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# Zeranol may increase the risk of leptin-induced neoplasia in human breast

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**Abstract.** Breast cancer and obesity are serious health problems and their relationship has been studied for many years. Leptin is mainly secreted by adipocytes and plays a key role in breast cancer development. Leptin expression is up-regulated in obese individuals and promotes breast cancer cell growth. On the other hand, exposure to environmental estrogens has been found to be directly related to breast cancer. Zeranol (Z) is a non-steroidal anabolic growth promoter used in the beef industry in the US. This study focused on the evaluation of Z and Z-containing sera (ZS) and its adverse health risk to human consumption of Z-containing meat produced from Z-implanted beef cattle. We hypothesized that Z increases the risk of breast neoplasia in women, particularly in obese women. A cell proliferation assay, ELISA analysis, RT-PCR and Western blot analysis were conducted. Our study demonstrated that Z and ZS collected from Z-implanted heifers stimulated the proliferation of primary cultured human normal breast epithelial cells (HNBECs) by up-regulating cyclin D1 expression. Leptin increased the sensitivity of HNBECs to Z, and Z increased the ability of HNBECs to secrete leptin. These results suggest an interaction between leptin and Z in HNBECs. Furthermore, Z may play a role in leptin-induced breast neoplasia.

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## Introduction

Breast cancer is a worldwide disease, which over 40,000 women succumb to each year in the US (1). Epidemiological studies suggest that there are numerous risk factors associated with breast cancer, including dietary fat and environmental estrogenic endocrine disruptors.

One of the known risk factors of breast cancer is obesity, which has become a major public health concern (2). The Centers for Disease Control and Prevention reported that about 2/3 of adults in the US are overweight and 1/3 are obese (2-4). The incidence of breast cancer is increased with obesity, and morbidity is increased in obese cancer patients as compared to cancer patients with normal or low weight (2). The relationship between breast cancer and obesity has been studied for more than 40 years (2,5).

Leptin, a transcriptional product of the *ob* gene, plays a key role in breast cancer development and has been studied since its discovery in 1994 (6). Besides its involvement in appetite regulation and energy balance by sending signals to the hypothalamus (7), leptin has a number of other regulatory functions including ensuring normal mammary gland development, bone development, fetal development, sex maturation, angiogenesis, lactation, hematopoiesis and immune responses (2,3). Additionally, leptin is required for normal mammary gland development in rodents (2,8). Animals and humans with defective leptin or with mutated leptin receptor genes are obese (3,9). In clinical studies, the serum leptin level in prostate cancer patients was found to be higher than that in a healthy control group, and was correlated with prostate-specific antigen (10,11). In a breast cancer research program, 60% (20/35) of the patients expressed leptin, while none out of four cases with normal breast tissue expressed leptin (11,12).

Leptin has a direct mitogenic effect on human breast cancer cells (12); therefore, the inhibition of leptin may contribute to the prevention and treatment of breast cancer (13,14). Leptin expression is up-regulated in obesity (3) and promotes breast cancer cell growth by directly affecting the estrogen receptor (ER) pathway (3). Similar to other growth

factors and cytokines, leptin is present in human serum and plays a role in human cancer development (3). Since leptin was found to be associated with breast cancer (3,15), investigators have attempted to determine the relationship and mechanisms of leptin action in breast cancer (16-21). Ishikawa *et al* found that leptin was overexpressed in breast cancer cells (13) and similarly concluded that high leptin levels in obese breast cancer patients may play a role in the development of antiestrogen resistance (16). Leptin is not expressed in normal breast tissue but exists near malignant breast lesions (12). In addition to its mitogenic effects, leptin promotes T47-D cell line proliferation (14) and a high level of leptin may contribute to the development of a more aggressive malignant phenotype (22). ICI 182,780 is a pure estrogen antagonist approved for the treatment of breast cancer patients who fail to respond to tamoxifen therapy. Treatment of cells with ICI 182,780 resulted in rapid degradation of membrane ER α, which reduced the nuclear expression of the receptor and ER α-dependent transcription, and produced significant growth inhibition. Leptin was able to counteract the cytostatic activity of ICI 182,780 as well as the effect of this compound on the expression of ER α and ER α-dependent transcription (23). The power of leptin to stimulate human MCF-7 cell growth and to counteract the effects of ICI 182,780 strongly suggests that leptin acts as a paracrine/endocrine growth factor towards mammary epithelial cells (16). Chen *et al* also found that leptin increases ZR-75-1 breast cell growth by up-regulating cyclin D1 and down-regulating P53 (24). Since it stimulates estrogen biosynthesis through the induction of aromatase activity and the modulation of ER α activity, leptin has been characterized as a growth factor for breast cancer (3,25). High levels of leptin in obese breast cancer patients may play a notable role in breast cancer cell proliferation, invasion and metastasis (2).

Estrogen has been regarded as a positive regulator of leptin production (26), and leptin levels in breast cancer patients treated with tamoxifen are significantly higher than those in the control group (27). Zearalenone, a stable natural product that mimics estrogen activity, is a carcinogen and thus hazardous to human health (28). Zeranol (Z), produced from zearalenone, is a non-estrogenic anabolic growth promoter used to stimulate cattle growth in the US beef industry (29). Zearalenone and Z bind to the active site of human ER α and ER β in a similar manner to 17 β-estradiol (30). Since it is a widespread food contaminant, it is difficult to avoid the intake of Z (28). Based on its toxicity information, the FDA approved the use of Z in the beef cattle industry. However, the European Union declined to import beef products with residues of hormonal implantation from the US due to the potential health concerns. It was reported that Z enhanced the proliferation of pre-adipocytes in beef heifers (31). A previous study found that Z did not change the serum leptin level in growing wethers (32). At low concentrations, it increases ER α-positive cell growth, but a high concentration of Z reduces the growth of ER α-positive and -negative cell lines (33). Moreover, previous data showed that Z was able to transform human normal breast epithelial cells and increase human breast cell growth in a dose-dependent manner (29). Additionally, Z down-regulated the estrogen-regulated human breast cancer candidate suppressor gene, protein tyrosine phosphatase γ expression (34).

Gossypol, another natural polyphenolic compound extracted from cottonseed and used as an anticancer chemopreventive agent, inhibits breast cancer cell growth (35,36). It is suggested that gossypol be used as a potential chemopreventive food component. It was also demonstrated that gossypol exhibits anticancer activity against multidrug resistant human breast cancer cells (36,37), with (-)-gossypol having the strongest effect among the three isoforms (data not shown).

This study aimed to investigate the interaction of leptin, Z and gossypol in breast cancer development, as well as the mechanisms involved in the suppression of Z- and leptin-induced proliferation of primary cultured human normal breast epithelial cells using (-)-gossypol as the main chemopreventive agent.

## Materials and methods

*Animal treatment and blood sampling*. Ralgro Magnum® (RM, commercial Z pallet) was purchased from Schering-Plough Corp, Kenilworth, NJ, USA, in the form of cartridges, each containing 72 mg Z. A total of 20 cross-bred Angus beef heifers (~1 year old) were purchased from the Department of Animal Science. The animals were randomly divided into two groups according to initial body weight. Animal treatment was described in our previous publication (31). Z-containing sera (ZS) dropped from the Z-implanted beef at day 0 (ZS-D0, prior to Z implantation) and 30 days post Z implantation (ZS-D30), and non-Z-containing serum (NZS) from non-Z-implanted beef at day 0 (NZS-D0, prior to Z implantation) and 30 days post Z implantation (NZS-D30) were sterilized using a 50 ml conical filter tube, and stored at -20˚C.

*Tissue culture*. Human normal breast tissues were sterilized in 70% ethanol for 30 sec, and then washed three times with fresh Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12). *In vitro* organ-cultured human normal breast tissues were treated with leptin at 0, 1.5, 3.0 and 6.0 nM in DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC)-stripped fetal bovine serum (FBS) and cultured in a 10 $cm^2$ cell culture plate in a humidified incubator (5% $CO_2$, 95% air, 37˚C) for 96 h. The medium was changed every 48 h.

*Primary-cultured human normal breast epithelial cell (HNBEC) isolation*. The cultured human normal breast tissues were minced and digested using a digestion buffer consisting of phenol red-free high calcium (DMEM/F12, 1:1) (1.05 mM $CaCl_2$) with 2% bovine serum albumin (BSA) (Invitrogen, Carlsbad, CA, USA) containing 10 ng/ml cholera toxin (Sigma, St. Louis, MO, USA) 6,300 U/ml collagenase (Invitrogen) and 100 U/ml hyaluronidase (Calbiochem, Gibbstown, NJ, USA). The mixture was incubated in a humidified incubator (5% $CO_2$, 95% air, 37˚C) overnight, and the solution was transferred to a 50 ml tube and centrifuged at 1,200 rpm for 5 min. The upper, middle and lower layers were separated and centrifuged again. The upper and middle layers, containing pre-adipocytes and stromal cells, respectively, were transferred to another 15-ml tube separately while the lower layer containing epithelial cells remained in the tube. The pellets were washed using DMEM/F12 with antibiotic-antimycotic (100 U/ml penicillin

G sodium, 100 $\mu$g/ml streptomycin sulfate and 0.25 $\mu$g/ml amphotericin B) (Invitrogen) and centrifuged again. The pellets were then washed three times. The final pellet in the tube contained HNBECs and a few stromal cells. The pellet was resuspended in 10 ml low calcium (0.04 mM $CaCl_2$) DMEM/F12 supplemented with 10% of low calcium FBS (Atlanta Biologicals, Norcross, GA, USA) and then transferred into a T75 flask for culturing.

*Cell culture*. The isolated HNBECs were cultured in a 75 $cm^2$ culture flask in a humidified incubator (5% $CO_2$, 95% air, 37˚C) with 10 ml low calcium (0.04 mM $CaCl_2$) DMEM/F12 mixture (Atlanta Biologicals) supplemented with 10% of Chelex-100- (Bio-Rad Laboratories, Richmond, CA, USA) treated FBS (Invitrogen). Low-calcium DMEM/F12 was changed every two days. Only HNBECs survived in this medium; thus, the growth of the stromal cells isolated from the same tissue stopped and the purity of the HNBECs was guaranteed. When the cells reached 85-90% confluence, they were washed with 10 ml of calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.3), and then trypsinized with 3 ml of 0.25% trypsin-5.3 mM EDTA (Invitrogen) for 10 min at 37˚C. Trypsinization was stopped by adding 10 ml of DMEM/F12 with 10% FBS. Following centrifugation, the dissociated cells were re-suspended in low-calcium DMEM/F12 with 10% low-calcium FBS and sub-cultured into 75 $cm^2$ culture flasks at a ratio of 1:5. The experiments were conducted on HNBECs not generated beyond the fourth passage.

*Cell proliferation (MTT) assay*. A total volume of 100 $\mu$l medium containing 4,000 HNBECs/well was seeded in 96-well plates in low-calcium DMEM/F12 and incubated in 37°C for 24 h. The following day, the medium was replaced with 100 $\mu$l low-calcium DMEM/F12 supplemented with 0.2% BSA and incubated in 37˚C for a further 24 h. Following the treatment, 1.5, 3.0 and 6.0 nM of leptin was administered to the HNBECs isolated from non-leptin treated human normal breast tissues for 0, 6, 12 and 24 h, and 0.1% DMSO was administered to the control group. The proliferation of HNBECs was measured by adding 20 $\mu$l of a fresh mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) (20:1) solution (Promega, Madison, WI, USA) to the wells. Following incubation at 37˚C for 1-5 h, the optical density (OD) values were measured using a kinetic microplate reader (Molecular Devices Cooperation, Menio Park, CA, USA) at a 490 nm wavelength, and the cell growth was compared.

The sera used in the MTT assay were: NZS-D0, NZS-D30, ZS-D0 and ZS-D30. The concentration for NZS and ZS was 0.2, 1.0 and 5.0% in the cultured medium and was administered to HNBECs isolated from the controls, as well as to 1.5 nM leptin-cultured human normal breast tissues for 6 h. The cell proliferation was measured as described above.

To investigate the effect of the combination of leptin with (-)-gossypol in HNBEC growth, 4,000/well HNBECs isolated from non-leptin cultured tissues were seeded in 96-well plates in low-calcium DMEM/F12 and incubated at 37˚C for 24 h. The following day, the medium was replaced by 100 $\mu$l of low-calcium DMEM/F12 supplemented with 0.2% BSA and incubated at 37˚C for a further 24 h. The treatment of 1.5, 3 and 6 nM leptin alone or in combination with 10 $\mu$M (-)-gossypol, with 0.1% DMSO as the control was administered, and the proliferation of HNBECs was measured using the methods described above.

*Cell treatment for RNA and PCR analysis*. Viable HNBECs ($10^5$/well) were seeded in 6-well plates in 5 ml low-calcium DMEM/F12 supplemented with 10% low-calcium FBS. After 24 h, the medium was replaced with low-calcium DMEM/F12 supplemented with 10% DCC-stripped low-calcium FBS. The cells were cultured overnight. After 24 h, the medium was changed and 24 h of treatment was administered. The concentration for leptin was 1.5, 3 and 6 nM and that for Z was 5, 10 and 20 nM. The combination of 1.5 nM leptin and 5 nM Z with or without 3 $\mu$M (-)-gossypol was also administered, with 0.1% DMSO being administered to the control group.

*RNA isolation, cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR)*. Following the treatment of HNBECs for 24 h, the cultured medium was collected for leptin measurement. Total RNA was isolated in 1 ml TRIzol reagent (Invitrogen) according to the manufacturer's instructions (37). The RT-PCR conditions were optimized for each primer and performed using a thermocycler Gene Amp PCR (Eppendorf®, Westbury, NY, USA). A volume of 2 $\mu$l of the newly-synthesized cDNA was used as a template for RT-PCR. The PCR conditions were optimized for the $MgCl_2$ concentration, annealing temperature and cycle number for the amplification of each of the PCR products (cyclin D1, 36B4). Under optimal conditions, 1 unit of platinum Taq DNA polymerase (Invitrogen) was added for a total volume of 25 $\mu$l.

The primers for cyclin D1 were: upper, 5'-GCT CCT GTG CTG CGA AGT GG-3' and lower, 5'-TGG AGG CGT CGG TGT AGA TG-3' (product size 372 bp). The PCR conditions were: denaturing at 95˚C for 5 min, followed by 27 cycles at 94˚C for 45 sec, 54˚C for 45 sec, 72˚C for 60 sec and extension at 72˚C for 10 min. The primers for 36B4 were: upper, 5'-AAA CTG CTG CCT CAT ATC CG-3' and lower, 5'-TTT CAG CAA GTG GGA AGG TG-3' (product size 563 bp). The PCR conditions were: denaturing at 95˚C for 5 min, followed by 24 cycles at 95˚C for 60 sec, 63˚C for 60 sec, 72˚C for 60 sec and extension at 72˚C for 10 min. Pure $H_2O$ was used as a negative control to detect genomic DNA contamination and 36B4 as the internal control whose RNA is unmodified by treatment.

The final RT-PCR products (10 $\mu$l) mixed with 1 $\mu$l 10X loading buffer were separated on 1.5% agarose gel and visualized by staining with ethidium bromide. Electronic images were captured by a Fujifilm LAS-3000 image system (Fuji Film Medical Systems USA, Inc. Stanford, CT, USA). The densities of specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The results were presented as the ratio of cyclin D1 to 36B4.

*Western blotting assay*. HNBECs isolated from non-leptin cultured tissue were plated in a 10-$cm^2$ culture dish with a density of $1x10^6$ viable cells/well with a 10 ml low-calcium DMEM/F12 supplemented with 10% low-calcium FBS and cultured overnight. The medium was then replaced

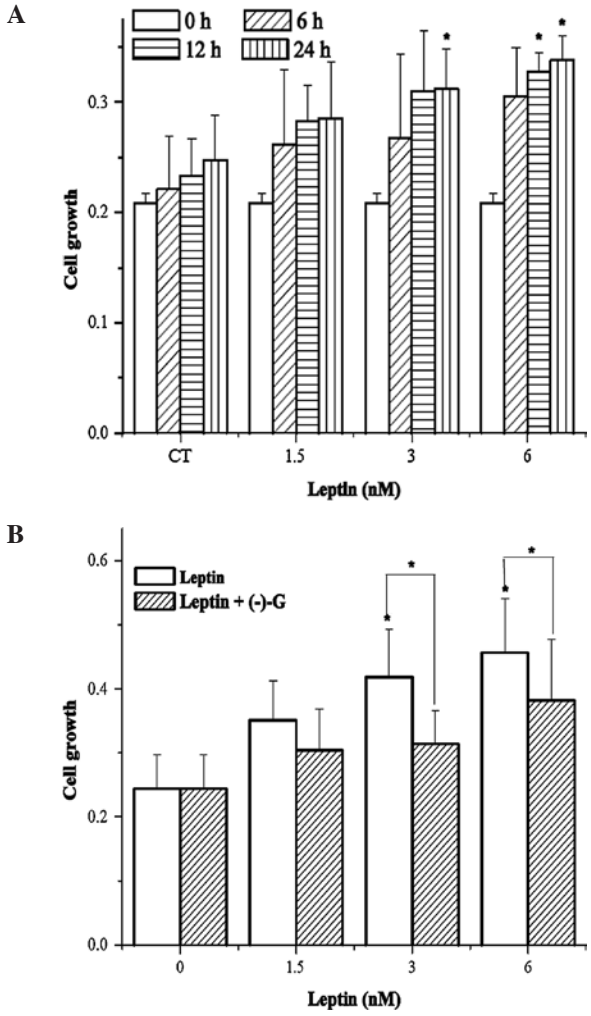


Figure 1. (A) Leptin stimulated HNBEC growth. A total volume of 100 $\mu$l medium containing 4,000 HNBECs/well isolated from control human normal breast tissues were treated with 1.5, 3.0, 6.0 or 12.0 nM of leptin and administered to HNBECs for 0, 6, 12 and 24 h. Additionally, 0.1% DMSO was administered to the control group and the proliferation of HNBECs was measured. *Significant difference compared to the control group ($p<0.05$). (B) (-)-Gossypol suppresses leptin-induced HNBEC growth. A total volume of 100 $\mu$l medium containing 4,000 HNBECs/well isolated from control human normal breast tissues were treated with 1.5, 3 or 6 nM of leptin alone or in combination with 10 $\mu$M (-)-gossypol for 24 h. Additionally, 0.1% DMSO was administered to the control group and the proliferation of HNBECs was measured. *Significant difference compared to the control group ($P<0.05$).

with low-calcium DMEM/F12 supplemented with 10% DCC-treated low-calcium FBS and cultured for a further 24 h. The primary cultured HNBECs were then treated with 1.5 and 3 nM leptin, 5, 10 and 20 nM Z or 0.1% DMSO as a vehicle control. Following 24 h of treatment, culture media were collected for the leptin measurement and proteins were isolated from the control and treatment groups using M-PER® mammalian protein extraction reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Culture media were collected for the leptin measurement, and 400 $\mu$l of extraction reagent was added to each dish. Each dish was then placed on an orbital agitator to be digested for 5 min. The digested products were collected and transferred to a 1.5-ml centrifuge tube. The mixture was centrifuged at 10,000 x g for 5 min, and the supernatant was then transferred to a new 1.5-ml centrifuge tube. The protein concentrations were measured using a Micro BCA™ protein assay reagent kit (Pierce) according to the manufacturer's instructions. Proteins (50 $\mu$g) from each treatment group were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidine fluoride membrane (Bio-Rad Laboratory, Hercules, CA, USA). The membrane was initially blotted in PBS-Tween 20 (PBST) containing 10% fat-free dry milk for 1 h and then incubated with primary antibody (cyclin D1 1:1,000 dilution, Cell Signaling Technology® Danvers, MA, USA; β actin, 1:2,000 dilution, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) for 1 h. The membrane was rinsed in PBST three times, each time for 5 min. The membrane was then incubated with the second antibody for 1 h. After the membrane had been washed three times in PBST, it was detected using the Fuji Imaging System (Fuji Film Medical Systems USA, Inc.). The images were captured by FujiFilm LAS-300 image system (Fuji Film). The protein ratios of cyclin D1 to β actin were calculated by measuring the density of the specific band using Multi Gauge (V3.0) software.

*Leptin measurement*. The culture media were collected prior to protein extraction as previously described. The human leptin immunoassay kit for leptin measurement was purchased from R&D Systems (Minneapolis, MN, USA). The particles in the cell culture supernates were removed by centrifugation at 1,200 rpm for 5 min and the assay was performed immediately according to the manufacturer's instructions. The assay was stopped by the addition of 50 $\mu$l of stop solution to each well. The OD values were then measured within 30 min using a kinetic microplate reader (Molecular Devices Cooperation, Menio Park, CA, USA) at a wavelength of 450 nm and the leptin concentration was compared.

*Statistical analysis*. The results for the cell proliferation assay are presented as mean ± standard deviation (SD) of 4 replicate culture wells. The analysis was performed using Minitab 15 (Minitab Inc., PA, USA). The statistical difference was determined using two sample t-test analyses for independent samples. $P<0.05$ was considered to be statistically significant.

## Results

*Leptin increased HNBEC proliferation*. As shown in Fig. 1A, leptin increased the growth of HNBECs. When compared to their respective control groups, it was found that 3 nM leptin with 24 h of treatment or 6 nM leptin with 12 h of treatment significantly increases HNBEC growth. Fig. 1B shows that (-)-gossypol inhibits HNBEC growth induced by 3 and 6 nM leptin. A significant difference between leptin and the combination of leptin with (-)-gossypol is noted.

*Z-containing serum increases primary cultured HNBEC growth*. Fig. 2A shows that NZS-D0 and NZS-D30 have no effect on HNBECs isolated from non-leptin cultured tissue at any dose, but stimulated HNBEC growth isolated from leptin cultured tissue at the same dose (Fig. 2B).

Fig. 2C shows that ZS-D0 and ZS-D30 at concentrations of 0.2, 1 and 5% increased HNBECs isolated from non-leptin cultured tissue as compared to the control group, respectively.

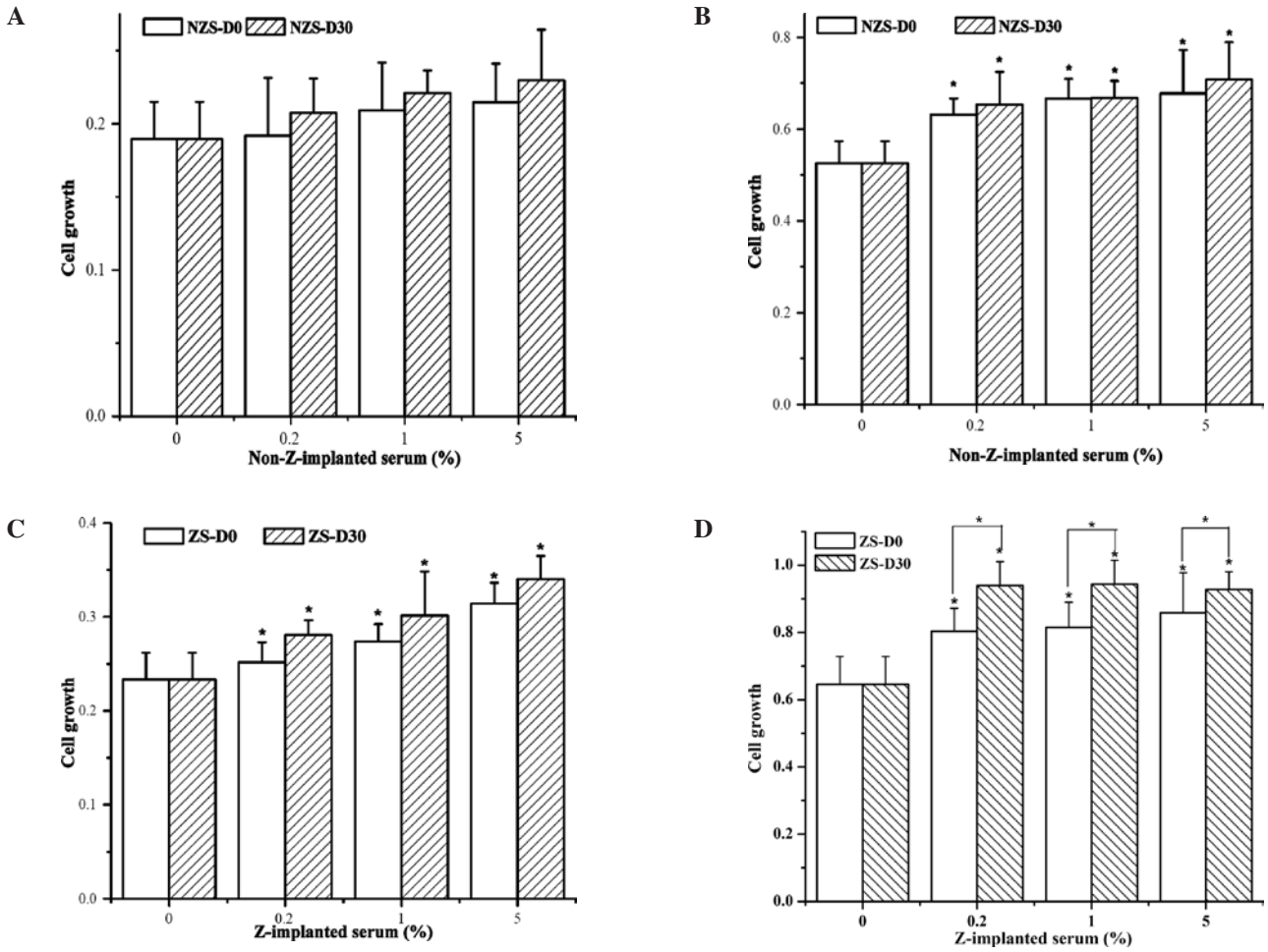


Figure 2. (A) Effects of non-Zeranol serum on HNBECs isolated from non-leptin cultured tissues. Primary human normal breast epithelial cells were isolated from non-leptin cultured tissues, treated with 0.2, 1 and 5% NZS-D0 and NZS-D30 in cultured medium for 6 h and the cell growth was measured. No significant difference was found in any group compared to the control group (0.0% group). (B) Effects of non-Zeranol serum on HNBECs from 1.5 nM of leptin cultured HNBECs. Primary human normal breast epithelial cells were isolated from 1.5 nM-leptin cultured tissues, treated with 0.2, 1 and 5% NZS-D0 and NZS-D30 in cultured medium for 6 h and the cell growth was measured. No significant difference was found between NZS-D0 and NZS-D30 at any dose. (C) Effects of Zeranol serum on HNBECs from non-leptin cultured tissues. Primary human normal breast epithelial cells were isolated from non-leptin cultured tissues, treated with 0.2, 1 and 5% ZS-D0 and ZS-D30 in cultured medium for 6 h and the cell growth was measured. No significant difference was found between ZS-D0 and ZS-D30 at any dose. (D) Effects of Zeranol serum on HNBECs from 1.5 nM of leptin cultured HNBECs. Primary human normal breast epithelial cells were isolated from 1.5 nM leptin cultured tissues, treated with 0.2, 1 and 5% ZS-D0 and ZS-D30 in cultured medium for 6 h and the cell growth was measured. Significant differences were found between ZS-D0 and ZS-D30 at all doses.

Fig. 2D shows that ZS-D0 and ZS-D30 at concentrations of 0.2, 1 and 5% increased HNBECs isolated from 1.5 nM of leptin cultured tissue with a significant difference between ZS-D0 and ZS-D30 at all doses.

*The effect of leptin, zeranol and (-)-gossypol in the cyclin D1 expression in breast cells*. HNBECs were treated with 1.5, 3 and 6 nM leptin, 5, 10 and 20 nM Z, a combination of 5 nM Z and 1.5 nM leptin with or without 3 $\mu$M (-)-gossypol for 24 h. Significant differences in cyclin D1 expression were found between the control group and the group treated with 3 and 6 nM leptin and 10 and 20 nM Z ($p<0.05$). In addition, the combination of 1.5 nM leptin with 5 nM Z significantly increased HNBEC growth as compared to the control group. However, the effective combination was inhibited by adding 3 $\mu$M (-)-gossypol (Fig. 3A). Consistent with the mRNA expression, cyclin D1 protein expression also increased with the 24 h treatment of Z in HNBECs (Fig. 3B).

*Zeranol increased HNBEC leptin secretion*. As shown in Fig. 4, 20 nM Z significantly increased leptin secretion in HNBECs, as compared to the control group ($p<0.05$).

## Discussion

Cyclin D1 is a cell cycle regulator that plays an important role in cell growth. It is the product of the CCND1 gene which is located on chromosome 11q13 (39). The cyclin-dependent kinases (CDKs) cannot modulate cell growth without the cyclin subunit. By binding to cyclin D, cyclin D-CDK 4/6 comprises the mechanism of the cell cycle and affects the $G_1$ phase in cell growth. The cyclin D1 level is modulated by changing growth factors in the medium used to culture the cells. Cyclin D1 was found to be overexpressed in over 50% of breast cancer patients and is known as one of the most overexpressed proteins in breast cancer (39). Leptin stimulates breast cancer cell growth by up-regulating the cyclin

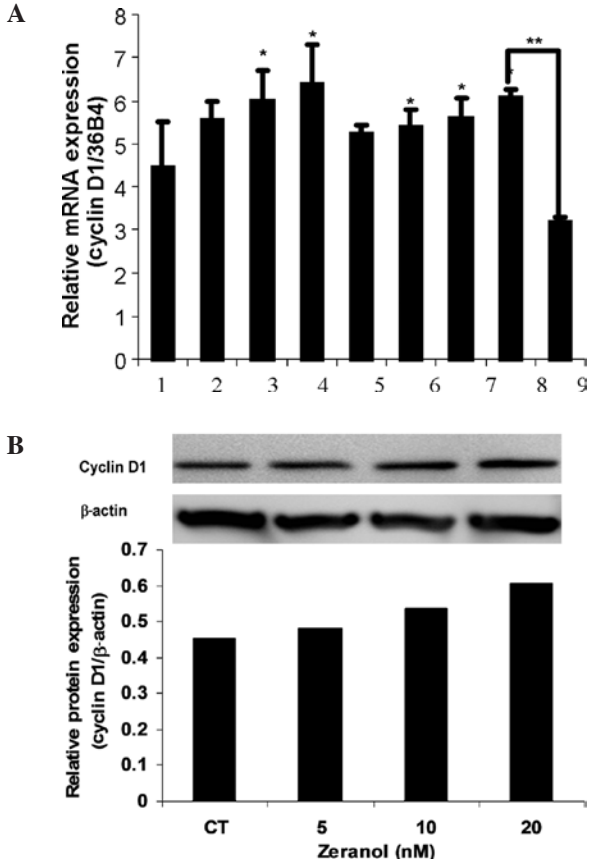


Figure 3. (A) Effects of leptin, zeranol and (-)-gossypol on the cyclin D1 expression in HBNECs. HBNECs were treated with 1.5, 3 or 6 nM leptin, 5, 10 or 20 nM zeranol, a combination of 5 nM zeranol and 1.5 nM leptin with or without 3 $\mu$M (-)-gossypol for 24 h. The bars show: 1, CT; 2, 1.5 nM lp; 3, 3 nM lp; 4, 6 nM lp; 5, 5 nM Z; 6, 10 nM Z; 7, 20 nM Z; 8, 5 nM Z + 1.5 nM lp; 9, 5 nM Z + 1.5 nM lp + 3 $\mu$M (-)-gossypol. The RNA was then extracted, cDNA was synthesized and cyclin D1 was amplified. *Significant differences in cyclin D1 expression compared to the control group ($P<0.05$). (B) Zeranol up-regulated the cyclin D1 protein expression in HBNECs. Primary cultured human normal breast epithelial cells were treated with 5, 10 and 20 nM zeranol, and 0.1% DMSO was administered as a control. After 24 h, protein was extracted, Western blot analysis was conducted and the cyclin D1 expression was compared.

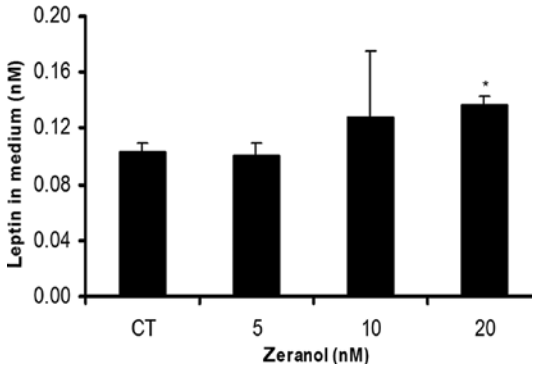


Figure 4. Zeranol increased leptin secretion in HNBECs. HNBECs were treated with 5, 10 and 20 nM zeranol for 24 h, the culture medium was collected and a human leptin immunoassay was conducted. Zeranol at 20 nM significantly increased leptin secretion in HNBECs compared to the control group. *$P<0.05$.

D1 expression. Moreover, Garofalo *et al* noted that leptin regulates estrogen synthesis and ER α activity (3). Besides regulating the cell cycle, it was noted that cyclin D1 correlates with ER (39). Cyclin D1 binds to ER and stimulates its transcriptional activities. The cyclin D1 and ER complex may play a role in stimulating tumor cell proliferation. It is crucial to elucidate whether leptin and Z affect the cyclin D1 expression in primary cultured normal breast epithelial cells.

Results of this study show that 6 nM leptin or 30 nM Z alone had no effect on the cyclin D1 expression. However, their combination significantly increased the cyclin D1 expression as compared to the control group. In our cell proliferation assay, cells isolated from leptin exposure tissues increased their sensitivity to Z. This increase is partially explained by the fact that the combination resulted in a high expression of cyclin D1. A previous study noted that the serum level of leptin in breast cancer patients is higher than that in controls (27). It is likely that if obese healthy women or breast cancer patients have higher leptin in their serum, the sensitivity of normal or cancerous breast cells is increased to the presence of Z in beef. Consequently, these results suggest a possible correlation between obesity and breast neoplasia and indicate a potential risk for breast neoplasia in obese individuals, particularly in those consuming beef from animals implanted with Z.

Of noteis that leptin secretion was increased by the treatment of Z in HNBECs; thus, Z amplified the leptin activity. It was reported that leptin affects transformed breast cancer cells to induce an alteration to a more aggressive phenotype, and leptin potentially serves as a tumor marker. This result shows that Z is potentially more harmful to obese individuals than those with normal weight by increasing the risk of breast neoplasia (33) since the consumption of Z-containing products by the obese individuals increases their chances of developing breast neoplasia. However, (-)-gossypol has been found to reverse the effect of the combination of leptin with Z on cell growth and may therefore be used in the treatment of breast cancer patients.

The effect of ZS in human normal breast epithelial cell growth was also evaluated. We found that 2.5% of ZS harvested from 60-day post 72 mg Z pellet-implanted beef heifers transformed the human normal breast epithelial cell line, MCF-10A, to breast neoplasia cells in a 21-day culture (unpublished data). Our current data showed that there was no observable proliferative stimulation from exposure to NZS for 6 h in HNBECs isolated from the non-leptin cultured tissues (Fig. 2A). However, the proliferation of HNBECs isolated from 1.5 nM leptin cultured tissues was significantly increased by treatment of ZS at all doses for 6 h (Fig. 2D). As shown in Figs. 2C and D, ZS increases HNBECs isolated from leptin cultured tissues more than that isolated from non-leptin cultured tissues. A comparison of Fig. 2A to Fig. 2B and Fig. 2C to Fig. 2D showed that HNBECs isolated from 1.5 nM of leptin cultured tissues developed more rapidly than those isolated from non-leptin cultured tissues with the same treatment of ZS or NZS. This result shows that leptin stimulates HNBEC growth.

On the other hand, a comparison of Fig. 2A to Fig. 2C and Fig. 2B to Fig. 2D showed that ZS increases HNBECs

isolated from leptin cultured tissues more than those isolated from non-leptin cultured tissues. The stimulatory effect of ZS is greater than that of NZS in HNBECs isolated from with or without leptin cultured tissues. A significant difference between the ZS-D0 and ZS-D30 at concentrations of 0.2, 1 and 5% was only found in the HNBECs isolated from 1.5 nM of leptin cultured tissues. The only difference between NZS and ZS was the implantation of Z pellets and the presence of metabolites in the blood. This result suggests that certain as yet undefined growth factors responsible for stimulatory action in HNBEC proliferation are secreted by the Z-implanted heifers in the blood. Therefore, we attribute the stimulatory effect of ZS on HNBECs to the implantation of Z.

It appears that leptin stimulates HNBEC growth, while ZS-D30 improves leptin-induced growth. Considering the leptin level is higher in obese women than in normal or lower weight women, this result suggests that obese women are more sensitive to Z. Additionally, it was shown that obese women may have a higher risk of breast neoplasia due to the consumption of beef products containing Z.

In conclusion, we found that the mitogenic activity of Z in human normal breast epithelial cells is enhanced by leptin and inhibited by gossypol. Z appears to increase HNBEC growth by increasing the cyclin D1 expression. Leptin improves HNBEC sensitivity to Z and Z strengthens the effect of leptin by increasing leptin secretion in HNBECs. Leptin and Z up-regulate the cyclin D1 expression in HNBECs. However, (-)-gossypol counteracts the growth of breast cancer cells induced by leptin alone or in combination with Z by down-regulating the cyclin D1 mRNA expression. Further mechanisms are currently being investigated and research on the Z metabolites that stimulate HNBEC growth is ongoing.

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