Journal of Poultry Science, 41 : 30–37, 2004

Estrogen Receptor Binding of Xenoestrogens and Phytoestrogens in Japanese Quail *(Coturnix japonica)*

Ahmed M. Hanafy[1)], Tomohiro Sasanami, Kouhei Ichikawa[2)], Kiyoshi Shimada[2)] and Makoto Mori

Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422–8529, Japan
[1)] United Graduate School of Agricultural Science, Gifu University, Yanagido, Gifu 501–1193, Japan.
[2)] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan.

Estrogenic compounds must bind to estrogen receptors (ER) and modulate estrogen-sensitive gene expression. However, various *in vivo* and *in vitro* assays have established that xenoestrogens and phytoestrogens are rather weak estrogens, with a 5,000- to 10,000-fold lower binding affinity to ER than estradiol-17β (E_2). The purpose of this study was to determine the binding affinity of various estrogen-like chemicals to bacterially expressed quail ERα. The first-strand cDNA was synthesized from total RNA isolated from mature female quail liver with oligo-dT primed reverse transcription. The cDNA included the hinge region, the ligand-binding domain, and the C-terminal domain of quail ERα. It was amplified by PCR, and the PCR product was ligated into GST fusion protein expression vector, and transfected to the *E. coli* DH5α strain. A binding assay using the supernatant of the cell lysate was performed by incubation with [^{3}H] E_2 and increasing concentrations of competitor at 4℃ for 18 h. Unbound steroids were removed by the addition of dextran-coated charcoal, followed by centrifugation for 15 min. The radioactivity of the supernatant was determined with a liquid scintillation counter. Quail ERα expressed bacterially showed binding affinity to E_2 with a dissociation constant of $1.74 \pm 0.34 \times 10^{-10}$ M. The competition studies indicated that the relative binding affinities for the synthetic estrogens, diethylstilbestrol and ethynylestradiol, are very high, but that of the xenoestrogens, bisphenol A and nonylphenol, are very low. The phytoestrogens, coumestrol and genistein, can compete with E_2 with a significant binding affinity.

Key words : estrogen receptor, endocrine-disrupting chemicals, phytoestrogens, Japanese quail, recombinant ERα

Introduction

Some synthetic chemicals are known to mimic the action of endogenous steroid hormones and initiate cellular steroid-dependent processes. These chemicals are called endocrine-disrupting chemicals (McLachlan, 2001). Among them, those chemicals that mimic the actions of estrogens have been an object of focus. Although most of the effects of estrogens are mediated through binding to an estrogen receptor (ER), the exact mode of action of endocrine-disrupting chemicals is not fully understood. The

Received : June 16, 2003, Accepted : August 18, 2003

most complex problem is how chemicals with different structures produce similar physiological responses.

Recently, the Organization for Economic Co-operation and Development (OECD) proposed the establishment of standard assay systems for evaluating the biological potencies of the endocrine-disrupting chemicals in birds and recommended the Japanese quail (*Coturnix japonica*) as a model animal. In designing a bioassay of endocrine-disrupting chemicals for estrogenic activity, expression of the ZP1 gene in the male quail has been shown to be a sensitive biomarker (Sasanami *et al.,* 2003). To understand the overall mechanism of action of the estrogenic chemicals, an *in vitro* assay system using quail recombinant ER is desired (Ichikawa *et al.,* 2003 b).

To investigate the ability of estrogenic chemicals to compete with estradiol-17β (E_2) for binding to quail ER, a competitive study was undertaken using bacterially expressed ER fusion protein. The estrogenic compounds we chose in the present study were diethylstilbestrol and ethynylestradiol for synthetic estrogens, which have been known to bind ER as strong as natural estrogens in other species (Blair *et al.,* 2000 ; Tollefsen *et al.,* 2002), and two xenoestrogens, nonylphenol and bisphenol A, which are the most probable candidates for endocrine-disrupting chemicals (Hiroi *et al.,* 1999 ; Matthews *et al.,* 2000). We also compared the binding affinity of genistein and coumestrol, both of which are known as phytoestrogens and which are estrogenic compounds naturally present in plant materials in milligram quantities and are constituents of animal foodstuffs (Jefferson *et al.,* 2002 ; Wuttke *et al.,* 2003).

Materials and Methods

Animals and Tissue Preparation

Female Japanese quail, *Coturnix japonica,* 15–30 weeks of age (Tokai-Yuki, Toyohashi, Japan), were maintained individually under a photoperiod of 14L : 10D (with the light on at 0500), and were provided with water and a commercial diet (Tokai-Kigyo, Toyohashi, Japan) *ad libitum*. The liver was removed after cervical dislocation and was flash-frozen in liquid nitrogen. All experimental procedures for the use and care of animals in the present study were approved by the Animal Care Committee of the Faculty of Agriculture, Shizuoka University.

Chemicals

Radiolabeled [2, 4, 6, 7-^{3}H]E_2 (^{3}H-E_2 ; 118.0 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). Unlabeled E_2, bovine serum albumin, diethylstilbestrol (4, 4'- (1, 2-diethyl-1, 2-ethene-diyl) bisphenol), ethynylestradiol (17 β-ethynyl-1, 3, 5 (10)-estratriene-3, 17β-diol) and genistein (4', 5, 7-trihydroxyisoflavone) were obtained from Sigma (St. Louis, MO, USA). Bisphenol A (4, 4'-isopropylidenediphenol) was obtained from ICN Biomedicals (Aurora, OH, USA) and *p*-nonylphenol (a mixture of isomers of monoalkyl phenols) was obtained from Kanto Chemical (Tokyo, Japan). Coumestrol (3, 9-dihydroxy-6H-benzofuro [3, 2-c]-[1] benzopyran-6-one) was obtained from Helix Research (Springfield, OR, USA).

All other chemicals were of the highest quality available from commercial sources.

Generation of ERα Expression Construct

Total RNA was extracted from a piece of liver (ca. 100 mg) with a commercial kit, ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. The first-strand cDNA was synthesized from 2 μg of total RNA using the SuperScript First-Strand Synthesis System for an RT-PCR kit (Gibco BRL, Rockville, MD, USA) with oligo (dT) primed reverse transcription. The entire cDNA products were amplified by PCR (30 cycles using the following conditions : 94℃ for 1 min, 62℃ for 1 min, and 72℃ for 1 min) with 0.2 μM primers (forward : 5'-AAAAGAATTCCC GAATGATGAAACAGAAACGTCAAAG-3' and reverse : 5'-AAAACTCGAGTTA TATTGTATTCTGCATACTCTCCTC-3') designed based on the reported cDNA sequence of quail ERα (GenBank Database Accession Number AF442965 ; Ichikawa *et al.,* 2003 b). A PCR product that included the hinge region (D domain), the ligand-binding domain (E domain), and the C-terminal domain (F domain) was digested with *EcoRI* and *XhoI* and ligated into the glutathione-S-transferase (GST) fusion protein expression vector, pGEX-6P-3 (Amersham Pharmacia Biotech, Piscateway, NJ, USA) treated with the same restriction enzymes (see Fig. 1). The resulting construct, qERα-def, was transformed into competent *Escherichia coli* (*E. coli*) strain DH5α (Takara, Tokyo, Japan), and the ampicillin-resistant clone containing qERα-def was selected after analysis of the nucleotide sequence was performed.

Expression of GST-qERα-def fusion protein

The *E. coli* containing qERα-def were cultured overnight at 37℃ with constant shaking in 2 m*l* of LB medium supplemented with 100 μg/m*l* ampicillin. The overnight culture was diluted 1 : 25 in 10 m*l* of LB medium and was grown to an optical density of approximately 1.0 at 600 nm. To express GST-qERα-def fusion protein in the cell, isopropylthio-β-D-galactoside at a final concentration of 1 mM was added to the medium, and the *E. coli* was incubated for 3 h at 28℃ with constant shaking. After the

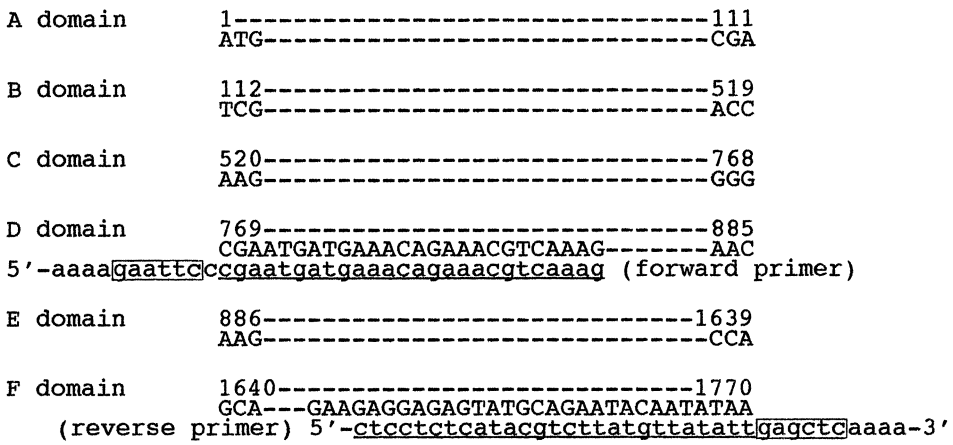

Fig. 1 A schematic representation of the nucleotide sequence of quail estrogen receptor α. Only the beginning and end of each domain is shown, except for the D domain and F domain, for which the corresponding nucleotides for the forward and reverse primers are shown with the restriction enzyme cleavage sites in boxes.

culture, the cells were collected by centrifugation at 10,000X *g* for 1 min, and were suspended in buffer A containing 50 mM HEPES, 3 mM EDTA, 5 mM DTT, 50 mM NaCl, and 10% glycerol, pH 7.5. The cells were lysed by sonication on ice 10 times for each 15 sec, separated by 10-sec intervals. Tween 20 was added to a final concentration of 0.1%, and the cell debris was incubated for 20 min at 4℃. Insoluble materials were removed by centrifugation at 10,000 X *g* for 10 min at 4℃, and clear supernatant was stored at −80℃ until use. The protein concentrations of the cell lysates were determined by the method of Lowry *et al.* (1951).

Binding Assay

The receptor binding assay was performed as previously described (Lazier and Jordan, 1982). Briefly, an aliquot (100 μg) of the cell lysates conaining GST-qERα-def fusion protein was incubated at 4℃ for 18 h with 10,000 dpm of ^{3}H-E_2 (38.2 fmol) in the presence of various concentrations (1 to 20 pmol) of unlabeled E_2 in a total reaction volume of 1 m*l*. The ethanol concentrations were fixed at 0.1% in each reaction tube. Unbound steroids were removed by the addition of a dextran-coated charcoal suspension (0.9% Norit A and 0.09% dextran T70 in TEDB buffer) followed by centrifugation at 1,000 x *g* for 15 min at 4℃. The radioactivity of the aliquot of supernatant was measured using a liquid scintillation counter. The amount of non-specific binding was calculated in the presence of a 100-fold excess of unlabeled E_2. The equilibrium dissociation constant (Kd) was calculated by the method of Scatchard (1949).

For the competition study, the test chemicals were dissolved in ethanol and incubated with the cell lysates (100 μg of protein) containing GST-qERα-def fusion protein in the presence of 10,000 dpm of ^{3}H-E_2 for 18 h at 4℃. Specific binding quantified as described above was plotted as the percentage of ^{3}H-E_2 binding versus the molar concentration of the test chemicals, and the equilibrium inhibitory concentration (IC50) was calculated as the concentration causing 50% inhibition of ^{3}H-E_2 binding. The relative binding affinity (RBA) was obtained by comparing the individual binding affinities (IC50) of the test chemicals to that of E_2, and the RBA value for E_2 was arbitrarily set at 100. All assays were replicated at least three times, and the IC50 values of the test chemicals are the means of the replicates.

Results

Scatchard analysis of E_2 binding to GST-qERα-def fusion protein showed a single class of high-affinity saturable binding sites with a Kd of $1.74\pm0.34\times10^{-10}$ M (Fig. 2).

Competition studies demonstrated that the receptor was highly specific for estrogens with less than 1% non-specific binding. The synthetic estrogens diethylstilbestrol and ethynylestradiol bound ER with slope curves similar to that of E_2 (Figs. 3-A, 3-B, and 3-C). IC50s for diethylstilbestrol and ethynylestradiol were $9.77\pm2.71\times10^{-9}$ M and $1.20\pm0.27\times10^{-8}$ M, respectively, while that of E_2 was $1.41\pm0.25\times10^{-8}$ M. The RBAs of diethylstilbestrol and ethynylestradiol were 143.8 and 117.1, respectively.

Competitive binding assays showed that nonylphenol and bisphenol A exhibited IC50s of $8.35\pm6.61\times10^{-5}$ M and $4.02\pm2.25\times10^{-4}$ M, respectively (Figs. 3-F and 3-G), and RBAs of 0.017 and 0.0035, respectively. In contrast, coumestrol and

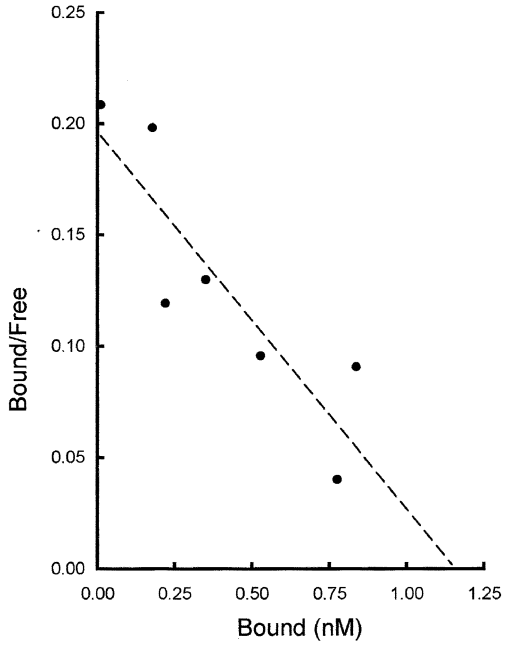

Fig. 2. Representative Scatchard plot of specific binding of estradiol-17β to GST-qERα-def fusion protein.

genistein bound to the GST-qERα-def fusion protein with 10- to 500-times higher affinity than xenoestrogens. The IC50s were $9.26\pm3.10\times10^{-7}$ M and $5.61\pm1.86\times10^{-6}$ M for coumestrol and genistein (Figs. 3-D and 3-E), and the RBAs were 1.52 and 0.25, respectively.

None of the other steroids tested (progesterone, testosterone, and corticosterone) caused competitive displacement of ^{3}H-E_2 binding at concentrations up to 100 nM (data not shown).

Discussion

The results of the present study demonstrate that the bacterially expressed ERα fusion protein exhibits the similar binding characteristics to those of native ER. ER-binding to E_2 was found to be saturable, and its Kd is comparable to the range of reported values (4×10^{-10} M to 1×10^{-9} M) for chicken liver ER (Lazier and Haggarty, 1979 ; Kloosterboer *et al.,* 1980). A recent study using GST-ER fusion protein from five different species (human, mouse, chicken, green anole, and rainbow trout) demonstrated that there exists a considerable species difference in the binding affinity to E_2, with Kd values ranging from 0.3 to 0.9 nM (Matthews *et al.,* 2000).

The subtype of ER used in the present study was ERα. Because a novel ER distinct from the classical ER has been identified in the rat prostate (Kuiper *et al.,* 1996) and is referred to as an ERβ subtype, the classical one is referred to as ERα. The discovery of ERβ had generated a considerable amount of interest because many of the endocrine-disrupting chemicals that have been suspected to disrupt normal endocrine functions

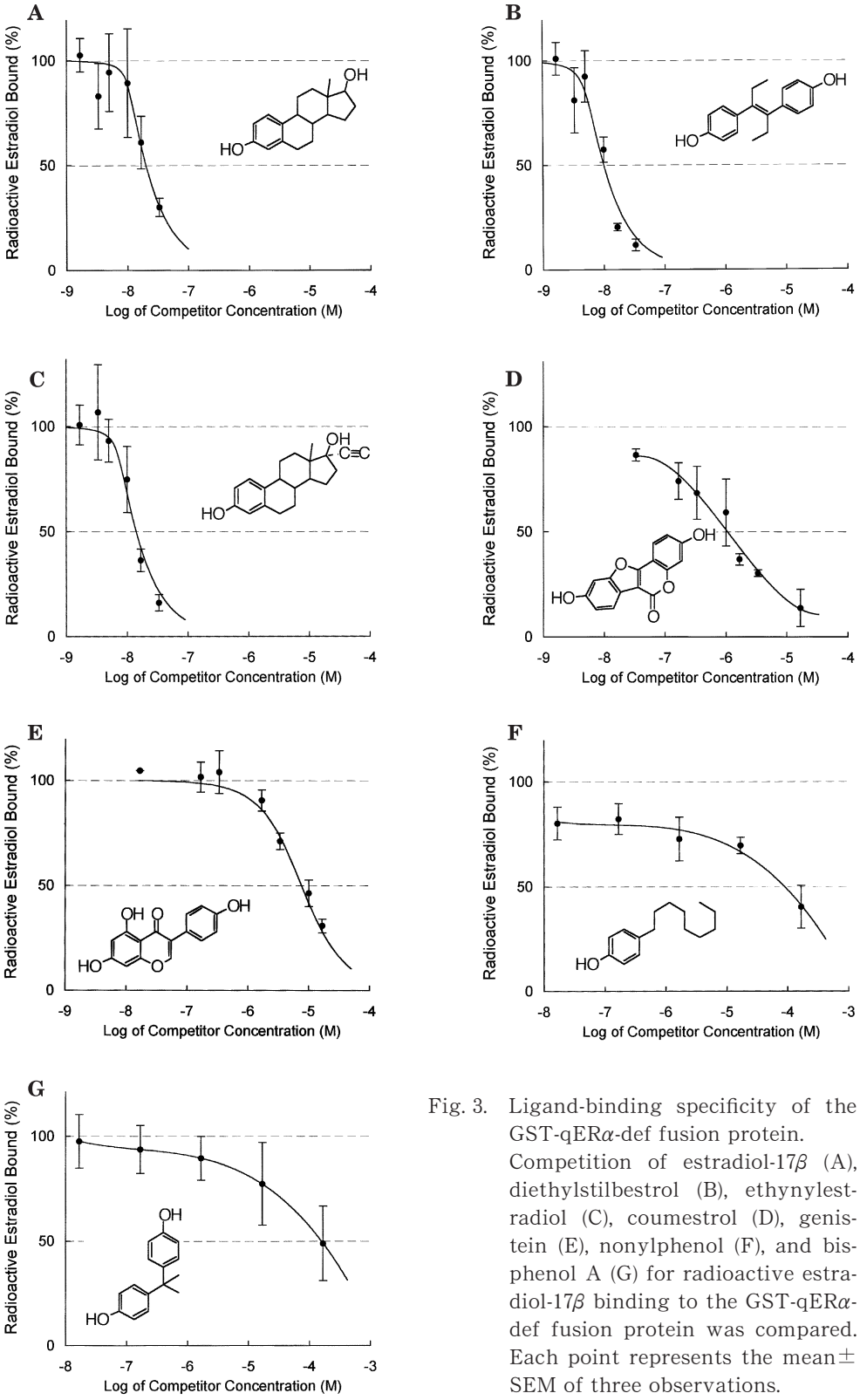

Fig. 3. Ligand-binding specificity of the GST-qERα-def fusion protein. Competition of estradiol-17β (A), diethylstilbestrol (B), ethynylestradiol (C), coumestrol (D), genistein (E), nonylphenol (F), and bisphenol A (G) for radioactive estradiol-17β binding to the GST-qERα-def fusion protein was compared. Each point represents the mean$\pm$SEM of three observations.

interact only weakly, if at all, with ERα. While both ER subtypes bind E_2 with comparable affinity, some phytoestrogens show a higher affinity for ERβ than for ERα (Kuiper *et al.,* 1997). Quail ERβ has also been cloned (Foidart *et al.,* 1999), but the tissue distribution of this subtype has revealed that extremely low levels of the mRNA are expressed in quail tissues (Ichikawa *et al.,* 2003 b).

We also report here that ERα binds a number of compounds that exhibit remarkably diverse structural features. Of the chemicals tested, diethylstilbestrol showed a higher binding affinity than E_2 for ERα, and ethynylestradiol had a similar binding affinity to E_2. Similar results have been obtained from a dissociation kinetic study of chicken liver ER (De Boer *et al.,* 1982). Genistein and coumestrol both of which have two aromatic rings with a hydroxyl group separated by two carbon atoms, similar to diethylstylbestrol, exhibited low but significant binding affinity to ERα, although the RBAs were 100- to 400-times lower than with E_2. Adverse effects of phytoestrogens on reproduction and development have been documented in quail (Leopold *et al.,* 1976), while scientific data supporting the beneficial effects of phytoestrogens are insufficient. It is therefore important to study the effects of exposure to these chemicals, as their content in plant material is far larger than that of synthetic estrogens or xenoestrogens.

Our results also demonstrate that xenoestrogens display considerably weaker binding affinity to ERα than do synthetic estrogens and phytoestrogens. As shown in Fig. 2, nonylphenol has a single ring structure and bisphenol A has two rings separated by one carbon atom. Because the effects of nonylphenol on estrogen-inducible mRNA expression have been confirmed in chicken (Sakimura *et al.,* 2001 ; 2002) and quail (Ichikawa *et al.,* 2003 a), the effects of concentrated distributions of these chemicals in the liver or alternative mechanisms of action of xenoestrogens besides via ERα should be considered.

Acknowledgments

Funding for this work was provided by the Ministry of Education, Science, Sports, and Culture of Japan (Grant-in-Aids for Scientific Research : 14042224 and 15380191).

References

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R and Sheehan DM. The estrogen receptor relative binding affinities of 188 natural and xenochemicals : Structural diversity of ligands. Toxicological Sciences, 54 : 138–153. 2000.

De Boer W, Snippe L, Ab G and Gruber M. Estrogen receptor in avian embryo and adult liver : Estrogen receptor activation and dissociation kinetics of estradiol, ethynylestradiol, and moxestrol. Endocrinology, 110 : 1217–1224. 1982.

Foidart A, Lakaye B, Grisar T, Ball GF and Malthazart J. Estrogen receptor-β in quail : Cloning, tissue expression and neuroanatomical distribution. Journal of Neurobiology, 40 : 327–342. 1999.

Hiroi H, Tsutsumi O, Momoeda M, Takai Y, Osuga Y and Taketani Y. Differential interactions of bisphenol A and 17β-estradiol with estrogen receptor α (ERα) and ERβ. Endocrine Journal, 46 : 773–778. 1999.

Ichikawa K, Ha Y, Tsukada A, Saito N and Shimada K. Effect of endocrine disrupters on mRNA expression of vitellogenin (VTG) II and very low density lipoprotein (apoVLDL) II in the liver of quail embryos. Journal of Poultry Science, 40 : 45–52. 2003 a.

Ichikawa K, Yamamoto I, Tsukada A, Saito N and Shimada K. cDNA cloning and mRNA expression of estrogen receptor α in Japanese quail. Journal of Poultry Science, 40 : 121–129. 2003 b.

Jefferson WN, Couse JF, Padilla-Banks E, Korach KS and Newbold RR. Neonatal exposure to genistein induces estrogen receptor (ER) α expression and multioocyte follicles in the maturing mouse ovary : Evidence for ERβ-mediated and nonestrogenic actions. Biology of Reproduction, 67 : 1285–1296. 2002.

Kloosterboer HJ, Ab G and Gruber M. Estradiol receptor in chicken liver cytosol. FEBS Letter, 111 : 356–360. 1980.

Kuiper GGJM, Enmark E, Pelto Huikko M, Nilsson S and Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proceeding of National Academy of Science, USA, 93 : 5925–5930. 1996.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Gaggblad J, Nilsson S and Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. Endocrinology, 138 : 863–870. 1997.

Lazier CB and Haggarty AJ. A high-affinity oestrogen-binding protein in cockerel liver cytosol. Biochemical Journal, 180 : 347–353. 1979.

Lazier CB and Jordan VC. High affinity binding of anti-oestrogen to the chick liver nuclear oestrogen receptor. Biochemical Journal, 206 : 387–394. 1982.

Leopold AS, Erwin M, Oh J and Browning B. Phytoestrogens : Adverse effects on reproduction in California quail. Science, 191 : 98–100. 1976.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193 : 256–275. 1951.

Matthews J, Celius T, Halgren R and Zacharewski T. Differential estrogen receptor binding of estrogenic substances : A species comparison. Journal of Steroid Biochemistry and Molecular Biology, 74 : 223–234. 2000.

McLachlan JA. Environmental Signaling : What embryos and evolution teach us about endocrine disrupting chemicals. Endocrine Reviews, 22 : 319–341. 2001.

Sakimura M, Hanzawa S, Tsukada A, Yamamoto I, Saito N, Usami M, Ohno Y and Shimada K. Effect of estradiol and nonylphenol on mRNA levels of vitellogenin II in the liver of chicken embryos. Journal of Poultry Science, 38 : 250–257. 2001.

Sakimura M, Tsukada A, Usami M, Hanzawa S, Saito N, Ohno Y and Shimada K. Effect of estradiol and nonylphenol on mRNA expression of estrogen receptors α and β, and cytochrome P450 aromatase in the gonad of chicken embryos. Journal of Poultry Science, 39 : 302–309. 2002.

Sasanami T, Pan J and Mori M. Expression of perivitelline membrane glycoprotein ZP1 in the liver of Japanese quail (*Coturnix japonica*) after in vivo treatment with diethylstilbestrol. Journal of Steroid Biochemistry and Molecular Biology, 84 : 109–116. 2003.

Scatchard G. The attractions of proteins for small molecules and ions. Annals of New York Academy of Science, 51 : 660–672. 1949.

Tollefsen KE, Mathisen R and Stenersen J. Estrogen mimics bind with similar affinity and specificity to the hepatic estrogen receptor in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). General and Comparative Endocrinology, 126 : 14–22. 2002.

Wuttke W, Jarry H, Becker T, Schultens A, Christoffel V, Gorkow C and Seidlova-Wuttke D. Phytoestrogens : Endocrine disrupters or replacement for hormone replacement therapy? Maturitas, 44 : S9–S20. 2003.