Original Paper

HORMONE RESEARCH

Horm Res 2006;65:276–288
DOI: 10.1159/000092591

Received: May 13, 2005
Accepted: February 20, 2006
Published online: April 6, 2006

Effects of Phytoestrogen Extracts Isolated from Rye, Green and Yellow Pea Seeds on Hormone Production and Proliferation of Trophoblast Tumor Cells Jeg3

A. Matscheski[a] D.-U. Richter[c] A.-M. Hartmann[a] U. Effmert[a] U. Jeschke[b] M.S. Kupka[b] S. Abarzua[a] V. Briese[c] W. Ruth[d] U. Kragl[d] B. Piechulla[a]

[a]Department of Biological Sciences, University of Rostock, Rostock, [b]First Department of Obstetrics and Gynaecology, Ludwig-Maximilians-University of Munich, Munich, Departments of [c]Obstetrics and Gynaecology and [d]Technical Chemistry, University of Rostock, Rostock, Germany

Key Words
Jeg3 · Rye · Green and yellow pea · Estradiol · Progesterone · 5-Bromo-2′-deoxy-Uridine

Abstract
Background: Phytoestrogens are a diverse group of non-steroidal plant compounds. Because they have chemical structures similar to estrogens they are able to bind on estrogen receptors in humans. ***Objectives:*** In this study, we tested the effects of crude phytoestrogen extracts from rye *(Secale cereale)*, green pea *(Pisum sativum)* and yellow pea seeds (*Pisum sativum* cv.) on cell proliferation and the production of progesterone in trophoblast tumor cells of the cell line Jeg3. ***Methods:*** Isoflavone extracts from green and yellow pea seeds and lignan extracts from rye seeds were obtained, using different extraction methods. Isolated extracts were incubated in different concentrations with trophoblast tumor cells. Untreated cells were used as controls. At designated times, aliquots were removed and tested for estradiol and progesterone production. In addition, we tested the effects of the phytoestrogen extracts on cell proliferation. ***Results:*** Cell proliferation is significantly inhibited by potential phytoestrogens isolated from rye, green and yellow pea seeds in trophoblast tumor cells of the cell line Jeg3. We found a correlation between the effects of proliferation and production of estradiol in isoflavone extracts from green and yellow pea seeds in Jeg3 cells. In addition, higher concentrations of isoflavones isolated from green pea seeds and lignans from rye showed also a inhibition of progesterone production whereas higher concentrations of rye lignans elevated estradiol production in Jeg3 cells. ***Conclusion:*** A useful indicator test system for potential phytoestrogens could be established. Based on the obtained results it is proposed that green and yellow pea seeds contain measurable concentrations of isoflavones and rye seeds contain lignans which can be isolated and used for special human diet programs.

Introduction

Phytoestrogens are a diverse group of non-steroidal plant compounds that occur naturally in many plants, e.g. fruits and vegetables. Because they show structural similarities to estrogens the binding to human estrogen recep-

A.M. and D.-U.R. contributed equally to this work.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0301–0163/06/0656–0276$23.50/0

Accessible online at:
www.karger.com/hre

Dr. U. Jeschke, PD
Ludwig-Maximilians-University of Munich, First Department of Obstetrics and Gynaecology, Maistrasse 11
DE–80337 Munich (Germany)
Tel. +49 8951 604 266, Fax +49 8951 604 916, E-Mail udo.jeschke@med.uni-muenchen.de

tors is possible. Phytoestrogens can bind to both types of estrogen receptors [estrogen receptor alpha (Erα) and estrogen receptor beta (ERβ)], however, with an higher affinity to the β estrogen receptor than steroidal estrogens [1]. Despite their ability to bind to estrogen receptors the physiological effect of phytoestrogens is reduced by 10^2–10^5 compared with human estrogens [2]. Especially the selectivity of genistein for ERβ is well understood using X-ray crystallography and computational methods [3]. Phytoestrogens have several potential anticarcinogenic activities. The proposed mechanisms by which they may inhibit cancer cells include inhibition of DNA topoisomerase, suppression of angiogenesis, induction of differentiation in cancer cell lines, and induction of apoptosis [4].

Two main types of phytoestrogens are presently known: isoflavones and lignans. The most-studied category of phytoestrogens is the isoflavones found in soybeans, chick peas, green and yellow peas and other legumes [5]. From more than 1,000 types of isoflavones genistein and daidzein are the most commonly investigated isoflavones [1]. Lignans are found in flax seed, lentils, whole grains, beans, fruits and vegetables. The mammalian lignans enterolactone and enterodiol, commonly found in human plasma and urine, are phytoestrogens that may contribute to the prevention of breast cancer and coronary heart disease. They are formed by the conversion of dietary precursors such as secoisolariciresinol and matairesinol lignans from rye or wheat bran by the colonic microflora [6].

Numerous in vitro cell culture studies and in vivo animal experiments have demonstrated that phytoestrogens can inhibit tumor growths [7]. In a comprehensive review on the potential of phytoestrogens to reduce tumor growth, Fournier et al. [8] noted, that in 16 of 17 animal studies the addition of soybean products reduced tumor incidence or multiplicity in tumor models of the breast, prostate, liver, esophagus, and lung. Many studies focused on the isoflavone genistein, which seems to be the primary anticancer soy-bean constituent. It has antioxidant properties that may also play a role in its anticancerogenic effects [9]. It can inhibit hydrogen peroxide-induced tumor promoter activity in vitro and in vivo [10] and has been shown to inhibit tyrosine kinase [11, 12]. Its activity as an anticancer agent probably results from its suppression of enzymes like vascular endothelial growth factor 165, platelet-derived growth factor, tissue factor, urokinase plasminogen activator, and matrix metalloprotease-2 and -9, respectively [13]. In addition phytoestrogens can inhibit steroid synthesis in bovine [14] and human granulosa cell cultures [15]. There are many reports in literature on inhibition of aromatase (estrogen synthetase) by phytoestrogens [15–27].

In a former study, we evaluated the interaction between phytoestrogens (genistein and daidzein) and chorion carcinoma cell lines (BeWo and Jeg3). These tumor cell lines express ERα and ERβ and in addition, produce a variety of specific hormones like progesterone and hCG [28]. We were able to show that high doses of the phytoestrogens genistein and daidzein (1–2 μmol/ml) reduced the cell proliferation and the production of the steroid hormone progesterone. In addition, high doses of daidzein reduced the production of hCG in both cell types tested. High doses of genistein had no influence on the hCG production in BeWo and Jeg3 cells. Surprisingly low doses of daidzein and genistein induced both cell types to produce high amounts of hCG. Therefore, we assumed that in parallel measurements of progesterone but not hCG and in addition cell proliferation give comparable results in dose dependent effects of phytoestrogens in Jeg3 and BeWo cell cultures. Another study of our group showed that crude flax seed extracts containing the lignan matairesinol and the isoflavone biochanin A decreased the proliferation and production of progesterone and hCG in Jeg3 cells [29]. We could also show that these cells express both ERα and ERβ. In another study, we evaluated the distribution patterns of steroid hormone receptors (ER, PR) and glycodelin A expression of proliferative endometrial glandular cells after stimulation with phytoestrogens genistein and daidzein. ER expression showed a significant decline with genistein, whereas PR expression increased significantly with genistein. Glycodelin A did not show any significant expression under genistein stimulation. Stimulation with daidzein resulted in no statistically relevant alterations in ER expression, whereas the PR and glycodelin A expression significantly increased [30].

The aim of the present study was: (1) isolation of potential isoflavones from green and yellow pea seeds *(Pisum sativum)* and lignans from rye seeds *(Secale cereale)*, and (2) testing the effects of isolated potential phytoestrogens on cell proliferation, estradiol and progesterone production and expression of estrogen- and progesterone receptors (PR) in Jeg3 trophoblast tumor cells.

Materials and Methods

Cell Culture

The chorion carcinoma cell line Jeg3 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in DMEM medium with 10% inactivated fetal

calf serum (FCS gold) with antibiotics and antimycotics. This cell culture medium with 10% FCS gold did not contain any measurable amounts of estrogen and progesterone as determined with the automated hormone analyzer Immulite (DPC Biermann, Freiburg, Germany)

Preparation of Isoflavone Extracts from Seeds of Green Pea (Pisum sativum) *and Yellow Pea (*Pisum sativum *cv.) and Lignan Extracts from Rye Seeds* (Secale cereale)

Seeds of green pea, *(Pisum sativum)*, sort Gloriosa were obtained from the regional institution for agriculture and horticulture (Halle/Saale, Germany) and seeds of yellow pea (*Pisum sativum* cv.), sort 'Harmas' and rye *(Secale cereale)*, sort 'Recrut' were purchased from the regional agricultural research institution (Rostock, Germany). The procedure for the extraction of isoflavones from green and yellow pea seeds was adapted from [31] and modified by [32]. 50 g of green and yellow pea seeds were ground using a commercial coffee grinder and extracted with 1 liter of methanol in a water bath for 10 min at 70°C under backflow. The hot solution was then filtered and 1 liter of distilled water was added and cooled to room temperature. 500 ml of the extract solution was combined with 125 ml petroleum ether in a separating funnel to separate concomitant substances. The aqueous-methanol solution was evaporated to dryness. The pellet was resuspended in phosphate buffer solution (PBS) or dimethylsulfoxide (DMSO) and then used in proliferation and progesterone production assays.

The method for the extraction of lignans from rye seeds was performed according to [33] and modified by [32]. 100 mg of rye seeds were ground in a commercial coffee grinder and extracted with 1 liter of methanol in a water bath for 15 min at 70°C under backflow. The hot extract solution was filtered, then cooled and evaporated to dryness. This extract was resuspended in 10 ml of distilled water and partitioned with ethyl acetate. The ethyl acetate soluble residue was evaporated to dryness and resupended in PBS or ethanol prior to application in proliferation and progesterone production assays.

Identification of Isolated Phytoestrogens with HPLC-MS

Chromatographic separation of the isolated phytoestrogene fractions was done by reversed-phase HPLC using a gradient elution gradient program: 0.2 ml/min, 20% methanol (A), 80% water with 0.1% formic acid (B), linearly to 80% A:20% B in 15 min followed by holding for 25 min and going to start conditions at additional 10 min. A Discovery C 18 (15 cm × 2.1 mm) column by Supelco (Taufkirchen, Germany) was used.

For MS analysis a LCQ-Advantage (Thermo Finnigan, San Jose, Calif.,USA) was used. Identification of the peaks was obtained by using ion trap technology; ESI mode and positive ion. Source voltage was 4.5 kV and a mass range of 150–2,000 amu was detected.

Cytotoxicity-Assay

Viability of the trophoblast tumor cell line Jeg3 treated with different concentrations of isoflavone extracts from seeds of green pea *(Pisum sativum)* and yellow pea (*Pisum sativum* cv.) and lignan extracts from rye seeds *(Secale cereale)* was tested by the trypan blue exclusion assay. IC_{50} values (phytoestrogen concentration which reduce viability of the cells to 50%) were read for each phytoestrogen extract from appropriate inhibition curves and compared to each other.

Proliferation-Assay

Cell proliferation was analyzed using a 5-bromo-2′-deoxy-uridine (BrdU) labeling and detection kit according to the instructions of the manufacturer. Jeg3 cells (5×10^3/0.1 ml supplemented cell culture medium) were grown in 96-well tissue culture plates for 48 h in the absence (controls) and presence of different phytoestrogen extracts. Extracts from green pea, yellow pea and rye seeds were dissolved in PBS, DMSO or ethanol before added to 100 μl of the Jeg3 cells giving final concentrations of 10, 50 and 100 μg/ml, respectively for isoflavone extracts and 10, 50, 100, 500 and 1,000 μg/ml for lignans. After labeling with BrdU for 3 h, the cells were fixed and BrdU incorporation into DNA was measured by an ELISA technique. Cellular proliferation inhibition is expressed relative to controls (100 %) ± SD.

Determination of Progesterone

Jeg3 cells (1×10^6/2 ml supplemented cell culture medium) were grown in 24-well tissue culture plates for up to 96 h in the absence (controls) and presence of the isolated phytoestrogen extracts. Extracts from green and yellow pea and rye seeds were resuspended in PBS, DMSO or ethanol before added to 2 ml of the Jeg3 cells giving final concentrations of 10, 50 and 100 μg/ml, respectively. In addition, estradiol (dextran encapsulated, Sigma, Deisenhofen, Germany) was added to Jeg3 cells giving final concentrations of 10, 50 and 100 μg/ml, respectively. After 96 h 1 ml cell culture supernatant was removed and samples were stored at –20°C. At the end of the tests all solutions were analyzed for production of progesterone. Secretion of progesterone was determined by the automated hormone analyzer SR1 from BioChemImmunoSystems GmbH. Progesterone itself was analyzed by competitive enzyme-immuno assay (EIA). Specific antibodies were labeled with fluorescein and after binding to magnetic particles the separation was performed. Color intensity was measured at three different wavelengths. Samples for progesterone had to be diluted 1:3 with hormone-free diluents, due to intense colored culture medium. Sensitivity and the intra-assay coefficient of variation were: progesterone EIA <6.4 nmol/l and 6.8% (at a medium value of 11.5 nmol/l; 5.4% at 65.6 nmol/l, respectively).

Determination of Estradiol

Jeg3 cells (1×10^6/2 ml supplemented cell culture medium) were grown in 24-well tissue culture plates for up to 96 h in the absence (controls) and presence of the isolated phytoestrogen extracts. Extracts from green pea, yellow pea and rye seeds were resuspended in PBS; DMSO or ethanol before added to 2 ml of the Jeg3 cells giving final concentrations of 10, 50 and 100 μg/ml, respectively for isoflavones and 10, 50, 500 and 1,000 μg/ml for lignan extracts. After 96 h, 1 ml cell culture supernatant was removed and samples were stored at –20°C. At the end of the tests all solutions were analyzed for production of estradiol. Secretion of estradiol was determined by the automated hormone analyzer Immulite (DPC Biermann, Freiburg, Germany). Estradiol itself was analyzed by competitive EIA. Specific antibodies were labeled with fluorescein and after binding to magnetic particles the separation was performed. Color intensity was measured at three different wavelengths. Samples for estradiol were diluted 1:3 with hormone-free diluents. Sensitivity and the intra-assay coefficient of variation were: estradiol EIA <20 pg/ml and 5.7% (at a medium value of 236 pg/ml; 5.6% at 1,237 pg/ml, respectively).

Immunocytochemistry

Jeg3 cells (1×10^6/2 ml supplemented cell culture medium) were grown on quadriperm tissue slides for up to 96 h in the absence (controls) and presence of the isolated phytoestrogen extracts. Extracts from yellow and green pea and rye seeds were resuspended in PBS, DMSO or ethanol before added to 3 ml of the Jeg3 cells giving final concentrations of 10, 50 and 100 μg/ml, respectively. In addition, estradiol (dextran encapsulated, Sigma, Deisenhofen, Germany) was added to Jeg3 cells giving final concentrations of 10, 50 and 100 μg/ml, respectively. For the immunocytochemical characterization of steroid hormone receptors in choriontrophoblast tumor cells of the cell line Jeg3 the Vectastain®Elite ABC-Kit (Vector Laboratories, Burlingame, USA) was used for visualization. Mouse monoclonal antibodies used for the experiments are: mouse antihuman PR (Dako, Hamburg, Germany), mouse antihuman ERα (Dako) and mouse antihuman ERβ (Serotec, Düsseldorf, Germany). For immunocytochemical evaluation the slides were air dried, washed in PBS, fixed in 4.0% formalin for 15 min, washed again in PBS for 5 min and incubated for 30 min in 0.3% H_2O_2. After this the slides were washed in distilled water. The slides were incubated with the ABC-Kit's normal serum for 30 min, followed by incubation with the described primary antibodies for 1 h and after washing with diluted biotinylated secondary antibody for another 30 min. After incubation with the Reagent ABC for 30 min, H_2O_2/3′3′diaminobenzidine (Dako) was added for 2 min until brown staining of 'positive' cells could be observed. The slides where not counterstained because of nuclear expression of hormone receptors. After xylol treatment the slides were covered. Positive cells showed a brownish color.

Statistical Analysis

Statistical analysis was performed using the Wilcoxon's signed rank tests for comparison of the means. The $p < 0.05$ value was considered statistically significant.

Results

Preparation of Isoflavone Extracts from Seeds of Green Pea (Pisum sativum) *and Yellow Pea (*Pisum sativum *cv.) and Lignan Extracts from Rye Seeds* (Secale cereale)

As a result of the isoflavone preparation from seeds of green pea *(Pisum sativum)* and yellow pea (*Pisum sativum* cv.) 3.0–11 g isoflavones could be isolated using 100 g seeds.

Lignan extract preparations from rye seeds *(Secale cereale)* in addition gave similar yields (0.5–1.5 g lignanes using 100 g seeds).

Identification of Isolated Phytoestrogens with HPLC-MS

Chromatographic separation and MS analysis of isoflavone extracts from seeds of green pea showed that this extract contained genistein (dimerized), daidzein (di-glycosidic), whereas isoflavone extracts from seeds of yellow pea contained genistein (dimerized) and daidzein (deoxy-di-glycosidic). Lignan fractions from rye seeds contain matairesinol (di-glycosidic, glycosidic dimerized), pinoresinol (deoxy-di-glycosidic), arctigenin (glycosidic dimerized) and lariciresinol (di-glycosidic).

Cytotoxicity-Assay

Viability of the trophoblast tumor cell line Jeg3 was reduced to 50% (IC_{50}) at concentrations of 250 μg/ml of both isoflavone preparations from seeds of green pea *(Pisum sativum)* and yellow pea (*Pisum sativum* cv.). Lignan extract preparations from rye seeds *(Secale cereale)* showed much higher IC_{50} values. Concentrations of 1,000 μg/ml lignan extract preparations from rye seeds reduced viability of trophoblast tumor cells to only 70%.

Isoflavone Extracts from Green and Yellow Pea Seeds (Pisum sativum) *Reduce Proliferation in Jeg3 Cells*

As demonstrated in figures 1 and 2, extracts from green and yellow pea seeds inhibit the proliferation of Jeg3 cells in a concentration dependent manner. The addition of extracts from green pea seeds (fig. 1) at 10, 50, and 100 μg/ml reduced cellular BrdU-uptake to 95.9, 93.5, and to 77% and yellow pea seeds (fig. 2) to 72.4, 60.2, and to 60.2%, respectively, relative to non-treated control cultures (100%). Significant decreases in cell proliferation were induced by treatment of Jeg3 cultures with extracts from green pea seeds at 100 μg/ml whereas incubation of Jeg3 cell cultures with extracts from yellow pea seeds significantly reduced BrdU-uptake with all three concentrations tested.

Lignan Extracts from Rye Seeds (Secale cereale) *Reduce Proliferation in Jeg3 Cells*

Significant decreases in cell proliferation were induced by treatment of Jeg3 cultures with extracts from rye seeds (*Secale cereale*, fig. 3). Incubation of Jeg3 cell cultures with extracts from rye seeds at 10, 50, 100, 500 and 1,000 μg/ml reduced cellular BrdU-uptake to 77, 77, 69, 64 and 52% relative to non-treated control cultures (100%). The effects of lignan extracts isolated from rye seeds are concentration dependent.

Determination of Estradiol and Progesterone

With this study, we could show that the production of the steroid hormones estradiol and progesterone in trophoblast tumor cells of the cell line Jeg3 is influenced by isolated crude isoflavone and lignan extracts. In this cell

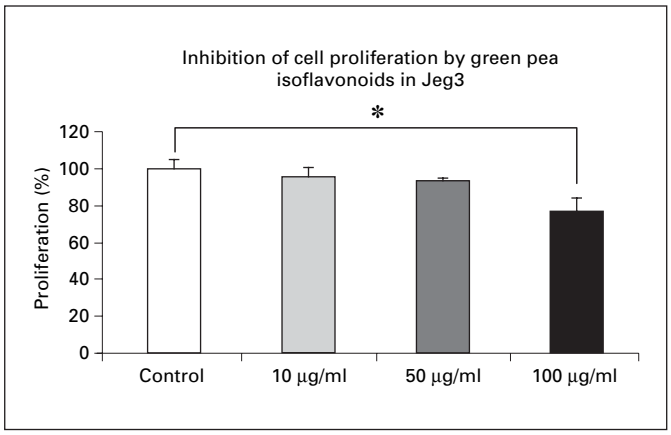

Fig. 1. Inhibition of proliferation in trophoblast tumor cells Jeg3 after addition of different concentrations of isoflavone extracts from green pea seeds. Data (mean ± SD) represent relative BrdU uptake obtained in at least 3 experiments. $p < 0.05$ indicate significant differences in isoflavone treated cultures and unstimulated controls marked with an asterisk (*).

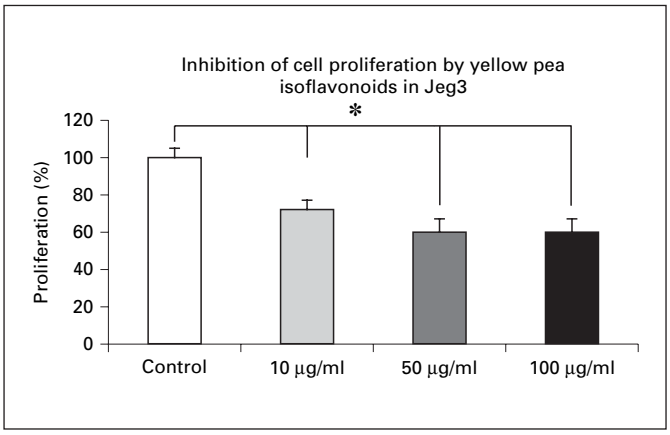

Fig. 2. Inhibition of proliferation in trophoblast tumor cells Jeg3 after addition of different concentrations of isoflavone extracts from yellow pea seeds. Data (mean ± SD) represent relative BrdU uptake obtained in at least 3 experiments. $p < 0.05$ indicate significant differences in isoflavone treated cultures and unstimulated controls marked with an asterisk (*).

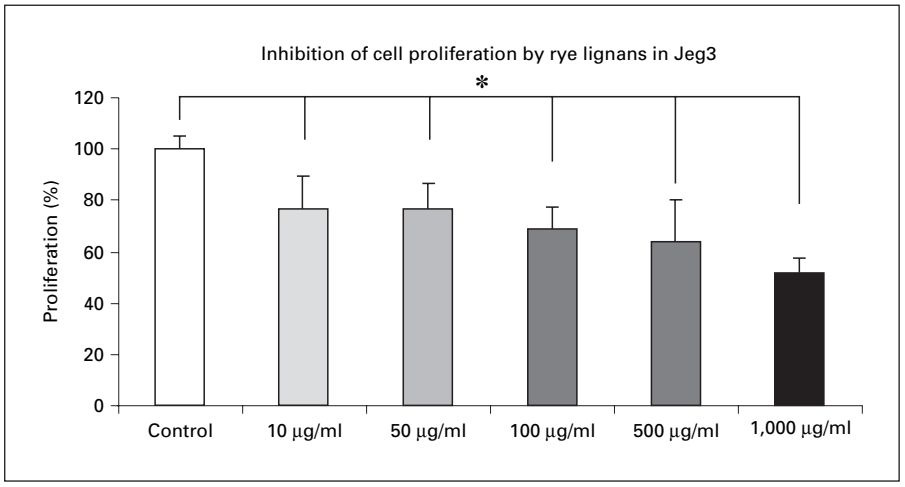

Fig. 3. Inhibition of proliferation in trophoblast tumor cells Jeg3 after addition of different concentrations of lignan extracts from rye seeds. Data (mean ± SD) represent relative BrdU uptake obtained in at least 3 experiments. $p < 0.05$ indicate significant differences in lignan treated cultures and unstimulated controls marked with an asterisk (*).

line, cultured in the presence of extracts from green pea seeds at 10, 50 and 100 µg/ml, the production of estradiol was significantly inhibited at concentrations of 50 and 100 µg/ml compared to unstimulated controls ($p = 0.043$ in both cases, fig. 4). In Jeg3 cells cultured in the presence of extracts from yellow pea seeds at 10, 50 and 100 µg/ml, the production of estradiol was also significantly inhibited at concentrations of 50 and 100 µg/ml compared to unstimulated controls ($p = 0.042$ and 0.043, fig. 5). Furthermore, the treatment of Jeg3 cells with crude lignan extracts from rye seeds at concentrations of 10, 50 and 100 µg/ml also led to a decrease in the production of estradiol. Differences between stimulated and unstimulated cells were significant at concentrations of 50 and 100 µg/ml ($p = 0.043$ in both cases, fig. 6). On the other hand, higher concentrations (500 and 1,000 µg/ml) of lignan extracts stimulated estradiol production in these cells (data not shown).

The addition of isoflavone extracts from green pea seeds at 10, 50 and 100 µg/ml reduced progesterone production in Jeg3 cells significantly ($p = 0.046$, 0.028 and 0.028, fig. 7). The addition of isoflavone extracts

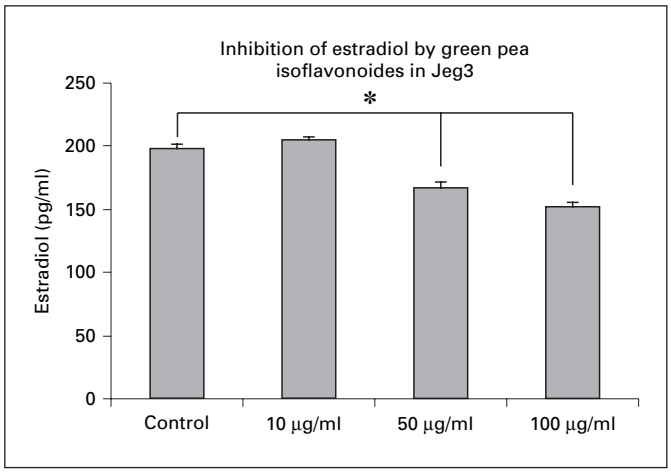

Fig. 4. Inhibition of estradiol production in tumor cells Jeg3 after addition of different concentrations of isoflavone extracts from green pea seeds. Data (mean ± SD) represent relative estradiol production obtained in at least 3 experiments.

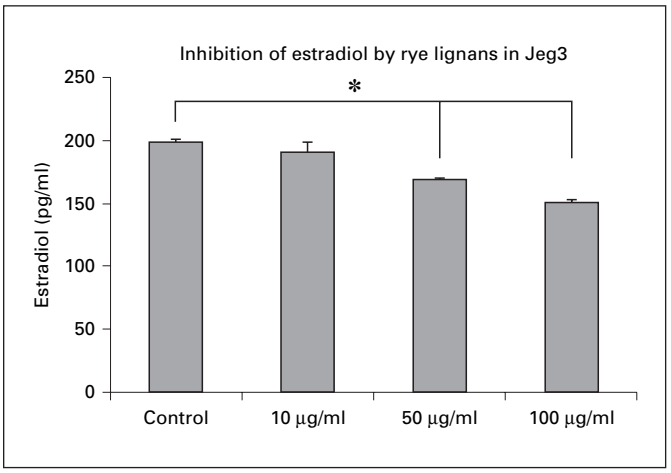

Fig. 6. Estradiol production in trophoblast tumor cells Jeg3 after addition of different concentrations of lignan extracts from rye seeds. Data (mean ± SD) represent relative estradiol production obtained in at least 3 experiments.

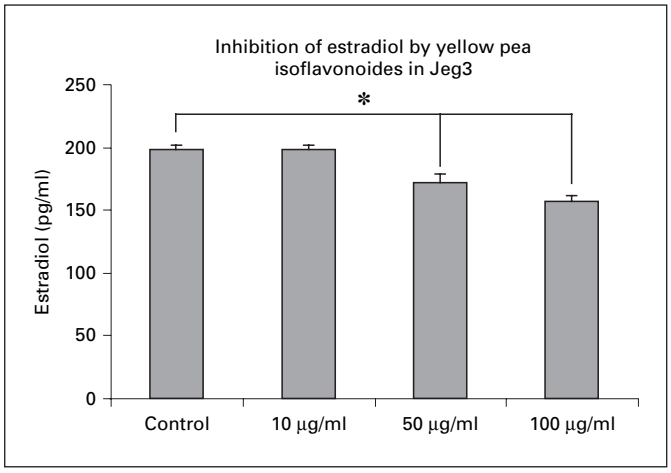

Fig. 5. Inhibition of estradiol production in tumor cells Jeg3 after addition of different concentrations of isoflavone extracts from yellow pea seeds. Data (mean ± SD) represent relative estradiol production obtained in at least 3 experiments.

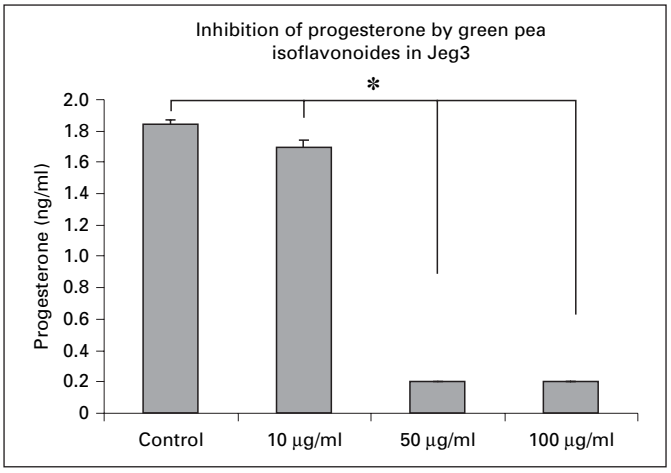

Fig. 7. Inhibition of progesterone production in tumor cells Jeg3 after addition of different concentrations of isoflavone extracts from green pea seeds. Data (mean ± SD) represent relative progesterone production obtained in at least 3 experiments.

from yellow pea seeds at 50 and 100 μg/ml also reduced progesterone production in Jeg3 cells significantly ($p = 0.028$ in both cases, fig. 8a). In addition, supplementation of lignan extracts from rye at 50 and 100 μg/ml also reduced progesterone production in Jeg3 cells significantly (data not shown). Estradiol added to Jeg3 cells also showed an inhibition effect on progesterone production. This inhibition effect was concentration dependent. Addition of 100 μg/ml estradiol showed the highest effect on progesterone inhibition, whereas addition of 10 μg/ml estradiol gave lowest progesterone inhibition (fig. 8b). Differences in progesterone production between unstimulated controls and estradiol substituted cell cultures were statistically significant ($p = 0.043$) in all cases.

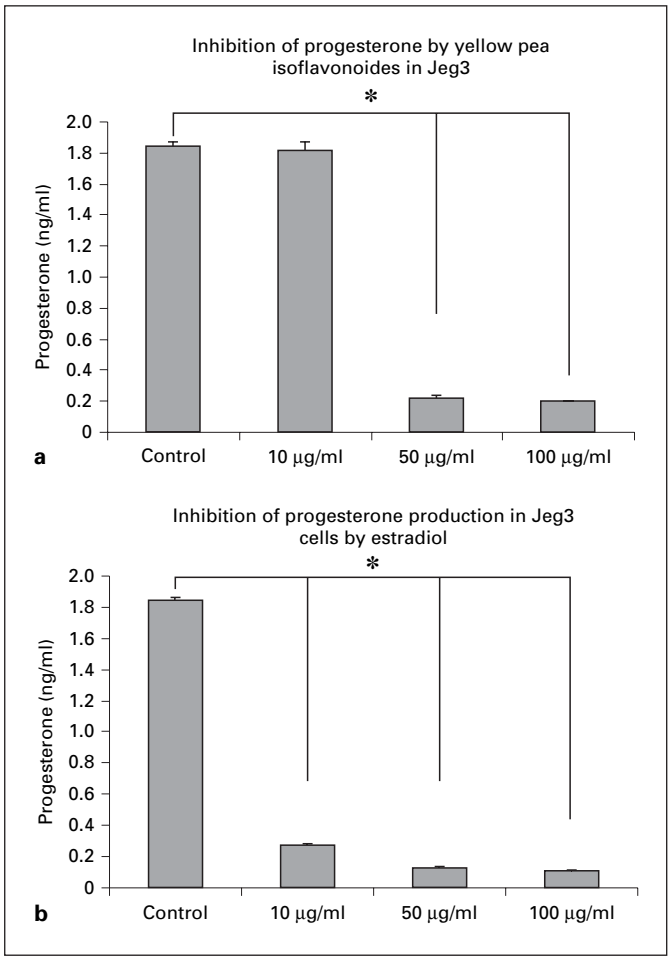

Fig. 8. Inhibition of progesterone production in tumor cells Jeg3 after addition of different concentrations of isoflavone extracts from yellow pea seeds. Data (mean ± SD) represent relative progesterone production obtained in at least 3 experiments.

Data presented demonstrate a correlation between cell proliferation and production of estradiol and progesterone in the presence of potential phytoestrogens. Crude isoflavone extracts from green and yellow pea seeds induced inhibition of estradiol and progesterone production and inhibition of cell proliferation in Jeg3 cell lines. Crude lignan extracts from rye seeds showed inhibition of estradiol, progesterone and cell proliferation but upregulation of estradiol production at higher concentrations (>500 mg/ml).

Immunocytochemistry

Jeg3 cells were analyzed for expression of PR, ERα and ERβ after stimulation with different concentrations of phytoestrogens, estradiol and in unstimulated controls. We could show that the expression of the ERα is low in untreated Jeg3 cells (fig. 9a). In Jeg3 cells stimulated with extracts from green pea seeds at 10, 50 and 100 μg/ml, we identified an up-regulation of the ERα expression in cells stimulated with 50 and 100 μg/ml green pea seed extracts (fig. 9c, d), whereas in Jeg3 cells stimulated with 10 μg/ml extracts the ERα expression is comparable to the unstimulated controls (fig. 9b). In Jeg3 cells stimulated with extracts from yellow pea seeds at 10, 50 and 100 μg/ml, we also identified an upregulation of the ERα expression in cells stimulated with 50 and 100 μg/ml, green pea seed extracts (fig. 9f, g), and also in Jeg3 cells stimulated with 10 μg/ml extracts the ERα expression is upregulated compared to the unstimulated controls (fig. 9e). In Jeg3 cells stimulated with lignan extracts from rye seeds at 10, 50 and 100 μg/ml, we identified an upregulation of the ERα expression in cells stimulated with 50 and 100 μg/ml lignan extracts (fig. 9i, j) whereas in Jeg3 cells stimulated with 10 μg/ml extracts the ERα expression is comparable to the unstimulated controls (fig. 9h). In Jeg3 cells stimulated with estradiol, we identified an upregulation of the ERα expression in cells stimulated with 50 and 100 μg/ml estradiol (fig. 9l, m), whereas in Jeg3 cells stimulated with 10 μg/ml (fig. 9k) extracts the ERα expression is comparable to the unstimulated controls (fig. 9h).

In addition, in Jeg3 cells stimulated with extracts from green pea seeds at 10, 50 and 100 μg/ml, we identified a down-regulation of the ERβ expression in cells stimulated with 50 and 100 μg/ml green pea seed extracts (fig. 10c, d) whereas in Jeg3 cells stimulated with 10 μg/ml extracts the ERβ expression is comparable to the unstimulated controls (fig. 10b). In Jeg3 cells stimulated with extracts from yellow pea seeds at 10, 50, and 100 μg/ml, we also identified an downregulation of the ERβ expression in cells stimulated with 50 and 100 μg/ml green pea seed extracts (fig. 10f, g). In Jeg3 cells stimulated with 10 μg/ml extracts the ERβ expression is not down regulated compared to the unstimulated controls (fig. 10e). In Jeg3 cells stimulated with lignan extracts from rye seeds at 10, 50 and 100 μg/ml we identified a downregulation of the ERβ expression in cells stimulated with 50 and 100 μg/ml lignan extracts (fig. 10i, j) whereas in Jeg3 cells stimulated with 10 μg/ml extracts the ERβ expression is comparable to the unstimulated controls (fig. 10h). In Jeg3 cells stimulated with estradiol we identified an upregulation of the ERβ expression in cells stimulated with 50 and 100 μg/ml estradiol (fig. 10l, m) whereas in Jeg3 cells stimulated with 10 μg/ml (fig. 10k) extracts the ERβ expression is comparable to the unstimulated controls (fig. 10h).

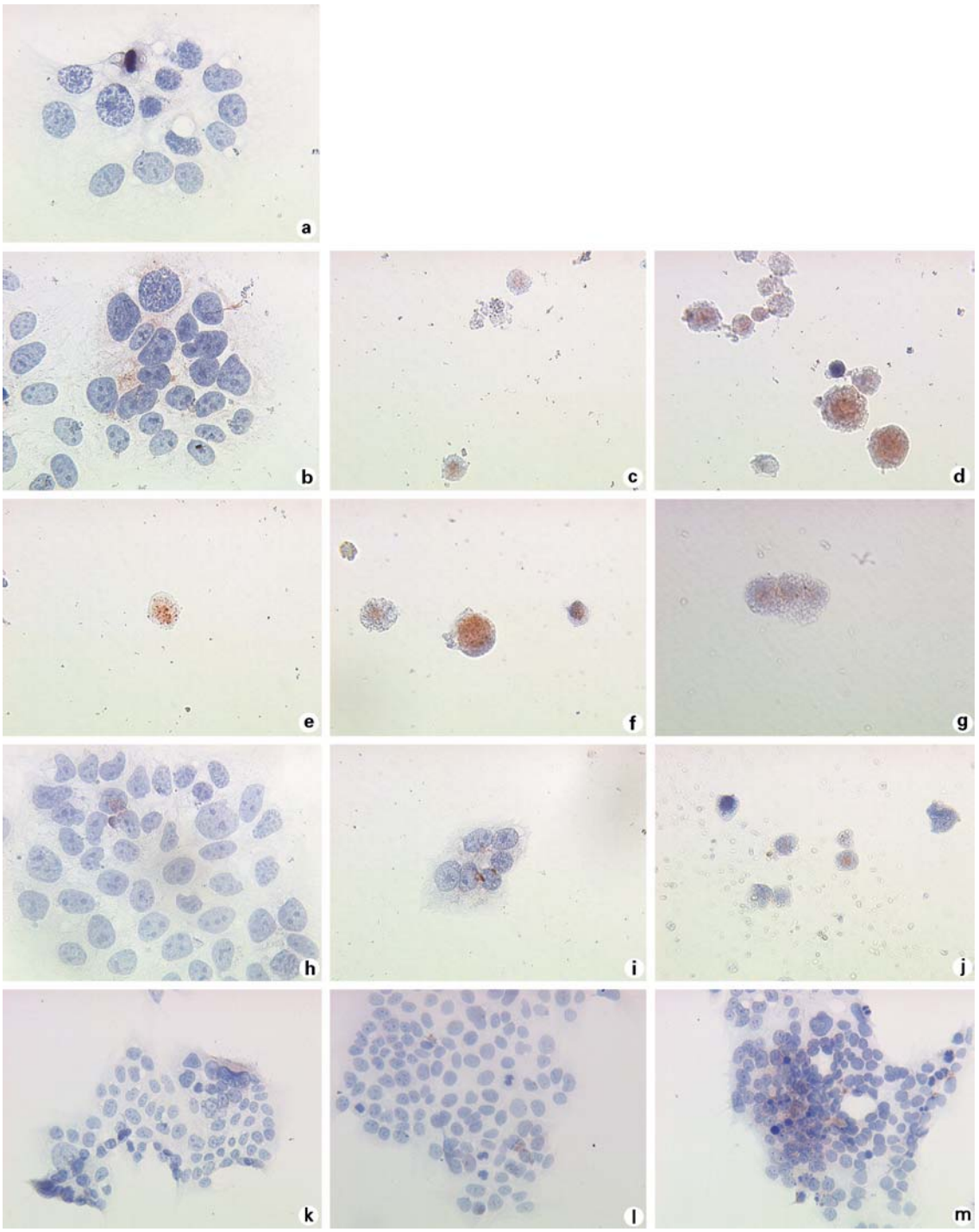

Fig. 9. Expression of ERα in Jeg3 cells in vitro, **a**: control; **b–d**: 10, 50, 100 μg/ml extracts from green pea seeds; **e–g**: 10, 50, 100 μg/ml extracts from yellow pea seeds; **h–j**: 10, 50, 100 μg/ml lignan extracts from rye seeds; magnification: 25× lens, **k–m**: 10, 50, 100 μg/ml estradiol; magnification: 10× lens.

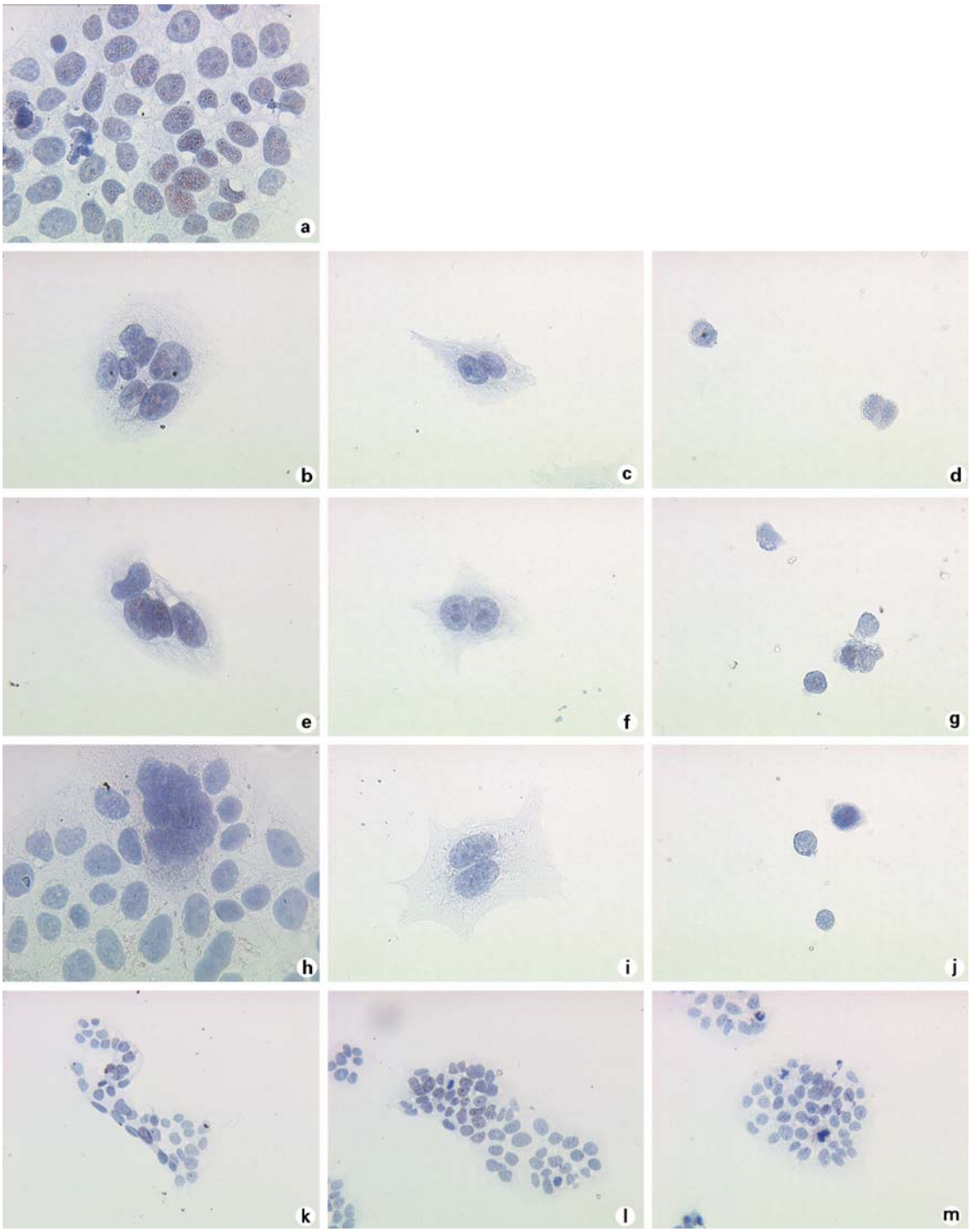

Fig. 10. Expression of ERβ in Jeg3 cells in vitro, **a**: control; **b–d**: 10, 50, 100 μg/ml extracts from green pea seeds; **e–g**: 10, 50, 100 μg/ml extracts from yellow pea seeds; **h–j**: 10, 50, 100 μg/ml lignan extracts from rye seeds; magnification: 25× lens, **k–m**: 10, 50, 100 μg/ml estradiol; magnification: 10× lens.

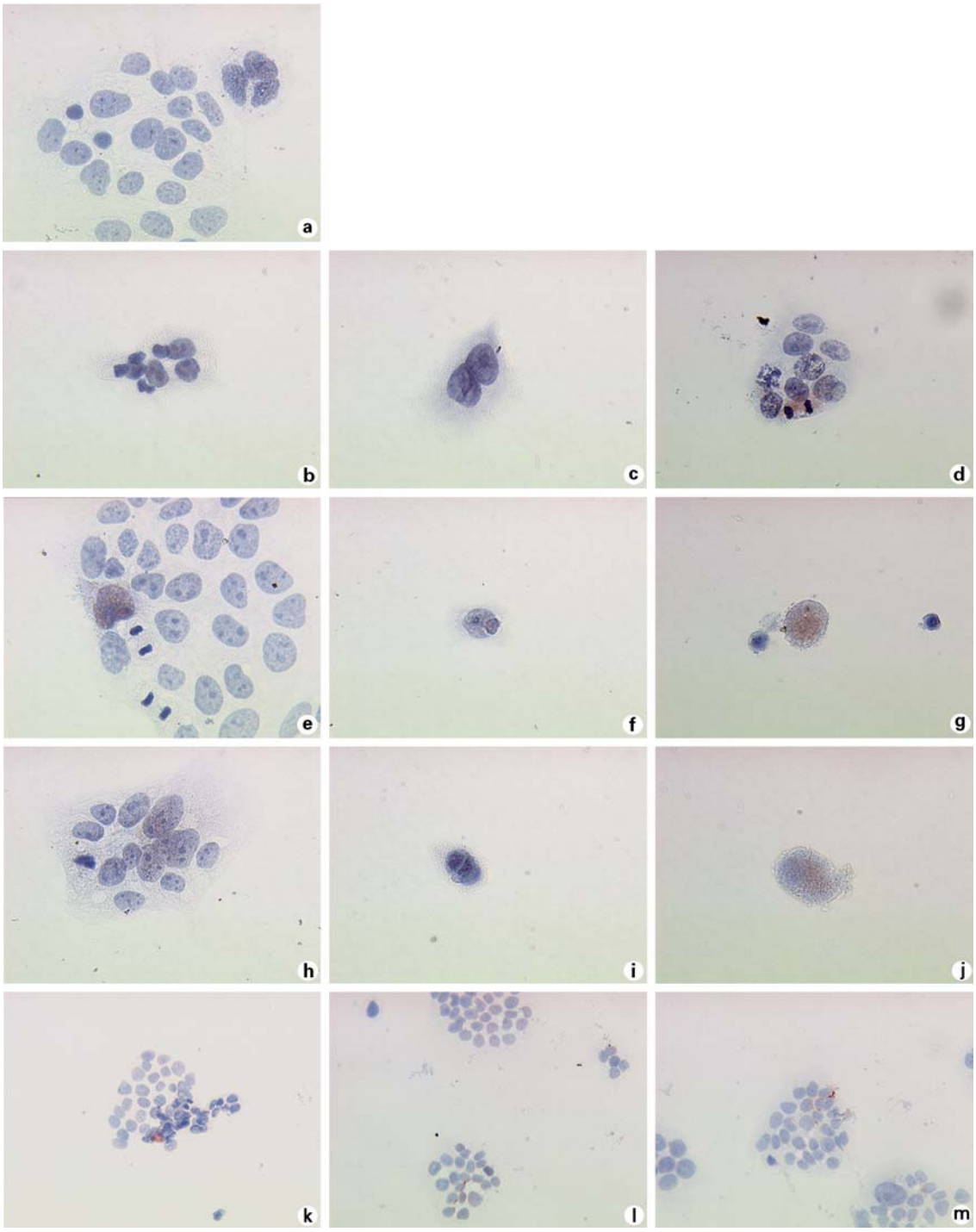

Fig. 11. Expression of PR in Jeg3 cells in vitro, **a**: control; **b–d**: 10, 50, 100 μg/ml extracts from green pea seeds; **e–g**: 10, 50, 100 μg/ml extracts from yellow pea seeds; **h–j**: 10, 50, 100 μg/ml lignan extracts from rye seeds; magnification: 25× lens, **k–m**: 10, 50, 100 μg/ml estradiol; magnification: 10× lens.

In Jeg3 cells stimulated with extracts from green pea seeds at 10, 50, and 100 μg/ml we identified an upregulation of the PR expression in cells stimulated with 50 and 100 μg/ml green pea seed extracts (fig. 11c, d) whereas in Jeg3 cells stimulated with 10 μg/ml extracts the PR expression is comparable to the unstimulated controls (fig. 11b). In Jeg3 cells stimulated with extracts from yellow pea seeds at 10, 50 and 100 μg/ml, we also identified an upregulation of the PR expression in cells stimulated with 50 and 100 μg/ml green pea seed extracts (fig. 11f, g). In Jeg3 cells stimulated with 10 μg/ml extracts the PR expression is not upregulated compared to the unstimulated controls (fig. 11e). In Jeg3 cells stimulated with lignan extracts from rye seeds at 10, 50, and 100 μg/ml, we identified an upregulation of the PR expression in cells stimulated with 50 and 100 μg/ml lignan extracts (fig. 11i, j) whereas in Jeg3 cells stimulated with 10 μg/ml extracts the PR expression is comparable to the unstimulated controls (fig. 11h). In Jeg3 cells stimulated with estradiol we identified an upregulation of the PR expression in cells stimulated with 50 and 100 μg/ml estradiol (fig. 11l and m), whereas in Jeg3 cells stimulated with 10 μg/ml (fig. 11k) extracts the PR expression is comparable to the unstimulated controls (fig. 11h).

Conclusion

Phytoestrogens have become increasingly popular among health conscious individuals over the past 10 years. Soybean based food has potential health benefits in a variety of areas including cancer, osteoporosis and coronary heart diseases as reported in different studies [34, 35]. Isoflavones are possible selective estrogen receptor modulators [36] with a higher binding affinity for ERβ compared to ERα [37]. Genistein, one of two major isoflavonoids in soy, has antiproliferative effects on mitogen-stimulated cell growth of human breast cancer cells in culture and is a candidate for use in the prevention of breast cancer. Soy protein preparations containing isoflavonoid conjugates have chemopreventive activity in carcinogen-induced rat models of breast cancer [38]. Recent experiments in these models with purified genistein have revealed that the timing of the exposure of rats to this isoflavonoid is critical [39]. Rats treated neonatally or prepuberally with genistein have a longer latency before the appearance of mammary tumors and a marked reduction in tumor number [40]. The mechanism of genistein's preventive action is in part dependent on its estrogenic activity, which causes a more rapid differentiation of the cells of the mammary gland, and analogous to the effects of an early pregnancy. Rats administered genistein after 35 days of age have smaller alterations in breast cancer risk, with a maximum reduction in mammary tumor number of 27%. In ovariectomized nude mice, dietary genistein increases cell proliferation of human breast cancer MCF-7 cell xenografts compared with a control diet [41, 42]. This estrogen-like effect of genistein is not observed in non-ovariectomized rats. Isoflavones have been shown to down regulate estrogen receptor expression as an indirect effect for reduced estrogenic functions and protein synthesis [43]. In addition, isoflavones have been shown to inhibit the activity of enzymes involved in estrogen metabolism, inhibition of aromatases and 17β-oxidoreduction of estrogens in vitro. Several studies directly examined the effects of isolated isoflavones on mammary tumor genesis [44], but chorion carcinomas have not been investigated so far. Our own investigations on phytoestrogens genistein and daidzein on steroid hormone receptor expression in endometrial cells showed that expression of ERα is significantly declined with genistein, whereas PR increased significantly with genistein. Stimulation with daidzein resulted in no statistically relevant alterations in ER, whereas the PR significantly increased [30].

In another study using genistein and daidzein in BeWo and Jeg3 cells we could show that high doses of the phytoestrogens genistein and daidzein (1–2 μmol/ml) reduced the cell proliferation and the production of the steroid hormone progesterone. In addition, high doses of daidzein reduced the production of hCG in both cell types tested. High doses of genistein had no influence on the hCG production in BeWo and Jeg3 cells. Surprisingly low doses of daidzein and genistein induced both cell types to produce high amounts of hCG. In contrast the production of progesterone was inhibited by low concentrations of phytoestrogens (0.1 μmol/ml) genistein and daidzein and the proliferation rate was weakly inhibited in both cell types tested [28]. In former studies it was found that both BeWo and Jeg3 cells express the ER [45]. In addition, Jiang et al. found that estradiol stimulated BeWo cell proliferation and increased the percentage of S-phase cells. Tamoxifen inhibited BeWo cell growth and antagonized the stimulatory actions of estradiol. The BeWo cell ER binds to an estrogen response element consensus sequence and the ER-estrogen response element complex is supershifted by antibodies directed against the ER. Jiang et al. [46] concluded that BeWo cells express a functional ER that is important for the control of BeWo cell proliferation, suggesting a potential role for estrogens in mediating

placental trophoblast growth and development. Within this study we could show that estradiol and phytoestrogens induced the expression of both the ERα and PR in Jeg3 cells in vitro. Expression of ERβ was only induced by estradiol. In addition, estradiol also inhibited progesterone production of Jeg3 cells. These results implicate that Jeg3 cells show an estrogenic response and in addition, proliferation inhibition by phytoestrogens investigated is mediated by the hormone receptors.

Therefore, we assumed that co-measurement of progesterone and in addition proliferation in Jeg3 cells after addition of different phytoestrogens can be seen as model to investigate the action of newly isolated phytoestrogens. In this study, we further developed this cell culture model system for testing the influence of different concentrations of potential phytoestrogens in isoflavone extracts from green and yellow pea seeds and crude lignan extracts from rye seeds. We were able to show that isoflavones isolated from green and yellow pea seeds reduced the cell proliferation in both cell cultures tested and inhibited production of the steroid hormones estradiol and progesterone in Jeg3 cells. In addition, lignans isolated from rye seeds reduced the production of progesterone and the proliferation rate in Jeg3 cells.

Interestingly, the effect in estradiol production by rye seed lignans is dose dependent. Lower concentrations up to 100 μg/ml showed an inhibition of estradiol whereas higher concentrations ≥500 μg/ml strongly upregulated estradiol production. Reversed effects on hormone production induced by different concentrations of phytoestrogens could already be described in one of our former studies [28]. High doses of daidzein reduced the production of hCG in Jeg3 cells. High doses of genistein had no influence on the hCG production in Jeg3 cells. Surprisingly low doses of daidzein and genistein induced Jeg3 cells to produce high amounts of hCG [28].

Results obtained in this study suggest that potential phytoestrogens isolated from pea and rye seeds can reduce in vitro proliferation and progesterone production of trophoblast tumor cells significantly. Based on the results obtained by MS analysis it is proposed that green and yellow pea seeds contain measurable concentrations of isoflavones and rye seeds contain lignans which can be isolated and used for further investigations. This study also demonstrates that a useful cell culture model for testing new types of phytoestrogens could be established. Because measurements of progesterone production could be correlated to proliferation of the cells used, measurement of progesterone in cell culture supernatants can therefore be used as an indicator for newly isolated potential phytoestrogens. Additional experiments have to be performed in future to identify the structure of the potential phytoestrogens.

Acknowledgments

We thank Dr. H. Walzel, Dr. T. Bittorf, Mrs. R. Miethchen, C. Bauer, C. Kuhn, S. Schulze, S. Kunze, V. Ackermann, N. Kolega and G. Gaede for their help and technical assistance.

References

1 Setchell KD: Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr 1998; 68:1333S–1346S.
2 Price KR, Fenwick GR: Naturally occurring oestrogens in foods – a review. Food Addit Contam 1985;2:73–106.
3 Manas ES, Xu ZB, Unwalla RJ, Somers WS: Understanding the selectivity of genistein for human estrogen receptor-beta using X-ray crystallography and computational methods. Structure (Camb) 2004;12:2197–2207.
4 Messina MJ, Persky V, Setchell KD, Barnes S: Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr Cancer 1994;21: 113–131.
5 Park D, Huang T, Frishman WH: Phytoestrogens as cardioprotective agents. Cardiol Rev 2005;13:13–17.
6 Begum AN, Nicolle C, Mila I, Lapierre C, Nagano K, Fukushima K, Heinonen SM, Adlercreutz H, Remesy C, Scalbert A: Dietary lignins are precursors of mammalian lignans in rats. J Nutr 2004;134:120–127.
7 Adlercreutz H: Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect 1995;7(suppl):103–112.
8 Fournier DB, Erdman JW Jr, Gordon GB: Soy, its components, and cancer prevention: a review of the in vitro, animal, and human data. Cancer Epidemiol Biomarkers Prev 1998;7: 1055–1065.
9 Wei H, Wei L, Frenkel K, Bowen R, Barnes S: Inhibition of tumor promoter-induced hydrogen peroxide formation in vitro and in vivo by genistein. Nutr Cancer 1993;20:1–12.
10 Glazier MG, Bowman MA: A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. Arch Intern Med 2001;161:1161–1172.
11 Akiyama T, Ogawara H: Use and specificity of genistein as inhibitor of protein-tyrosine kinases. Methods Enzymol 1991;201:362–370.
12 Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y: Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987;262: 5592–5595.
13 Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, Liu HS, Hsu PY, Chow NH: The novel targets for anti-angiogenesis of genistein on human cancer cells. Biochem Pharmacol 2005;69:307–318.
14 Kaplanski O, Shemesh M, Berman A: Effects of phyto-oestrogens on progesterone synthesis by isolated bovine granulosa cells. J Endocrinol 1981;89:343–348.
15 Whitehead SA, Cross JE, Burden C, Lacey M: Acute and chronic effects of genistein, tyrphostin and lavendustin A on steroid synthesis in luteinized human granulosa cells. Hum Reprod 2002;17:589–594.

16 Basly JP, Lavier MC: Dietary phytoestrogens: potential selective estrogen enzyme modulators? Planta Med 2005;71:287–294.
17 Chen S, Zhang F, Sherman MA, Kijima I, Cho M, Yuan YC, Toma Y, Osawa Y, Zhou D, Eng ET: Structure-function studies of aromatase and its inhibitors: a progress report. J Steroid Biochem Mol Biol 2003;86:231–237.
18 Morrissey C, Watson RW: Phytoestrogens and prostate cancer. Curr Drug Targets 2003;4: 231–241.
19 Papiez M, Gancarczyk M, Bilinska B: The compounds from the hollyhock extract (Althaea rosea Cav. var. nigra) affect the aromatization in rat testicular cells in vivo and in vitro. Folia Histochem Cytobiol 2002;40:353–359.
20 Almstrup K, Fernandez MF, Petersen JH, Olea N, Skakkebaek NE, Leffers H: Dual effects of phytoestrogens result in u-shaped dose-response curves. Environ Health Perspect 2002; 110:743–748.
21 Eng ET, Williams D, Mandava U, Kirma N, Tekmal RR, Chen S: Suppression of aromatase (estrogen synthetase) by red wine phytochemicals. Breast Cancer Res Treat 2001;67:133–146.
22 Zhao J, Mak P, Tchoudakova A, Callard G, Chen S: Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. Gen Comp Endocrinol 2001;123:180–191.
23 Kirk CJ, Harris RM, Wood DM, Waring RH, Hughes PJ: Do dietary phytoestrogens influence susceptibility to hormone-dependent cancer by disrupting the metabolism of endogenous oestrogens? Biochem Soc Trans 2001;29: 209–216.
24 Krazeisen A, Breitling R, Moller G, Adamski J: Phytoestrogens inhibit human 17beta-hydroxysteroid dehydrogenase type 5. Mol Cell Endocrinol 2001;171:151–162.
25 Chen S: Aromatase and breast cancer. Front Biosci 1998;3:d922–d933.
26 Kao YC, Zhou C, Sherman M, Laughton CA, Chen S: Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: a site-directed mutagenesis study. Environ Health Perspect 1998;106:85–92.
27 Adlercreutz H, Bannwart C, Wahala K, Makela T, Brunow G, Hase T, Arosemena PJ, Kellis JT Jr, Vickery LE: Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J Steroid Biochem Mol Biol 1993;44:147–153.
28 Plessow D, Waldschlager J, Richter DU, Jeschke U, Bruer G, Briese V, Friese K: Effects of phytoestrogens on the trophoblast tumour cell lines BeWo and Jeg3. Anticancer Res 2003; 23:1081–1086.
29 Waldschlager J, Bergemann C, Ruth W, Effmert U, Jeschke U, Richter DU, Kragl U, Piechulla B, Briese V: Flax-seed extracts with phytoestrogenic effects on a hormone receptor-positive tumour cell line. Anticancer Res 2005; 25:1817–1822.
30 Mylonas I, Jeschke U, Makovitzky J, Winkler L, Richter DU, Friese K, Briese V: Immunohistochemical expression of steroid receptors and glycodelin A in isolated proliferative human endometrial glandular cells after stimulation with tamoxifen and phytoestrogens (genistein and daidzein). Anticancer Res 2003;23: 1119–1125.
31 Franz G KH: Allgemeine Nachweismethoden für Flavonoide in Drogen; in Franz G KH (ed): Drogen und Naturstoffe. Grundlagen und Praxis der chemischen Analyse.Berlin, Heidelberg, Springer Verlag, 1992, p 129.
32 Matscheski A: Untersuchungen zur Wirkung von Pflanzenextrakten aus Linum usitatissimum, Secale sereale und Pisum sativum auf die Zellproliferation und Progesteronproduktion von humanen Trophoblasttumorzellen; in Fachbereich Biowissenschaften, Biochemie. Rostock, Universität Rostock, 2003.
33 Luyengi L SN, Fong HHS, Pezzuto JM, Kinghorn AD: A lignan and four terpenoids from Brucea javanica that induce differentiation with cultured HL-60 promeolytic leukemia cells. Phytochemistry 1996;43:409–412.
34 Adlercreutz H, Mazur W: Phyto-oestrogens and Western diseases. Ann Med 1997;29:95–120.
35 Anderson JJ, Garner SC: Phytoestrogens and bone. Baillieres Clin Endocrinol Metab 1998; 12:543–557.
36 An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC: Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 2001;276:17808–17814.
37 Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA: Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252–4263.
38 Barnes S: The chemopreventive properties of soy isoflavonoids in animal models of breast cancer. Breast Cancer Res Treat 1997;46:169–179.
39 Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J, Mentor-Marcel R, Elgavish A: Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. J Nutr 2002;132:552S–558S.
40 Lamartiniere CA, Zhang JX, Cotroneo MS: Genistein studies in rats: potential for breast cancer prevention and reproductive and developmental toxicity. Am J Clin Nutr 1998;68: 1400S–1405S.
41 Allred CD, Allred KF, Ju YH, Goeppinger TS, Doerge DR, Helferich WG: Soy processing influences growth of estrogen-dependent breast cancer tumors. Carcinogenesis 2004;25:1649–1657.
42 Allred CD, Allred KF, Ju YH, Virant SM, Helferich WG: Soy diets containing varying amounts of genistein stimulate growth of estrogen-dependent (MCF-7) tumors in a dose-dependent manner. Cancer Res 2001;61:5045–5050.
43 Sathyamoorthy N, Wang TT: Differential effects of dietary phyto-oestrogens daidzein and equol on human breast cancer MCF-7 cells. Eur J Cancer 1997;33:2384–2389.
44 Zaizen Y, Higuchi Y, Matsuo N, Shirabe K, Tokuda H, Takeshita M: Antitumor effects of soybean hypocotyls and soybeans on the mammary tumor induction by N-methyl-n-nitrosourea in F344 rats. Anticancer Res 2000;20: 1439–1444.
45 Ho CK, Chi CW, Yu KJ, Wang SY: Tamoxifen-mediated anti-cellular effect against a choriocarcinoma cell line. Int J Oncol 1998;12: 1171–1176.
46 Jiang SW, Lloyd RV, Jin L, Eberhardt NL: Estrogen receptor expression and growth-promoting function in human choriocarcinoma cells. DNA Cell Biol 1997;16:969–977.