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Effects of different progestins on prostaglandin biosynthesis in human endometrial explants☆,☆☆,★,★★

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ABSTRACT

Objective: To compare the effects of chlormadinone acetate (CMA), dienogest (DNG) and drospirenone (DRSP) on prostaglandin biosynthesis in a human endometrial explants model.
Study design: Human endometrial explants obtained by aspiration curettage and human endometrial YHES cells were stimulated with interleukin-1β (IL-1β) and exposed to CMA, DNG, DRSP or dexamethasone (DEX; YHES cells). Cellular messenger RNA (mRNA) levels of cyclooxygenase-2 (COX-2) were analyzed by reverse-transcription quantitative real-time polymerase chain reaction. Concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in culture supernatants were measured by enzyme-linked immunosorbent assay.
Results: CMA exerted after IL-1β stimulation a stronger down-regulation of COX-2 mRNA compared to DNG and DRSP in human explants (−55% vs. −40% and 46%, respectively). The effect of CMA on COX-2 mRNA was significantly stronger ($p=.025$) than that of DNG. Moreover, the effect of CMA was independent from cycle phase or presence of endometriosis. In order to evaluate the impact of the investigated progestins on effector molecules, $PGF_{2\alpha}$ release was determined in supernatants. Again, CMA reduced the $PGF_{2\alpha}$ release significantly by an average of −60% ($p<.01$). In contrast, no significant reduction was found for DNG and DRSP. In YHES cells, only DEX but not the progestins under study exerted a significant down-regulating effect (−79%, $p<.01$) on COX-2 mRNA after IL-1β stimulation.
Conclusion: Among the tested progestins, CMA displayed the most consistent suppression of prostaglandin biosynthesis in human endometrial explants.
Implication: Among three tested progestins, chlormadinone acetate had the most consistent suppressive effect on prostaglandins in endometrial explants. These findings support clinical observations about the efficacy of chlormadinone acetate in dysmenorrhea treatment.

1. Introduction

About 40%–80% of young women suffer from primary dysmenorrhea [1,2]. An increased prostaglandin synthesis plays a major role in the pathophysiology of dysmenorrhea, and thus, the prostaglandin system is a key target in the treatment of this disorder [1,3–6]. Elevated levels of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) were reported in dysmenorrheic patients. In particular, $PGF_{2\alpha}$ was suggested to be a pivotal player in mediating uterine ischemia, myometrial contractions and pain [7–10]. The biosynthesis of endometrial prostaglandins is a complex process under the control of local steroid hormones and catalyzed by phospholipases (mainly phospholipase A_2) as

Abbreviations: CMA, chlormadinone acetate; COX-2, cyclooxygenase-2; DEX, dexamethasone; DNG, dienogest; DRSP, drospirenone; IL-1β, interleukin-1β; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

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well as by the constitutive cyclooxygenase-1 (COX-1) and the inducible cyclooxygenase-2 (COX-2). Estrogens stimulate the synthesis of endometrial prostaglandins, whereas progestins suppress this process [11,12]. Progesterone withdrawal in the late secretory phase of the menstrual cycle promotes nuclear factor κB accumulation in the endometrial stroma that induces COX-2 expression and elevates prostaglandin biosynthesis. Due to this sex-steroid-dependent regulation, uterine prostaglandin levels vary during the menstrual cycle [6,13,14].

The first line therapy for dysmenorrhea is nonsteroidal anti-inflammatory drugs [4,5]. In women with demand for contraception, combined oral contraceptives are the therapy of choice. Their progestin components reduce endometrial thickness and suppress uterine prostaglandin synthesis [3,4]. While no results from randomized studies exist, limited clinical data support a more favorable response with chlormadinone acetate (CMA), a progestin that also has glucocorticoid activity [15–20]. Beside CMA, dienogest (DNG) and drospirenone (DRSP) have been clinically used for treatment of dysmenorrhea [21–24].

Due to its complex structure and functions, studies on human endometrium demand a variety of culture methods for assessment of its functions and dysfunctions. These include permanent cell lines, primary cell cultures and explants cultures. The closest approach to in vivo conditions are cultures of human endometrial explants in which tissue integrity and communication between cells are maintained (e.g., paracrine interactions between stromal and epithelial cells) [25,26]. Besides epithelial and stromal cells, other functionally important cell types (e.g., endothelial cells, macrophages) are preserved. Endometrial explants from nondysmenorrheic patients stimulated with IL-1β can be regarded as a short-time model for dysmenorrhea since activation of the arachidonic acid cascade is a general feature in dysmenorrheic patients [6]. In a recent study, we have demonstrated a suppressive effect of CMA on COX-2 mRNA expression and $PGF_{2\alpha}$ levels in human IL-1β-stimulated endometrial explants [27]. In the present study, we compare effects of CMA on the activated prostaglandin system in cell and tissue culture models of human endometrium with those of DNG and DRSP.

2. Materials and methods

2.1. Tissue collection

For this study, 34 women between 18 and 50 years undergoing surgery for benign gynecological disorders were recruited with their written informed consent. There was no hormonal treatment within the last 3 months. Endometrial tissue was taken by aspiration curettage (Pipelle De Cornier; Laboratoire C.C.D., Paris, France) before starting any surgical intervention. No patients with endometrial pathologies were included into the study. The sampling of endometrial tissues was approved by the local Ethical Committee (No. 251/15). Cycle phase determination was performed by menstrual cycle anamnesis and hormone profiles of LH, FSH, 17β-estradiol and progesterone. FSH serum levels <15 mIU/mL indicated a premenopausal status.

2.2. Explants culture

Endometrial explants were cultivated as described previously [26]. After preincubation (1 h) in DMEM/Ham's F-12 medium, they were placed on MilliCell-PCF inserts (12 μm; Millipore, Eschborn, Germany) and incubated in DMEM/Ham's F-12 medium (Sigma-Aldrich, Taufkirchen, Germany) containing 2 mM L-glutamine (Fisher Scientific; Schwerte, Germany) at 37°C with 5% CO_2 for 6 h in a humidified incubator (Heraeus BBD 6620, Thermo Fisher Scientific). Explants were treated for 6 h with CMA (Gedeon Richter, Budapest, Hungary), DRSP (Sigma-Aldrich) or DNG (Santa Cruz Biotechnology, Heidelberg, Germany) (10^{-6} M each). This concentration is approximately two orders of magnitude higher than the in vivo serum levels of progestins after therapeutic use [28,29]. Human recombinant IL-1β (final concentration 1 ng/mL; R&D, Wiesbaden, Germany) was added after 1 h. Vehicle controls were run with 0.1% DMSO (Sigma-Aldrich) with or without IL-1β ("DMSO"; "IL-1β"). After incubation, the explants were submerged overnight into RNA later (Qiagen, Hilden, Germany) and stored at −80°C. Supernatants were kept at −20°C.

2.3. YHES cells

The progesterone receptor positive, immortalized Yale human endometrial stromal cell line (YHES cells *syn.* T HESC) was from ATCC® (CRL-4003™; LGC Standards, Wesel, Germany). Cells were grown for 3 days to subconfluency in a phenol red-free DMEM/Ham's F-12 medium with 3.1 g/L glucose, 1 mM sodium pyruvate, 500 ng/mL puromycin dihydrochloride (Sigma-Aldrich), 1% ITS Premix (BD Biosciences, Heidelberg, Germany), 2.5 mM L-glutamine (Thermo Fisher Scientific) and 10% charcoal-stripped fetal bovine serum (Hyclone SH30068.03; GE Healthcare Life Sciences, Freiburg, Germany). Cells were cultivated on 6-well plates (48,000 cells seeded in 4 mL; Fisher Scientific, Schwerte, Germany) at 37°C in a humidified incubator (Heracell 150, Thermo Fisher Scientific) with 3% CO_2.

Cells were treated for 6 h in triplicates with CMA, DRSP, DNG or DEX (Sigma-Aldrich) in a concentration of 10^{-6} M each. IL-1β (1 ng/mL) was added after 1 h of culture. Vehicle controls were run with 0.1% DMSO (Sigma-Aldrich) with and without IL-1β ("DMSO"; "IL-1β"). All experiments were performed with cells from passage 3 or 4.

2.4. Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Endometrial explants were homogenized by a Precellys homogenizator (PeqLab, Erlangen, Germany). Total RNA was extracted by spin column-based nucleic acid purification with on-column DNase digestion (E.Z.N.A. Total RNA Kit I; VWR-Omega Bio-Tek, Darmstadt, Germany). Quantification of total RNA was performed by measurement of absorbance at 260 nm in a NanoDrop spectrophotometer (PeqLab). Quality of total RNA was controlled on a microchip electrophoresis system (MultiNA, Shimadzu, Duisburg, Germany).

cDNA synthesis from total RNA (1 μg) was carried out in a reaction volume of 20 μL containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate mix, 200 U SuperScript™ III reverse transcriptase (all from Thermo Fisher Scientific) and 1 μL random primers (hexanucleotide mix, 10×; Roche Diagnostics, Mannheim, Germany) at 50 °C for 50 min. cDNA was stored at −20°C.

qPCR analysis was performed as described previously [26] on a LightCycler® 480 instrument (Roche Diagnostics). Run-to-run differences were equalized by carrying along the same calibrator in all runs of the study. External standard curves were created in the beginning of the project for all genes under study with standard DNA that had been generated by conventional PCR. The DNA standard solutions contained the specific target sequences of the respective UPL assays and a carrier-RNA (MS2 RNA, 10 ng/μL; Roche Diagnostics). No template controls and no-reverse transcription controls were included for each gene. Results of qPCR analyses are given as normalized ratios in relation to the reference genes G6PDH, ALAS1 and PGK1.

2.5. $PGF_{2\alpha}$ enzyme immunoassay

$PGF_{2\alpha}$ concentrations in supernatants of endometrial cell and explants cultures were determined by a specific EIA kit (AssayDesigns; Supplier: ENZO Life Sciences, Lörrach, Germany) according to the manufacturer's instructions. Evaluation was performed on a μQuant spectrophotometer with Gen5 data analysis software (BioTek Instruments, Bad Friedrichshall, Germany). The $PGF_{2\alpha}$ concentrations are presented in

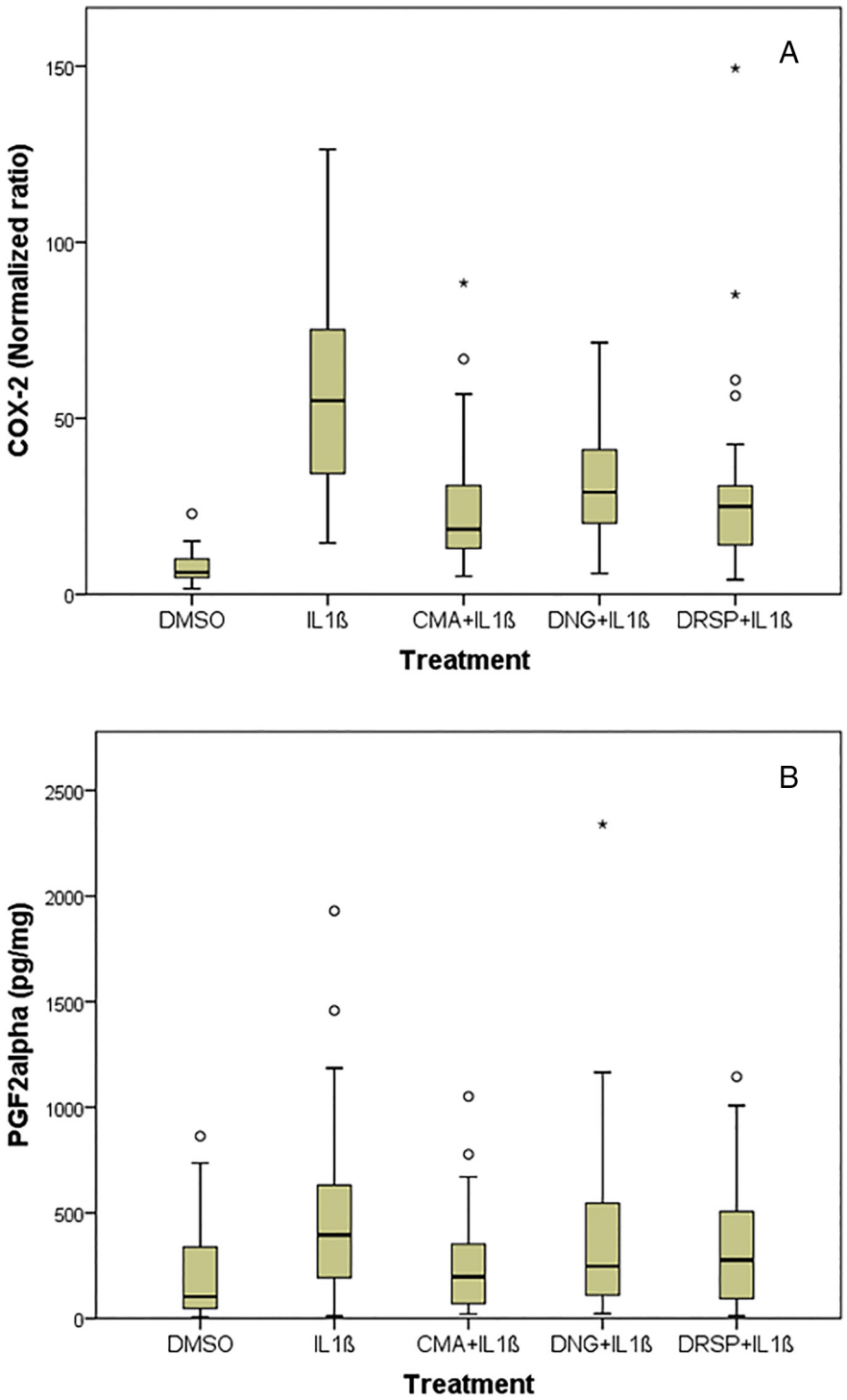

Fig. 1. Effects of progestins on COX-2 mRNA and $PGF_{2\alpha}$ levels in human endometrial explants. Explants (n=31) were exposed to CMA, DNG and DRSP and stimulated with IL-1β. Boxes represent lower and upper quartiles with median. (A) COX-2 mRNA levels. The differences between DMSO and IL-1β, as well as between all progestin treatments and IL1β, were significant ($p<.01$). The difference between CMA and DNG was also significant ($p=.025$). Mean normalized ratios were 7.9 (DMSO), 56.2 (IL-1β), 25.0 (CMA), 33.7 (DNG) and 30.4 (DRSP). (B) $PGF_{2\alpha}$ levels in supernatants related to tissue weight (pg/mg wet weight). One outlier (5083 pg/mg) in the IL1β group is not shown. The differences between DMSO and IL-1β, as well as CMA and IL1β, were significant ($p<.01$). Mean concentrations were 213 (DMSO), 645 (IL-1β), 256 (CMA), 430 (DNG) and 362 pg/mg (DRSP).

pg/mg wet weight of explants. The sensitivity of the assay was 7 pg/mL. The wet weight was determined after removal of the explants from RNAlater. Intraassay coefficients of variation were 10.0% for control 1 (mean 824 pg/mL) and 16.5% for control 2 (mean 126 pg/mL) (n=8). Interassay coefficients of variation were 11.0% for control 1 and 20.2% for control 2 (n=5) and thus within the acceptance criteria for immunoassays [30,31].

2.6. Statistical evaluation

Statistical analysis of all experiments was carried out by *Masem Research Institute GmbH* (Wiesbaden, Germany). Since the data were not normally distributed the Mann–Whitney *U* test for independent samples was applied. The results were considered significant at $p<.05$.

3. Results

3.1. Endometrial explants

Effects of CMA, DNG and DRSP on prostaglandin biosynthesis were investigated in human endometrial explants from 34 patients who underwent surgery for benign gynecological disorders. All participants were within the reproductive age and had a mean age of 35± 6.5 years (21–46 years). The menstrual cycle phase was determined by the history of the menstrual cycle prior to the surgery and hormone serum levels. Eleven patients were within the proliferative; 23 patients were within the secretory phase. Endometriosis was diagnosed in 10 participants. Three patients were excluded retrospectively due to extremely high basal COX-2 mRNA levels in DMSO and poor IL-1β stimulatability. According to the available menstrual cycle history and the respective hormone serum levels, these three patients had menstrual cycle abnormalities.

Mean COX-2 mRNA tissue levels were significantly increased by IL-1β by an average of 7.1-fold ($p<.01$). This stimulatory effect of IL-1β was suppressed in the presence of progestins. The strongest effect among the tested progestins was exerted by CMA (−55%, $p<.01$) followed by DRSP (−46%, $p<.01$) and DNG (−40%, $p<.01$). Moreover, the CMA effect on COX-2 mRNA was significantly stronger ($p=.025$) than that of DNG (Fig. 1A). $PGF_{2\alpha}$ levels in supernatants were significantly increased by stimulation with IL-1β. CMA did reduce the $PGF_{2\alpha}$ release significantly by 60% under IL-1β stimulation ($p<.01$). No significant reduction was found for DNG and DRSP (Fig. 1B).

When we compared results from explant tissues obtained during the secretory and proliferative phase of the cycle, or from subjects with and without a diagnosis of endometriosis, we observed the same general pattern favoring CMA (see Supplemental Figs. 1 and 2).

3.2. YHES cells

The results of YHES experiments are shown in Fig. 2. COX-2 mRNA levels were significantly up-regulated by IL-1β compared to the DMSO control. In IL-1β-stimulated cells, COX-2 mRNA was significantly suppressed only by DEX (−79%, $p<.01$). Neither CMA nor DNG and DRSP exerted significant effects on COX-2 mRNA levels. Relative COX-2 mRNA levels were several orders of magnitude lower than in explants cultures. Further, a decline of COX-2 mRNA expression with higher passages was observed in the YHES experiments (data not shown). This phenomenon was not caused by loss of IL-1β activity since IL-1β stimulation in contemporaneous explants experiments was not affected.

In contrast to COX-2 mRNA, $PGF_{2\alpha}$ release in IL-1β-stimulated YHES cells was not modulated by the drug treatments. $PGF_{2\alpha}$ concentrations were in the low range of the standard curves, and $PGF_{2\alpha}$ release was not stimulated by IL-1β. Likewise, in two control experiments run over 24 h, no differences between treatments were found (data not shown).

In pilot experiments with two human endometrial epithelial cell lines (Ishikawa and AN3-CA cells), COX-2 mRNA levels and $PGF_{2\alpha}$ did not respond to IL-1β and progestin treatments (data not shown).

4. Discussion

The main finding of this study is a stronger suppressive effect of CMA on COX-2 mRNA and $PGF_{2\alpha}$ release in comparison to DNG and DRSP in human IL-1β-stimulated endometrial explants as dysmenorrhea model. In the entire cohort of 31 explants, COX-2 mRNA tissue levels and $PGF_{2\alpha}$ release were significantly increased by IL-1β, and this IL-1β effect was significantly suppressed by CMA. The DNG and DRSP effects were significant only on COX-2 mRNA expression but not on $PGF_{2\alpha}$ release. The observed significant differences were found in spite of a high variability of measured values, which is a consequence of the heterogeneity of the study population (menstrual cycle phase, surgery indication, age) and the complexity of the explants model. The response patterns for COX-2 mRNA and $PGF_{2\alpha}$ were similar. Our findings correspond to clinical observations that CMA is an effective treatment for dysmenorrhea.

Clinical improvement or remission of dysmenorrhea was found after switching from another contraceptive to a combination of CMA and ethinylestradiol [17,18]. In direct comparison, a CMA-containing

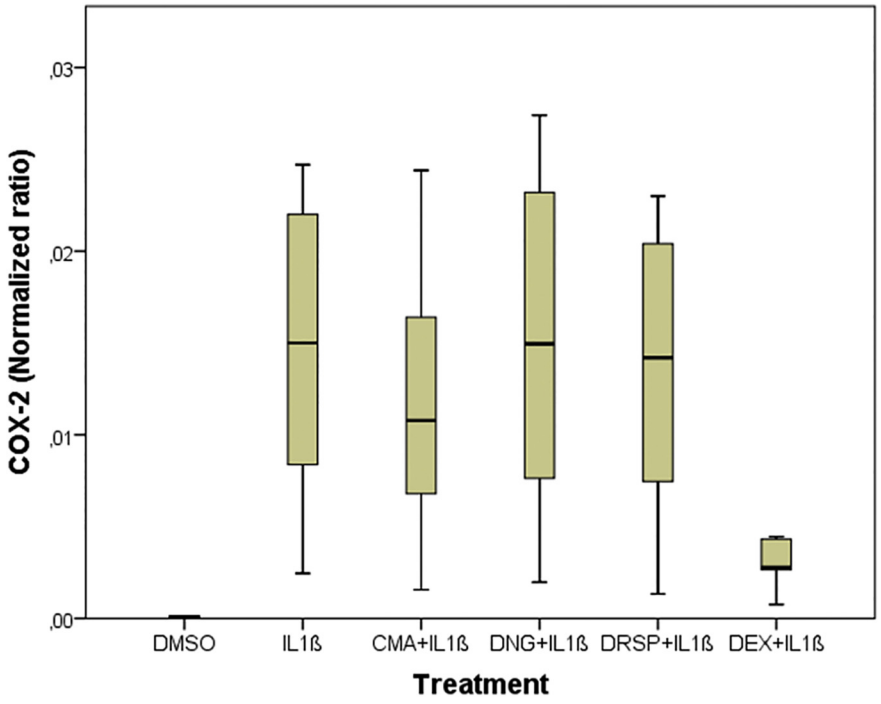

Fig. 2. Effects of steroid hormones on COX-2 mRNA levels in YHES cells. The box plots show the relative COX-2 mRNA levels of six independent cell culture experiments. Cells were exposed to CMA, DNG, DRSP or DEX and stimulated with IL-1β. Boxes represent lower and upper quartiles with median. Significance was demonstrated only for IL-1β vs. DMSO and DEX + IL1β vs. IL1β ($p<.01$, respectively). Mean normalized ratios were 0.00008 (DMSO), 0.015 (IL-1β), 0.011 (CMA), 0.015 (DNG), 0.013 (DRSP) and 0.003 (DEX). The normalized ratios indicate that COX-2 mRNA levels in YHES cells are lower than in endometrial explants (Fig. 1).

formulation had a more favorable effect on dysmenorrhea than a DRSP-containing pill [21]. These observations cannot be explained by a further reduction of endometrial thickness by CMA. It was speculated that the observed CMA effect may be due to its glucocorticoid activity [4,15,17,18]. However, in a previous study, we have demonstrated that the synthetic glucocorticoid DEX does not significantly down-regulate COX-2 mRNA and $PGF_{2\alpha}$, and neither CMA nor DEX significantly affects the mRNA level of the anti-inflammatory annexin A1 in human endometrial explants. These results suggested that suppression of prostaglandin synthesis by CMA in human endometrium by CMA is mainly mediated by progesterone receptor-dependent down-regulation of COX-2 rather than by a glucocorticoid off-target effect [27]. The differential effects of CMA, DNG and DRSP on prostaglandin formation observed in this study may be due to different progestogenic potencies [33,34] being mediated via the progesterone receptor by inhibiting the binding of NF-κB to the response element of the COX-2 promoter [32]. However, it cannot be excluded that the glucocorticoid activity of CMA contributes to the suppression of prostaglandin biosynthesis. In our experimental design, a glucocorticoid effect may be favored by an enhanced GR-binding since the applied concentration was higher than the pharmacological CMA level. Stromal cells may be involved in a glucocorticoid effect as indicated by the strong DEX effect in YHES cells. To our knowledge, there exist no data backing an additional regulation of endometrial COX-2 expression via androgen or mineralocorticoid receptors.

The response patterns towards the progestins under study were retained when explants from the proliferative and the secretory menstrual cycle phase were evaluated separately. This finding demonstrates that the suppressive effect of progestins on the prostaglandin biosynthesis in human endometrium can be found in both cycle phases. The higher variability in the secretory phase may be explained by the more dynamic functional alterations during this cycle phase (window of implantation, subsequent decidualization). We also evaluated separately a subgroup of patients suffering from endometriosis since an augmented inflammatory milieu was reported in the endometrium of endometriosis patients [35]. However, the response patterns were similar between endometrium donors with and without endometriosis.

Apart from the endometrial explants culture, we carried out similar experiments with CMA, DNG, DRSP and DEX in the IL-1β-stimulated human endometrial YHES cell line. In this monolayer cell culture model, only stromal cells are represented. DEX treatment was included since endometrial stromal cells express the glucocorticoid receptor. In YHES cells, only DEX exerted a significant down-regulating effect on IL-1β-stimulated COX-2 mRNA. None of the progestins under study had a significant effect on COX-2 mRNA. This indicates that, in the YHES model, the COX-2 mRNA expression is more susceptible to the action of glucocorticoids than to the action of progestins. None of the tested steroids exerted an effect on $PGF_{2\alpha}$ release. Literature data on prostaglandin biosynthesis in YHES cells are scarce. In nonstimulated YHES cells, a low production of prostaglandin E_2 but no COX-2 protein expression was reported [36]. To our knowledge, no data exist on $PGF_{2\alpha}$ biosynthesis in YHES cells. We conclude that YHES cells, the only commercially available immortalized endometrial stromal cell line, are not a suitable model to study progestin effects on endometrial prostaglandin biosynthesis. In another, commercially not available endometrial stromal cell line with extended life span, $PGF_{2\alpha}$ and PGE_2 were stimulated by IL-1β and suppressed by DEX [37].

In general, it would be more appropriate to select an endometrial epithelial cell line as model for dysmenorrhea research since in human endometrial tissue COX-2 expression in stromal cells is less prominent than in epithelial cells [38]. However, permanent progesterone-sensitive human epithelial cell lines such as Ishikawa cells have no functional prostaglandin systems [37,39]. The establishment of immortalized epithelial cell lines is generally difficult, and therefore, no such cell line is commercially available.

The discrepancy between our findings in explants cultures and the YHES cell line indicates that progestin actions on entire human endometrial tissues are more complex than in monolayer cell cultures. It is known that progestins display differential actions in endometrial stromal and epithelial cells [6]. Further, the overall progestin effect may involve paracrine interactions of both cell types [40]. This in vivo environment cannot be mimicked by monolayer cell cultures. Therefore, it is important to study overall endometrial effects of synthetic steroid hormones in tissue cultures or advanced cell culture models.

The advantages of using human explants are their close proximity to the in vivo situation due to the maintenance of tissue integrity and communication between different cell types [25] as well as their derivation from individual persons. This is in contrast to tumor and immortalized cell lines which differ from normal cells and tissues in many aspects. The drawbacks of explants cultures are their limited supply and amount of tissue, cellular heterogeneity, contaminations, limited viability and interindividual variation. Therefore, functional studies can be conducted only over a limited time period. A sufficient viability of epithelial and stromal cells has been demonstrated for a culture period of 6–8 h [25,26]. The limitations and applicability of human endometrial explant cultures are discussed in detail in Schäfer et al. 2011 [26]. A limitation of this study was the use of samples from women undergoing surgery for benign gynecological disorders and not from normal healthy volunteers. RT-qPCR is a suitable and accurate method to investigate effects of exogenous substances on gene expression during the limited ex vivo life span of the vulnerable endometrium.

In conclusion, our findings regarding the action of CMA, DNG and DRSP in human endometrial explants demonstrate that these progestins are able to suppress endometrial prostaglandin biosynthesis by down-regulation of COX-2. This mode of action provides an explanation for the therapeutic effects of these progestins in dysmenorrheic women. Among the tested progestins, CMA displayed the most consistent suppression of prostaglandin biosynthesis in human endometrial explants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.contraception.2018.08.001.

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