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Abstract

Many women would prefer fewer bleeding episodes while taking oral contraceptives. For this reason and with the intention of reducing menstruation-associated symptoms, an extended-cycle contraceptive is considered in the present paper. However, it remains unknown whether this long-term treatment is associated with a different breast cancer risk from that of the usual treatment. Therefore, in the present in vitro work we intend to compare the effect of these different treatment regimens on breast cancer risk. MCF-7 cells (human estrogen- and progesterone-receptor-positive metastatic breast cancer cells) and HCC1500 cells (human estrogen- and progesterone-receptor-positive primary breast cancer cells) were incubated with physiological concentrations of ethinylestradiol (EE). Usual and extended cycles were mimicked by incubation periods of 18 hours with EE followed by 6 hours without EE and 24 hours with EE for 3 days, respectively. In both cell lines, EE elicited a significant increase in the proliferation rate. No significant difference was found between the two incubation periods. Our results indicate that continuously administered ethinylestradiol may not increase breast cancer risk in comparison to intermittent application. However, clinical studies are necessary to prove these in vitro results.
Comparison of the proliferative effects of ethinylestradiol in an intermittent and a continuous dosing regime on human breast cancer cells

Running title: Continuous and intermittent ethinylestradiol and breast cancer cell proliferation

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Summary

Many women would prefer fewer bleeding episodes when taking oral contraceptives. For this reason and with the intention to reduce menstruation associated symptoms an extended – cycle contraceptive is currently considered. However it remains unknown whether this long-term treatment is associated with a different breast cancer risk as compared to the usual treatment. Therefore in the present in vitro work we intended to compare the effect of these different treatment regimens on breast cancer risk.

MCF-7 cells (human estrogen- and progesterone-receptor positive metastatic breast cancer cells) and HCC1500 cells (human estrogen- and progesterone-receptor positive primary breast cancer cells) were incubated with physiologic concentrations of ethinylestradiol (EE2). Usual and extended cycle were mimicked by incubation periods of 18 h with EE2 followed by 6 h without EE2 and 24 h EE2 for 3 days, respectively.

In both cell lines EE2 elicited a significant increase in the proliferation rate. No significant difference was found between the two incubation periods.

Our results indicate that continuously administered ethinylestradiol may not increase breast cancer risk as compared to intermittent application. However clinical studies are necessary to prove these in vitro results.

**Key Words:** Ethinylestradiol, breast cancer cells, proliferation, continuous incubation, intermittent incubation
Introduction
The role of estrogens in tumor development was marked out as a promoting rather than an initiating one since estrogens are able to stimulate the proliferation of target cells such as breast epithelial cells. This can result in an increase in DNA mutations due to the high mitotic rate [1]. Recent studies provide evidence, that estrogens not only can promote cancer, but also may initiate mutations by certain metabolites [1]. Whether compounds may have a stimulatory or inhibitory effect on breast cancer development or growth is often evaluated in studies with established breast cancer cell lines, like MCF-7. Estrogens stimulate breast cell proliferation and established breast cancer risks such as early age at menarche, late menopause and hormone replacement therapy are indicative of a greater lifetime estrogen exposure.

Combined oral contraceptives (COC) contain ethinylestradiol (EE2), a potent synthetic estrogen with higher affinity to the estrogen receptor and longer persistence in the body than estradiol (E2) [2]. Epidemiologic studies of the association between COC and breast cancer are not consistent. A pooled analysis combining 54 studies reported no association between past COC use and breast cancer, however breast cancer may increase with use of COC at younger ages and COC use might cause earlier diagnosis [3-5]. The studies included women using the conventional treatment regimes of COC with 21 days treatment followed by a treatment free interval of 7 days.

The 21-day regime of oral contraceptives mimics the natural cycles by causing a monthly menstrual bleeding. However there is no scientific reason for a 21- day regime of combined oral contraceptives (COC). Many women would prefer fewer bleeding episodes [6]. For this reason and with the intention to reduce menstruation associated symptoms an extended – cycle contraceptive has been developed and approved in the U.S (Seasonale ®). The dosing regime consists of 84 days of active
pills followed by 7 days of placebo. Many gynaecologists in Germany and Switzerland use an extended regime of COC in women with menstrual problems or symptomless women, who find an extended regime with less menstruations more comfortable [7]. Because in European countries extended cycle pills regimes are not approved different ethinylestradiol-progestagen preparations are prescribed containing 20-30mcg ethinylestradiol. The duration of continuous pill intake varies from 42-168 days [8]. Although these regimes are described to be more comfortable for many women it has to be considered, that extended-pill-cycles are associated with an about 30% increase of the monthly hormone dosage. Clinical trials on the safety of the new regimes particularly the risk of breast cancer are not available [9]. To receive long-term epidemiologic data will take years. Breast cancer initiation occurs many years before presentation of the disease. Therefore it is important to study the effect of the new dosage regime of EE2 on pre-existing breast cancer cells. With the present study we intended to further explore these uncertainties in vitro. For the first time we investigated the effect of ethinylestradiol, the estrogen currently used in combined contraceptives, in an intermittent and a continuous regime on the well-known breast cancer cell line MCF7 cells and estrogen-receptor positive HCC 1500 primary breast cancer cells.
Materials and Methods

17α-ethinylestradiol (EE2) was purchased from Sigma Chemical Co., Munich, Germany. The compound was dissolved in ethanol and was stored as concentrated stock solution at -20°C.

The MCF-7 cells were acquired from DSMZ, Braunschweig, Germany. Prior to the experiment, the MCF-7 cells were maintained in 5% FCS in DMEM (Gibco BRL, Eggenstein, Germany) supplemented with 0.3 mg/ml glutamine, 5 ng/ml bovine insulin and 100 U/ml penicillin plus 100 µg/ml streptomycin.

HCC1500, a human estrogen and progesterone receptor-positive primary breast cancer cell line was purchased from ATCC. Cells were maintained in RPMI-1640 medium (without phenol red) purchased from Sigma, which was modified to contain 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g/L glucose, 10% (v/v) heat inactivated foetal bovine serum and 100 U/ml penicillin plus 100 µg/ml streptomycin.

All assays were conducted using charcoal/dextran-treated serum-containing medium (without phenol red) for MCF-7 and HCC1500 cells. Stock concentrations of EE2 were further diluted with these assay media during working experiments to give a final ethanol concentration of less than 0.01% per well.

To simulate the two in vivo conditions, i.e. intermittent and continuous therapy, the following time frames were used: For intermittent incubation the cells were incubated for 3 days with the steroid for 18 h and then steroid-free medium was used for 6 h. For imitating continuous therapy the cells were incubated with EE2 for 24 h for 3 consecutive days.

Ninety-six well plates were seeded with approximately 1000 cells per well. The cells were incubated for the appropriate time frame at 37°C in culture medium. EE2 was tested in the concentrations of 0.1 nM (10⁻¹⁰M) and 1nM (10⁻⁹M).
Cell proliferation was measured by the ATP chemosensitivity test, where proliferation is quantified by measuring light emitted during the bioluminescence reaction of luciferene in the presence of ATP and luciferase. This assay has been validated in the routine laboratory of our hospital, where it has been in use for several years to evaluate the efficacy of chemotherapy agents for the treatment of breast cancer patients. In previous experiments we have compared this assay with established proliferation assays such as crystal violet assay and BrdU assay and found equal changes, but a more better reproducibility for the ATP assay.

**Statistical analysis**

Proliferation was measured versus controls and performed in triplicates in three independent experiments. Within and between statistical analysis was done by ANOVA with the logarithmic values followed by Dunnett’s procedure for within group comparisons and Bonferroni’s correction for between group comparisons. The overall alpha level was set at 0.05. The statistical software used was JMP (Version 3.2.1) by SAS Institute Inc.
Results

As seen in Figure 1 for the MCF-7 cells, EE2 significantly stimulated the proliferation of the cells by both incubation schemes. The values were 49 and 54 % at the concentration of 0.1 nM and 28 and 39% at the concentration of 1 nM for the intermittent and continuous incubation time, respectively. There was no significant difference between both schemes.

In Figure 2 the results for the HCC 1500 cells are depicted. Similarly to the MCF-7 cells, both incubation modes elicited a significant stimulation of the proliferation. The corresponding values were 31.5 and 38.2 % for 0.1 nM and 29.4 and 33.6% for 1 nM. Accordingly to the MCF-7 cells no significant difference was found between the two incubation modes.
Discussion

Nowadays it is well known, that estrogens promote the growth of breast tumor cells [10]. The results of our in vitro experiments show, that EE2 elicits a pattern of proliferation on the receptor-positive breast cancer line MCF-7 and on HCC1500 cells in both an intermittent and a continuous dosing regime. The ethinylestradiol concentrations used for stimulation in our study were in the range from $10^{-9} – 10^{-10}$ M, because clinically relevant concentrations of ethinylestradiol found in COC users are in the order of $4.6 \times 10^{-10}$ M [11].

Various breast cancer cell lines (MCF-7; T47D) have been used to study the role of both estrogens and progestagens on cellular proliferation in order to determine their potential tumor risk. These studies clearly showed a stimulating effect by estrogens [12-15]. Proliferation of malignant cells is under control of estrogen and growth factors. The current meaning is, that estrogens act proliferatively in a paracrine fashion by inducing production of stromal derived growth factors and cytokines or their receptors via activation of epithelial or stromal estrogen receptors [15].

Only few data are available on the effect of ethinylestradiol on breast cancer cell lines. Ethinylestradiol differs substantially from estradiol, because it suppresses the endogenous estradiol synthesis and the ethinylgroup on C-17 of ethinylestradiol blocks C-17 and C-16 oxidation [16]. In the liver EE2 induces the synthesis of proteins controlling coagulation and fibrinolysis. Furthermore the synthesis of binding-globulins is increased. Sex hormone–binding globulin which has been suggested to reduce estradiol-mediated cell growth in MCF-7 breast cancer cells increases by more than 100% in users of contraceptives pills [17].
In vivo ethinylestradiol plasma levels reach a steady-state between day seven and ten of the pill cycle and decrease during the 7-day pause of the normal pill cycle [18]. Since in users of the long-cycle this decrease of ethinylestradiol is missing, it can be hypothesised that the pharmacokinetic of SHBG is different during the long-cycle. Different SHBG levels may on the other hand cause different effects on the growth of MCF-7 cells.

Previously it has been shown, that in comparison to estradiol EE2 elicits a weaker effect on MCF-7 cells than estradiol at the concentration of $10^{-10}$M [19]. Interpreting these data it has to be considered, that in women using COC breast cells are exposed to ethinylestradiol and the endogenous produced estrogens, too.

In the present study we focused on different dosing regimen of ethinylestradiol, because a new continuous contraceptive pill for the long-cycle has been approved. Lippert et al. demonstrated, that the continuous and the sequential combined hormone replacement therapy exert different effects in vitro on MCF-7 cells [20-22]. These regimen do investigate changes in the addition of a progestagen to estradiol, while the estrogen is administered continuously. In contrast to these results with a combined regime, we did not find different stimulation of MCF-7 and HCC1500 cells using continuous or intermittent stimulation with EE2 alone.

Taking our results into account it has to be considered for future studies, that synthetic progestagens too exert variable effects on proliferation of tumor cell lines depending on the dose and the type of progestagen. There is evidence, that the 19-nortestosterone derivatives in COC have estrogenic properties and activation of estrogen receptor is the growth stimulatory mechanism for these synthetic progestins [12,23]. The progestagen levonorgestrel used in the long-cycle regime is a 19-nortestosterone derivate and breast cancer cell stimulation caused by ethinylestradiol in different dosage regimes might be modified by this progestagen.
Limitations of our study are, that cell culture studies are a means of observing trends and mechanistic effects which are not easily obtained otherwise. However these studies require careful interpretation since culture conditions play an important role in determining the results. Using the same cell model and conditions as in the present experiment we could verify similar results e.g. for actions of estradiol in different experiments [20-22]. A cell culture model cannot reproduce the complex clinical situation, but can reflect many characteristics of the original tissue such as enzyme and receptor types so that one can focus on individual factors possibly involved in the in vivo situation. A model can attempt to approximate the clinical situation and help in the elucidation of possible mechanisms involved but never replace prospective clinical or epidemiological studies. Insofar our results are preliminary and do not allow yet the recommendation of the long-cycle to healthy women. On the other hand our data do not force us, to change the clinical practice of prescribing the long-cycle in women with gynaecological problems like severe dysmenorrhoea, menstrual-related headache, premenstrual syndrome and severe endometriosis.

Conclusion

Our results indicate that continuously administered ethinylestradiol stimulates breast cell proliferation to the same degree as intermittent application of ethinylestradiol. In order to evaluate further which COC - regimen might be more safe for breast cancer additional studies are needed which take into account the effect of progestogens too.
References


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Legends:

Figure 1: Changes of proliferation of MCF-7 cells after addition of ethinylestradiol continuously (Cont) or intermittently (Inter) for 3 consecutive days as compared to controls (C). (Means ± SD)

Figure 2: Changes of proliferation of HCC 1500 cells after addition of ethinylestradiol continuously (Cont) or intermittently (Inter) for 3 consecutive days as compared to controls (C). (Means ± SD)
Figure 1

![Bar chart for Figure 1](chart.png)

Figure 2

![Bar chart for Figure 2](chart.png)