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Exogenous estrogen protects mice from the consequences of obesity and alcohol

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Abstract

Objective—Breast cancer is the second leading cause of cancer death among American women. Risk factors for breast cancer include obesity, alcohol consumption and estrogen therapy. In the present studies we determine the simultaneous effects of these three risk factors on Wnt-1 mammary tumor growth.

Methods—Ovariectomized female mice were fed diets to induce different body weights (calorie restricted, low fat, high fat), provided water or 20% alcohol, implanted with placebo or estrogen pellets and injected with Wnt- mouse mammary cancer cells.

Results—Our results show that obesity promoted the growth of Wnt-1 tumors and induced fatty liver. Tumors tended to be larger in alcohol consuming mice and alcohol exacerbated fatty liver in obese mice. Estrogen treatment promoted weight loss in obese mice which was associated with suppression of tumor growth and fatty liver.

Conclusions—In summary, we show that estrogen protects against obesity which is associated with inhibition of fatty liver and tumor growth.

Keywords

obesity; alcohol; estrogen; postmenopausal breast cancer; fatty liver

Introduction

Breast cancer is the second most frequent cancer afflicting American women and accounts for nearly 25% of all diagnosed cancers¹. The American Cancer Society predicted approximately 207,000 new cases of invasive breast cancer in 2010; 40,000 of which would succumb to the disease¹. Estrogen receptor alpha (ER α)-positive breast cancer is more prevalent among postmenopausal women than premenopausal women^{2, 3}. Modifiable risk factors for postmenopausal breast cancer, such as alcohol consumption, obesity and estrogen

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plus progestogen therapy, have been shown to increase the risk of ER α -positive breast cancers⁴⁻⁶.

Both obesity and alcohol consumption are established risk factors for breast cancer^{1, 5, 7-11}. Postmenopausal obesity increases the risk of morbidity and mortality from breast cancer^{7, 8, 10, 11}. Excess body weight accounts for nearly 30–50% of all breast cancer deaths¹¹. Adipose tissue can produce a multitude of factors known to influence the carcinogenic process, such as estrogen, leptin, adiponectin, insulin-like growth factor-1 (IGF-1) and cytokines¹²⁻¹⁵. Obesity increases the risk of developing ER α -positive cancers⁹. Obesity also increases the risk of developing fatty liver or hepatic steatosis¹⁶. Epidemiological studies have reinforced the positive association between alcohol consumption and breast cancer risk¹⁷. ER α -positive breast cancers are influenced by alcohol use although the mechanism is unknown⁵. Alcohol consumption increases breast cancer risk in a dose dependent manner, 10% for every drink consumed per day¹⁸. The hormonal status in postmenopausal women is also affected by alcohol; circulating blood estrogen levels increase in women who consume alcohol¹⁹⁻²¹. Moreover, chronic alcohol consumption increases the risk of developing hepatic steatosis²².

Evidence suggests both obesity and alcohol increase breast cancer risk via the hormone estrogen^{5, 9, 21, 23-25}. In premenopausal women, estrogen is produced largely by the ovaries²⁶. However, after menopause, primary estrogen production shifts to peripheral tissues (e.g. adipose tissue) where aromatase converts androgen to estrogen¹⁴. As a result, obese postmenopausal women have higher estrogen levels than lean postmenopausal women²⁴. Elevated estrogen levels are considered a risk factor for breast cancer^{25, 27, 28}. Based on this phenomenon, it is reasonable to understand why obesity tends to increase the risk of estrogen responsive cancers²⁹. Alcohol consumption also increases systemic estrogen levels^{20, 21, 25, 30}. Circulating blood estrogen levels may increase by as much as 20% in women who consume alcohol³⁰. It is possible alcohol increases systemic estrogen levels by increasing the expression level of aromatase^{31, 32}. Studies also show alcohol exposure increases the expression of ER α in breast cancer cells^{31, 32}. Therefore, not only does alcohol increase systemic estrogen levels but also increases the expression of ER α , which may explain why breast cancer cells are more sensitive to estrogen in the presence of alcohol. This suggests alcohol consumption in conjunction with estrogen therapy may increase breast cancer risk to a higher degree than either treatment alone. In fact, Chen et al. show that alcohol in combination with estrogen therapy increases breast cancer risk to a greater extent than estrogen therapy or alcohol only²⁵.

Even though studies show obesity, estrogen and alcohol consumption can increase breast cancer risk, the simultaneous effects of these factors on mammary cancer have not been determined in an experimental setting. To assess the simultaneous effects of these three risk factors on tumor growth, we injected Wnt-1 mammary tumor cells subcutaneously into mice fed a 30% calorie restricted, a low fat diet or a high fat diet, mice consumed either water or alcohol in the drinking water. To determine the combine effects of body weight, alcohol and estrogen on tumor growth, control and obese mice consuming water and alcohol were implanted with pellets delivering estrogen, and then injected with Wnt1 tumor cells.

Methods

Mouse husbandry and diets

All animal procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee. Mice were housed according to National Institutes of Health guidelines. 180 ovariectomized, 6 week old female C57Bl/6J mice were purchased from the Jackson Labs (Bar Harbor, MA, USA). The animals were maintained at 24°C on a

12-hour light/dark cycle. Mice were allowed to acclimate for 2 weeks, followed by randomization into treatment groups (9 groups, 20 mice per group). At this point different diets were fed and alcohol treatment commenced for 27 weeks. Mice were fed a 30% calorie restricted (CR), low fat (LF; 10% kcal from fat) or high fat (HF; 60% kcal from fat) diet. The CR diet is a modified version of the LF diet in which the mice receive 70% of the average daily calories and 100% of the vitamins and minerals of the LF diet. All diets have been previously described and used to mimic lean, overweight and obese postmenopausal women³³. Water or 20% weight per volume alcohol in the water were given to the animals *ad libitum* throughout the entire study. Body weight, food consumption and liquid consumption were measured weekly.

Pellet implantation

Estrogen (17 β -estradiol) or placebo pellets were implanted 19 weeks into the study when the mice were 27 weeks old. Implantation occurred under the skin at the dorsal area between the neck and shoulder of the mice. We used estrogen pellets manufactured to deliver 0.72mg of estrogen for 90 days (Innovative Research of America, Sarasota, FL, USA). Therefore, the mice received estrogen/placebo treatment from the time the pellets were implanted until the end of the study when the mice were euthanized; this was approximately 8 weeks.

Wnt-1 transgenic mouse model of breast tumor growth

Mammary tumor cells were generated from mouse mammary tumor virus (MMTV)-Wnt-1 transgenic mice on a C57Bl/6J genetic background. Tumor cells were isolated from transgenic females as previously described³⁴. Wnt-1 mouse tumors are estrogen receptor alpha-positive like the majority of human breast tumors in postmenopausal women³⁵. For implantation, 1×10^5 cells were implanted subcutaneously in 100 μ l serum-free RPMI 1640 medium into the lower back, halfway between the middle of the back and the base of the tail at 22 weeks into the study when the mice were 30 weeks of age. Once tumors became palpable, tumor volume was determined three times a week with calipers by measuring length, width and depth of the tumor.

Body composition and uterine weight

Baseline percent body fat was measured on un-anesthetized mice using an Echo MRI (Echo Medical Systems, Houston, TX, USA) when the mice were 8 weeks old. Percent body fat and bone mineral density (BMD) were analyzed by dual energy x-ray absorptiometry (DXA) using a GE Lunar Piximus II densitometer (Madison, WI, USA) on the mouse carcasses at the end of the study when they were 35 weeks old. To evaluate uterine weight mouse uteri were removed from the carcasses and weighed at the end of the study when the mice were 35 weeks of age.

Serum analysis and blood chemistry

Serum was collected from the animals at baseline (24 weeks old) and at the end of the study (35 weeks old). Insulin (pg/ml; Mercodia, Uppsala, Sweden) and 17 β -estradiol (pg/ml; Alpha Diagnostic, San Antonio, TX, USA) ELISA kits were used to measure serum insulin and estrogen, respectively. Similarly, serum adiponectin (ng/ml), vascular endothelial growth factor (VEGF; pg/ml), leptin (pg/ml) and insulin-like growth factor-1 (IGF-1; ng/ml) were also analyzed using ELISA kits (R&D Systems, Minneapolis, MN, USA). Alanine aminotransferase (ALT) levels were measured in the serum with an ELISA kit (BIO Scientific, Austin, TX, USA). A nicotinamide adenine dinucleotide (NAD)/reduced form of NAD (NADH) kit (Sigma, St. Louis, MO, USA) was used to quantify blood alcohol levels (BAC; mg/dl). All kits were used according to the manufacturer instructions.

Histology

Tissues were collected at sacrifice, 35 weeks of age, and fixed in 10% neutral buffered formalin for 24 h followed by 70% ethanol until embedded in paraffin. Tissues were cut into 4 μ m sections and stained with hematoxylin and eosin by standard procedures at the Histology and Tissue Processing Core at the University of Texas MD Anderson Science Park (Smithville, TX, USA).

Statistical analysis

Statistical comparisons for all analyses (body composition, tumor volume, uterine weight, BMD, serum analysis) were determined using multivariate analysis of variance and *post hoc* comparison of the means using Tukey's Honestly Significant Difference. SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA) was used to perform statistical evaluations. All results are presented as the mean \pm standard error of the mean (SEM) and statistical significance was considered $p < 0.05$.

Results

Body weight and percent body fat were unaffected by alcohol and drastically modified by exogenous estrogen

At the start of the experiment, baseline body weights were comparable among all groups ($p > 0.05$), as shown in Fig. 1A. Baseline body weights were taken when the mice were 8 weeks old and immediately before diet and food regimens were implemented (depicted as week 1 on the graph). After administration of the different diet regimens, a nice shift in body weight was observed between CR, LF and HF mice on water. Specifically, CR mice weighed the least, LF mice had an intermediate weight and HF mice were the heaviest. Mice receiving the LF diet and water served as the control group for these experiments. Similar to the water group, mice receiving alcohol displayed the same trend in body weight; alcohol had no affect on body weight ($p > 0.05$). These results were surprising as mice receiving alcohol consumed additional calories from alcohol which provided 1.42 kcal/g; no differences in food consumption ($p > 0.05$) were observed as highlighted in Table 1. The blood alcohol concentration (BAC) of mice consuming 20% alcohol are also depicted in Table 1 which ranged from 57–90 mg/dl (0.057–0.09%), levels equivalent to physiological levels known to increase breast cancer risk in women^{18, 36, 37}. After exogenous estrogen supplementation, the HF group ($p < 0.05$), but not the CR group ($p > 0.05$), underwent drastic weight loss, regardless of water or alcohol consumption. Exogenous estrogen modified body weight but had no affect on alcohol consumption.

Moreover, much like body weight, no differences in percent body fat were seen between groups at baseline (8 weeks old), as shown in Fig. 1B. However, after treatment (35 weeks old), notable variations were detected between CR and HF groups; CR mice had less percent body fat than HF mice for the placebo groups ($p < 0.05$). Alcohol consumption did not affect body fat levels. However, upon exogenous estrogen supplementation percent body fat drastically decreased, especially for mice in the HF groups, compared to placebo groups.

Effect of exogenous estrogen on physiology

Estrogen has been shown to increase uterine weight and increase bone mineral density³⁸. Because the animals used for the current study have been ovariectomized and lack endogenous estrogen, we decided to verify the exogenous estrogen treatment was effective by analyzing the aforementioned physiological parameters in our mice. Fig. 2A depicts serum estrogen levels at baseline (24 weeks of age) and after treatment (end of study, 35 weeks old) with estrogen and placebo pellets. No statistically significant difference in serum estrogen was noted among groups before estrogen supplementation at baseline ($p > 0.05$).

However, after estrogen treatment, serum estrogen levels greatly increased compared to the placebo groups ($p<0.01$). After estrogen treatment, uterine weight (35 weeks old, Fig. 2B) significantly increased for both CR and HF mice on water and alcohol compared to the placebo group ($p<0.01$). BMD also increased in the estrogen group compared to the placebo (Fig. 2C, 35 weeks of age, $p<0.01$). Collectively, the increased circulating blood estrogen levels, uterine weight and BMD, along with the decreased percent body fat suggest the estrogen implantation was successful.

Obesity and alcohol tended to promote while exogenous estrogen suppressed tumor growth

As portrayed in Fig. 3, diet tended to promote mammary tumor growth. HF mice developed larger tumors than CR mice; though the difference was not significant for the water group ($p=0.153$) but was significant for the alcohol group before estrogen treatment ($p<0.032$). Alcohol treatment had a tendency to increase tumor volume in obese mice compared to mice on water for both the placebo and estrogen groups even though the difference was not significant ($p>0.05$). In contrast, alcohol did not impact the growth of CR tumors compared to water animals ($p>0.05$). Fascinatingly, compared to placebo, tumor growth was considerably suppressed in HF mice ($p<0.05$) after estrogen pellet implantation. No distinctions were noticed for CR mice in either the estrogen or placebo group ($p>0.05$). Estrogen treatment inhibited the effects of both obesity (HF) on tumor growth.

Effect of alcohol, obesity and exogenous estrogen on growth factors

A multitude of growth factors, including but not limited to insulin, IGF-1, leptin and VEGF, have been implicated in cancer³⁹⁻⁴⁷. Therefore, we decided to analyze the serum levels of these hormones to determine the role they may play in tumor growth in the present study. Serum insulin and IGF-1 levels increased in Fig. 4A, B as body fat levels increased; CR mice had significantly lower levels of IGF-1 compared to HF mice, after estrogen treatment ($p<0.01$). Alcohol had no influence on insulin or IGF-1 compared to mice receiving water. However, supplementation of estrogen tended to significantly decrease the circulation of insulin in the blood of HF mice compared to placebo ($p<0.01$). IGF-1 levels were also greatly diminished in CR mice post-estrogen treatment ($p<0.05$). Much like insulin and IGF-1, serum leptin levels increased with body adiposity as portrayed in Fig. 5A, $p<0.05$. Alcohol did not modify the effect of obesity on leptin expression. Nevertheless, serum leptin levels were dramatically reduced after estrogen treatment for both CR ($p<0.05$) and HF ($p<0.01$) mice in contrast to placebo. Adiponectin levels, on the other hand, have been shown to inversely correlate with body fat⁴⁸. Adiponectin levels in Fig. 5B support this idea after treatment (35 weeks old) where CR mice have higher serum adiponectin than HF mice in all groups but the alcohol/placebo group ($p<0.05$). This was not the case at baseline (24 weeks old). In addition, estrogen treatment caused a down shift in adiponectin expression for both CR and HF animals versus placebo ($p<0.01$). Finally, we determined blood levels of the pro-angiogenic growth factor VEGF. Fig. 6 shows diet had no affect on VEGF levels after treatment. Among alcohol fed mice, HF diet compared to CR diet stimulated VEGF levels after placebo treatment ($p<0.05$). The addition of estrogen tended to block the effects of alcohol on VEGF levels in the HF mice post treatment, although the difference was not significant ($p>0.05$).

Alcohol and obesity negatively affected liver histopathology which was reversed by exogenous estrogen

Fatty liver disease, or hepatic steatosis, is commonly associated with alcohol consumption and obesity⁴⁹⁻⁵². It is unclear how exogenous estrogen affects the disease. We qualitatively evaluated liver histology in the current study to determine if estrogen has a deleterious or protective affect on hepatic steatosis. Fig. 7A shows the induction of fatty liver with

increasing adiposity as characterized by fat droplets in the hepatocytes. HF displayed more fatty liver than CR as shown in the literature⁵³. Moreover, the condition seemed to worsen after alcohol consumption in HF animals compared to HF mice on water. Alcohol even had a negative effect on CR livers compared to the water group; however, not near the extent of the HF animals. Importantly, estrogen treatment alleviated the fatty liver phenotype for all body weights and liquid groups and provided a protective effect. ALT is used as a marker of liver damage and overall liver health⁵⁴. High levels of ALT are suggestive of fatty liver, as well as a myriad of other liver pathologies. To confirm the histology results, serum ALT levels were analyzed as depicted in Fig. 7B. Serum ALT levels were consistent with the microscopic findings of the liver; ALT levels were higher in obese mice ($p < 0.01$), even higher in obese mice consuming alcohol (not significant) and ultimately suppressed to baseline levels (24 weeks of age, $p > 0.05$) after estrogen treatment, especially in HF mice.

Discussion

Postmenopausal women come in all sizes, lean, overweight and obese, many of which drink alcohol and even take estrogen to prevent the side effects of menopause. It is unclear if body weight or exogenous estrogen modulates the effects of alcohol on breast cancer growth. To address this in an experimental setting, we determined the simultaneous effects of alcohol, obesity and exogenous estrogen on tumor growth and fatty liver using mouse models in this study. Mice of different body weights (calorie restricted, low fat, high fat) consuming water or alcohol were injected subcutaneously with Wnt-1 cancer cells and implanted with placebo or estrogen pellets. Our results show that obesity promoted Wnt-1 tumor growth, which was ameliorated by estrogen supplementation. We also show that exogenous estrogen treatment resulted in significant weight loss in obese mice. Others have shown similar effects of exogenous estrogen on body weight⁵⁵. We correlate the dramatic loss in body weight to Wnt-1 tumor growth suppression. Obese mice consuming alcohol tended to have elevated tumor growth compared to obese mice consuming water. However, the difference was not significant and therefore suggests alcohol does not modulate the effects of body weight on tumor growth. Moreover, our results show that both obesity and alcohol consumption promoted fat accumulation in the liver and exogenous estrogen completely blocked the accumulation of fat in the livers of obese and alcohol consuming mice.

Here, alcohol consumption did not significantly modulate the effect of obesity on tumor growth. This does not rule out alcohol alone as a player in tumor growth. Evidence shows that alcohol accelerates tumor growth in mice⁵⁶ and in cell culture studies alcohol increases the metastatic potential of breast cancer cells in a dose dependent manner^{57, 58}. Moreover, alcohol and obesity are associated with high levels of hormones, including leptin and VEGF, which have been linked to breast cancer^{10, 15, 18, 43, 45, 59–63}. In cell culture studies, leptin promotes the proliferation and increases the invasiveness of breast cancer cells^{43, 44}. Leptin also up-regulates aromatase activity, suggesting leptin may affect breast cancer via estrogen⁵⁹. Gonzalez et al. demonstrated that blocking the effects of leptin, with a pegylated leptin peptide receptor antagonist, inhibited tumor growth in mice, suggesting leptin plays an important role in breast cancer growth⁶⁰. We reasoned that alcohol and obesity may influence mammary tumor growth via systemic hormones, such as leptin and estrogen. VEGF could also play a role. VEGF is a major player in angiogenesis, a process essential for tumor growth and metastasis^{45, 64, 65}. We originally assumed factors, like VEGF, produced by adipose tissue were associated with tumor growth; however, we cannot rule out the involvement of tumor-derived VEGF. VEGF levels were the highest in the HF placebo group receiving alcohol. This was interesting since alcohol had no effect on body weight nor body fat suggesting it must come from the tumor itself, rather than the adipose tissue. Moreover, estrogen treatment decreased VEGF expression in these mice. This data is in contrast to the literature which shows estrogen increases serum levels of VEGF⁶⁶. In our

study, body weight and body fat significantly decreased after estrogen treatment which was associated with a decrease in tumor growth. This suggests that the decreased VEGF expression could correspond to the tumor growth inhibition⁶⁷.

Much like alcohol consumption, obesity increases the risk of developing and dying from breast cancer in post-menopausal women^{7, 8, 10, 68, 69}. Obesity can lead to multiple physiological alterations that can influence breast cancer susceptibility. Obesity is also associated with a plethora of co-morbidities, such as hepatic steatosis¹⁶. Our results show that obesity promoted tumor growth. Moreover, obesity promoted liver steatosis. Serum insulin, IGF-1, leptin and VEGF levels increased with increasing body weight. This suggests obesity may affect tumor growth via these growth factors.

Estrogen is a steroid hormone produced predominantly by the ovaries in premenopausal women²⁶. However, production shifts to peripheral tissues (e.g. adipose tissue) in postmenopausal women where aromatase converts androgen to estrogen²⁶. Estrogen-producing cells, such as adipocytes, contain aromatase²⁶. Obese postmenopausal women have higher estrogen levels than lean postmenopausal women because they have more adipose tissue and aromatase²⁴. Elevated estrogen levels are considered a risk factor for breast cancer^{25, 27, 28}. Surprisingly, estrogen treatment suppressed tumor growth in both CR and HF mice. This was unexpected since estrogen is a growth factor for breast cancer cells. The present data challenge the central dogma regarding estrogen and breast cancer; however, these effects of estrogen on tumor growth may be dependent on the presence of obesity. This notion is supported by findings from Nkhata *et al.* who showed that estrogen treatment inhibited T47-D human breast cancer cell progression in obese mice⁵⁵. Thus, it is feasible that if estrogen treatment leads to a loss of body weight it may protect against breast cancer. On the other hand the timing of estrogen supplementation may be the key to whether estrogen is a risk factor or a protector against breast cancer. For example, one study from the Woman's Health Initiative highlighted the benefit of initiating estrogen therapy five or more years after menopause and emphasized the resultant decrease in breast cancer⁷⁰. Most animal studies do not consider this factor when designing experiments and supplement estrogen immediately before ovariectomy⁷¹⁻⁷³. In doing this they ignore physiological changes that occur after long term estrogen deprivation, as seen in postmenopausal women. Others have shown under cell culture conditions that estrogen inhibited cell growth and induced apoptosis (cell death) of MCF-7 breast cancer cells, after long-term estrogen deprivation⁷⁴. All of these studies highlight the delicate balance between hormone therapy timing and breast cancer susceptibility. Alternatively, it is possible that estrogen inhibits tumor growth, but promotes breast cancer metastasis⁷². In our studies we only evaluated primary tumor growth, thus it is feasible that estrogen may increase the metastatic ability of breast cancer cells, while it inhibits tumor growth.

Previous research has shown that estrogen treatment results in weight loss⁵⁵. Therefore, the inhibition of tumor growth may be due to weight loss after estrogen treatment and not the estrogen treatment itself. In our studies, estrogen supplementation triggered a drastic decrease in body weight and percent body fat in obese mice. What is more, circulating insulin and leptin levels were reduced after estrogen treatment. Thus, the decrease in systemic growth factors, as a result of weight loss, may be responsible for the inhibition of tumor growth by estrogen treatment. The key factor for estrogen to inhibit tumor growth may be dependent in whether or not it promotes weight loss, or more specifically loss of body fat, leading to a drastic decrease in systemic hormone levels (e.g., leptin, insulin and IGF-1).

Consistent with the literature, our studies show both alcohol consumption and obesity promote liver steatosis (fatty liver)⁴⁹⁻⁵². Fatty liver is a reversible condition where lipids

accumulate in hepatocytes⁷⁵. Fatty liver is strongly associated with obesity^{16, 76, 77}. In our studies, consumption of alcohol by obese mice exacerbated liver steatosis to a greater extent than either alcohol or obesity alone. Liver steatosis was alleviated by estrogen treatment. Interestingly, fatty liver disease is more prevalent in men and postmenopausal women than in premenopausal women, suggesting that indeed estrogen is a protective factor against the development of liver steatosis^{78, 79}. Additionally, Ogawa et al. reported a dramatic induction of fatty liver in breast cancer patients treated with tamoxifen, an estrogen receptor antagonist⁸⁰. Thus, these studies along with our findings support the notion that indeed estrogen may protect against the development of fatty liver. Although interesting, the data presented here is limited to the Wnt-1 allograft mouse model of breast cancer. The results reflect the effects of exogenous estrogen on this model and were restricted to 20% alcohol.

Conclusions

We show that both alcohol and obesity promoted tumor growth and fatty liver. Surprisingly, estrogen treatment inhibited the effects of obesity and alcohol consumption on tumor growth and fatty liver. This is the first study to show that estrogen is a protective factor to the effects of alcohol and obesity on mammary tumor growth and fatty liver development. It is interesting that estrogen can behave in such a dichotomous fashion as both a risk factor for breast cancer and suppressor of tumor growth in obese mice. Studies are warranted to determine if indeed certain estrogen therapies can be used in postmenopausal women without leading to an increased risk of breast cancer.

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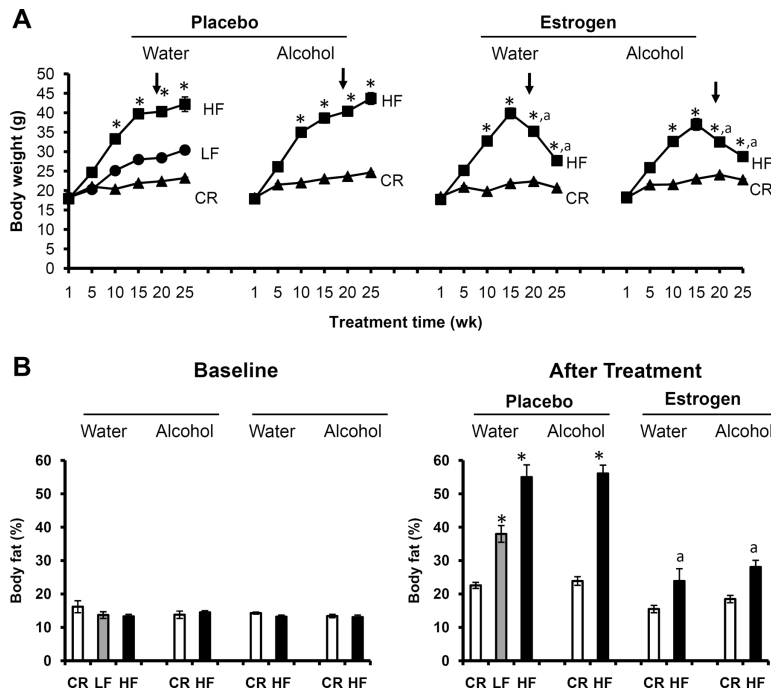


Fig. 1. Exogenous estrogen dramatically affected body weight and percent body fat. 20 mice per group; HF: high fat, LF: low fat, CR: caloric restriction. **A.** Body weight. Measurements were recorded weekly after diet and liquid regimens were given and throughout the course of the 25 week treatment. Graph depicts averages recorded for every 5 weeks of treatment. Arrow indicates pellet implantation at week 19 when the mice were 27 weeks old, *Significantly different from corresponding CR, ^aSignificantly different from corresponding placebo. **B.** Percent body fat. Pre-estrogen treatment body fat levels were recorded at baseline when the mice were 8 weeks old and before pellets were implanted. Post-estrogen treatment body fat levels were evaluated at the end of the study when the mice were 35 weeks of age. *Significantly different from corresponding CR, ^aSignificantly different from corresponding placebo.

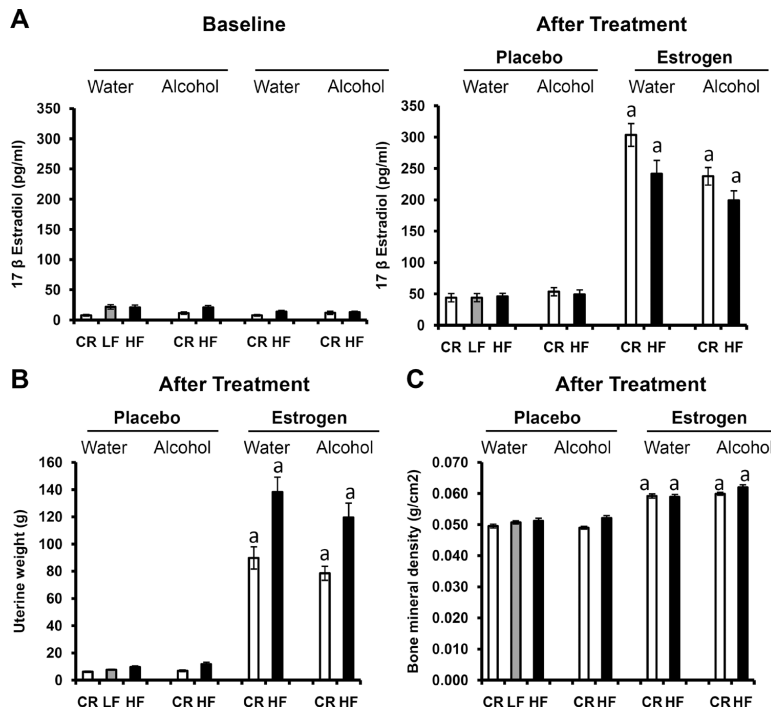


Fig. 2. Physiological effects of exogenous estrogen treatment. HF: high fat, LF: low fat, CR: caloric restriction, 17 β estradiol: estrogen. **A.** Levels of circulating serum estrogen. Estrogen levels were recorded at baseline when the mice were 24 weeks old and before pellets were implanted. Post-estrogen treatment levels were evaluated at the end of the study when the mice were 35 weeks of age and after pellets were implanted. 10 mice per group, ^aSignificantly different from corresponding placebo. **B.** Uterine weight was analyzed at the end of the study when the mice were 35 weeks old. 12 mice per group, ^aSignificantly different from corresponding placebo. **C.** Bone mineral density was evaluated when the mice were 35 weeks of age. 20 mice per group, ^aSignificantly different from corresponding placebo.

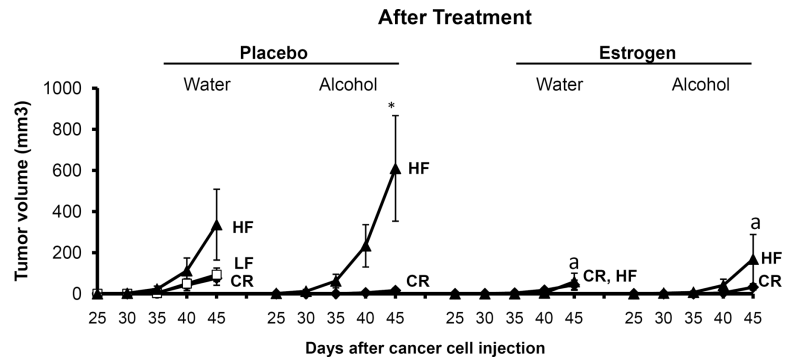


Fig. 3. Tumor volume. Estrogen or placebo treatments began when the mice were 27 weeks of age. Cancer cells were injected when the mice were 30 weeks old. Following cancer cell injection, tumor volume was measured 2–3 times per week until the mice were euthanized at the end of the study. The graph depicts the average tumor volume recorded every 5 days once tumors became palpable around 25 days after cancer cell injection. 20 mice per group; HF: high fat, LF: low fat, CR: caloric restriction; ^aSignificantly different from corresponding placebo, *Significantly different from corresponding CR.

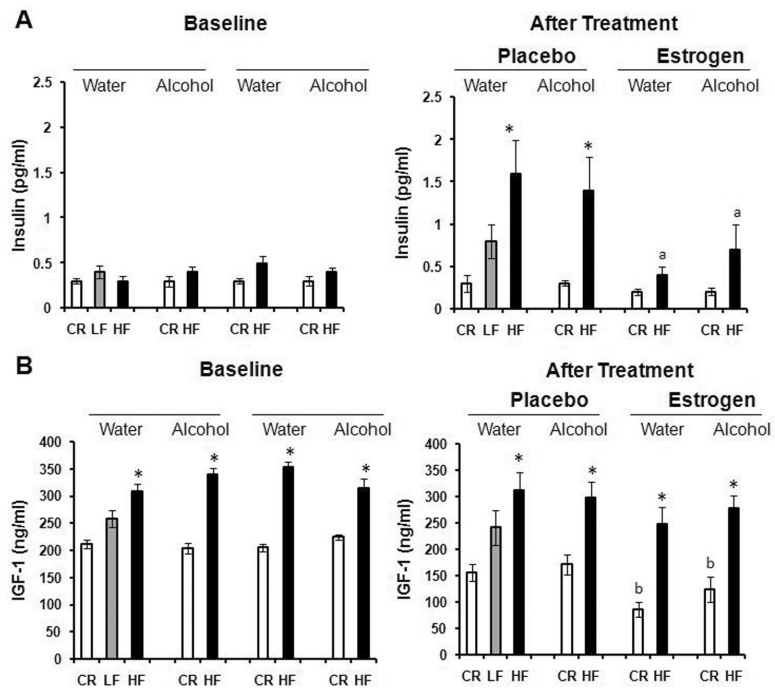


Fig. 4. Modulation of hormones by alcohol, obesity and exogenous estrogen. Hormones were measured at the end of the study when the mice were 35 weeks old. 10 mice per group; HF: high fat, LF: low fat, CR: caloric restriction. **A.** Levels of circulating Insulin. *Significantly different from corresponding CR, ^aSignificantly different from corresponding placebo. **B.** Levels of circulating IGF-1. *Significantly different from corresponding CR, ^bSignificantly different from corresponding baseline.

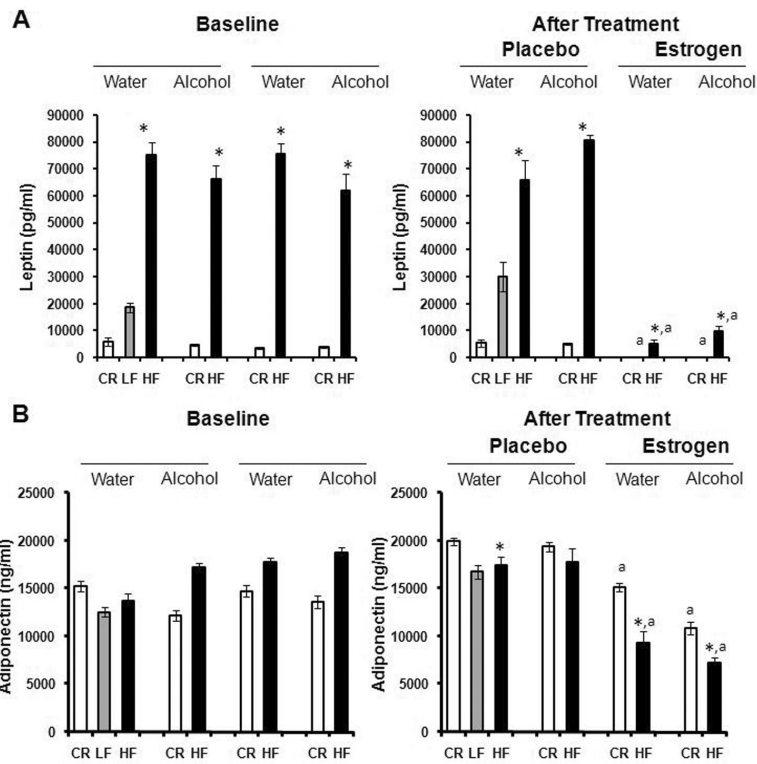


Fig. 5. Modulation of adipokines by alcohol, obesity and exogenous estrogen. Hormone levels were analyzed at the end of the study when the mice were 35 weeks of age. 10 mice per group; HF: high fat, LF: low fat, CR: caloric restriction. **A.** Levels of circulating Leptin. *Significantly different from corresponding CR, ^aSignificantly different from corresponding placebo. **B.** Levels of circulating Adiponectin. *Significantly different from corresponding CR, ^aSignificantly different from corresponding placebo.

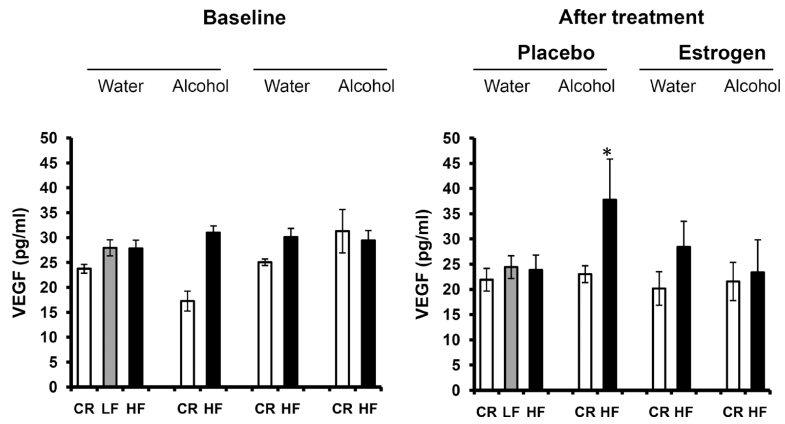


Fig. 6. Levels of circulating VEGF. VEGF levels were measured at the end of the study when the mice were 35 weeks old. 10 mice per group; HF: high fat, LF: low fat, CR: caloric restriction. *Significantly different from corresponding CR.

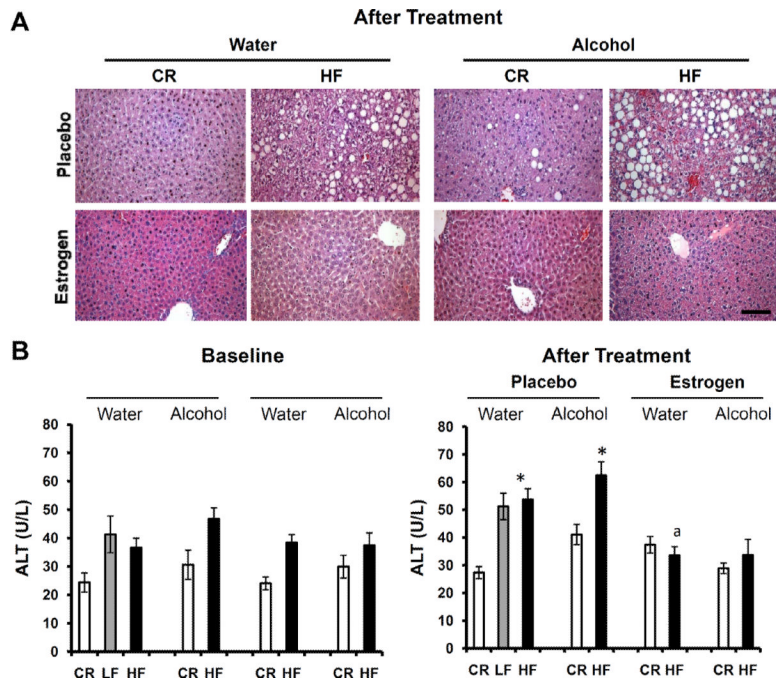


Fig. 7. Effects of alcohol, obesity and exogenous estrogen on liver histopathology. HF: high fat, LF: low fat, CR: caloric restriction. **A.** Hematoxylin and Eosin stained sections of Fatty Liver. Livers were fixed and analyzed when the mice were 35 weeks old. 3 mice per group, 20× magnification, 100µm bar. **B.** Levels of circulating ALT. 10 mice per group; *Significantly different from corresponding CR, ^aSignificantly different from corresponding placebo. Pre-estrogen treatment ALT levels were recorded at baseline when the mice were 24 weeks old. Post-estrogen treatment levels were evaluated at the end of the study when the mice were 35 weeks of age.

Table 1

Final caloric intake and blood alcohol levels

	Placebo						Estrogen					
	Water			Alcohol			Water			Alcohol		
	CR	LF	HF	CR	HF	LF	CR	HF	LF	CR	HF	LF
Food (kcal)	7.6 ± 0.0	12.2 ± 0.4	15.0 ± 0.6	7.6 ± 0.0	13.6 ± 0.7	15.0 ± 0.6	7.6 ± 0.0	13.9 ± 0.5	15.0 ± 0.6	7.6 ± 0.0	13.9 ± 0.5	15.0 ± 0.6
Alcohol (kcal)				3.3 ± 0.1	3.1 ± 0.3					4.6 ± 0.1	4.6 ± 0.1	
Total (kcal)	7.6 ± 0.0	12.2 ± 0.4	15.0 ± 0.6	10.9 ± 0.1	16.6 ± 0.7	15.0 ± 0.6	7.6 ± 0.0	13.9 ± 0.5	10.9 ± 0.1	12.2 ± 0.1	13.9 ± 0.5	14.9 ± 0.3
BAC (mg/dl)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	90.4 ± 24.6 ^c	62.4 ± 12.7 ^c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	84.2 ± 19.0 ^c	0.0 ± 0.0	57.1 ± 5.8 ^c

Values are represented as the mean ± SEM (n=10/group)

BAC: blood alcohol concentration, CR: caloric restriction, LF: low fat, HF: high fat

^cSignificantly different from corresponding water group