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Review

# In vivo test systems for the quantitative and qualitative analysis of the biological activity of phytoestrogens

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**Abstract**

Many compounds of plant origin with the ability to bind to the estrogen receptor have been identified in the last decades. One of the most extensively used in vivo assays to characterise the estrogenic potency of these phytoestrogens and mechanisms of their action is the rodent uterotrophic assay. Various protocols exist for this test system, using immature, hypophysectomized, or ovariectomized rats and mice and oral or subcutaneous administration of the test compound. However, just monitoring the ability of a compound to stimulate uterine growth is not sufficient to characterize its estrogenicity. Over the last decades, an increasing number of estrogen sensitive tissues has been identified. Moreover, a variety of different molecular mechanisms have been discovered for the action of estrogens, including non-genomic actions. Therefore, an in vivo test design for estrogenicity should include an analysis of several estrogen sensitive parameters in different estrogen sensitive tissues. To distinguish between agonistic and antagonistic properties of a substance, combinations of the test compound with estrogens and antiestrogens should be analyzed. A reasonable supplement to this enhanced uterotrophic assay are selected estrogen sensitive tumor models, which can be used to test for potential chemopreventive properties of phytoestrogens.


*Keywords:* Reviews; Uterotrophic assay; In vivo test systems; Phytoestrogens

## Contents



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## 1. Introduction

Over the last decades, a variety of plants chemicals have been identified which bind to the estrogen receptor and may induce many mechanisms of estrogen action [1]. The most potent of these phytoestrogens are the isoflavonoids, diphenolic compounds detected in the bean subfamily of Leguminosidae. Even though the potency of most of these natural estrogens is low compared to endogenous or synthetic steroid estrogens like 17β-estradiol or ethinylestradiol, significant quantities of these nonsteroidal estrogens are detectable in human urine [2]. Epidemiological data suggest that the consumption of phytoestrogens may have beneficial effects like protection against breast and prostate cancer [3,4]. On the other hand, there is the possibility that these compounds may act as endocrine disrupters which could affect the endocrine system and may cause developmental [5,6] and reproductive disturbances [6,7]. For this reason there is a need to characterize the hormonal potency of natural compounds with the ability to bind to the estrogen receptor. A variety of sophisticated in vitro test systems have been developed in the last decades to determine the estrogenic potency of synthetic compounds. These systems are also suitable to detect and quantify the estrogenicity of natural products and helpful to provide mechanistic insights. However, it is necessary to point out that even a combination of different in vitro test systems is not able to predict the action of a substance in the organism. In vivo a substance is exposed to multiple metabolic transformations and integrated in complex endocrine interactions. To get information about a potential pharmaceutical benefit and to assess a potential risk for human health, which may be caused by such compounds, the use of in vivo test systems is essential.

## 2. Physiology and molecular mechanisms of estrogen action

According to the classical definition, estrogens are steroid hormones with important functions for the regulation of specific sexual processes in the female organism. Therefore, the highest amounts of estrogen receptors (ER) are found in tissues with reproductive functions. These estrogen target organs are the mammary gland, the ovaries, the vagina and the uterus. In these tissues estradiol stimulates the cell proliferation and the biosynthesis of the progesterone receptor (PR) [8]. Estrogens also act in the male organism. ER can be detected in the prostate and is believed to have an important function during the development of this organ [9]. In addition, there is knowledge about the action of estrogens in other, non-classical target tissues like the brain, the bone, the cardiovascular system, the kidney, the immune system and the liver (Table 1). Estrogen deficiency seems to be involved in many pathologic processes like arteriosclerosis [10], osteoporosis [11], the loss of cognitive abilities [12] and degenerative processes

Table 1
Estrogen sensitive tissues and cells

| Classic targets | Non-classical targets |
|---|---|
| • Ovary | • Kidney |
| • Vagina | • Islets of Langerhanus |
| • Uterus | • Liver |
| • Mammary gland | • Bone |
| • Adrenal gland | • Cardiovascular system |
| • Prostate | • Macrophages |
| • Pituitary gland | • Thymocytes |
| • Hypothalamus | • Lymphoid cells |
| • Leydig cells | • Endothecial cells |
| | • Osteoblastic cells |
| | • Glia cells |
| | • Schwann cells |
| | • Adipose tissue |

in the central nervous system [13], whereas elevated estrogen levels are believed to support the development and promotion of tumors [14].

The classical concept of the action of steroid hormone receptors was established by Jensen in 1968 [15,16]. The protein structure of steroid hormone receptors, their biochemical properties and the molecular mechanisms of their action have been intensively characterized during the last 25 years. In the classical concept, the ER is rapidly transferred to the nucleus [17], where it is stored in an inactive but primed state until a proper stimulus is received. The priming step is accomplished by the association of the ER with a variety of heat shock proteins, including hsp 90 and hsp 56 [18]. This complex of the ER and heat shock proteins rapidly dissociates after binding to a hormone. However, it has to be mentioned that the mechanism of activation, long believed to be hormone-dependent, is in question due to the observation that ER-mediated activity can be induced by signal transduction pathways. Growth factors like epidermal growth factor (EGF) and other members of cAMP-mediated signal transduction pathways are able to activate the ER without binding to a hormone. These mechanisms are defined as so-called cross talk mechanisms [19]. After activation, protein–protein interactions between ER monomers occur to form homodimers. These ER dimers are required for high affinity DNA-binding [20]. The receptor interacts via specific DNA-binding domains with ER binding sides, the estrogen receptor responsive elements (EREs), in the promotor region of sensitive genes; this binding leads to the initiation of transcription [21]. The organisation in functional domains like the DNA-binding domain, the hormone-binding domain and the transactivation domains has been demonstrated to be highly conserved [22,23]. The recent progress in steroid hormone research made it necessary to modify the classical concept of steroid hormone action in many aspects (Fig. 1). One of the most important findings, which is believed to be of utmost significance for the understanding of estrogen action, was the discovery of a second type of ER, the so-called ER β by Gustafsson and coworkers in 1996 [24]. Some compounds of plant origin, like the isoflavone genistein, have been demonstrated to bind with higher affinity to this type of receptor than to ER α [25].

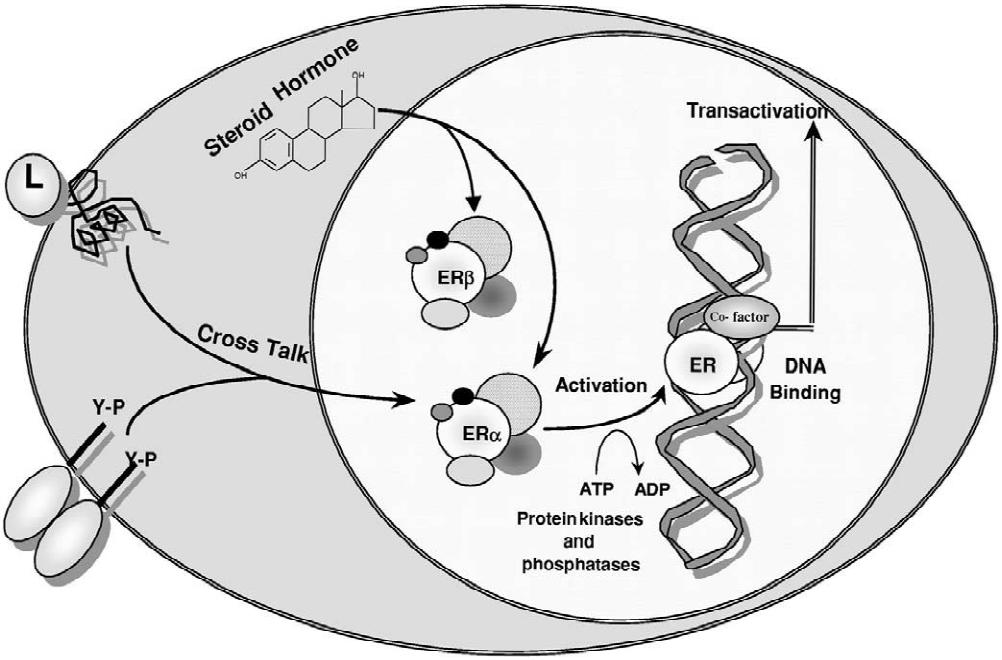


Fig. 1. Mechanisms of estrogen receptor activation.

There are speculations that the tissue distribution of ER β and the interaction with ER α may be relevant for the regulation of cell proliferation [26] and also for distinct physiological functions, especially in non-classical target tissues like the vascular system [27]. An other important finding was the discovery of so-called co-activators [28] and co-repressors [29], which are involved in the initiation and regulation of gene transcription by the ER. Such co-activators are believed to determine the agonistic and antagonistic properties of compounds and to be responsible for the tissue specific action of so-called selective estrogen receptor modulators (SERMs) like tamoxifen or raloxifene. Besides these very spectacular findings it is known that the action of steroid hormone receptors on the initiation of transcription of sensitive genes can be modulated by a number of additional factors. In general, it seems that the term estrogenicity has to be defined today in a much more complex manner than 25 years ago. This fact has to be taken into account when we test for the estrogenic activity of a compound.

## 3. The rodent uterotrophic assay: a classical in vivo test system for the determination of estrogenicity

One of the most extensively used in vivo assays for estrogenicity is the rodent uterotrophic assay in which the ability of chemicals to stimulate uterine growth is determined [30,31]. There are various protocols for this assay, using immature, hypohysectomized, or ovariectomized rats and mice, and oral or subcutaneous administration of the test compound [32,33]. In a typical uterotrophic assay, either immature female rats (21–22 days old) or ovariectomized adult female rats (6–8 weeks old) are treated for 3 days orally or subcutaneously with the test chemicals. On day 4, the animals are sacrificed and the absolute uterus weights are determined. In some cases, the duration of treatment is shorter or extended up to 14 or even 28 days. The choice of the animal species is probably not a critical protocol variable, as evidenced by a similar sensitivity of rats and mice to a variety of different chemicals [34]. However, in many cases rats are favored over mice for reason of laboratory practice. The question whether immature animals are superior to adult ovariectomized animals cannot be clearly answered. Recently it has been demonstrated for distinct compounds that immature animals are more sensitive than ovariectomized animals [35]. Another major advantage of juvenile animals is their easy handling. Therefore the uterotrophic assay with juvenile animals is commonly used in toxicologic investigations. The design of the uterotrophic assay is also suitable for investigating potential antiestrogenic properties of test chemicals. In such a test design, the ability of test chemicals to reduce or abolish the uterotrophic response to estradiol is determined [36].

### *3.1. Limits of the rodent uterotrophic assay*

Doubts whether the classical uterotrophic assay is suitable in all cases to identify chemicals with estrogenic properties have been raised with the development and the pharmacological characterization of drugs like raloxifene, which proved to be a tissue-selective estrogen agonist. Raloxifene counteracts estrogen effects in the breast while acting like an estrogen in the bone, but it does not promote excess growth of uterine tissue [37,38]. The observation that raloxifene is a systemic ER agonist but is devoid of activity in the uterine tissue has important implications for the rodent uterotrophic assay as a primary screen for estrogenic activity. It is very likely that some phytoestrogens may also possess properties of selective ER modulators (SERMs). For example, it has been shown very recently that genistein exerts properties very similar to raloxifene [39,40]. This fact has to be considered when the uterotrophic assay is used for the identification and the pharmacological characterisation of chemicals.

### *3.2. The enhanced uterotrophic assay*

The sensitivity of the uterotrophic assay and its scientific significance can be enhanced dramatically if an analysis of estrogen sensitive endpoints in the uterus and other estrogen sensitive target tissues is included [41]. Such an enhanced uterotrophic assay should combine the analysis of morphological, histological, biochemical and molecular endpoints (Fig. 2). Important target tissues should include the uterus,

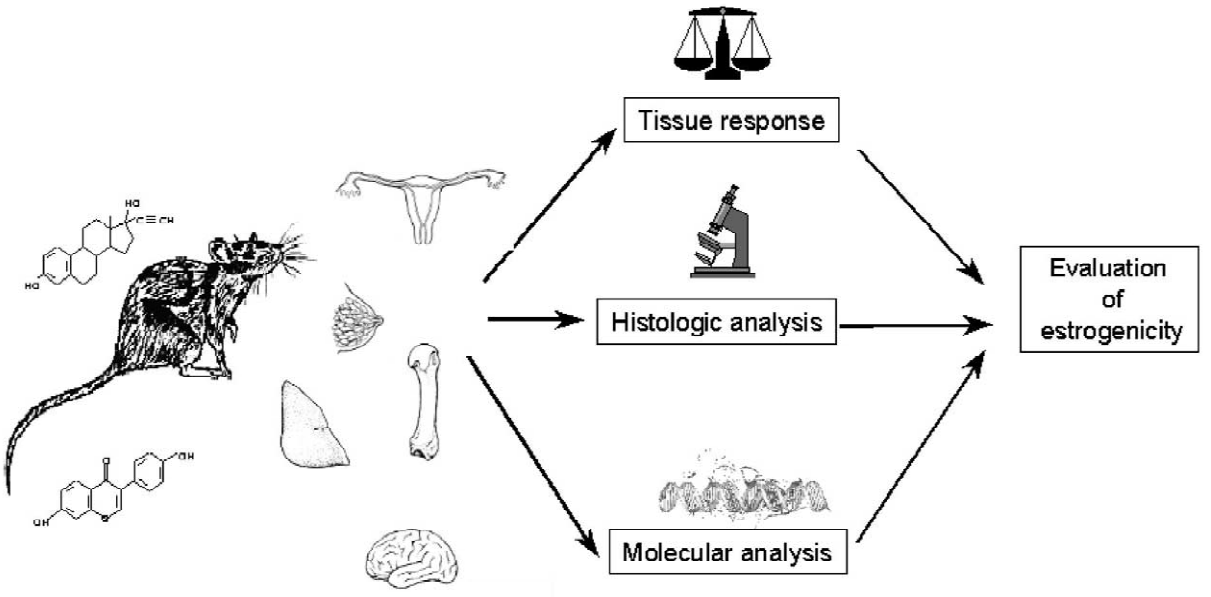


Fig. 2. Principle of the enhanced uterotrophic assay.

the vagina, the mammary gland, the liver, the bone, the cardiovascular system and the brain.

*3.2.1. Suitable endpoints for estrogenicity*

There are various morphological, histological, biochemical and molecular endpoints in the mentioned target tissues suitable to analyze the estrogenic properties of a test substance. Often organ weights and the tissue composition are investigated. Morphological parameters which are frequently analyzed are the uterine dry and wet weight, the number of glands in the uterus, the tissue composition of the uterus (epithelium, myometrium and stroma), the weight and the cornification of the vagina, the vaginal opening, the branching of the ducts in the mammary gland, the trabecular structure in the bone, and, for toxicological reasons, the weight of the liver. Among commonly used histological endpoints are the height of the uterine (Fig. 3) and vaginal epithelium (Fig. 4). Great pharmacological and pathological importance has been given to the uterine epithelium as an endpoint. It has been demonstrated that the ability of a substance to stimulate the height of the uterine epithelium is correlated with an enhanced risk to promote the development of endometrium carcinoma [42]. This has been demonstrated for the first time for the partial agonist tamoxifen, a substance widely used in the therapy of breast cancer. The vaginal epithelium has been demonstrated to be a very sensitive endpoint for estrogenicity. In many cases the vaginal epithelium responds at doses at which no stimulation of the uterus is detectable at all [41]. In the mammary gland, suitable parameters for estrogenicity are the expression and regulation of the progesterone receptor (PR) which has been demonstrated to be a sensitive estrogen regulated gene [43]. An analysis of the PR expression can be performed either by the determination of the receptor content in this tissue by biochemical methods like receptor binding [43], by immunohistochemistry [44] or by an analysis of the PR mRNA expression [41]. The same techniques can also be used in the uterus were an analysis of the PR expression and determination of its localisation can supplement the endpoints described earlier. Another very suitable endpoint for estrogenicity is cell proliferation. Estrogens are known to act as mitogens and stimulate cell proliferation, especially in breast and uterine tissues. This ability is believed to correlate with the promotion of tumor development in these tissues. Suitable techniques to detect and quantify proliferation are either cell cycle analysis by flow

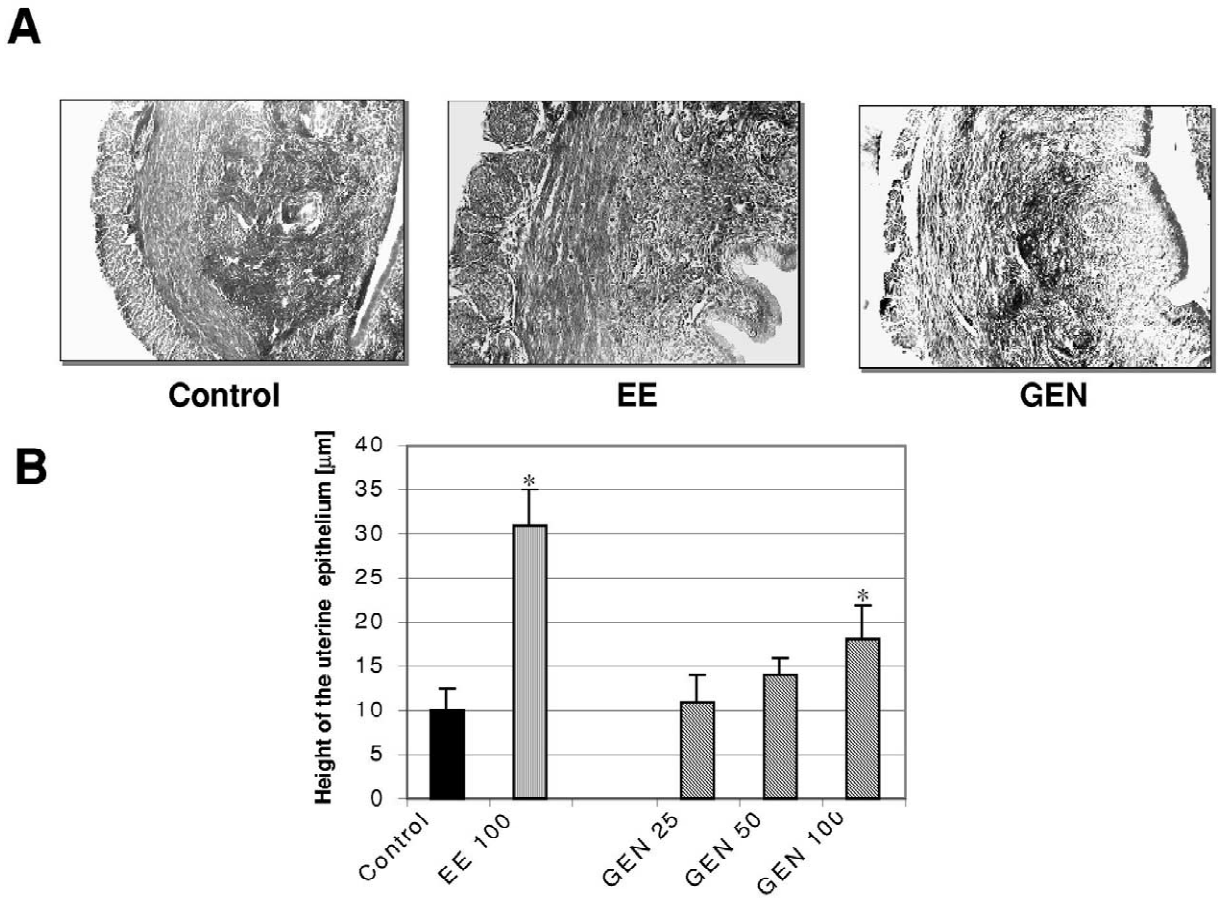


Fig. 3. Analysis of the uterine epithelium height following genistein treatment of ovariectomized DA/Han rats. Rats were treated orally for 3 days with ethinylestradiol (EE) or genistein (GEN). Dosage for EE is given in μg/kg per day, whereas doses for GEN represent mg/kg per day. Shown are uterine morphology (A) and semiquantitative evaluation of uterine epithelium height by morphometry (B). * means significant against control group, $P \leq 0.05$, Mann–Whitney *U*-test. For experimental details see Ref. [77].

cytometry or immunohistochemical analysis of proliferation markers like Ki67 or PCNA [45]. In the liver, estrogens influence the synthesis of a variety of factors. Estrogens are believed to protect the cardiovascular system. It has been demonstrated that estrogen administration lowers the serum cholesterol concentration and the serum composition of high- and low-density lipoproteins [46]. In addition, there are some genes which are known to be estrogen-regulated in the liver and can be included in such investigations [47]. In the vascular system it has been further demonstrated that the gene expression in the vessels [48] and the blood flow [49] are modulated by estrogens. In the bone, important markers for estrogen action are the bone density and the expression of specific genes. It is important to point out that analysis of estrogenic effects on bone density needs a modification of the classical design of the uterotrophic assay. An extension of the treatment period is necessary [50]. A suitable gene involved in the estrogen-dependent molecular mechanisms of bone remodelling is the IL6 receptor [51]. Analysis of the action of estrogens in the brain is very difficult. Indirect parameters for estrogen action in the brain, which are comparatively easy to monitor and provide information in regard to the ability of a test substance to cross the blood–brain barrier, are the analysis of the modulation of gonado-

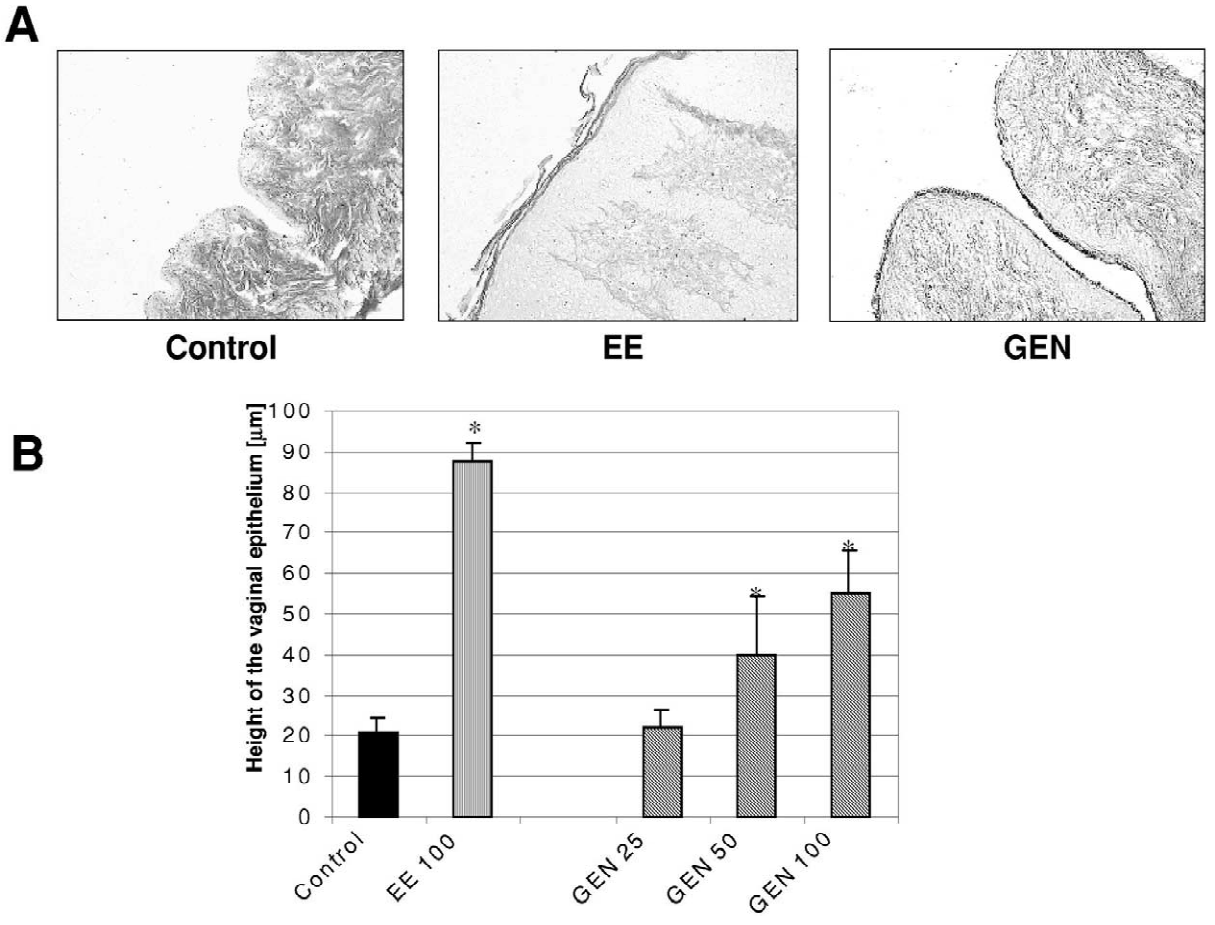


Fig. 4. Analysis of the vaginal epithelium height and cornification following genistein treatment of ovariectomized DA/Han rats. Rats were treated orally for 3 days with ethinylestradiol (EE) or genistein (GEN). Dosage for EE is given in μg/kg per day, whereas doses for GEN represent mg/kg per day. Shown are vaginal morphology (A) and semiquantitative evaluation of vaginal epithelium height by morphometry (B). * means significant against control group, $P \leq 0.05$, Mann–Whitney *U*-test. For experimental details see Ref. [77].

tropin and prolactin secretion [52,53]. This can be monitored by analysis of the prolactin levels in the serum and/or by a direct analysis of prolactin secretion in the hypothalamus by immunohistochemistry. Table 2 provides a survey of tissue specific estrogenic endpoints.

### *3.2.2. The gene expression fingerprint, a powerful and sensitive tool for the analysis of tissue specific estrogenic action*

The analysis of the regulation of endogenous estrogen sensitive genes is one of the most valid procedures to characterize the estrogenicity of a substance in vivo. The response to estrogens involves the activation of a large pattern of estrogen sensitive genes. Analysis of the tissue specific pattern of expressed genes offers the opportunity to quantify the estrogenic potency of a substance and in parallel to elucidate molecular mechanisms of its action. In contrast to in vitro test systems like reporter gene assays, where the expression of artificial gene constructs in an artificial environment is analyzed, endogenous estrogen sensitive genes are imbedded in their native environment [54]. The genes are integrated in the genome, in their natural copy number at the appropriate position on the chromosome. They possess complete promotors and they are imbedded in the complex regulatory ma-

Table 2
Suitable endpoints for estrogenicity

| Tissue | Morphological endpoints | Histological endpoints | Molecular and biochemical endpoints |
|---|---|---|---|
| Uterus | • Uterine wet and dry weight | • Uterine epithelium<br>• Uterine glands | • Gene expression: C3, Clusterin, PR<br>• Localisation and expression of PR and ER<br>• Cell proliferation |
| Mammary gland | – | • Branching of the ducts | • Gene expression: PR<br>• Expression of PR and ER<br>• Cell proliferation |
| Vascular system | – | – | • Gene expression: PR, ER<br>• HDL–LDL<br>• Blood volume flow |
| Vagina | • Opening of the vagina<br>• Weight of the vagina | • Cornification<br>• Vaginal epithelium | • Gene expression: PR |
| Liver | • Weight of the liver | – | • HDL–LDL<br>• Cholesterol<br>• Gene expression: IGFBP1 |
| Brain | – | – | • Gonadotropin and prolactin secretion |
| Bone | – | • Trabecular structure | • Bone density<br>• Gene expression: IL6 receptor |

chinery of the cell. The analysis of the expression of endogenous genes in different types of organotypic cell lines offers the possibility to recognize organ selective effects of substances [41,55]. A disadvantage of this method is the tedious experimental procedure necessary to perform such an assay. However, if methods like quantitative polymerase chain reaction (PCR) are used [41,55], it is possible to standardize the test systems. An example for a PCR-based gene expression fingerprint of estrogen sensitive genes is given in Fig. 5. A highly sophisticated form of the gene expression fingerprint is the gene array technology [56]. Using cDNA arrays, the activity of a large number of genes can be analyzed in one experiment, which is a very helpful tool in toxicological and mechanistic studies of estrogens.

## 4. Tumor models

Tumor models are mainly used to test for potential chemopreventive properties of phytoestrogens. Several experimental approaches have been described which can be subdivided into three categories: (1) models of spontaneous carcinogenesis; (2) chemical carcinogen-induced tumor models; and (3) tumor models by xenotransplantation of tumor cells.

### *4.1. Spontaneous carcinogenesis*

For prostate and endometrial carcinogenesis, animal models are available with a high incidence of spontaneous tumors of the respective organs. Male Lobund–Wistar rats spontaneously develop metastasizing adenocarcinoma in the anterior prostate seminal vesicle complex with an incidence of approximately 30%. Carcinogenesis of these tumors could be prevented by feeding animals a soy-protein/isoflavone diet [57,58]. Two rat strains have been described as potential models for the carcinogenesis of the endometrium. Female DA/Han rats die from endometrial adenocarcinoma with an incidence rate $>60\%$ if bred to their natural life end [59], and female BDII/Han rats with an incidence rate $>90\%$ [60]. Although it has been clearly shown by ovariectomy of juvenile animals [60] or by hormonal treatment [61] that these tumors exhibit an estrogen-dependent growth characteristic, they have never been used in studies investigating the preventive activities of phytoestrogens.

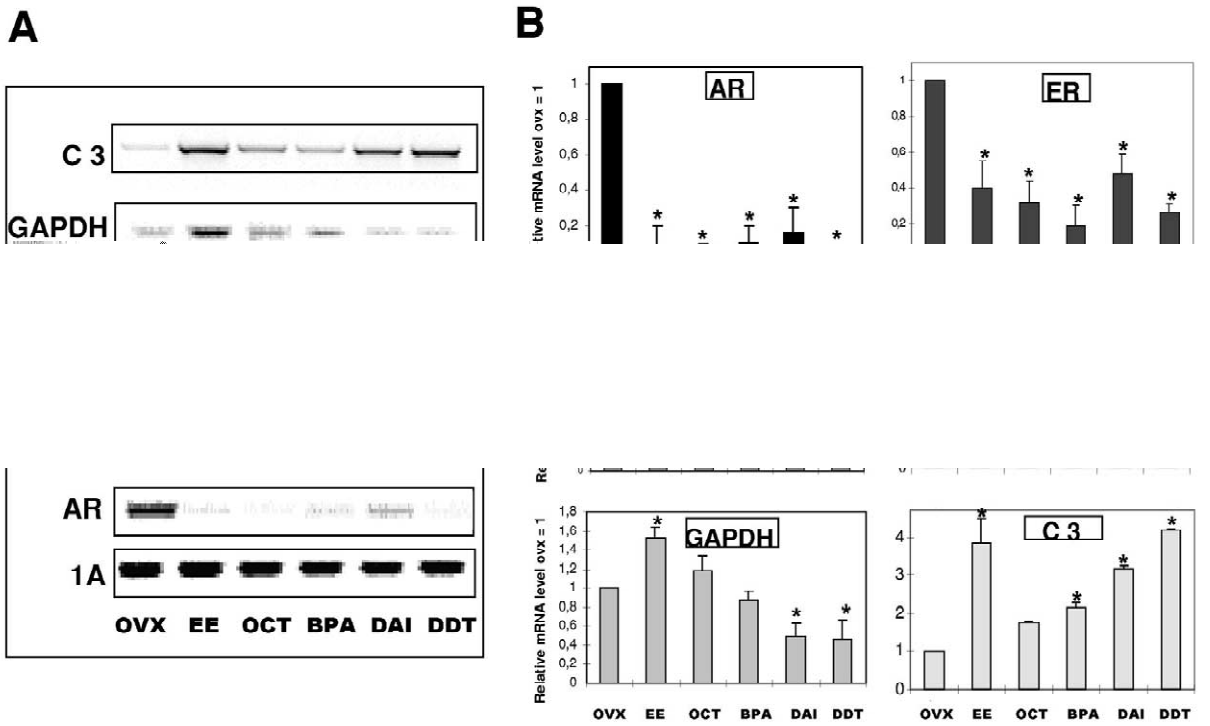


Fig. 5. Example for a gene expression fingerprint. Analysis of uterine clusterin (CLU), androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), GAPDH and C3 mRNA expression after treatment with daidzein (DAI, 500 mg/kg per day), *o,p′*-DDT (DDT, 500 mg/kg per day), *p-tert*.-octylphenol (OCT, 200 mg/kg per day), bisphenol A (BPA, 200 mg/kg per day) and ethinylestradiol (EE, 100 μg/kg per day). Analysis by semiquantitative PCR. OVX=ovariectomized vehicle-treated animal. For each treatment group, the pooled RNA of six uteri was analyzed. cDNA synthesis and semiquantive PCR analysis was performed independently four times. The results are shown as mean±SD. The mRNA expression of the ovariectomized vehicle-treated animal group was defined as 1. Statistical significant differences ($P \leq 0.05$) of the mRMA expression from the ovariectomized vehicle-treated animal group are indicated by *. For experimental details see Ref. [41].

### *4.2. Chemical carcinogen-induced tumor models*

Two animal models to test for tumor promoting or tumor preventing activities of phytoestrogens in the mammary gland have been described. Following exposure to 7,12-dimethylbenz(a)anthracene (DMBA) or nitrosomethylurea (NMU), Sprague–Dawley rats develop chemically induced mammary carcinomas. These models have preferentially been used for chemoprevention studies. Genistein, a potent isoflavone, has been described as a chemopreventive agent for breast cancer in the DMBA-induced tumor model. It had been shown in a series of studies that treatment with genistein during perinatal, neonatal, or prepubertal periods prevents breast cancer [62–65]. In contrast, maternal exposure to genistein during pregnancy induced susceptibility of the offspring to DMBA-induced mammary carcinogenesis [66].

Results described for NMU-induced mammary carcinoma are conflicting. Inhibition of tumorigenesis by soybean isoflavones has been observed in one study [67], whereas another study reported a trend towards inhibition using a similar approach [68]. A third study in this NMU-induced mammary tumor model, investigating effects of perinatal exposure to genistein alone, concludes that genistein is an endocrine disrupter and increases the multiplicity of NMU-induced mammary carcinoma in rats [69].

A similar model has been described for the prostate. Biweekly treatment of inbred F344 rats with a combination of 3,2′-dimethyl-4-amino-

biphenyl (DMAB) and testosterone propionate for 20 weeks induces prostate carcinogenesis. Feeding studies with mixtures of genistein and daidzein suggested that these isoflavones have the potential to prevent carcinogenesis of the prostate [70].

### *4.3. Xenotransplantation of tumor cells*

Some tumor cell lines, if xenotransplanted to either immunodeficient nude mice or rats or to syngeneic animals, have the property to grow tumors at the ectopic site and eventually metastasize through blood or lymphogenic pathways. In connection with studies on potential chemopreventive effects of phytoestrogens, this approach has been used as an experimental model for breast, prostate and endometrial tumors.

Following xenotransplantation of LNCaP prostate adenocarcinoma cells into nude mice, delayed tumor growth in combination with increased apoptosis rates could be demonstrated for diets containing either low fat and soy protein with isoflavones [71] or combinations of rye bran and soy protein [72].

Contradictory results have been obtained in prevention studies following xenotransplantation of estrogen-sensitive MCF-7 or estrogen-insensitive MDA-MB mammary carcinoma cells into nude mice. Whereas all these studies report growth inhibition of cultured MCF-7 breast cancer cells by genistein, the studies come to totally different conclusions for xenotransplanted breast cancer cells. Genistein has been described to induce maturation of both estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-468 cells following xenotransplantation into nude mice, thereby preventing tumor growth [73]. This suggests that the differentiation-inducing effect of genistein is independent of its estrogenic/antiestrogenic properties. Santell et al. [74] reported no significant response of the growth of xenotransplanted MDA-MB-231 cells to treatment with genistein. A third study even reported a dose-dependent growth stimulation of xenotransplanted MCF-7 cells by genistein [75].

Finally, from tumors of two inbred rat strains, from accessory sex gland tumors of male Lobund–Wistar rats and from endometrial adenocarcinoma of female DA/Han rats, the K1 cell line and RUCA-I cell line have been established. These cell lines can be xenotransplanted into corresponding syngeneic animals and give rise to either prostate-like carcinoma or endometrial adenocarcinoma at the ectopic site. Whereas genistein inhibited the growth and metastasis of transplantable rat accessory sex gland carcinoma [76], no effect on the growth of xenotransplanted RUCA-I cells could be detected [77], although the same model dramatically responded by growth inhibition to the treatment with the pure antiestrogen ICI 182,780 [78].

In summary, there are different in vivo models available to study potential effects of phytoestrogens on carcinogenesis and on growth of hormone-dependent tumors. Application of these models in chemoprevention studies lead to partly contradictory results. Isoflavones appear to protect against prostate and DMBA-induced mammary carcinogenesis. Conversely, effects of the same substances on either NMU-induced mammary carcinoma or already initiated tumors of the breast and the endometrium are either not detectable (endometrial carcinogenesis) or are contradictory (NMU-induced tumors or xenotransplanted MCF-7 cells). In general, there exists an urgent need for tumor models to study the antitumor effects of phytoestrogens. However, since it is difficult to establish new models and since the existing models produce conflicting data, there is also the need for experimental efforts which aim to stringently enhance the power of existing experimental models and their applicability in investigations on phytoestrogens. From the experiences made with normal tissues, it is proposed to examine tumors not only for their growth properties but also for their gene expression pattern and ultimately establish gene expression fingerprints.

## 5. Conclusion

During the last years, international agencies like the Organisation for Economic Cooperation and Development have validated the rodent uterotrophic assay with the aim to screen compounds for estrogenic activity [79]. The main focus of these investigations was to develop standard test systems for risk assessment and the identification of endocrine disruptors. Many helpful informations regarding the experimental conditions (influence of the

strain, diet, housing protocol, bedding and vehicle) are provided by theses studies. However, to analyse the suitability of phytoestrogens as pharmaceuticals for the treatment or prevention of specific diseases, a modification, adaptation and supplementation of these standard protocols is necessary. Therefore, a test designed for the analysis of specific pharmaceutical properties of phytoestrogens should include an analysis of several estrogen-sensitive parameters in different estrogen-sensitive tissues and combinations of the test compound with estrogens and antiestrogens to distinguish between agonistic and antagonistic properties. In order to identify molecular mechanisms of the action of phytoestrogens, it make sense to combine in vivo and in vitro test systems. In vivo tumor models are of limited use for risk assessment and the quantitative determination of the estrogenicity of phytoestrogens. Nevertheless, they are helpful tools to test these substances for potential chemopreventive properties.

## 6. Nomenclature

| | |
|---|---|
| DMAB | 3,2′-dimethyl-4-aminobiphenyl |
| DMBA | 7,12-dimethylbenz(a)anthracene |
| ER | estrogen receptor |
| NMU | nitrosomethylurea |
| PR | progesterone receptor |
| SERM | selective estrogen receptor modulator |

## References


[1] S.R. Davis, F.S. Dalais, E.R. Simpson, A.L. Murkies, Recent Prog. Horm. Res. 54 (1999) 185.
[2] H. Adlercreutz, T. Fotsis, R. Heikkinen, J.T. Dwyer, M. Woods, B.R. Goldin, S.L. Gorbach, Lancet 2 (1982) 1295.
[3] H. Adlercreutz, Y. Mousavi, J. Clark, K. Hockerstedt, E. Hamalainen, K. Wahala, T. Makela, T. Hase, J. Steroid Biochem. Mol. Bio. 41 (1992) 331.
[4] H. Adlercreutz, P. Rozen (Ed.), Front. Gastrointestinal Res 14 (1988) 165.
[5] K.D. Setchell, S.P. Borriello, P. Hulme, D.N. Kirk, M. Axelson, Am. J. Clin. Nutr. 214 (1984) 569.
[6] J.A. McLachlan, R.R. Newbold, Environ. Health Perspect. 75 (1987) 25.
[7] K.D. Setchell, S.J. Gosselin, M.B. Welsh, J.O. Johnston, W.F. Balistreri, L.W. Kramer, B.L. Dresser, M.J. Tarr, Gastroenterology 93 (1987) 225.
[8] K.B. Horwitz, Y. Koseki, W.L. McGuire, Endocrinology 103 (1978) 1742.
[9] G. S Prins, L. Birch, J.F. Couse, I. Choi, B. Katzenellenbogen, K.S. Korach, Cancer Res. 61 (2001) 6089.
[10] T.S. Mikkola, T.B. Clarkson, Cardiovasc. Res. 53 (2002) 605.
[11] R.T. Turner, B.L. Riggs, T.C. Spelsberg, Endocr. Rev. 15 (1994) 275.
[12] B.S. McEwen, J. Appl. Physiol. 91 (2001) 2785.
[13] H. Honjo, N. Kikuchi, T. Hosoda, K. Kariva, Y. Kinoshita, K. Iwasa, T. Ohkubo, K. Tanaka, T. Tamura, M. Urabe, M. Kawata, J. Steroid Biochem. Mol. Biol. 76 (2001) 227.
[14] G.A. Colditz, Cancer 71 (1993) 1480.
[15] E. Jensen, H. Jacobson, Rec. Prog. Horm. Res. 18 (1962) 387.
[16] E.V. Jensen, T. Suzuki, T. Kawashima, W.E. Stumpf, P.W. Jungblut, E.R. DeSombre, Proc. Natl. Acad. Sci. USA 59 (1968) 632.
[17] W.J. King, G.L. Greene, Nature 307 (1984) 745.
[18] G.P. Rossini, J. Theor. Biol. 166 (1994) 339.
[19] C.L. Smith, O.M. Conneely, B.W. O'Malley, Proc. Natl. Acad. Sci. USA 90 (1993) 6120.
[20] Y. Wang, R.J. Miksicek, Mol. Endocrinol. 5 (1991) 1707.
[21] H. Gronemeyer, FASEB J. 6 (1992) 2524.
[22] W.W. Grody, W.T. Schrader, B.W. O'Malley, Endocr. Rev. 3 (1982) 141.
[23] B.A. Liebermann, Crit. Rev. Eukaryot. Gene Expr. 7 (1997) 43.
[24] G.G. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.A. Gustafsson, Proc. Natl. Acad. Sci. USA 93 (1996) 5925.
[25] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Endocrinology 139 (1998) 4252.
[26] Z. Weihua, S. Saji, S. Makinen, G. Cheng, E.V. Jensen, M. Warner, J.A. Gustafsson, Proc. Natl. Acad. Sci. USA 98 (2001) 6330.
[27] B.O. Nilsson, E. Ekblad, T. Heine, J. Gustafsson, J. Endocrinol. 166 (2000) R5.
[28] S.A. Onate, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Science 270 (1995) 1354.
[29] R.M. Lavinsky, K. Jepsen, T. Heinzel, J. Torchia, T.M. Mullen, R. Schiff, A.L. Del-Rio, M. Ricote, S. Ngo, J. Gemsch, S.G. Hilsenbeck, C.K. Osborne, C.K. Glass, M.G. Rosenfeld, D.W. Rose, Proc. Natl. Acad. Sci. USA 95 (1998) 2920.
[30] J.R. Reel, J.C. Lamb IV, B.H. Neal, Fund. Appl. Toxicol. 34 (1996) 288.
[31] J.C. O'Connor, J.C. Cook, S.C. Craven, C.S. Van Pelt, J.D. Obourn, Fund. Appl. Toxicol. 33 (1996) 182.
[32] H. Tinwell, A.R. Soames, J.R. Foster, J. Ashby, Environ. Health Perspect. 108 (2000) 631.
[33] J. Odum, P.A. Lefevre, S. Tittensor, D. Paton, E.J. Routledge, N.A. Beresford, J.P. Sumpter, J. Ashby, Regul. Toxicol. Pharmacol. 25 (1997) 176.
[34] W.N. Jefferson, R.R. Newbold, Nutrition 16 (2000) 658.
[35] K.S. Kang, H.S. Kim, D.Y. Ryu, J.H. Che, Y.S. Lee, Toxicol. Lett. 118 (2000) 109.

[36] L. Kangas, Acta Oncol. 31 (1992) 143.
[37] L.J. Black, M. Sato, E.R. Rowley, D.E. Magee, A. Bekele, D.C. Williams, G.J. Cullinan, R. Bendele, R.F. Kauffman, W.R. Bensch, J. Clin. Invest. 93 (1994) 63.
[38] G.L. Evans, H.U. Bryant, D.E. Magee, R.T. Turner, Endocrinology 137 (1996) 4139.
[39] A.C. Pike, A.M. Brzozowski, R.E. Hubbard, T. Bonn, A.G. Thorsell, O. Engstrom, J. Ljunggren, J.A. Gustafsson, M. Carlquist, Structure 9 (2001) 145.
[40] P. Diel, S. Olff, S. Schmidt, H. Michna, Planta Med. 67 (2001) 510.
[41] P. Diel, T. Schulz, K. Smolnikar, E. Strunck, G. Vollmer, H. Michna, J. Steroid Biochem. Mol. Biol. 73 (2000) 1.
[42] F.E. van Leeuwen, J. Benraadt, J.W. Coebergh, L.A. Kiemeney, C.H. Gimbrere, R. Otter, L.J. Schouten, R.A. Damhuis, M. Bontenbal, F.W. Diepenhorst, Lancet 343 (1994) 448.
[43] K.B. Horwitz, Y. Koseki, W.L. McGuire, Endocrinology 103 (1978) 1742.
[44] C. Lohmann, E. Gibney, G. Cotsonis, D. Lawson, C. Cohen, Appl. Immunohistochem. Mol. Morphol. 9 (2001) 49.
[45] F. Barzanti, M. Dal Susino, A. Volpi, D. Amadori, A. Riccobon, E. Scarpi, L. Medri, L. Bernardi, S. Naldi, M. Aldi, M. Gaudio, W. Zoli, Cell Prolif. 33 (2000) 75.
[46] S. Samman, P.M. Lyons Wall, G.S. Chan, S.J. Smith, P. Petocz, Atherosclerosis 147 (1999) 277.
[47] P. Diel, A. Walter, K.H. Fritzemeier, C. Hegele-Hartung, R. Knauthe, J. Steroid Biochem. Mol. Biol. 55 (1995) 363.
[48] R. Knauthe, P. Diel, C. Hegele-Hartung, A. Engelhaupt, K.H. Fritzemeier, Endocrinology 137 (1996) 3220.
[49] C. Hegele-Hartung, K.H. Fritzemeier, P. Diel, J. Steroid Biochem. Mol. Biol. 63 (1997) 237.
[50] C. Picherit, V. Coxam, C. Bennetau-Pelissero, S. Kati-Coulibaly, M.J. Davicco, P. Lebecque, J.P. Barlet, J. Nutr. 130 (2000) 1675.
[51] S.C. Lin, T. Yamate, Y. Taguchi, V.Z. Borba, G. Girasole, C.A. O'Brien, T. Bellido, E. Abe, S.C. Manolagas, J. Clin. Invest. 100 (1997) 1980.
[52] A. Tohei, S. Suda, K. Taya, T. Hashimoto, H. Kogo, Exp. Biol. Med. 226 (2001) 216.
[53] S.S. Yen, Y. Ehara, T.M. Siler, J. Clin. Invest. 53 (1974) 652.
[54] M. Jorgensen, R. Hummel, M. Bevort, A.M. Andersson, N.E. Skakkebaek, H. Leffers, APMIS 106 (1998) 245.
[55] D.A. Zajchowski, K. Kauser, D. Zhu, L. Webster, S. Aberle, F.A. White III, H.L. Liu, R. Humm, J. MacRobbie, P. Ponte, C. Hegele-Hartung, R. Knauthe, K.H. Fritzemeier, R. Vergona, G.M. Rubanyi, J. Biol. Chem. 275 (2000) 15885.
[56] A.R. Green, E.L. Parrott, M. Butterworth, P.S. Jones, P. Greaves, I.N. White, J. Endocrinol. 170 (2001) 555.
[57] M. Pollard, Prostate 39 (1999) 305.
[58] M. Pollard, W. Wolter, Prostate 45 (2000) 101.
[59] F. Deerberg, S. Rehm, K.G. Rapp, in: J. Archibald, J. Ditchfield, H.C. Rowsell (Eds.), The Contribution of Laboratory Animal Science to the Welfare of Man and Animals, Fischer Verlag, Stuttgart, 1985, p. 171.
[60] F. Deerberg, J. Kaspareit, J. Natl. Cancer Inst. 78 (1987) 1245.
[61] F. Deerberg, G. Pohlmeyer, K. Lorcher, V. Petrow, Oncology 52 (1995) 319.
[62] W.A. Fritz, L. Coward, J. Wang, C.A. Lamartiniere, Carcinogenesis 19 (1998) 2151.
[63] C.A. Lamartiniere, X.J. Zhang, M.S. Cotroneo, Am. J. Clin. Nutr. 68 (1998) 1400S.
[64] C.A. Lamartiniere, W.B. Murrill, P.A. Manzaolillo, J.X. Zhang, S. Barnes, X. Zhang, H. Wei, N.M. Brown, Proc. Soc. Exp. Biol. Med. 217 (1998) 358.
[65] S. Barnes, Breast Cancer Res. Treat. 46 (1997) 169.
[66] L. Hilakivi-Clarke, E. Cho, I. Onojafe, M. Raygada, R. Clarke, Oncol. Rep. 6 (1999) 1089.
[67] A.I. Constantinou, R.G. Metha, A. Vaughan, Anticancer Res. 16 (1996) 3293.
[68] L.A. Cohen, Z. Zhao, B. Pittman, J.A. Smicea, Carcinogenesis 21 (2000) 929.
[69] J. Yang, H. Nakagawa, K. Tsuta, A. Tsubara, Cancer Lett. 149 (2000) 171.
[70] M. Onozawa, T. Kawamori, M. Baba, K. Fukuda, T. Toda, H. Sato, M. Ohtani, H. Akaza, T. Sugimura, K. Wakabayashi, Jpn. J. Cancer Res. 90 (1999) 393.
[71] W.J. Aronson, C.N. Tymchuk, R.M. Elashoff, W.H. McBride, C. McLean, H. Wang, D. Heber, Nutr. Cancer 35 (1999) 130.
[72] A. Bylund, J.X. Zhang, A. Bergh, J.E. Damber, A. Widmark, A. Johansson, H. Adlercreutz, P. Aman, M.J. Shepherd, G. Hallmans, Prostate 42 (2000) 304.
[73] A.I. Constantinou, A.E. Krygier, R.R. Mehta, Am. J. Clin. Nutr. 68 (1998) 1426S.
[74] R.C. Santell, N. Kieu, W.G. Helferich, J. Nutr. 130 (2000) 1665.
[75] C.D. Allred, K.F. Allred, Y.H. Ju, S.M. Virant, W.G. Helferich, Cancer Res. 61 (2001) 5045.
[76] R.L. Schleicher, C.A. Lamartiniere, M. Zheng, M. Zhang, Cancer Lett. 136 (1999) 195.
[77] P. Diel, K. Smolnikar, T. Schulz, U. Laudenbach-Leschowski, H. Michna, G. Vollmer, Human Reprod. 16 (2001) 997.
[78] W. Wünsche, M.P. Tenniswood, A.-C. Hopert, M.R. Schneider, G. Vollmer, Int. J. Cancer 76 (1998) 684.
[79] J. Kanno, L. Onvon, J. Haseman, P. Fenner-crisp, J. Ashby, W. Owens, Environ. Health Perspect. 109 (2001) 785.