological studies, which state that ERα protein is found in the nucleus (Clark and Markaverich, 1982; Knobil and Neill, 1994; Hiroi *et al.,* 1999). However in the pseudopregnant (H) and pregnant (G)

treated sections (Fig.3.10) ERα expression is found within the nuclei, both in the absence of hormones and in the hormone treated sections, thus supporting Hiroi *et al.,* (1999) that ERα expression is nuclear, independent of the presence of hormones.

In the CC treated sections (Fig.3.4D), expression is again mostly in the luminal epithelium. It appears that the expression of ERα protein is reduced in the stroma and glandular epithelium in comparison to the control (Figs.3.4A and B) and E_2 treated (Fig.3.4E) sections, which would correlate with the results obtained in the Western blots. However expression appears to be increased, although only slightly, in the luminal epithelium in comparison to the control sections (Figs.3.4A and B) and therefore may suggest that CC has different effects in the different uterine compartments, as has been observed in previous studies (Clark and Markaverich, 1982; Hosie and Murphy, 1992; 1995).

In all treatments there was no difference in the expression of ERα protein in the myometrium (myo). In summary although ERα is detected in the stroma, luminal and glandular epithelium, it is mostly present in the luminal epithelium. In addition expression of ERα protein may be affected differently in the different uterine compartments by the same SERM or ovarian hormone.

Figure 3.5A-E shows the expression of Hsp90 protein within sections of ovariectomized rat uteri treated with CC or E_2. The controls sections (Fig.3.5A, B and C) were almost identical to the negative controls (NegI and II), which only showed autofluorescing red blood cells in the stroma (str). Expression of Hsp90 protein was located in the stroma, luminal and glandular epithelium in the CC (Fig.3.5D) and E_2 (Fig.3.5E) treated sections, but was mostly found in the luminal epithelium. It also appeared to be upregulated mostly in the luminal epithelium by E_2 (Fig.3.5E) and CC (Fig.3.5D), compared to the control sections, with little expression in the glandular epithelium and stroma, indicating that these compartments may be differentially regulated by these compounds.
The fluorescence ICC results for Hsp90 expression support those of the Western blots in that E_2 increased the expression of Hsp90 protein greater than what CC did, compared to the control sections. However in the Westerns, this difference was not significant. In addition the expression of Hsp90 appears to be cytoplasmic and is mostly found in the luminal epithelium. Therefore in terms of the latter finding it appears that Hsp90 protein expression is co-expressed with ERα expression in the rat uterus.

3.1.1.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR) of ERα mRNA expression in the ovariectomized rat uterus 24 hours after a single dose of E_2 or CC

In order to strengthen the results obtained with the Westerns and ICC on the effect of CC on ERα expression, as well as to test whether this effect on ERα is at the translational or transcriptional level, RT-PCR was performed. Total RNA was extracted from ovariectomized rat uteri 24 hours after treatment with a single dose of 0.25mg CC in saline or 0.5μg E_2 in oil. Six RT-PCR runs were performed with the PCR mix containing primers for ERα and β-actin mRNA, the latter being used as an internal control to account for sample to sample variation. The PCR products were then loaded onto a 1.6% agarose gel, which was stained with ethidium bromide and the results viewed under UV light, as set out in the Materials and Methods (section 2.8).

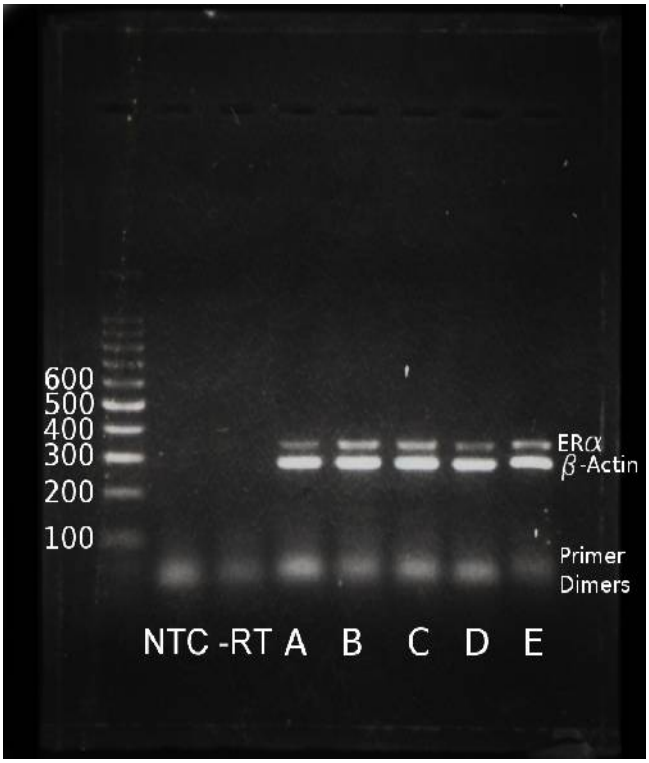


Fig.3.6 Agarose gel representing results of RT-PCR showing expression of ERα mRNA, in the ovariectomized rat uterus, 24 hours after a single treatment. Ovariectomized rats were either untreated, vehicle treated, treated with 0.25mg CC or treated with 0.5µg E_2 and then sacrificed 24 hours later and the uterus removed. 0.04µg of total RNA was converted to cDNA and then amplified by PCR, as per materials and methods (two-step RT-PCR). 5µl of PCR product was loaded onto a 1.6% agarose gel, stained with Ethidium Bromide and viewed under UV light. Lanes represent a "no template control" (NTC), where cDNA in one sample was replaced with nuclease free water; a "minus reverse transcriptase control" or "non-amplification control" (-RT) where the reverse transcriptase in one sample was replaced with nuclease free water; untreated (A), saline treated (B), oil treated (C), CC treated (D), and E_2 treated (E) samples. A molecular weight marker in increments of 100bp is also shown on the left of the gel. Bands present at 344bp represent ERα and bands present at 280bp represent β-actin, which served as an internal control.

A “no template control” (NTC), in which no cDNA template was added to the PCR reaction mixture, was added in every run to account for any cross contamination during the PCR reaction. In addition a “minus reverse transcriptase control” (-RT), in which no reverse transcriptase was added to the first strand synthesis reaction mixture, was also included in each run to account for any genomic DNA present in the total RNA extract. The bands representing expression of ERα mRNA in the different treatments groups within each gel were visually compared. The band with the weakest signal in each gel was assigned a + sign and those with increasingly strong signals were assigned an increasing number of + signs. One of the gels showed results that were very inconsistent with the others, and therefore it was eliminated. Table 3.1 represents the results of 5 gels and Fig.3.6 represents the best representative gel describing the results from the table.

Table 3.1 Table representing semiquantitative results from RT-PCR agarose gels showing expression of ERα 24 hours after a single treatment. A-E represent the different treatments from 5 gels. A + sign represents the lowest expression in that gel and an increasing number of + signs represents an increasing expression. In all gels there were no bands in the NTC or -RT lanes, indicating that there was no cross contamination and no genomic DNA was present in the initial RNA samples respectively. In addition it appeared that the expression of β-actin did not alter with any of the treatments in any of the gels and hence appeared to be an appropriate internal control. Gel 1 is represented in Fig.3.6.

Gel Number	Untreated (A)	Saline (B)	Oil (C)	CC (D)	E_2 (E)
1 (Fig.3.6)	+	++	++	+	++
2	+	+++	++	++	++
3	++++	++++	++	+	+++
4	+++	+	++	++	+++
5	++	+	++	++	+++
Total	**11**	**11**	**10**	**8**	**13**
Average	**2.2**	**2.2**	**2**	**1.6**	**2.6**

In all gels analysed, there appeared to be no difference in the expression of β-actin mRNA in the different treatments, suggesting that it was an appropriate internal control. There were also no bands present in the NTC and -RT control lanes. This indicates that no contamination was present in the samples and that there was no genomic DNA in the original RNA extract.

The expression of ERα mRNA in the different treatments seemed to differ between the different gels and in no case was the ERα mRNA expression equal between all the control samples. It should however be emphasized that the results were not quantified and therefore had the densities of the bands been quantified, in relation to β-actin mRNA expression, a more reliable result may have been achieved and the differences observed in the control bands would, most likely, be not significant.

From Table 3.1, it can be seen that in three of the five gels performed, E_2 (E) upregulated ERα mRNA expression compared to its vehicle control (oil; C), whilst in the other two gels, no change was shown. In addition in three of the gels, E_2 (E) upregulated ERα mRNA expression compared to the untreated control (A), including the gel represented in Fig.3.6. This suggests that E_2 upregulates ERα expression in the rat uterus, 24 hours after its administration, which correlates with the Western blots and ICC results above.
In four of the gels, CC (D) downregulated ERα mRNA expression compared to the E_2 treated samples (E), including Fig.3.6, which correlates with the results obtained in the Western blots. In addition, three of the gels showed that CC downregulated ERα mRNA expression compared to its vehicle control (saline; B), including Fig.3.6, whilst the other two gels showed upregulation by CC. However only two gels showed that CC (D) downregulated ERα mRNA expression compared to the untreated control (A), with the other two gels indicating no difference, including Fig.3.6, and only one showed an increase in expression of ERα mRNA by CC. It is therefore difficult to determine how CC is affecting ERα at the mRNA level. However, when considering the most repetitive results, CC downregulates ERα mRNA, thus correlating with the Western blot results and indicating that CC downregulates ERα at the transcriptional level. However due to the large decrease observed in the Western blots in response to CC, it is possible that CC also downregulates ERα at the translational level. In summary it appears as if E_2 upregulates ERα mRNA and CC downregulates ERα mRNA, 24 hours after their administration in the ovariectomized rat uterus.

3.1.2 Effect of CC on the Expression of ERα and Hsp90 in the Pseudopregnant Rat Uterus in Relation to Implantation

Rationale: CC has been shown in previous studies to interfere with implantation thus reducing the number of successful pregnancies in women using this drug. This is despite CC being able to induce superovulation in 80% of women who use it (Prasad and Kalra, 1967; Poteat, 1981; Birkenfeld *et al.,* 1985; Huet and Dey, 1987; Navot and Laufer, 1989; Nelson *et al.,* 1990; Cohen, 1991; Cummings *et al.,* 1991; Eley *et al.,* 1991; Dickey and Holtkamp, 1996; Fujii *et al.,* 1997; Hughes *et al.,* 2000). In addition E_2 has been shown to be essential in preparing a receptive uterus for implantation, as donor embryos will only implant into an ovariectomized rat uterus that has been primed with P_4, within 24 hours of administering a small amount of E_2 (Psychoyos, 1973). It was therefore of interest to test the effect of CC on two E_2 sensitive genes that form part of the estrogen signaling pathway in an attempt to try and elucidate how CC interferes with implantation at the molecular level, especially in light of the present findings that a single dose of CC appears to downregulate ERα expression in the ovariectomized rat uterus. In this section ovariectomized rats were treated with CC prior to inducing a receptive uterus for implantation and therefore a closer experiment to the situation used in patients is established, in that CC is taken before pregnancy is achieved. In addition this study will be able to test the direct effect of CC on implantation in the uterus without the interference of embryos.

3.1.2.1 Western blot analysis of the ERα and Hsp90 protein expression, in the pseudopregnant rat uterus, at the time of implantation

Western blotting was performed to determine the effects of 0.25mg CC on the protein expression of ERα and Hsp90, in the pseudopregnant rat uterus, at the time of implantation. Only 3 blots (n=3) were performed for each gene (ERα and Hsp90). Primary antibodies raised against actin were also used as an internal control. The expression of ERα and Hsp90 protein in CC treated pseudopregnant (CCPPPE treated; group I) rat uteri were compared to the expression of these proteins in pseudopregnant rat uteri without CC treatment (PPPE treated; group H), as well as to the vehicle control (SOOO treated; group F), untreated (group A), and 5½ day pregnant (Pregnant; group G) rat uteri.

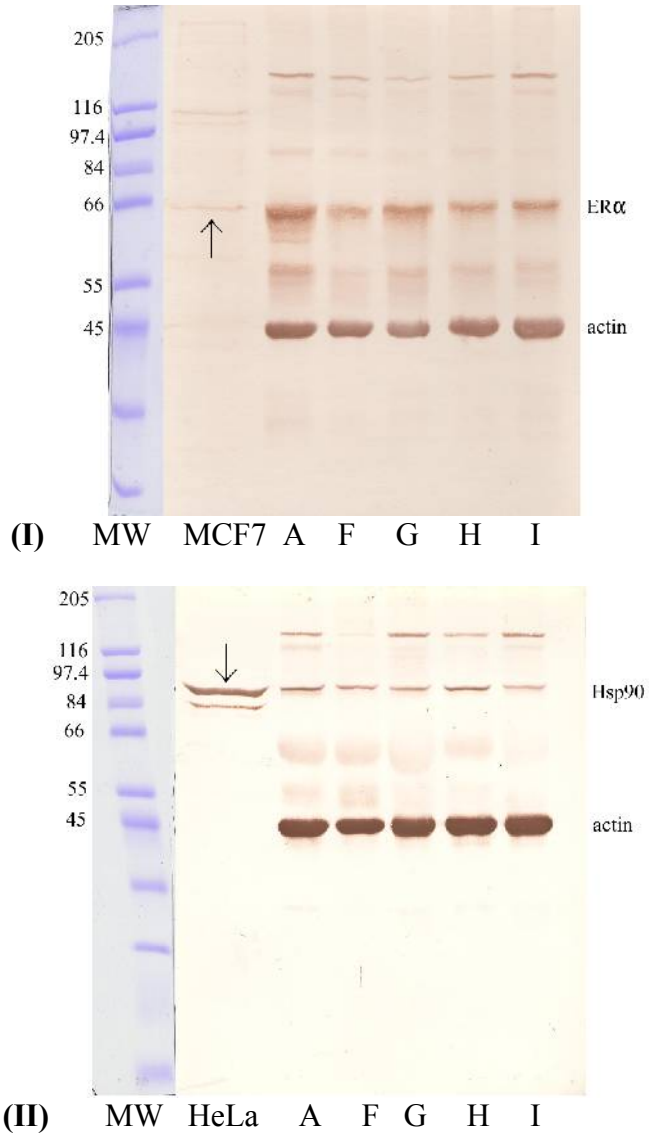


Fig.3.7 Western blots representing expression of ERα (I) and Hsp90 (II) protein, in the ovariectomized, pregnant or pseudopregnant rat uterus, with or without treatment with CC. Six mature female rats were mated with a fertile male and sacrificed on day 5½ of pregnancy (Pregnant), which is the time of implantation. In the other groups, ovariectomized rats were left untreated; treated with vehicle (1 day saline, 3 days oil [SOOO]); treated with 1 day saline, 2 days 5mg P_4 and 1 day 5mg P_4 and 0.5µg E_2 (PPPE), which induces a receptive uterus for implantation according to (Psychoyos, 1973); or treated with 1day 0.25mg CC, 2 days 5mg P_4, and 1day 5mg P_4 and 0.5µg E_2 (CCPPPE), and then sacrificed 24hours after the last injection. 17.5µg of uterine protein was loaded onto an SDS polyacrylamide gel and Western blotting was performed, according to materials and methods. Lanes represent molecular weight marker in kDa (MW), untreated (A), SOOO (F), Pregnant (G), PPPE (H) and CCPPPE (I) treated samples. Bands represent ERα at 67kDa, Hsp90 at 90kDa and actin at 42kDa, which served as an internal control. Arrows indicate positive control bands: MCF7 breast carcinoma whole cell lysate (sc-2206 Santa Cruz Biotech) in (I); and HeLa whole cell lysate (sc-2200 Santa Cruz Biotech) in (II). Negative controls for ER α and Hsp90 were shown in Fig.3.1.

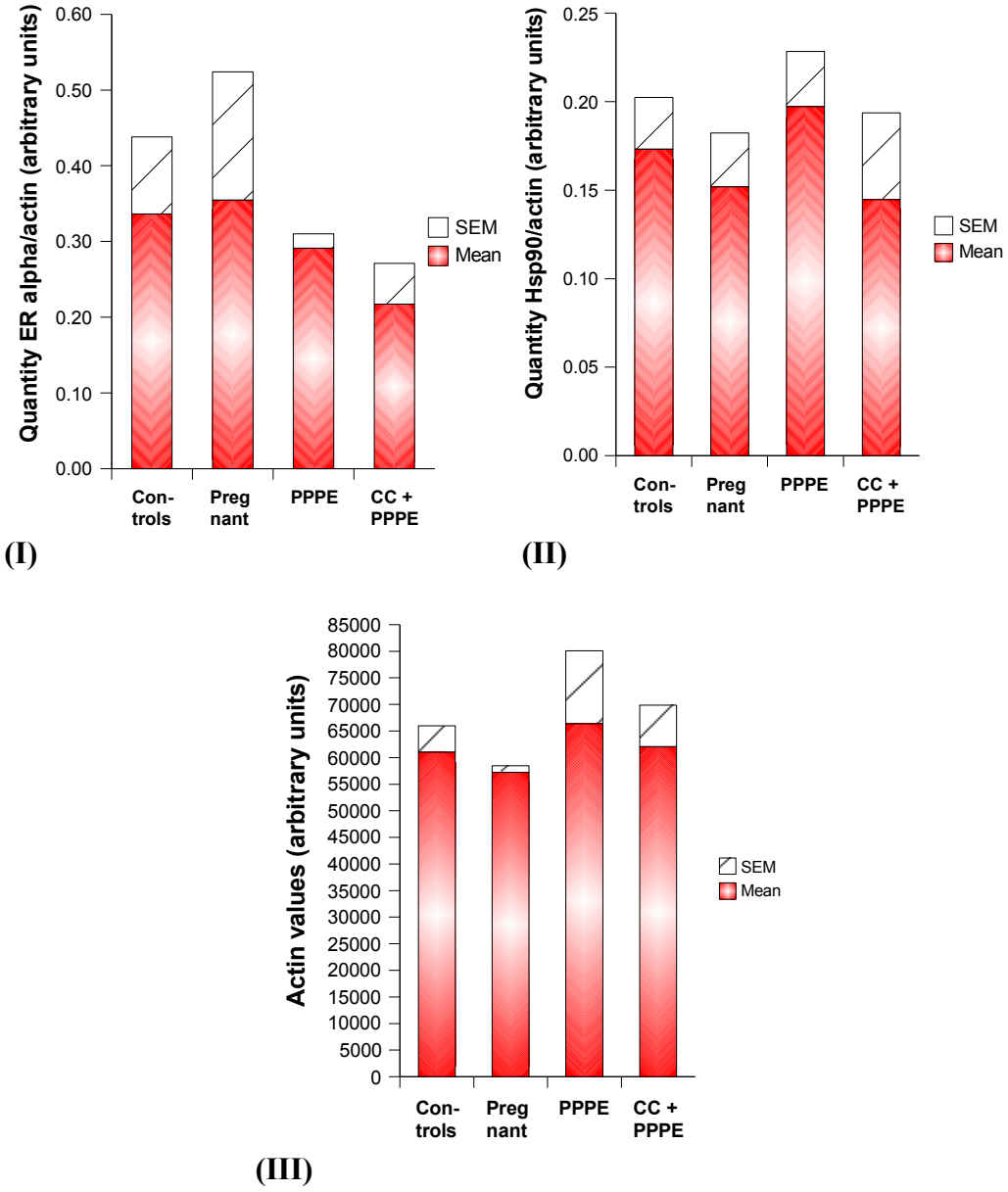


Fig.3.8 Graphs representing Mean ± SEM of ERα (I); Hsp90 (II) and actin (III) protein expression in the ovariectomized, pregnant or pseudopregnant rat uterus and the effect of CC on their expression, at the time of implantation, as obtained from scans of the Western blots. Ovariectomized rats were treated with 0.25mg CC (ip) prior to a hormone treatment regime to induce implantation (CC + PPPE), or they were treated with vehicle for 1 day followed by 2 days of P_4 and then 1 day of P_4 and E_2 to induce implantation (PPPE). Five and a half day pregnant rats were also obtained. Animals were sacrificed 24 hours after the last treatment or on day 5½. The controls group consists of ovariectomized rats that were untreated or vehicle treated for 4 days (SOOO) and are taken together as one group, as their values were not significantly different to each other ($p>0.05$ Appendix III [5.11.2 & 5.12.2]). Values in (I) and (II) are expressed as the quantity of ERα or Hsp90 expression over the quantity of actin expression, in arbitrary units, in the rat uterus. In (I), (II) and (III) none of the treatments were significantly different to each other ($p>0.05$).

Figure 3.7 represents Western blots showing the protein expression of ERα (I) and Hsp90 (II) with the different treatments: untreated (A), vehicle treated (SOOO) (F), 5½ day pregnant (Pregnant) (G), pseudopregnant (PPPE) (H), and CC treated pseudopregnant (CCPPPE) (I) rat uteri. In Fig.3.7(I) bands at 67kDa and 42kDa are detected and represent ERα and actin protein respectively. A positive control in the form of the MCF7 breast carcinoma cell line whole cell lysate, was also included, which is known to express ERα. The bands in lanes A, F-I representing ERα protein aligned with a band at 67kDa in the positive control lane (arrow in Fig.3.7(I)), and hence these bands do represent ERα protein. The same non-specific bands between 116 and 205kDa and half way between 55 and 66kDa are also present in Fig.3.7(I) as they were in the single dose treated ERα blots (Fig.3.1(I)). However they do not align with the known molecular weights of ERα or actin.

In Fig.3.7(II), bands at 90kDa and 42kDa are detected, representing Hsp90 and actin protein respectively. A positive control was also added, in the form of a whole cell lysate from the HeLa cell line (a uterine cervical adenocarcinoma), recommended by Santa Cruz as a positive control for Hsp90. The band in the HeLa lane, at 90kDa (arrow in Fig.3.7(II)), aligned with the bands in lanes A, F-I at 90kDa, indicating that these bands do represent Hsp90 protein. The same non-specific bands present, between 116 and 205kDa, in the single dose Hsp90 blots (Fig.3.1(III)) are also present in Fig.3.7(II). Negative control blots, for both ERα and Hsp90 expression, were already shown in the single dose treatment groups (Fig.3.1II and IV), indicating specificity of the primary antibodies to the bands in Fig.3.7(I and II).

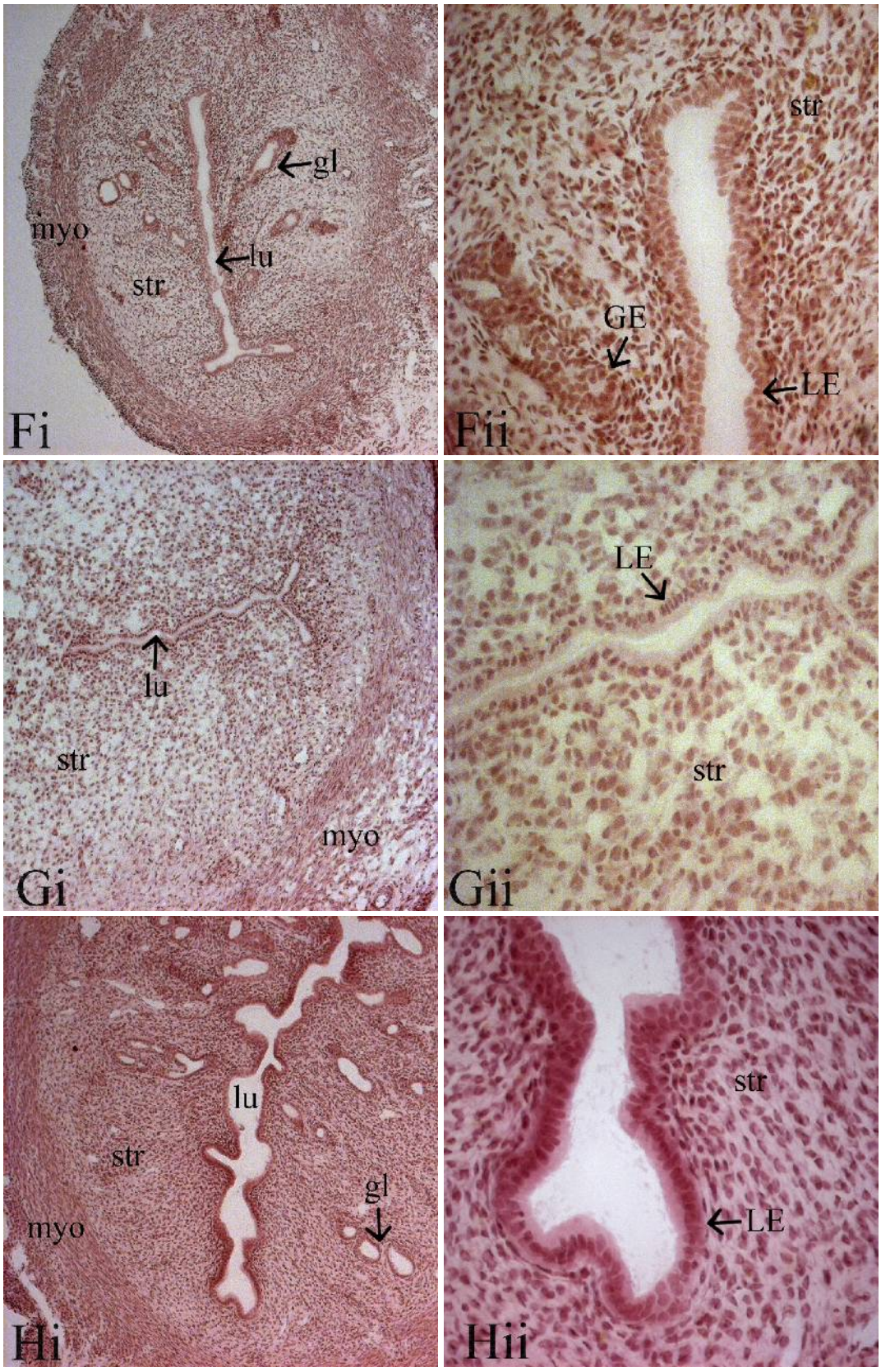
In Fig.3.8 the expression of ERα (I) and Hsp90 (II) protein are represented in graphical form as the mean ± SEM in arbitrary units. The observations for the control groups [untreated (A) and SOOO treated (F)] were pooled into one group, as their means were not significantly different to each other ($p>0.05$ Appendix III [5.11.2 & 5.12.2]) and this supports the validity of saline and oil as vehicle controls. Therefore the number of observations (n) for the controls is six [3 for the untreated (A) and 3 for the SOOO treated (F) groups] and for the remainder: pregnant (G), PPPE (H) and CCPPPE (I) treated groups, it is three each.

Western blot analysis (Fig.3.8(I)) shows that there is no significant difference between the mean ERα protein expression for any of the treatment groups ($p>0.05$ Appendix III [5.11.3]), even though in Fig.3.7(I) it appears as if the untreated (A) and pregnant (G) samples have a higher expression than the other treatment groups. This is most likely due to small sample to sample variation and hence reinforces the need for an internal control.
Figure 3.8(I) appears to show that CC (0.22 ± 00.05) lowers the expression of ERα protein, in the pseudopregnant rat uterus, at the time of implantation, compared to the other groups (control: 0.34 ± 0.10; pregnant: 0.35 ± 0.17; and PPPE: 0.29 ± 0.02 treated samples). Although this was not shown to be significantly different, it possibly suggests a trend and supports the results, in the present study, obtained with the single dose CC treatment in section 3.1.1.1.

In Fig.3.8(II), the expression of Hsp90 protein is shown. No significant difference was found between the mean Hsp90 protein expression for the different treatment groups ($p>0.05$ Appendix III [5.12.3]), particularly between the pregnant (0.15 ± 0.03); control (0.17 ± 0.03); and PPPE (0.197 ± 0.03) treated groups.
Although no significant difference was found between the PPPE and CCPPPE treated groups, there appears to be a decreased expression of Hsp90 protein in the CCPPPE treated samples (0.14 ± 0.05) compared to the PPPE (0.197 ± 0.03) treated samples. Since the only difference between these two treatment groups is the CC treatment, it is possible that, in combination with ovarian hormones, CC may downregulate Hsp90 protein, in the rat uterus, at the time of implantation.

In Fig.3.8(III) the actin protein expression in the different treatments groups is shown and no significant difference was found between these groups ($p>0.05$ Appendix III [5.11.4 & 5.12.4]), indicating that actin is an appropriate internal control.

3.1.2.2 Histology of pseudopregnant and pregnant rat uteri at the time of implantation
The general histology of the pseudopregnant rat uteri were observed by Light Microscopy and staining with H&E. Changes in the morphology of the uteri with CC treatment were also observed and compared to previous studies done.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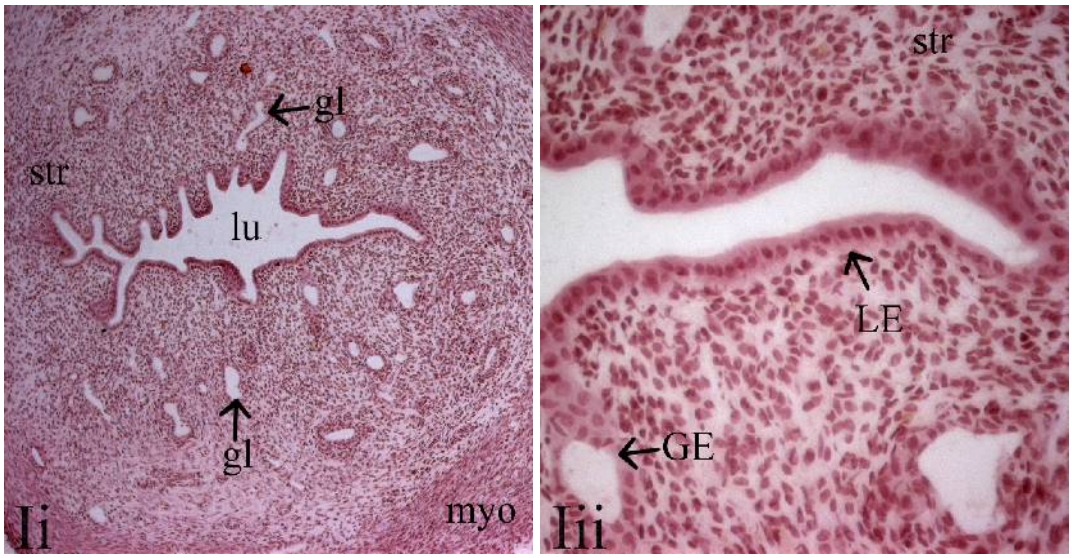


Fig.3.9 Light microscopy images representing crossections of ovariectomized, pregnant or pseudopregnant rat uteri at the time of implantation with or without treatment with CC and stained with Haematoxylin and Eosin. Six mature female rats were mated with a fertile male and sacrificed on day 5½ of pregnancy (Pregnant) at the time of implantation. In the other groups ovariectomized rats were treated with a hormone regime to induce implantation, consisting of 1 day of saline, 2 days P_4 in oil, and then 1 day P_4 and E_2 in oil (PPPE); or treated with 1 day of 0.25mg CC in saline, 2 days P_4 and then 1 day P_4 and E_2 (CCPPPE); or vehicle treated (SOOO), and then sacrificed 24 hours after the last injection. The uteri were frozen in liquid nitrogen and 10μm crossections cut and stained with Haematoxylin and Eosin (H&E), as per the Materials and Methods. Sections represent SOOO (F), pregnant (G), PPPE (H), and CCPPPE (I) treated rat uteri. LE = luminal epithelium, GE = glandular epithelium, str = stroma, lu = lumen, gl = glands, myo = myometrium. (i) = 100X; (ii) = 400X

F (SOOO treated): Similar to untreated section in Fig.3.3A. Small diameter with straight lumen, which is relatively closed. No stromal edema is evident. Although this section is obliquely cut, the glandular and luminal epithelium are cuboidal in shape (arrows in ii).

G (Pregnant)**:** Diameter of uterus is large with slightly convoluted lumen in which the luminal surfaces are closely opposed. There is evidence of stromal edema. Luminal epithelium is columnar with basal nuclei.

H (PPPE treated)**:** Diameter of uterus is large with convoluted lumen, which is relatively closed, but not as closed as pregnant (G) section. No stromal edema is present. Luminal epithelium is columnar with basal nuclei (see arrow in ii)

I (CCPPPE treated)**:** Diameter of uterus is large with a very convoluted lumen, which is more open than the control (F), pregnant (G) and PPPE treated (H) sections. No stromal edema is present. Luminal epithelium is columnar but with central nuclei.

In Fig.3.9, low (i) and high (ii) power light microscopy images of crossections of uteri that represent vehicle treated (SOOO) (F), 5½ day pregnant (G), PPPE treated (H) and CCPPPE treated (I) rat uteri, are shown.

Section F (SOOO treated) represents a typical crossection of uterus that is very similar in morphology to the untreated section in Fig.3.3Ai. It has a small diameter, which is round to oval in shape. The lumen (lu) is relatively open, is slit-like or straight with no convolutions, except for two indentations towards the mesometrial end. The section chosen is cut slightly obliquely as the luminal epithelium appears stratified, however, in parts (LE and arrow), it can seen that the lumen has a simple cuboidal epithelium lining its surface. The nuclei of the luminal epithelium are centrally placed. The stroma is approximately twice as thick as the myometrium (myo), is very cellular and contains blood vessels and glands (gl), which invaginate from the lumen. Although obliquely cut the glandular epithelium is also a simple cuboidal epithelium (GE). The myometrium consists of two smooth muscle layers: an inner circular and outer longitudinal layer. Overall the results correlate with the hormone understimulated rat uteri observed in the studies by Ljungkvist, (1971a).

In G (Pregnant), the diameter of the uterus is much larger than in the control (SOOO treated) (F) or PPPE treated (H) sections. The lumen is however quite small, and almost completely closed or obliterated. The luminal epithelium (LE) is relatively straight and lined by a simple columnar epithelium, with basal nuclei. The stroma contains some edema, possibly indicating the beginning of decidualization although no decidual cells are seen yet. Although no glands are visible in this section, they are generally lined by a simple cuboidal to low columnar epithelium.

In H (PPPE treated), the diameter of the uterus is larger than in the control (SOOO treated) (F) but smaller than the pregnant (G) sections. The lumens (lu) of the PPPE treated rat uteri are more closed and irregular in shape, than in the SOOO treated (F) sections, although the PPPE treated sections have more irregular shaped lumens than in the pregnant (G) sections. The lumen of the PPPE treated section is lined by a simple columnar epithelium with basal nuclei (LE), although in parts the

section is obliquely cut. No stromal edema is observed, in contrast to the pregnant (G) uterine sections. However the stromal cell nuclei are not as small as in the control (SOOO treated) (F) rat uteri. The morphological features, of both the pregnant and PPPE treated rat uteri, support those of previous studies done indicating the importance of P_4 and E_2, in stimulating an increase in luminal epithelial cell height (hypertrophy) and increase in epithelial and stromal cell proliferation (hyperplasia), in preparation for implantation in the uterus (Clark *et al.,* 1974; Finn and Porter, 1975; Knobil and Neill, 1994; Hewitt and Korach, 2000). The glands of the PPPE treated sections are lined by a simple cuboidal epithelium (GE), similar to the control (SOOO) (F) and pregnant (G) sections.

In I (CCPPPE treated), the diameter of the uterus is roughly the same size as in the PPPE treated sections (H), however the lumen is more open and more convoluted than in the other treatment groups. The lumen is also lined by a simple columnar epithelium (LE), but the nuclei are more centrally placed. The stroma is similar to the PPPE treated sections (H) and the glands are also lined by a simple cuboidal epithelium (GE).

3.1.2.3 Fluorescence ICC of ERα and Hsp90 protein expression in the pseudopregnant rat uterus at the time of implantation

As with the single dose treatments, fluorescence ICC was performed to show the spatial expression of ERα and Hsp90 protein, within the pseudopregnant and pregnant uteri, and how this may change with CC treatment at the time of implantation.

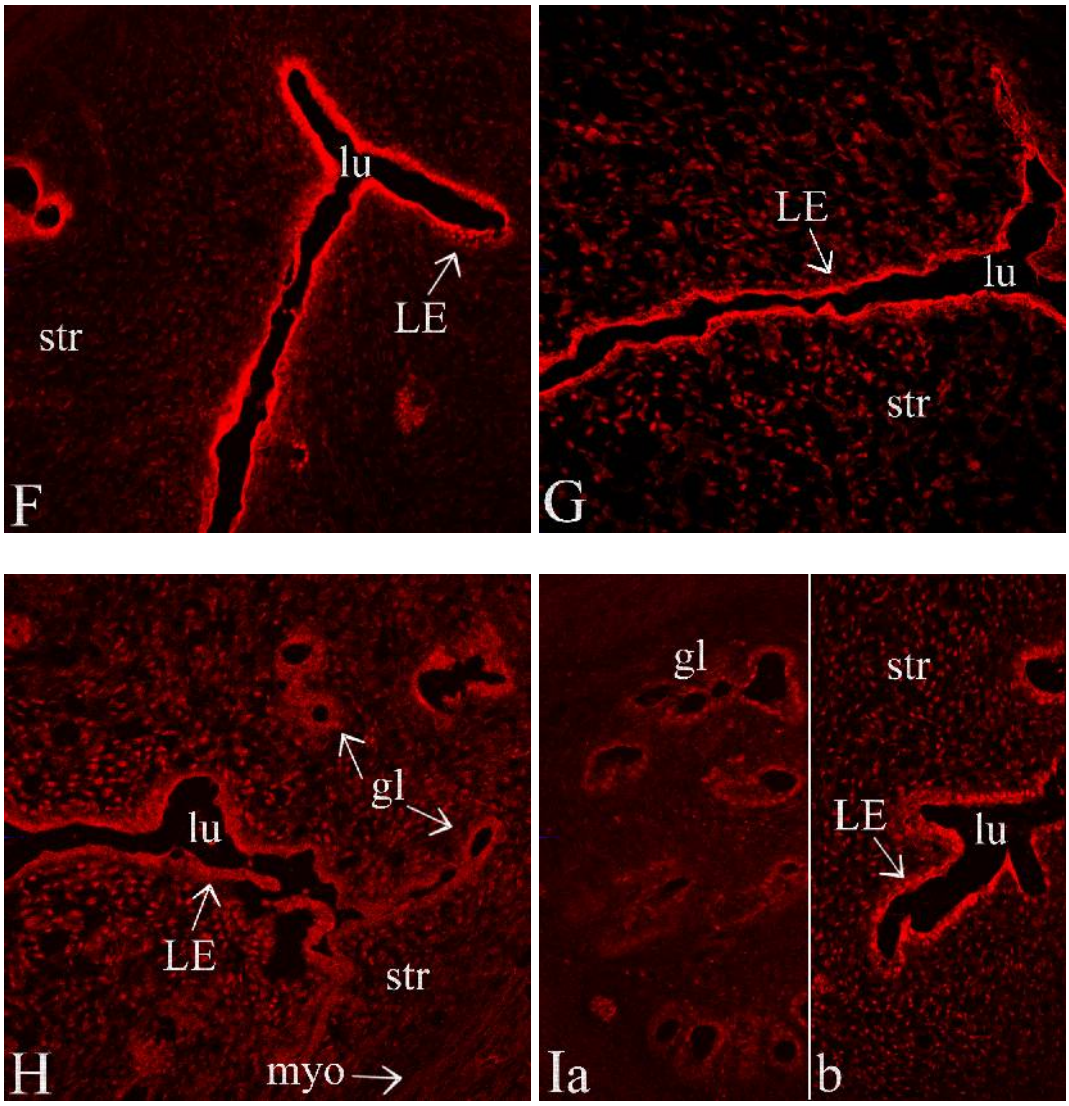


Fig.3.10 Fluorescence ICC confocal images representing ERα protein expression, in the ovariectomized, pregnant or pseudopregnant rat uterus, at the time of implantation, with or without treatment of CC. Crossections (10 μm) of frozen rat uteri were subjected to fluorescence ICC, as per materials and methods, using primary antibodies against ERα. Fluorescence was observed using secondary antibodies conjugated to Texas Red. Sections represent vehicle treated (SOOO) (F), pregnant (G), pseudopregnant (PPPE) (H), or CC treated pseudopregnant (CCPPPE) (I) rat uteri. Negative controls were shown in Fig.3.4. LE = luminal epithelium, lu = lumen, str = stroma, gl = glands, RBC = red blood cells. Magnification = 250x.

F (SOOO treated), **G** (Pregnant), **H** (PPPE treated), **I** (CCPPPE treated): No obvious difference between sections. Staining localizes to luminal and glandular epithelium and appears to be nuclear. Ia and b represent two sections showing glands (a) and lumen (b).

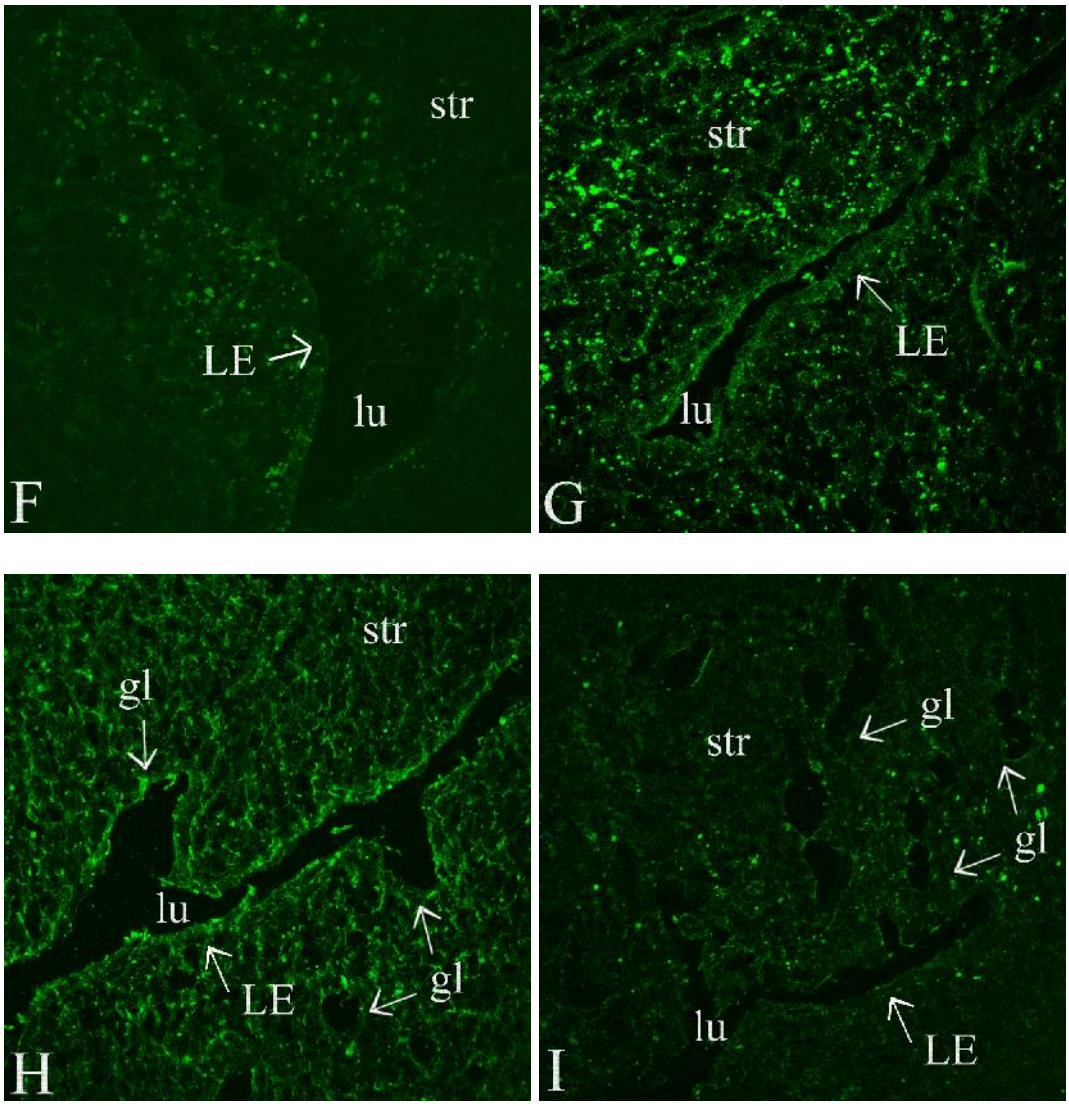


Fig.3.11 Fluorescence ICC confocal images representing Hsp90 protein expression, in the ovariectomized, pregnant or pseudopregnant rat uterus, at the time of implantation, with or without treatment of CC. Crossections (10 μm) of frozen rat uteri were subjected to fluorescence ICC, as per materials and methods, using primary antibodies against Hsp90. Fluorescence was observed using secondary antibodies conjugated to FITC. Sections represent vehicle treated (SOOO) (F), pregnant (G), pseudopregnant (PPPE) (H), or CC treated pseudopregnant (CCPPPE) (I) rat uteri. Negative controls were shown in Fig.3.5. LE = luminal epithelium, lu = lumen, str = stroma, gl = glands, RBC = red blood cells. Magnification = 250x.

F (SOOO treated), **G** (Pregnant), **H** (PPPE treated): Other than background staining being higher in the pregnant (G) and pseudopregnant (PPPE treated) (H) rat uteri, due to increased red blood cells in the stroma, there appears to be no difference in staining between the control (F) pregnant (G) and pseudopregnant (PPPE treated) (H) sections. Staining appears to be mostly in the stroma and is cytoplasmic.

I (CCPPPE treated): Staining appears to be less intense in the CC treated sections compared to the control (F), pregnant (G) and pseudopregnant (PPPE treated) (H) sections without CC treatment.

In Fig.3.10 F-I, the expression of ERα protein is shown in the 5½ day pregnant (G), PPPE treated (H), CCPPPE treated (I) and in the vehicle treated (SOOO treated) (F) rat uteri, with fluorescence ICC. The negative controls may be seen in Fig.3.4.

In F (SOOO treated), nuclear staining of ERα protein is observed in the stroma, luminal and glandular epithelium. This supports previous studies indicating that ERα expression is nuclear, independent of whether there are hormones present or not (Hiroi *et al.*, 1999). The strongest intensity of ERα expression is observed in the luminal and glandular epithelium. Although this image appears to be more intensely stained than the control sections in Fig.3.4A, the images were taken with a new laser in the confocal microscope. It may therefore be possible that under these conditions, better images were taken.

In G (Pregnant), expression of ERα is also present in the luminal and glandular epithelium and stroma. Although in this section (Fig.3.10G) ERα appears to be cytoplasmic in location, in other pregnant sections, expression of ERα was nuclear in location. There appears to be no difference in the expression of ERα between the vehicle (SOOO) treated (F) and the pregnant rat uteri (G).

In H (PPPE treated), expression is present in the luminal and glandular epithelium as well as in the stroma, and appears to be largely nuclear in location. The pseudopregnant (PPPE treated) (H) and pregnant (G) samples do not appear to differ in their expression of ERα protein.

In I (CCPPPE treated), expression of ERα protein appears to be located in the nuclei of the stroma, glandular and luminal epithelium and does not appear to be different to the pseudopregnant (PPPE treated) (H) or pregnant (G) sections.

In summary it appears that the expression of ERα protein does not differ, in the pseudopregnant rat uterus, with the treatment of CC at the time of implantation and therefore correlates with the results obtained in the Westerns blots (Fig.3.8(I)).

In Fig.3.11 F-I, the expression of Hsp90 protein is shown in the 5½ day pregnant (G), PPPE treated (H), CCPPPE treated (I), and in vehicle treated (SOOO) (F) rat uteri with fluorescence ICC. The negative controls may be seen in Fig.3.5.

In F (SOOO treated), expression of Hsp90 protein does not appear to be different to the untreated control in Fig.3.5 A, with only autofluorescing red blood cells present in the stroma.

In G (Pregnant), although there is some cytoplasmic expression of Hsp90 protein in the luminal epithelium, the section does not appear to be much different from the control section (SOOO) (F), except that there are more autofluorescing red blood cells in the stroma. This is most likely due to the increase in vascular permeability that is induced at implantation (Psychoyos, 1973; Knobil and Neill, 1994; Rockwell *et al.,* 2002).

In H (PPPE treated), there is no difference in the expression of Hsp90 protein, compared to the control (SOOO) (F) and pregnant (G) sections, although some cytoplasmic expression of Hsp90 protein can be seen in the luminal epithelium and stroma.

In I (CCPPPE treated), the expression appears to be slightly reduced, compared to the pseudopregnant rat uteri without CC treatment (PPPE) (H), indicating that CC decreases Hsp90 protein expression at the time of implantation, or when administered in combination with P_4 and/or E_2. However these differences may only be present in some parts of the uterus, and may not be significant when taking the whole uterus into context, therefore suggesting that CC does not affect Hsp90 expression, in the rat uterus, at the time of implantation.

In summary as with the Western blot results, there appears to be no change in the expression of Hsp90 with CC, in the pseudopregnant rat uterus, at the time of implantation.

3.1.2.4 Reverse Transcription-Polymerase Chain Reaction of ERα mRNA expression, in the pseudopregnant rat uterus, at the time of implantation

To further elucidate the effect of CC on ERα expression in the pseudopregnant rat uterus and to strengthen the results obtained with the Western blots and ICC, RT-PCR was performed on RNA extracted from vehicle treated (SOOO) (F), 5½ day pregnant (G), CCPPPE treated (I) and PPPE treated (H) rat uteri. Six runs were performed with primers for ERα and β-actin mRNA, the latter was used as an internal control. The PCR products were loaded onto a 1.6% agarose gel, which was stained with ethidium bromide and viewed under UV light. An "NTC" control and "-RT" control was included in every run, to account for any contamination or genomic DNA present in the RNA extract respectively. The bands representing ER α mRNA expression in the different treatment groups, within the same gel, were visually compared and assigned scores according to the intensity of the bands, in each gel, as described in section 3.1.1.4. Table 3.2 shows the results from 5 gels, as one gel showed results that were inconsistent with the others and was therefore excluded. In Fig.3.12 the best representative gel describing the results from the table, is shown.

Table 3.2 Table representing semiquantitative results from RT-PCR agarose gels representing the expression of ERα mRNA in the pregnant and pseudopregnant rat uteri and the effect CC has on its expression at the time of implantation. F-I represent different treatments from 5 gels. A + represents the lowest expression, in each gel, and an increasing number of + signs, represents increasing expression. In all gels, no bands were observed in the NTC or –RT control lanes, indicating that there was no cross contamination nor was there genomic DNA present in the initial RNA samples. It also appeared that the expression of β-actin did not alter with any of the treatments in any of the gels and thus appears to be an appropriate internal control. Gel 1 is represented in Fig.3.12.

Gel Number	SOOO (F)	Pregnant (G)	PPPE (H)	CCPPPE (I)
1 (Fig.3.12)	++	++	++	+
2	++	++	++	+
3	++	+++	++	+
4	++	+	+	+
5	++++	++	+++	+
Total	**12**	**10**	**10**	**5**
Average	**2.4**	**2**	**2**	**1**

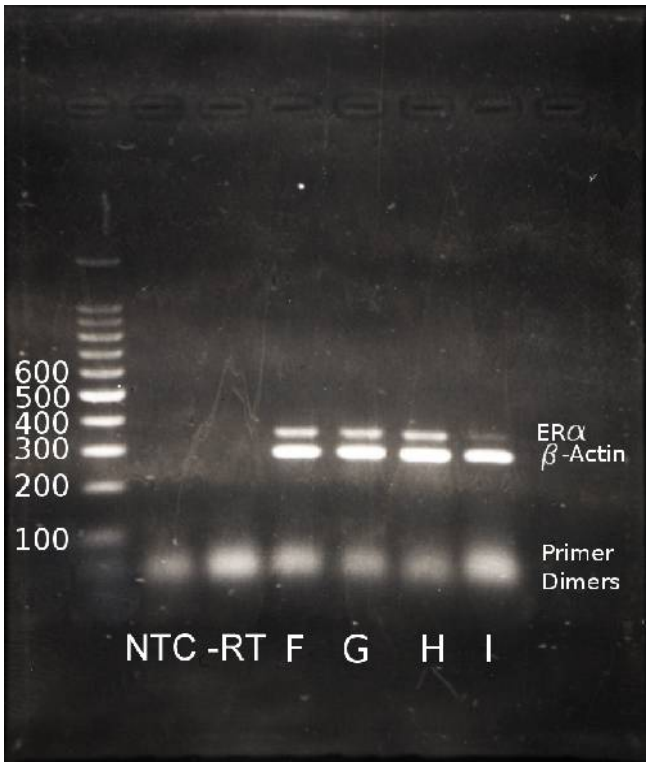


Fig.3.12 Agarose gel representing results of RT-PCR showing expression of ERα mRNA, in the ovariectomized, pregnant or pseudopregnant rat uterus, at the time of implantation, with or without CC treatment. Ovariectomized rats were treated with 0.25mg CC (CCPPPE) or saline (PPPE) prior to a hormone regime to induce implantation in the uterus, consisting of 2 days P_4 treatment and 1 day P_4 and E_2 treatment, and then sacrificed 24 hours after the last injection. Another group of ovariectomized rats were treated with saline for 1 day, and oil for 3 days as a vehicle control (SOOO) and a group of mature female rats were mated and sacrificed on day 5½ of pregnancy, at the time of implantation (Pregnant). 0.12μg of total uterine RNA was converted to cDNA and then amplified by PCR, as per materials and methods (two-step RT-PCR). 5μl of PCR product was loaded onto a 1.6% agarose gel, stained with Ethidium Bromide and viewed under UV light. Lanes represent a "no template control" (NTC), where cDNA in one sample was replaced with nuclease free water; a "minus reverse transcriptase control" (-RT), where the reverse transcriptase in one sample was replaced with nuclease free water; vehicle control (SOOO) (F), pregnant (G), pseudopregnant (PPPE) (H) and CC treated pseudopregnant (CCPPPE) (I) rat uterine samples. A molecular weight marker in increments of 100bp is also shown on the left of the gel. Bands present at 344bp represent ERα and bands present at 280bp represent β-actin, which served as an internal control.

In all gels analysed, β-actin amplification appeared to have been the same, indicating that it is an appropriate internal control. In addition, none of the gels showed bands in the NTC or -RT control lanes, therefore indicating that there was no cross-contamination during the procedure or DNA in the RNA extract respectively. Although, not all of the gels showed the same expression pattern, it must be emphasized that these results were not quantified. However two of the gels showed exactly the same expression pattern, one of which is shown in Fig.3.12.

From Table 3.2 and Fig.3.12, it appears that there is no difference between the SOOO (F), pregnant (G) and PPPE (H) treated groups, with four of the five gels showing equal expression between at least two of these groups.

Clomiphene appears to downregulate the expression of ERα mRNA in the pseudopregnant rat uterus, since except for one gel, the CCPPPE treated group represented the lowest expression of ERα mRNA and in four of the six gels the CCPPPE treated group (I) had a lower expression of ERα mRNA than the PPPE (H) treated group.

In summary it appears as if CC lowers mRNA expression of ERα, in the pseudopregnant rat uterus, at the time of implantation.

3.2 EFFECT OF CC ON HOXA10 EXPRESSION IN THE RAT UTERUS

3.2.1 Effect of a Single Dose of E_2 or CC on the Expression of Hoxa10 in the Ovariectomized Rat Uterus 24 Hours after its Administration

In addition to determining how CC affects the estrogen pathway, it was of interest to test the effect of CC on a gene known to be involved in implantation, to see if the observed effect that CC has on implantation may be brought about by gene expression changes of known implantation genes. For this reason Hoxa10 was chosen, which has been shown to be important for implantation and is upregulated by E_2 (Satokata *et al*., 1995; Benson *et al.*, 1996; Taylor *et al*., 1998; 1999; Gui *et al*., 1999; Lim *et al*., 1999; Bagot *et al*., 2000; Cermik *et al*., 2001).

First the effect of a single dose of CC was used to test the effects of this drug on Hoxa10 expression, in the absence of other ovarian hormones, and to compare its effect to the effects of a single dose of E_2.

Secondly the effects of CC, on Hoxa10 expression, was tested in the pseudopregnant rat uterus, at the time of implantation, to test whether CC affected Hoxa10 expression during implantation, thus explaining the poor implantation and pregnancy outcomes of women using this drug.

In both sections the results were examined by Western blotting and fluorescence ICC.

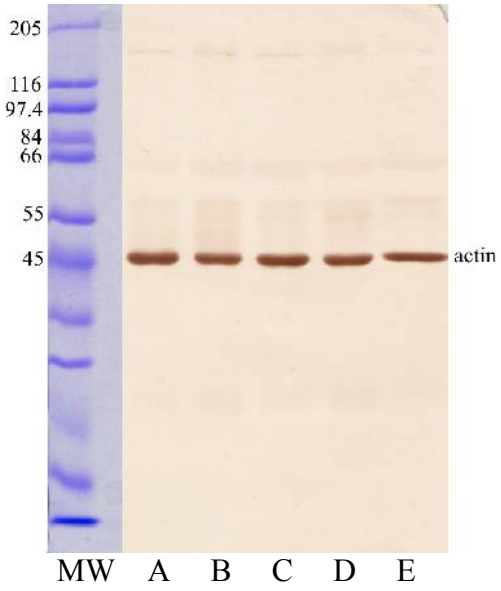
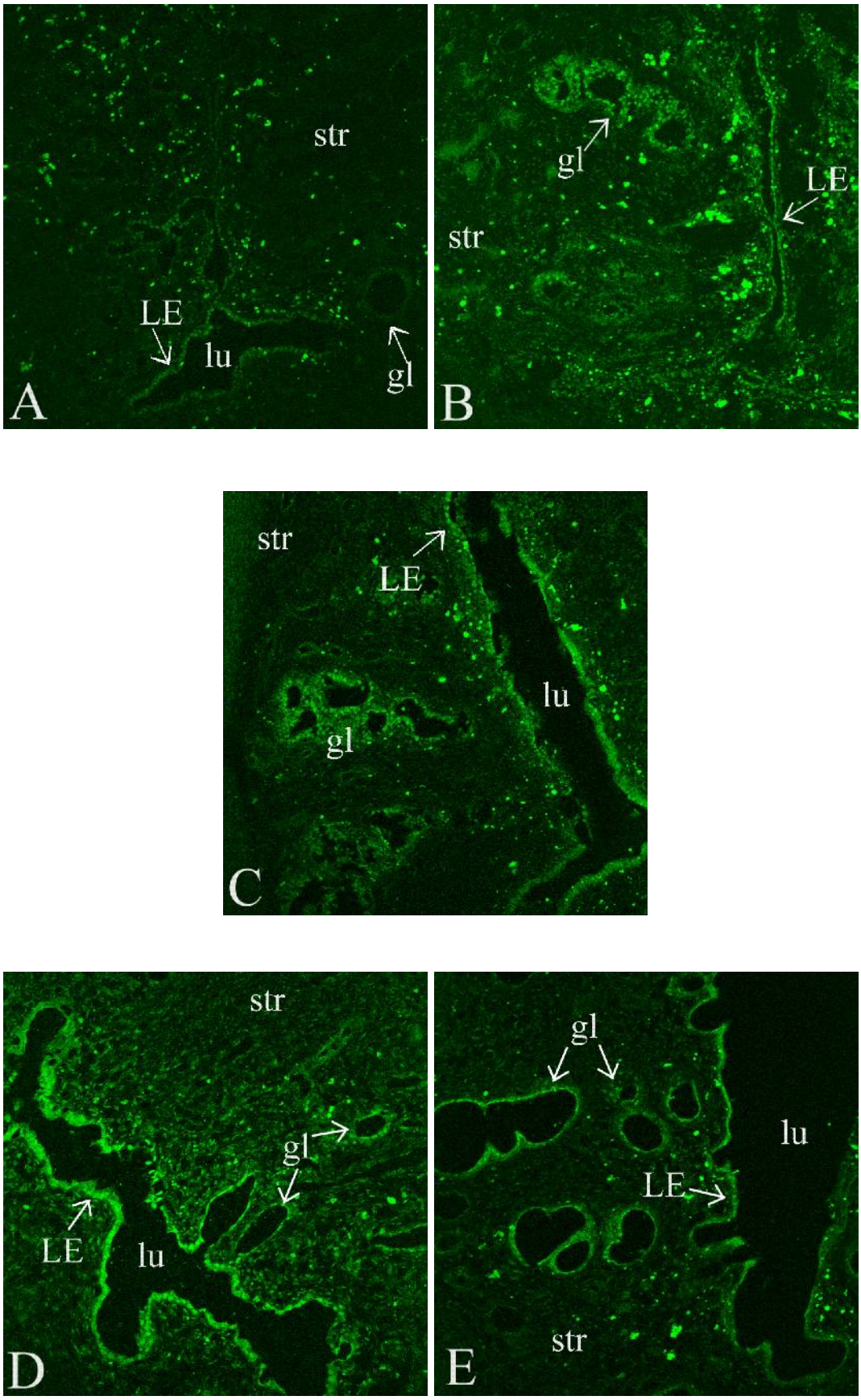


Fig.3.13 Western blot representing expression of Hoxa10 protein in the rat uterus. Ovariectomized rats were untreated, saline, oil, CC, or E_2 treated and then sacrificed 24 hours later. Total uterine protein was extracted and 35µg was loaded onto a 10% SDS polyacrylamide gel and Western blotting was performed, as per materials and methods. The primary antibodies used were raised against Hoxa10 or Actin protein, with the latter being used as an internal control. No bands corresponding to Hoxa10 protein (55kDa) were visible. Bands that are visible, only belong to Actin (45kDa) or to non-specific bands that bound to the anti-actin primary antibody, as they are also present on the other blots performed in this study for the analysis of ERα or Hsp90 protein expression.
Lanes represent: molecular weight marker (MW), untreated (A), saline treated (B), oil treated (C), CC treated (D) and E_2 treated rat uterine samples. No blots were performed for the pseudopregnant and pregnant samples.

Figure 3.13 represents a Western blot indicating the protein expression of Hoxa10 in the rat uterus with the different treatments A-E. A represents untreated, B: saline treated, C: oil treated, D: CC treated and E represents E_2 treated rat uteri. No bands corresponding to Hoxa10's known molecular weight of 55kDa were found, in spite of increasing the concentration of primary antibody, raised against Hoxa10 from the recommended starting dilution of 1:100 to 1:50, as well as increasing the concentration of total uterine protein loaded, from 17.5µg to 35µg (double). In addition, these 4 different concentration experiments were each repeated twice to account for any experimental error. The fact that, no visible bands for Hoxa10 protein expression were present, may suggest that the protein concentration of Hoxa10 in the uterus was too small to be detected by the antibody, or that the antibody was unable to detect its antigen. Since the bands at 42kDa, representing actin protein, are present, it indicates that the transfer during the blotting process and all other techniques used were successful.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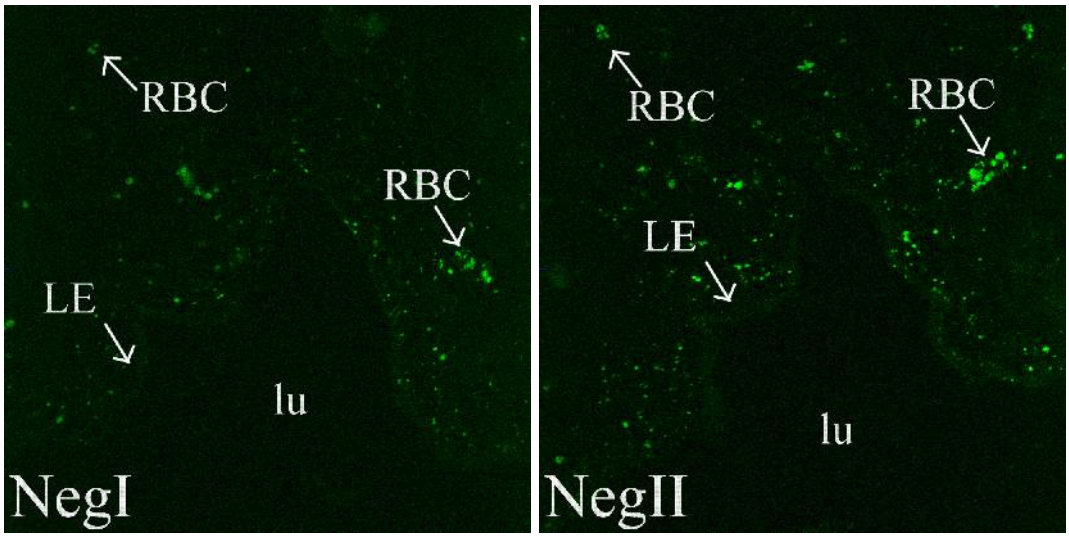

Fig.3.14 A-E, NegI and II. Immunofluorescence confocal images representing Hoxa10 protein expression, in the ovariectomized rat uterus, 24 hours after a single treatment. Crossections (10 μm) of frozen rat uteri were subjected to fluorescence ICC, as per materials and methods, using primary antibodies against Hoxa10 protein. Fluorescence was observed using secondary antibodies conjugated to FITC. Sections represent untreated (A), saline treated (B), oil treated (C), CC treated (D) and E_2 treated (E) rat uteri. Negative controls are represented by NegI (primary antibody replaced with PBS + 0.1% BSA) and NegII (secondary antibody replaced with PBS). LE = luminal epithelium, lu = lumen, str = stroma, gl = glands, RBC = red blood cells. Magnification = 250x.

A (untreated), **B** (saline), **C** (oil) **(controls):** Staining primarily found in the nuclei of the luminal and glandular epithelium, although staining is also evident in the stroma. Staining within the luminal and glandular epithelium appears slightly more intense in the saline (B) and oil (C) control sections, but is otherwise similar to the untreated control (A).

D (CC treated)**:** Although staining appears slightly more intense in the CC treated sections compared to the untreated (A) control, it does not appear to be different to its vehicle (saline) (B) control.

E (E_2 treated)**:** Does not appear to be different to the control (A and C) or CC treated (D) sections

NegI and II: Only autofluorescing red blood cells visible, which are present in the same position in these two serial sections (arrow and RBC).

In Fig.3.14, expression of Hoxa10 protein is shown in ovariectomized rat uteri, treated with CC or E_2, with fluorescence ICC. In the negative controls (NegI and II), only autofluorescing red blood cells are visible, indicating that the fluorescence seen, in the treated sections, is specific to Hoxa10. In addition these two sections are serial to each other and therefore it is observed that the autofluorescing red blood cells are in the same position in both sections (RBC and arrows).

In the control sections (Fig.3.14A-C), nuclear staining of Hoxa10 protein is observed, correlating with the fact that Hoxa10 is a transcription factor (McGinnis and Krumlauf, 1992; Krumlauf, 1994). Expression of Hoxa10 is observed in the stroma, but is more prominently observed in the luminal and glandular epithelium. Although the saline (B) and oil (C) vehicle treatments appear to have increased Hoxa10 expression compared to the untreated section (A), the multiple dose SOOO vehicle treated section, in Fig.3.15F, indicates that they do not have a significant effect as this section appears to be identical to the untreated section (Fig.3.14A). There appears to be no change in the expression of Hoxa10 protein, with E_2 treatment (E), when compared to its vehicle control (oil) (C), and its expression still appears to be located mostly within the luminal and glandular epithelium.

In the CC treated sections (D), there appears to be no difference in the staining intensity, of Hoxa10 protein expression, when compared to its vehicle control (saline) (B) and to the E_2 treated rat uterine sections (E). Hoxa10 protein continues to be expressed within the nuclei of the luminal and glandular epithelium of the CC treated group as with the other treatment groups.

3.2.2 Effect of CC on Hoxa10 Protein Expression in the Pseudopregnant Rat Uterus in Relation to Implantation

Since the Western blots proved to be unsuccessful in determining a quantitative effect of CC on Hoxa10 protein expression in the rat uterus, with the single dose treatments, they were not performed with the pseudopregnant and pregnant samples, at the time of implantation. However it was still possible to determine whether CC had an affect on the spacial expression of Hoxa10 protein at the time of implantation, in the pseudopregnant rat uterus, using fluorescence ICC.

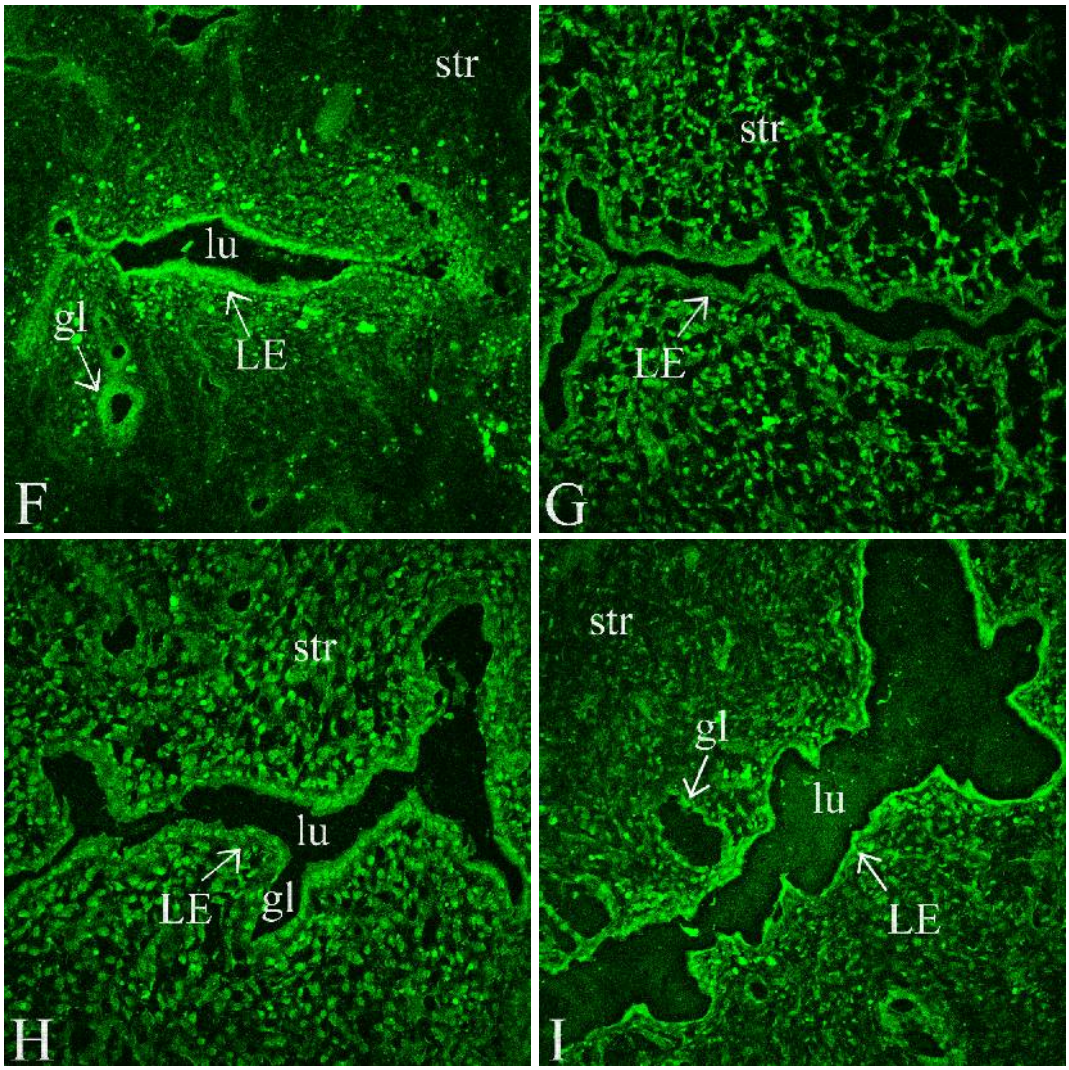

Fig.3.15 Fluorescence ICC confocal images representing Hoxa10 protein expression, in the ovariectomized, pregnant or pseudopregnant rat uterus, at the time of implantation, with or without CC treatment. Crossections (10 μm) of frozen rat uteri were subjected to fluorescence ICC, as per materials and methods, using primary antibodies against Hoxa10 protein. Fluorescence was observed using secondary antibodies conjugated to FITC. Sections represent vehicle treated (SOOO) (F), pregnant (G), pseudopregnant (PPPE) (H), or CC treated pseudopregnant (CCPPPE) (I) rat uteri. Negative controls were shown in Fig.3.14. LE = luminal epithelium, lu = lumen, str = stroma, gl = glands, RBC = red blood cells. Magnification = 250x.

F (SOOO treated)**:** Staining was most prominent in the luminal and glandular epithelium

G (Pregnant) **and H** (PPPE treated): Staining was more prominent in the stroma of these sections than in the control sections (SOOO) (F) and appears to be less intense in epithelia compared to the control sections (SOOO) (F).

I (CCPPPE treated): Expression of Hoxa10 protein was similar to the PPPE treated sections (H) in staining intensity and in terms its location within the uterus.

In Fig.3.15, the expression of Hoxa10 protein is shown in the pregnant and pseudopregnant rat uteri, with or without CC treatment, by fluorescence ICC. The negative controls may be seen in Fig.3.14.

In the vehicle treated sections (SOOO) (F), the nuclear staining of Hoxa10 protein appears to be similar to the untreated control, in Fig.3.14A, in that it is predominantly observed in the luminal and glandular epithelium, although some Hoxa10 protein is also observed in the stroma.

In the pregnant (G) and pseudopregnant (PPPE) (H) sections, expression of Hoxa10 protein is still observed in the nuclei of the luminal epithelium, but expression now appears to be more intense in the stroma than in the vehicle treated control uteri (SOOO) (F).

In the CCPPPE treated sections (I), expression does not appear to differ from the PPPE treated (H) and pregnant (G) sections at the time of implantation, in that it is mostly present in the nuclei of the stroma. Therefore CC does not appear to affect Hoxa10 expression, in the pseudopregnant rat uterus, at the time of implantation. However it must still be noted that the results were not quantified and since the results from the fluorescence ICC were visually compared, it is not a reliable technique to quantify protein expression.