

Serum Derived from Zeranone-implanted ACI Rats Promotes the Growth of Human Breast Cancer Cells *In Vitro*

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Abstract. *Background:* Zeranone (Z) is a non-steroidal anabolic growth promoter with potent estrogenic activity that is widely used as a growth promoter in the US beef industry. Consumption of beef derived from zeranone-implanted cattle may be a risk factor for breast cancer. *Materials and Methods:* The effect of serum on the proliferation of human breast cancer MCF-7 cell line and primary cultured human breast epithelial cells (PCHBECs) was investigated. ACI rats were implanted with 12 mg zeranone pellet and the serum was harvested at day 110 after implantation. The effect of zeranone-serum on mRNA expression of cell cycle regulating gene (cyclin D1) and tumor suppressor genes (p53, and p21) was evaluated using real-time RT-PCR. *Results:* The serum derived from ACI rats 110 days post-zeranone implantation significantly promoted the proliferation of MCF-7 cells and primary cultured human breast epithelial cells compared to control serum. Zeranone-serum up-regulated cyclin D1 and down-regulated p53 and p21 expression in PCHBECs compared with control serum. *Conclusion:* Bio-active zeranone metabolites contained in meat produced from cattle after zeranone implantation may be a risk factor for breast cancer.

Breast cancer is the mostly commonly diagnosed cancer in women and the leading cause of cancer death in women between the ages of 25 to 60 years. It is expected to account for 28% of all new cancer cases among women in the United States in 2010 (1). Estrogen exposure is one of the established risk factors for breast cancer. Higher concentrations of circulating estrogens, as well as prolonged exposure to estrogen, are associated with increased breast cancer risk (2). The mechanisms through which estrogens contribute to breast cancer initiation and progression are complex. Evidence suggests the participation of genotoxic estrogen metabolites and estrogen-receptor mediated genomic and non-genomic signaling that affect cell proliferation and apoptosis in mammary tissue (3, 4). Estrogens induce proliferation of epithelial cells in the mammary gland, mainly by binding to estrogen receptors (ER) and modulating expression of downstream genes (5). It is well established that a woman's lifetime risk of breast cancer increases with cumulative exposure to estrogens, including both endogenous and exogenous sources (6).

Research has shown that numerous environmental pollutants cause mammary gland tumors in animals. Some of these chemicals are hormonally active and mimic estrogen, which is a breast cancer risk factor (7). In recent years, environmental estrogens and synthetic xenoestrogens found in food products (10) have been established as carcinogens (8, 9). However, there has been scant research on the effects of these chemicals or their metabolites in humans (7, 11-13).

Zeranone (Ralgro[®]) is a non-estrogenic agent with potent estrogen-like activity. This compound was approved 40 years ago by the U.S. Federal Drug Administration (FDA) as an anabolic growth promoter for use in the U.S. beef industry to improve feed efficiency, weight gain, meat-to-fat ratio and carcass quality. Around 95% of the U.S. beef industry uses growth-promoters, and zeranone's market share is a significant 60%. Zeranone is typically administered to beef heifers by a

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subcutaneous implantation in the ear of six pellets containing a total of 72 mg zeranol.

The safety of using zeranol as a growth promoter has been debated for many years. Consumption of beef containing residues of zeranol and its metabolites may pose a breast cancer risk. Studies have shown that zeranol is able to bind to the active site of human estrogen receptor ER α and ER β in a similar manner as 17 β -estradiol (E2) (14). Epidemiological investigations found that the sperm quality in offspring of 'high beef consumers' was lower than that in males whose mothers consumed less beef during their pregnancy (15). Previous studies have shown that meat and serum from zeranol-implanted cattle possess heat-stable mitogenicity for cultured breast cells, and that both human normal breast and cancerous cells exhibit estrogenic responses to zeranol (16-18, 19-22). Previous data also showed that zeranol transforms the human normal breast epithelial cell line MCF-10A and increases growth of primary cultured human normal and cancerous breast epithelial cells in a dose-dependent manner (23). Furthermore, it was previously found that implantation of zeranol in beef cattle greatly increases growth of beef pre-adipocytes by up-regulating cyclin D1 and down-regulating P53 expression (24).

Based on manufacturer's information, following implantation of beef cattle with zeranol pellets (Ralgro[®]), zeranol is completely released 90 days after implantation. Whether bio-active zeranol metabolites persist in the circulation after 90 days post zeranol-implantation is a critical question that needs to be answered. This study investigated the effect of serum on the proliferation of MCF-7 cell line and primary cultured human breast cancer epithelial cells (PCHBCECs) and possible mechanisms responsible for increased proliferation.

Materials and Methods

Animal implantation and serum sample preparation. Ralgro Magnum[®] (RM, commercial zeranol product), obtained from Merck Schering-Plough Corp, NJ, USA, was used in the form of six pellets per cartridge, each containing 12 mg Zeranol. Six- to eight-week-old female ACI rats were housed in the Laboratory Animal Facility at the College of Veterinary Medicine, the Ohio State University under controlled temperature, humidity and lighting conditions. After the acclimatization period, six rats were divided randomly into two groups. The rats of the experimental group were implanted with a zeranol pellet subcutaneously into their back region, while those in the control group were not implanted with a zeranol pellet. Ten milliliters of blood was harvested at day 110, and allowed to clot overnight at 4°C, then the serum was separated by centrifugation of the blood in a 50 ml centrifuge tube at 4000 g for 20 min at 4°C. The separated serum was filter sterilized and stored at -20°C. Part of the Zeranol serum and control serum was treated with dextran-coated charcoal (DCC) and stored at -20°C.

DCC treatment of serum. Zeranol and its metabolites were removed from the serum samples by charcoal-dextran stripping. Briefly, a suspension of 5% activated charcoal (Sigma C9157) with 0.5%

dextran T-70 (Sigma, St. Louis, MO, USA) was prepared. DCC was added to the serum (5% final concentration) and the suspension was mixed at 4°C for 16 h, and then centrifuged at 5000 \times g for 15 min to recover the serum. This procedure was repeated and the resulting serum was filter sterilized through a 0.22 μ m filter (Millipore Corporation, Bedford, MA) and stored at -20°C.

Cell culture. Human breast cancer tissue was obtained from the Tissue Procurement Program of The Ohio State University Comprehensive Cancer Center Hospital. PCHBCECs were isolated from the human breast tissue as described (24) and cultured in low calcium (0.04 mM CaCl₂) Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM/F12) mixture (Atlanta Biologicals, Norcross, GA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO Cell Culture[™], Grand Island, NY, USA) previously treated with Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA). MCF-7 cells were obtained from the American Type Culture Collection and cultured in phenol red-free high calcium DMEM/F12 (1.05 mM CaCl₂) containing 5% FBS and antibiotic/antimycotic (100 unit/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate and 0.25 μ g/ml amphotericin B) (GibcoBRL, Bethesda, MD, USA).

Non-radioactive cell proliferation assay (MTS assay). MCF-7 cells and PCHBCECs were cultured separately in 100 μ l of either phenol red-free high-calcium DMEM/F12 medium supplemented with 5% FBS or low-calcium DMEM/F12 medium supplemented with Chelex-100-treated 10% FBS in 96-well culture plates at an initial density of 5 \times 10³ viable cells/well. After being cultured overnight, the medium was replaced with either DMEM/F12 medium or low calcium DMEM/F12 medium supplemented with 0.2% bovine serum albumin and cultured overnight. MCF-7 cells and PCHBCECs were treated with 0.1, 0.5, 2.5, and 5% zeranol-serum or control serum for 48 h. Cell proliferation was determined by non-radioactive cell proliferation assay according to the manufacturer's instructions. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium (Promega, Madison, WI, USA) was mixed with phenazine methosulfate (Sigma) (20:1), and 20 μ l of the mixture was added to each well and incubated for 1.5 h. The color density was measured at 490 nm using a kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA, USA) and Softmax Pro software (version 2.1.1).

Cell treatment, RNA isolation and cDNA synthesis. An initial density of 1 \times 10⁵ cells/well of PCHBCECs were seeded with 5 ml low-calcium (0.04 mM CaCl₂) DMEM/F12 in six-well plates and incubated for 24 h. The medium was replaced with low-calcium DMEM/F12 supplemented with 10% DCC stripped Chelex-100-treated FBS overnight. The cells were then treated with different concentrations of control or zeranol-serum for another 24 h. Total RNA was isolated using 1ml TRIZOL Reagent[®] (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were measured using a DU-70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The reverse transcription reaction consisted of total RNA (1 μ g), 200 U M-MLV Reverse Transcriptase (Invitrogen), 0.2 mM dNTP (1 μ l mixture of 10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH, Invitrogen), 1 μ M random hexamers (Amersham, Piscataway, New Jersey, USA), 10 μ l 5 \times First Strand buffer, 5 μ l 0.1M DTT and 40 U RNase Inhibitor (Invitrogen) in a total volume of 50 μ l. The reaction was incubated at 37°C for 50 min followed by inactivation at 70°C for 15 min in a gradient mastercycle (Eppendorf[®], USA).

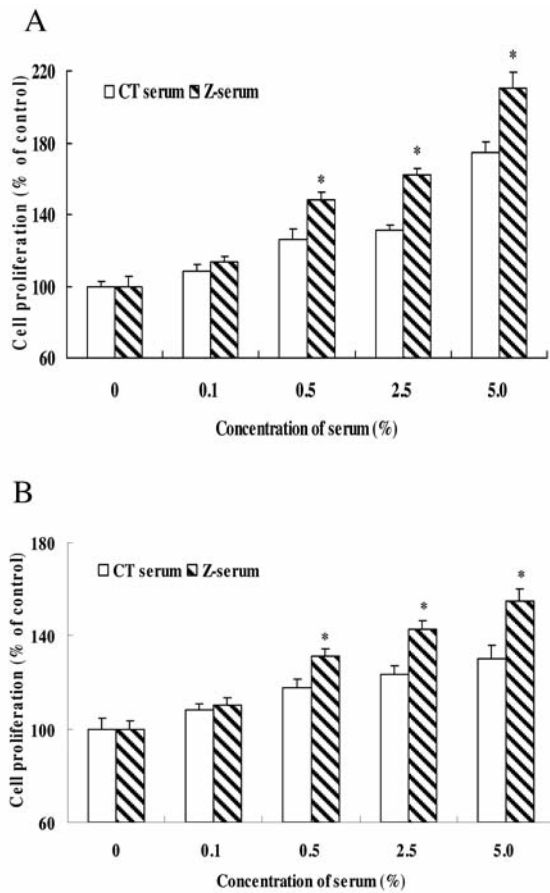


Figure 1. Effects of serum derived from the ACI rats 110 days post-zeranol implantation on the proliferation of human breast cancer cell line MCF-7 (A) and primary cultured human breast cancer epithelial cells (PCHBCECs) (B). *Significant difference of cell growth between zeranol-serum (Z-serum) and control-serum at the same concentration.

Quantitative real-time PCR. Real-time PCR was used to measure cyclin D1, p53 and p21 expression. Conditions were optimized for each primer pair and performed in Stratagene Mx3005p (Agilent Technologies, TX, USA). Briefly, newly synthesized cDNA (2 μ l) was used as a template for the reaction in a total volume of 20 μ l reactants, which included 10 μ l of 2 \times real-time master mix (Applied Biosystems, Warrington, UK), 3 μ l ultra-pure water and 5 μ l of primer mixer. The reactants were first incubated at 95°C for 10 min, and then 40 cycles of amplification were carried out with each cycle consisting of denaturing at 95°C for 30 s, annealing at 55°C for 1 min and elongation at 72°C for 1 min. Dissociation curves were created at the completion of each run to ensure that the PCR reactions produced the correct products as anticipated. The primer sequences for cyclin D1 were 5'-TTGGTTACAGTAGCGTAG-3' (sense) and 5'-TTATAGTAGCGTATCGTAGG-3' (antisense). Primer sequences for p53 were 5'-GACAATGGCAGCATCTAC-3' (sense) and 5'-GAAGGTGTAATCAGT CTCC-3' (antisense). The primer sequences for p21 were 5'-GGAAGGAAGCAGGAAGAC-3' (antisense) and 5'-AGCAGATACAAGGAAGG-3' (antisense). Primer sequences for 18S were 5'-TCCGATAACGGAACGAGAC-3' (sense) and 5'-CTAAGGGCATCACAGACC-3' (antisense). The

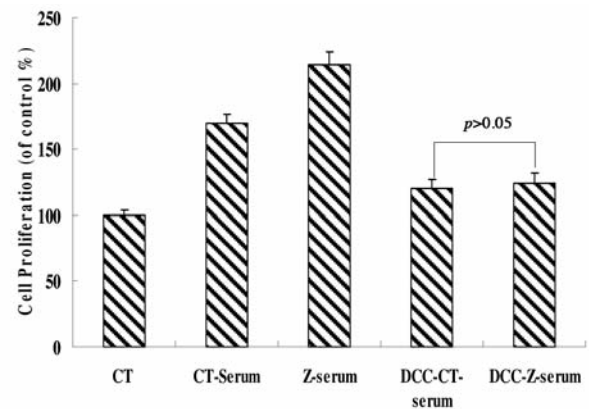


Figure 2. Effects of DCC treatment on MCF-7 cell proliferation promoted by control and Zeranol-serum (z-serum) at 5.0% in culture medium. No significant difference was noted between the growth of MCF-7 cells treated by control and Z-serum ($p > 0.05$).

results of the relative mRNA expression (cyclin D1, p53 and p21 to 18S) in cells were analyzed using the $\Delta\Delta$ Ct method (25).

Statistical analysis. The results are presented as the mean \pm standard deviation (SD) of four replicate culture wells. Analysis was performed using SAS for window (SAS Institute Inc. Cary, NC, USA). Statistical differences were determined using the Student's *t*-test for independent groups. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Effects of zeranol-serum on the proliferation of MCF-7 and PCHBCECs. In order to investigate whether the serum derived from ACI rats after 110-day implantation exhibits biological mitogenic activity, the proliferation of MCF-7 cells and PCHBCECs were evaluated by non-radioactive cell proliferation assay. After MCF-7 and PCHBCECs were treated with zeranol-serum and control serum for 48 h, zeranol-serum at concentrations between 0.5-5.0% significantly ($p = 0.010-0.031$) increased the proliferation of MCF-7 and PCHBCECs in a dose-dependent manner as compared with the control serum (Figure 1). In MCF-7 cells (Figure 1A), 0.5, 2.5 and 5% of zeranol-serum increased cell growth to 16.9, 20.6 and 23.5% of the control serum, respectively. In PCHBCECs (Figure 1B), the same concentrations of zeranol-serum promoted cell growth to 11.8, 15.7 and 18.9% of the control serum, respectively. These results suggest that zeranol-serum is a more effective in the promotion of MCF-7 cell proliferation than of PCHBCECs.

DCC treatment alleviated the stimulatory effect of zeranol-serum on MCF-7. In order to determine whether the serum from ACI rats after zeranol implantation at 110 days still contained bioactive residues or its metabolites, zeranol-serum and control serum were treated with DCC, which effectively

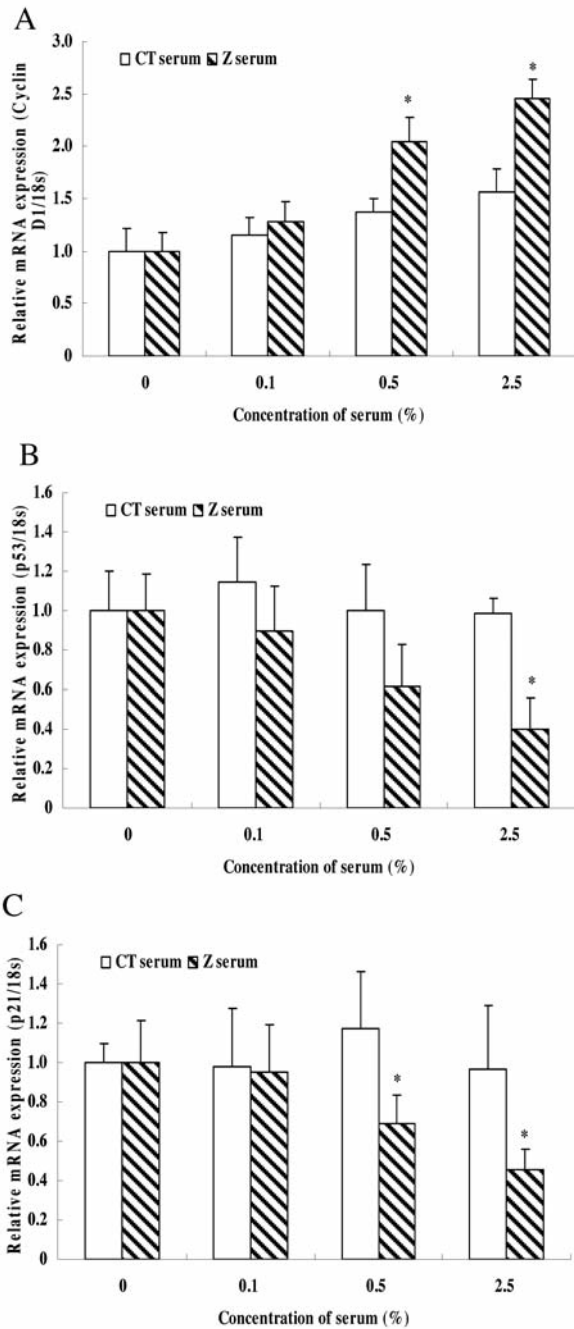


Figure 3. Comparison of the effects of control and Z-serum on the mRNA expression of cyclin D1 (A), p53 (B) and p21 (C) in primary cultured human breast cancer epithelial cells (PCHBCECs) after 24 h of treatment. *Significant difference of mRNA expression between Z-serum and control-serum at the same concentration.

removes hormones and growth factors from serum (16), and their effect on the growth of MCF-7 cells was tested using non-radioactive cell proliferation assay. DCC-treatment significantly ($p=0.009$) reduced the stimulatory effect of

zeranol- and control serum, and there was no significant difference ($p=0.114$) in the proliferation of MCF-7 cells after treatment with DCC-treated zeranol-serum compared to control serum (Figure 2).

Zeranol-serum up-regulates cyclin D1 and down-regulates p53 and p21 mRNA expression in PCHBCECs. In order to explore the mechanisms responsible for the growth stimulation of breast cancer cells by zeranol-serum, the mRNA expression of cyclin D1, p53 and p21 in PCHBCECs after treatment with different concentrations of zeranol-serum was investigated using real-time PCR. Zeranol-serum treatment at 0.5 and 2.5% significantly increased cyclin D1 mRNA expression ($p=0.012$ and 0.009) in the PCHBCECs by approximately 48% and 57%, respectively, compared to control serum treatment (Figure 3A). Figure 3B and C shows that zeranol-serum treatment of PCHBCECs down-regulated p53 and p21 mRNA expression in a dose-dependent fashion. p53 was down-regulated by 2.5% zeranol-serum treatment (Figure 3B) while 0.5% and 2.5% zeranol-serum treatment reduced p21 (Figure 3C) mRNA expression at a significant level ($p=0.002-0.011$) by 60, 41 and 53%, respectively.

Discussion

Recently, the public at-large and government regulatory agencies expressed great interest in the hypothesis that exposure to certain hormonally active environmental agents such as xenoestrogens and mycoestrogens induce carcinogenesis.

Zeranol (Ralgro[®]) is a non-steroidal agent with potent estrogenic activity that is widely used as a growth promoter in the U.S. beef industry. The U.S. FDA approved its use and reassured the public that if it is used by professional veterinarians in accordance with governmental regulatory guidelines and the manufacturer's instructions, the residues of the growth promoter in the meat of treated beef cattle should pose no risk to human health. Despite this, the occurrence of compounds possessing estrogenic activity in the environment and in food products, either as natural constituents or as contaminants, has received increased attention because of their potential to adversely affect human and animal endocrinology and their possible etiological role in estrogen-related carcinogenesis (7, 27, 28).

The use of anabolic growth promoters has been controversial since the introduction of diethylstilbestrol (DES), a synthetic estrogen that was approved and administered to millions of women as a preventive agent for miscarriage and was also utilized as a growth-promoting agent in animal feed for the enhancement of weight gains in meat-producing farm animals. DES was subsequently banned by the FDA after it was demonstrated to be carcinogenic (29).

A previous investigation among workers occupationally exposed to zeranol was conducted due to a variety of reported breast symptoms, including sharp pain, tingling, burning, aching and irritation (30). In addition, two former workers (one male, one female) had sons aged under 5 years who developed gynecomastia and presented unusual growth spurts. These two boys were exposed to zeranol through their parents' workclothes. The symptoms in the two boys abated after their parents changed work to control the zeranol exposure (30). This investigation clearly illustrated the relationship between breast symptoms and exposure to zeranol, particularly in children since they may be more sensitive to zeranol than adults. Human exposure to zeranol occurs through ingestion of beef products that contain pharmacological active residuals or its metabolites.

There is a concern regarding potential breast cancer risks from consuming beef products containing zeranol residues derived from cattle implanted with zeranol. This is largely based on its demonstrated estrogenic activity (21). Previous data showed that zeranol transforms the human normal breast epithelial cell line MCF-10A and increases proliferation of primary cultured human normal breast epithelial and cancer cells in a dose-dependent manner (23). Furthermore, it was previously found that implantation of zeranol in beef cattle greatly induces pre-adipocyte growth by up-regulating cyclin D1 and down-regulating p53 expression (20).

Based on manufacturer's information, the zeranol in 72 mg Ralgro[®] pellets in beef cattle will be completely released 90 days after implantation. However, the bioactivity of zeranol or its metabolites especially 90 days after implantation in animal remains unclear.

To examine the effect of zeranol-serum on the growth properties of human breast cancer cells *in vitro* and provide a biochemical background for understanding the biological properties of zeranol or its metabolites in circulation system *in vivo*, ACI rats were implanted with a zeranol pellet and the serum was harvested at day 110 after implantation. The present results revealed that the serum derived from the ACI rats 110 days post-zeranol implantation significantly promoted the proliferation of MCF-7 cell and PCHBCECs compared to control serum. The results imply that the residues of biologically active zeranol or its metabolites contained in the serum exhibit a stimulatory effect on human breast cancer cells, and this mitogenic activity can be abolished by DCC treatment.

Tumor growth is ultimately the net result of deregulated cell proliferation in relation to cell death. Regulation of cell cycle depends on the complex association between cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors, influencing the cell passage through the critical G₁ restriction point and its commitment to S-phase and completion of the cell cycle (31). Cyclin D1 gene is a critical mitogen-regulated cell-cycle control element whose transcriptional modulation plays a

crucial role in breast cancer growth and progression, whereas the p53 gene is a well-studied tumor suppressor gene which is directly involved in cell-cycle regulation and one of the most commonly mutated genes described in human neoplasia (32, 35). It was reported that the occurrence of p53 mutations in sporadic breast carcinomas was approximately 20% and p53 expression status was a significant molecular marker of the response to first-line endocrine therapy and for predicting time to endocrine therapy failure in recurrent breast cancer with hormone-sensitive disease (33, 34). p21 (WAF1/Cip1) is a cyclin-dependent kinase inhibitor involved in cell division and survival. It is activated by p53 and is a downstream effector for p53 function by inducing G₁ arrest when normal breast cells are exposed to DNA-damaging agents (32, 35).

In the current study, it was demonstrated that zeranol-serum up-regulated cyclin D1 and down-regulated p53 and p21 expression in primary cultured human breast cancer epithelial cells compared with control serum. This implicates zeranol-serum as being responsible for breast cancer cell proliferation.

However, the compound(s) in zeranol-serum responsible for this proliferation of cancer cells remain(s) unknown. Zeranol or its metabolites or other growth factors which are induced by zeranol implantation could be responsible; further studies are needed.

In summary, bio-active zeranol-serum promoted the proliferation of human breast cancer cell line MCF-7 cells and primary cultured human breast cancer cells. This clearly demonstrates that residual bio-activity of zeranol metabolites or growth factors induced by zeranol implantation still circulated in the bloodstream of ACI rats. The findings reported here extend previous work that bio-active zeranol metabolites contained in meat produced from beef cattle 90 days after zeranol implantation may still pose a risk to human health.

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