The hormonal induction of cervical remodeling in the common marmoset monkey (Callithrix jacchus)

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Abstract

Controversy still exists regarding the involvement of relaxin (RLX) in cervical reorganization throughout parturition in the human, despite its well-known role in facilitating extensive extracellular matrix (ECM) remodeling in diverse organs. Therefore, the aim of the present study was to examine the influence of RLX and estrogen (E2) on the cervical tissue of the common marmoset monkey. Two experimental designs were used: 1) in vivo analysis of the intracervical diameter under locally applied RLX and 2) ovariectomized (ov) marmosets were treated systemically with either recombinant human (rh) RLX, E2 or rhRLX + E2 to examine their action on the cervix. In vivo locally applied rhRLX induced a distinct and significant widening of the cervix (before: 4.8 ± 1.1 mm versus after: 5.7 ± 0.9 mm in diameter; \( P < 0.030, \text{MV} \pm \text{S.E.M.} \)). This widening effect was most pronounced in animals without previous pregnancies. In vitro investigation of cervical tissue showed significantly increased wet weights after all three hormone treatments (E2: 0.27 ± 0.07 g, RLX: 0.25 ± 0.04 g, E2 + RLX: 0.30 ± 0.11 g; all \( P < 0.05; \text{MV} \pm \text{S.E.M.} \) versus controls (0.10 ± 0.04 g). Furthermore, morphological changes such as loosening of the connective tissue structure and decline in collagen content, an increase in the number of eosinophils, increased the expression of matrix metalloproteinases (MMP1) and MMP2, as well as gene and protein expression of the RLX receptor RXFP1 could be detected in the cervical tissue after all hormone treatments, compared with controls. In summary, RLX has a potent widening effect on the cervix of the common marmoset monkey. Although E2 is not required for this RLX effect, a combined application of E2 and RLX induced the most prominent cervical ripening.

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Introduction

The cervix as the caudal part of the uterus plays an important role throughout pregnancy and parturition. It provides a barrier to protect the intrauterine composition from the outside milieu, to hold the embryo or fetus in the uterine cavity and to prevent premature delivery of the fetus by its relatively firm structure (Maul et al. 2002). However, the cervix has to soften and widen immediately during parturition to deliver the fetus (Leppert 1995). For these opposed functions, a progressive remodeling process, which is under endocrine control (Samuel et al. 2007a, Word et al. 2007), is essential. In humans and primates, there is considerable evidence that the hormones estrogen (E2) and relaxin (RLX) are involved in the process of cervical remodeling during parturition, although their precise roles are not well understood (Word et al. 2007). Exogenously applied porcine RLX to women throughout pregnancy induced cervical ripening and caused a shorter labor time (Evans et al. 1983, MacLennan et al. 1986). By contrast, the experiments repeated with rhRLX showed no positive effects of RLX on the human cervix (Bell et al. 1993, Brennand et al. 1997). In a review of four studies involving a total of 267 women (Kelly et al. 2001), it was summarized that the role of RLX as a cervical ripening agent remained unclear due to different application form and substance used, and suggested that further investigations were urgently needed to understand the true effects of RLX in the human cervix. While several experiments have been carried out in rodents to address this issue (Samuel et al. 2007a, 2007b), there are limitations with these models due to the fact that they have a totally different endocrine situation compared with humans. Since RLX is known for its species-specific effects, data from human-relevant animal models (such as primates) are required.

Therefore, the aim of the present study was to investigate the influence of RLX and E2 on the cervical tissue with respect to a potential softening and remodeling effect. For this purpose, two sets of experiments were carried out: first, an in vivo experiment to determine changes in cervical diameter under local RLX treatment; and, secondly, an in vitro analysis of tissue remodeling by systemic hormone treatment. The experiments were performed using
female common marmoset monkeys (Callithrix jacchus) as an animal model for the human situation in reproduction (Einspanier & Gore 2005).

Results

In vivo experiment: RLX measurement of intracervical diameter

Determination of the basic intracervical diameter (pre-measurement; n=13 animals) showed a mean value (MV) of 4.8±1.1 mm (MV±S.E.M.) in average. The animals were then separated into two groups: those with previous pregnancies (p+; n=7) and those without previous pregnancies (p−; n=6). The basic cervical diameter of the p+ animals (5.1±1.2 mm) was significantly greater (P=0.039) than that measured in the p− animals (4.2±0.5 mm).

Following the evaluation of the basal value, local intracervical rhRLX treatment was applied for a 30 min incubation period. Following the application of RLX, a significant increase in the average intracervical diameter (5.7±0.9 mm) was measured versus the 4.8±1.1 mm (P=0.030), which was determined before treatment. Again, for further statistical analysis, the animals were separated according to their breeding histories. The p+ animals showed a mean diameter of 5.9±1.1 mm (P=0.032 versus pretreated measurement), whereas the p− animals showed a mean diameter of 5.3±0.5 mm after RLX treatment (P=0.025). Repeated application of RLX to the same animal after 1 month resulted in similar measurements.

In summary, the cervical diameter had increased significantly after RLX treatment of both groups of animals. However, the widening effect was higher in the animals without previous pregnancies (p−; 1.1±0.4 mm) when compared with animals that had given birth to offspring several times (p+; 0.8±0.3 mm), although their general cervical diameter was enlarged.

In vitro experiment: investigation of cervical tissues

Serum levels of progesterone, E2 and RLX of the ov animals

The detection of hormone levels from the ov animals was acquired at the time of tissue collection, 30 min after the last hormonal injection, due to the short half-life of RLX. In the control animals, low progesterone (5.5±1.1 ng/ml) and E2 levels (4.5±1.2 ng/ml) were detected, while the levels of RLX were undetectable (<0.025 ng/ml). The E2-treated animals showed high E2 levels (43.9±11.2 ng/ml; P value <0.05 versus control group) and similar low hormone concentrations of progesterone (7.7±0.7 ng/ml) and RLX (<0.025 ng/ml) as in the control group. In the RLX-treated group, concentrations of 0.41±0.06 ng/ml RLX (P value <0.05 versus control group and E2 group) and 12.1±1.7 ng/ml E2 (P value <0.05 versus control group) could be detected, whereas progesterone stayed at low levels (8.1±1.6 ng/ml). The group with combined E2+RLX treatment showed increased E2 (52.0±3.45 ng/ml; P value <0.05 versus control group) and RLX (0.35±0.05 ng/ml; P value <0.05 versus control group) contents, whereas progesterone (3.15±0.6 ng/ml) was similar to the levels in the control group.

Cervical wet weights

The wet weights from all cervices of hormonal-treated and non-treated ov animals were determined: cervical wet weights from the E2-treated (0.27±0.07 g; P=0.007), RLX-treated (0.25±0.04 g; P=0.002) and E2+RLX-treated (0.30±0.11 g; P=0.001) groups were all significantly higher (P<0.05) than measurements from the control group (0.10±0.04 g). Maximal increases in tissue wet weight were detected after combined E2+RLX treatment. Nevertheless, there was no significant variation between the hormonally treated groups (E2: P=0.256; RLX: P=0.544; E2+RLX: P=0.216).

Histology and hydroxyproline analysis of collagen

The effect of RLX on the cervix has been mainly described for rodents (Sherwood et al. 1993) and pigs (Lenhart et al. 2001). However, the findings obtained in these species cannot easily be translated to humans due to their different endocrine situations.

The histology of the control group (Figs 1A and 2A and E) represented a small cervical diameter with narrow and compact collagen fibers, as demonstrated by the blue
staining from Masson’s trichrome-stained tissues. There were only few glandular structures without any secretory activity. Both Masson trichrome (Fig. 2A) and Sirius red staining (Fig. 2E) showed intense collagen staining with blood vessels and very few eosinophil granulocytes. The compact collagen fibers demonstrated by histology were also reflected by hydroxyproline analysis of collagen content (Fig. 3). Hydroxyproline is a major component of the protein collagen and has been used as an indicator to determine collagen and/or gelatin amount.

The cervixes from the E2 group (Figs 1B and 2B and F) showed high prismatic columnar epithelium with vacuoles and secretory activity. The expanded glands were of complex structure. In contrast to the control group, the connective tissue had expanded with hypertrophic fibroblasts and loosened stromal tissue with edema (Fig. 2B). The collagen fibers were very dense directly underneath the epithelium and became much looser below this area. Furthermore, a noticeable accumulation of eosinophil granulocytes could also be detected (Figs 2F and 4), which was not significantly different from that in the control group. Mean collagen levels were lower following E2 treatment versus levels in the control group (Fig. 3).

In the RLX group (Figs 1C and 2C and G), additional and complex glandular structures were present compared with controls (Figs 1A and 2A), combined with increased uterine size. The connective tissue layer was not as narrow and compact as in the control group but less expanded and loosened compared with the E2 group with hypertrophic fibroblasts. Decreased collagen staining was present compared with the control group, and the density and organization of the collagen fiber bundles were less compared with controls. The measured hydroxyproline content of collagen in relation to

Figure 2 Histology of cervixes from (A and E) control, (B and F) E2-treated, (C and G) RLX-treated and (D and H) E2 + RLX-treated marmoset monkeys using (D) Masson’s trichrome staining and (E–H) Sirius red staining. Masson’s trichrome-stained cell nuclei (black), cytoplasm (red) and collagen fibers (blue); Sirius red-stained cell nuclei (black) and granula of eosinophil cells (red); scale bars = 50 μm.

Figure 3 Total collagen concentration (percent collagen content/dry weight) in the cervix from control (C), E2-, RLX- and E2 + RLX-treated marmoset monkeys. For each group, two animals were analyzed.

Figure 4 Number of eosinophil granulocytes in cervical tissue from systemically hormone-treated ovariectomized marmoset monkeys. Per group six animals were analyzed and eight fields from each animal were analyzed. Mean value ± S.E.M., *P < 0.05.
to weight was less than controls, but higher than that measured in the E2 and E2+RLX groups (Fig. 3). As shown in Figs 2G and 4, there was an increase in the number of eosinophils (not significant) and blood vessels in comparison to controls, which was further supported by the RLX-induced increase in vascular endothelial growth factor (VEGF) gene expression (Fig. 5).

The E2+RLX-treated group (Figs 1D and 2D and H) showed the maximal size and cervical wet weight compared with the other treated groups. The luminal surface of the cervical mucosa was lined with a mainly high prismatic epithelium containing distinct secretory activity and vacuoles. The connective tissue showed an expanded and loosened structure as well as compact areas with massive accumulations of eosinophil granulocytes, which was significant compared with the control and RLX-treated groups (P<0.014/P<0.02). The morphology of the cervix in the E2+RLX-treated group was representative of the combination of both single treatments, E2 and RLX. The combination of both hormones resulted in the most prominent decline in collagen fiber density as well as orientation, as confirmed by collagen measurement (Fig. 3). The fibroblasts were hypertrophic compared with the controls, however, the levels were not significant (P=0.056).

In general, the main feature of all hormonal treated groups was a gradual change from a compact extracellular matrix (ECM)-dominated stromal tissue towards an enlarged soft, permissible structure. However, the single treatments induced different cellular patterns: E2 showed loosening and disorganization of the net-like collagen fiber structure with edema below the first layers following the epithelium, whereas the RLX group represented a general reduced density and organization of collagen fibers within the whole cervix. The E2+RLX group showed a combined picture with dense and arranged areas of fibers as in the RLX group alternated with large areas of loosened and disorganized, net-like fiber structures similar to the E2 group. All hormonal treated primates showed activated fibroblasts and inflammatory-like features, such as an invasion and increase in the number of eosinophil cells.

**Immunohistochemistry**

The changes obtained by histology were further analyzed by immunohistochemistry using the schema described previously (Brummer et al. 2002). The expression of ESR1 (ERα) showed the following results: in the sections of the hormonally treated groups, many positive red-stained epithelial and stromal cell nuclei (+ ++ +) were apparent. In the control group, a weaker (+ − + +) expression was detected. The same trend was determined for PRAR. Immunostaining of the RLX receptor RXFP1 showed a similarly increased expression pattern as that of the steroid receptors (with hormonal treatment), with only weak expression of RXFP1 in the stromal cells of the control group, while this expression was markedly increased in several positively stained cells (+ +) in the single treated E2 and RLX groups, and to a further extent (+ + +) in the combined E2 + RLX group.

The expressions of MMP1, MMP2 and MMP9 were all increased in the hormone-treated groups, compared with their relative levels in the control group, but their distribution was varied in the four groups studied. The expressions of MMP1 and MMP2 were mainly detected in cells with the morphology of granulocytes, especially in the lamina propria. Staining for these MMPs was weak in the control group, but more prominent in the RLX group and was most prominent in the E2 and E2+RLX groups. MMP9 was only weakly expressed in the connective tissue of all groups, while the muscular cells of the tunica muscularis showed a distinct positive reaction for MMP9 in all hormone-treated groups.

**Molecular biology**

Following analysis of protein expression, RT-PCR was carried out. Consistent with the changes in their protein expression, the ESR1 (ERα) and PRAR mRNA expression was clearly up-regulated in all hormone-treated groups, compared with controls (Fig. 5). Analysis of three cervices from the RLX group indicated strong RXFP1 transcript expression, which was also consistent with the immunohistological data obtained. RXFP1 was expressed in two out of three samples from the E2 group and in two out of four primates in the combined E2 + RLX group. These results are not surprising, as in human patients there has been a high degree of variation in the expression of RXFP1 mRNA described (Luna et al. 2004, Lowndes et al. 2006).

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**Figure 5** RT-PCR for the determination of the expression of the 26S housekeeping gene, estradiol receptor α (ESR1; ERα), progesterone receptor (PRAR) and relaxin receptor (RXFP1; LGR7), and vascular endothelial growth factor (VEGF) in the cervix after systemic hormone treatment: C, control group; E2, application of 17β-estradiol; RLX, treatment with relaxin; E2+RLX, combined treatment with estradiol and relaxin.
Since histological examination demonstrated an increase of blood vessels in some of the hormonal treated groups, the gene expression of VEGF was analyzed. VEGF expression was present in three out of four different animals in the RLX+E2-treated group and in one out of three animals in the RLX group. No VEGF expression was apparent in the E2 group.

Discussion

Our main focus in this study was to examine the effects of RLX as a remodeling factor on the primate cervix, due to the recent controversial clinical results involving RLX as a cervical ripening factor in the human. With this work, we were able to show that the single application of RLX or E2 as well as the combined application of both hormones in vivo and in vitro had a prominent remodeling effect on the marmoset cervix. The short-term cervical application of RLX in vivo induced a significant widening of the tissue, although marmoset monkeys with previous pregnancies (p+) had a much wider basic cervical diameter. However, this group showed lesser widening action under RLX treatment than monkeys without previous pregnancies (p−). This reduced cervical action of p+ animals is probably due to repair mechanisms after lesions or rupture of connective tissue during parturition.

In this study, all hormonal treatments (single RLX or E2 as well as their combined application) showed a significant increase in cervical wet weight in an equal range, suggesting similar molecular mechanisms. However, their morphology was very typical for each hormone applied: edema development under E2 treatment, reduced density and organization of collagen fibers for RLX application and for the combined application of RLX+E2 a massive eosinophil cell invasion, as well as a moderate focal edema development with reduced density and organization of collagen fibers. Cervical ripening is dependent on the disruption of ordered collagen bundles (Sherwood et al. 1993) and polymorphonuclear leukocyte invasion in the cervix of humans (Junqueira et al. 1980). In our experiments, the combined application of RLX and E2 resulted exactly within these actions. It is possible that this cooperation of RLX and E2 due to the invasion of eosinophil cells could be related to the ability of E2 to increase the production of granulocyte/macrophage-colony stimulating factor (GM-CSF), which is acting chemotactically on the appropriate cells (Robertston et al. 1996). Moreover, it has been suggested that RLX as a novel leukocyte stimulatory agent can affect adhesion and chemomigration (Figueiredo et al. 2006). Furthermore, the polymorphonuclear leukocytes can increase the permeability of certain tissues by the formation of intercommunicating canals through the cervical ECM (Hibbs et al. 1982, Milks et al. 1986). These canals would then allow a rapid diffusion of hormones or diffusible molecular mediators, such as cytokines, collagenase-activating factors or nitric oxide. This would also explain the fast widening action of locally applied RLX on the marmoset cervix obtained in our study, while RLX has been shown to stimulate a number of intracellular pathways, including those involved with NO synthase II (Samuel et al. 2007a, 2007b). Therefore, RLX seems to act differently on cervical ripening: a mediator of collagen turnover by stimulating the breakdown of collagen through MMP stimulation (Palejwala et al. 2001), activation of fibroblasts, which are important for tissue remodeling and attraction of neutrophils (Malmström et al. 2007), but also to limit collagen production as well as its reorganization, while stimulating increased collagen degradation (Unemori & Amento 1990, Bryant-Greenwood & Schwabe 1994, Palejwala et al. 2001). Furthermore, RLX modulates the phenotype of fibroblasts, as demonstrated by our obtained results, and this modulation is important for ECM remodeling (Malmström et al. 2007).

In all hormonal treated groups, a decline (not significant) in cervical collagen content was detected by histological and biochemical means. This reduction of collagen was due to different mechanisms, involving an increased local MMP release, number of eosinophil cells and RLX responsibilty of the cervical tissue by increasing RXFP1 expression. These combined events led to the softening of the cervix, as evidenced by a decrease in cervical resistance. Although combined application of RLX and E2 resulted in maximal cervical ripening, from the histological picture, RLX appeared to have a regulative function on some E2 actions, such as edema formation and softening of the marmoset cervical tissue, while widening and remodeling of the cervix are apparently limited to a certain extent during pregnancy (Winkler & Rath 1999). The combined RLX and E2 application resulted in a less hypertrophic cervix with reduced edema than the single E2 application in our experiments, suggesting that RLX could have an anti-hypertrophic effect in this model. This anti-hypertrophic effect could be a self-protective action of the cervix in relation to the actual endocrine milieu, and is consistent with the anti-hypertrophic effects of RLX in cultured neonatal rat cardiomyocytes (Moore et al. 2007). In another organ, the rat cervix, a supportive interaction between RLX and E2 has been described, where RLX is a ligand-independent activator of ERs inducing edema and uterine growth (Pillai et al. 1999). However, this action of RLX could not be confirmed in our study, again supporting RLX’s difference among species and organs. This is probably due to the different tissue compositions, cooperation with various hormones/factors, variable peripheral RLX levels and different intracellular pathways used by RLX, which therefore will induce different actions.

The systemically applied RLX induced a strong gene and protein expression of ESR1, PRAR and RXFP1 in the
marmoset cervix, while RXFP1 was expressed in all RLX-treated primates, but only in some animals from the E2- and E2/RLX-treated groups, which can be due to the actual endocrine status of the primates. By correlating the expression of RXFP1 with peripheral hormone levels of the cervical samples, marmoset monkeys with RXFP1 mRNA expression always had high E2 levels, whereas primates with low peripheral E2 content showed no or weak RXFP1 expression, suggesting a possible priming effect of E2 on the RLX receptor expression. Furthermore, it is known that RLX stimulates VEGF expression by human endometrial cells in vitro (Unemori et al. 1999, 2000). This would be in line with findings in a clinical trial, in which RLX was tested as a treatment for wound healing (Unemori et al. 2008) with high VEGF expression and for progressive systemic sclerosis, where some patients showed menorrhagia or irregular menstrual bleeding (Unemori et al. 1999). However, in our study, all animals in the combined application of RLX and E2 showed some VEGF expression; in the RLX-group, just one animal was positive for VEGF mRNA expression, whereas the E2 group was completely negative. These findings could support again the necessary direct or indirect interaction between E2 and RLX to obtain the optimal effect.

In summary, the data evaluated from the present in vivo and in vitro experiments support the role of RLX as a cervical tissue remodeling factor, achieving its strongest action in combination with E2. Although these hormones act through different pathways to promote cervical ripening, the combined actions of E2 and RLX suggest that they complement one another or possibly control each other to obtain an optimal physiological situation.

Materials and Methods

Animals and treatments

Adult female marmoset monkeys (C. jacchus) from an animal colony of the German Primate Centre in Göttingen, Germany, with known breeding history were used. The animals were housed in pairs of fertile males and females kept under controlled environmental conditions (Einspanier & Hodges 1994). Two different experiments were set up to examine the softening effect of RLX and E2 alone or in combination on primate cervical tissue.

In vivo experiment

Determination of the intracervical diameter with or without local intracervical treatment of rhRLX (courtesy gift from Corthera Inc., Alta, CA, USA) was carried out to investigate the widening effect on the primate cervix. Thirteen females of the above-described marmoset colony aged between 2 and 5 years with body weights of 350–440 g and with previous pregnancies (p+; n=7) or without previous pregnancies (p−; n=6) were used in this experiment. The animals were anesthetized with Saffan (Alfaxalone/Alfadolone; Mallinckrodt Veterinary Ltd, Middlesex, UK) using i.m. injection of 0.15 ml per 100 g body weight at a time point when peripheral RLX and E2 levels were high in the marmoset monkey (Hearn 1983, Einspanier et al. 1999). The reason why this time point was chosen is due to the fact that this endocrine situation reflects the human situation throughout parturition and, due to animal right reasons, no late pregnant marmoset monkeys should have been used.

Measurements of the intracervical diameter were obtained by using 12 different sized stainless steel tubes ranging from an outer diameter of 1–12 mm in 1 mm steps (Dostal-Maschenbau, Schlitz, Germany), a similar system as described for marmoset blastocyst flushing (Thomson et al. 1994). Firstly, to determine the basic cervical value of each animal, the smallest tube was inserted and, afterwards, the size of tube was progressively increased until a specific measured resistance pressure was attained (= pre-measurement). The measurements were always performed by the same person and controlled by another person. After analysis of the basic cervical values (pre-measurements), 400 μg rhRLX/400 g (RLX dissolved in acetate) were applied locally at the cervix for a 30 min incubation period. The second cervical measurement followed after the 30 min incubation period of RLX or placebo. In general, the duration of the anesthesia was around 45 min followed by 1 h wake up phase before females were re-paired with their male partners again. This procedure was followed up three times for each animal.

In vitro experiment

In the second set of experiments, the effects of systemically applied rhRLX, E2 or E2+RLX on cervical tissue were analyzed. Twenty-three female marmosets of the same colony with body weights between 350 and 400 g averaging 5 years of age were used. The animals were ov at least 3 month before hormonal treatment and removal of the uterus as well as the cervix. Ovariectomy was carried out to delete the main physiological source of the hormones RLX and E2 and to allow controlled exogenous hormone application. Before removal of the cervices, the serum levels of progesterone, E2 and RLX were determined as described (Einspanier et al. 1999). Four groups were set up: 1) control group without hormonal treatment (vehicle, n=6); 2) E2: treatment with 35 μg 17β-estradiol per 400 g i.m. (M. semitendinosus) daily for over 10 days (n=6); 3) RLX: 100 μg rhRLX application/400 g (i.m., M. semitendinosus) twice a day (12 h apart) over 3 days (n=5); and 4) E2+RLX: combined treatment with 35 μg E2/400 g i.m. over 10 days and, additionally in the last 3 days, 100 μg rhRLX/400 g twice a day (n=6). All hormonal concentrations were calculated due to their pregnancy concentrations already published (Hearn 1983, Einspanier et al. 1999). After the end of all hormonal treatments, blood samples were collected and then the uterus with the cervix was immediately removed under Saffan anesthesia (Mallinckrodt; 0.15 ml per 100 g body weight i.m.). Cervices were then separated from the whole uterus under the stereomicroscope. Cervical wet weights were immediately analyzed after removal. The main part of the removed cervices was fixed in 4% buffered formaldehyde and embedded in paraffin for histological and immunohistochemical examination. The remaining part was...
cryoconserved at −80°C for molecular biological investigation or hydroxyproline analysis of collagen. When the cervices were too small to be divided, only a fixed sample was prepared, therefore RNA preparation or hydroxyproline analysis was not available from all samples. All experiments were approved by the appropriate animal ethics committee in Braunschweig, Germany under no. 509.42502/08-03.99(V1).

**Histology, immunohistochemistry and hydroxyproline analysis of collagen**

The paraffin-embedded cervices were cut into 5 μm pieces followed by deparaffinization of the sections. Sections were then stained with hematoxylin and eosin for an overview of the cervical morphology and Masson's trichrome staining for connective tissue structure. Furthermore, Sirius red staining was used to identify eosinophil granulocytes (Reibiger & Spanel-Borowski 2000). Representative images from each sample were captured with a Sony video camera, connected to a computer with an image analysis system (Zeiss Imaging System, Jena, Germany), and were used to quantitate changes in collagen density, as previously described (Samuel et al. 2007a, 2007b). The Sirius red staining area was calculated as a percentage of the total area within each field, and expressed as the mean ± S.E.M., with P<0.05 described as statistically significant. The protein expression of steroid receptors and factors involved in tissue remodeling (MMPs and RXFP1) were analyzed in the various treatment groups. The technique of indirect immunohistochemistry was used to demonstrate the expression of estradiol receptor α (ERα, ESR1; Euromedex, Souffelweyersheim, France), progesterone receptor A (PRAR; Immunotech, Hamburg, Germany), RLX receptor RXFP1 (previously LGR7) and MMP1, MMP2 and MMP9 (MMP1, MMP2; Santa Cruz Biotechnology Inc., Heidelberg, Germany; MMP9; MICROM International GmbH, Walldorf, Germany). For the visualization of steroid receptors and MMPs, a pre-incubation with citrate buffer (pH 6.0) and trypsin (0.0125%; Gibco BRL Life Technology) at 120°C for 30 min was required. Immunohistochemical staining with or without pretreatment contained the following steps: the sections were washed in PBS (PBS; Biochrom KG, Berlin, Germany), incubated with 3% H2O2 (Carl Roth GmbH+Co.) for 20 min and treated with 1% normal human serum for 30 min in a humidified chamber. Incubation with the primary antibodies followed overnight at 4°C. The antibodies were diluted 1:100 (ESR1 and PRAR), 1:1000 (RXFP1), 1:5 (MMP1 and MMP2) and 1:200 (MMP9). On the following day, sections were washed in PBS again and subsequently incubated with the secondary, horseradish peroxidase-conjugated antibody (Dako Cytomation; Hamburg, Germany) for 40 min. To visualize the reaction, the 3-amino-9-ethylcarbazole-peroxidase substrate kit (Vector Laboratories, Burlingame, Canada) was used. For the detection of the cytoplasmic expression of MMPs and RXFP1, slides were additionally placed into Mayer's hematoxylin to stain the cell nuclei. Finally, all sections were mounted with Immu-Mount (Shandon, Pittsburg, USA) and analyzed under light microscopy (Axioskop 2 mot plus; Axiovision 4, Zeiss). Negative controls were also included in all immunohistochemical assays, where the respective primary antibodies were replaced by an equivalent concentration of normal IgG.

For hydroxyproline analysis of collagen, the collected cervices from two primates per group were lyophilized to dry weight and handled as previously described (Bergman & Loxley 1963, Samuel et al. 2007a). The tissues were rehydrated in buffer (0.05 M Tris/HCl, pH 7.5, 0.15 M NaCl, N-ethylmaleimide (10 mM), phenylmethylsulfonyl fluoride (0.1 mM), benzamidine hydrochloride (1 mM) and EDTA (10 mM)) for 24 h at 4°C. The samples were defatted in 2:1 chloroform:methanol mixture for 24 h before being rehydrated again for 60 h at 4°C. The samples were then frozen in liquid nitrogen and chopped finely before the overall collagen content was determined by hydrolyzing the finely diced tissue in 1.0 ml, 6M HCl at 110°C for 24 h. The hydrolyzates were evaporated to dryness in the presence of sodium hydroxide, and the residues redissolved in 2.5 ml of 0.1 M HCl. Duplicate of 10 and 20 μl aliquots from each sample were analyzed for hydroxyproline, as described by Bergman & Loxley (1963). The obtained hydroxyproline values were converted to collagen, as described by Samuel et al. (2007a), and further expressed as a proportion of the dry weight tissue respectively.

**Molecular biological techniques**

To complement the results obtained by immunohistochemistry, various analyses of gene expression were carried out. RNA was prepared from frozen cervical tissue fragments using the RNeasy Mini Kit (Qiagen) by following the user’s instructions. In situations where cervical tissue was too small to be divided for multiple analyses, sufficient levels of RNA could not be obtained. The isolated RNA was applied to RT-PCR for the detection of receptor expression (ESR1, PRAR, RXFP1) and VEGF at the mRNA level. The reverse transcriptase superscript III and oligo(dt)12–18 primers (Invitrogen GmbH) were used to produce cDNA from the entire RNA. Afterwards, the house-keeping gene 26S was detected for all cDNA samples using Taq polymerase (Invitek GmbH). The results were quantified (Gene Tools; GeneGenius 2, Syngene, Frederick, MD, USA) to determine the necessary volumes of the cDNA solution to obtain equal amounts of cDNA for the following PCRs. For the detection of ESR1, PRAR, RXFP1, and VEGF, Taq polymerase (Invitek) was used as well. Every PCR was carried out with 35 cycles of denaturation, annealing, and elongation.

<table>
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<tr>
<th>Primer</th>
<th>Sequence (from 5’ to 3’*)</th>
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<tr>
<td>26S rRNA</td>
<td>26Stor: AAT GGT CGT GCC AAA AAG GGC</td>
</tr>
<tr>
<td>ESR1</td>
<td>hera268rev: CGG AGA CAC GCT GTT GAG T</td>
</tr>
<tr>
<td>PRAR</td>
<td>hera268rev: CGG AGA CAC GCT GTT GAG T</td>
</tr>
<tr>
<td>RXFP1</td>
<td>hPRABror: GGT CTA CCC GCC CTA TCT CA</td>
</tr>
<tr>
<td>VEGF</td>
<td>hVEGFrev: TTC CTG TCC TCA TCT CAC CA</td>
</tr>
</tbody>
</table>

Primer from: MWG Biotech Ag, Ebersberg, Germany.

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The annealing temperature was 65 °C at the beginning, decreasing 1 °C every cycle to 55 °C from 11th till the last cycle. In order to demonstrate the PCR products, gel electrophoresis was used with 1.5% agarose gels (70 ml) stained with 1 μl ethidium bromide (1 g/100 ml aqua bidest) to visualize DNA bands under u.v. light exposure. The gels were evaluated and photographed using the gel documentation system GeneGenius2 (Syngene). The sequences of the primers used are shown in Table 1.

**Statistical analysis**

For simple statistics comparing MV and s.d., Microsoft Excel software was used. For comparisons of the standard error of means (S.E.M.), paired t-tests or the signed-rank test were used for the in vivo data obtained, while t-tests or the Mann–Whitney rank-sum test were used for the in vitro data obtained, using SigmaStat2.0 (Jandel). For further statistical analysis, animals of the in vivo experiment were divided into groups due to previous pregnancies, with (p+; n=7) and without previous pregnancies (p−; n=6).

Histological and immunohistochemical staining was analyzed under a light microscope (Axioskop 2 mot plus, Zeiss). The number of eosinophils was evaluated by counting the cells in eight visual fields per slide in three cervical tissue sections of six different animals per group at 400 × magnification.

For the evaluation of the immunohistochemically stained sections, the following modified semi-quantitative scheme (developed by Brummer et al. 2002) was used: 0, no positive staining; (+), only sporadic positive cells, <10%; +, a few cells with positive reaction, ca. 10–40%; ++, several positive cells, ca. 40–70%; ++++, many positively stained cells, >70%.

The data presented in this paper represent mean ± S.E.M., with P values < 0.05 described as significant.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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