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Tocopherols Inhibit Oxidative and Nitrosative Stress in Estrogen-Induced Early Mammary Hyperplasia in ACI Rats

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Abstract

Oxidative stress is known to play a key role in estrogen-induced breast cancer. This study assessed the chemopreventive activity of the naturally occurring γ -tocopherol-rich mixture of tocopherols (γ -TmT) in early stages of estrogen-induced mammary hyperplasia in ACI rats. ACI rats provide an established model of rodent mammary carcinogenesis due to their high sensitivity to estrogen. Female rats were implanted with 9 mg of 17 β -estradiol (E2) in silastic tubings and fed with control or 0.3% γ -TmT diet for 1, 3, 7 and 14 days. γ -TmT increased the levels of tocopherols and their metabolites in the serum and mammary glands of the rats. Histological analysis revealed mammary hyperplasia in the E2 treated rats fed with control or γ -TmT diet. γ -TmT decreased the levels of E2-induced nitrosative and oxidative stress markers, nitrotyrosine and 8-oxo-dG, respectively, in the hyperplastic mammary tissues. 8-Isoprostane, a marker of oxidative stress in the serum, was also reduced by γ -TmT. Noticeably, γ -TmT stimulated Nrf2-dependent antioxidant response in the mammary glands of E2 treated rats, evident from the induced mRNA levels of Nrf2 and its downstream antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase. Therefore, inhibition of nitrosative/oxidative stress through induction of antioxidant response is the primary effect of γ -TmT in early stages of E2-induced mammary hyperplasia. Due to its cytoprotective activity, γ -TmT could be a potential natural agent for the chemoprevention of estrogen-induced breast cancer.

Keywords

estrogen; oxidative stress; breast cancer; vitamin E

Introduction

Breast cancer is the second leading cause of cancer death among women in the U.S. [1]. While the role of estrogens in breast carcinogenesis is widely recognized [2–5], the

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molecular mechanisms underlying the initiation and progression of breast cancer have not been fully elucidated. Several studies suggest that estrogens exert their carcinogenic effects via two distinct mechanisms- estrogen receptor (ER)-dependent and independent [2–7]. The ER-dependent mechanism involves stimulation of cell growth and proliferation [5, 8]. In the ER-independent pathway, metabolism of estrogen generates highly reactive genotoxic metabolites and reactive oxygen species (ROS) capable of damaging cellular macromolecules [6, 9]. Estrogen metabolism produces catechol estrogens, which are oxidized to electrophilic quinones and semiquinones. These quinone intermediates can form depurinating DNA adducts, generating highly mutagenic apurinic sites [2]. In addition, redox cycling of quinones and semiquinones results in the formation of ROS, enhancing the probabilities of DNA damage [10]. Oxidative stress exerted by the metabolites of estrogen is thought to act in concert with ER signaling to promote genetic alterations and deregulated cell proliferation [7]. A clear understanding of these processes is essential for the development of successful preventive strategies for breast cancer.

Naturally occurring antioxidants are considered as promising chemopreventive agents against various human cancers. Vitamin E is a family of fat soluble phenolic compounds, consisting of a chromanol ring and a side chain [11]. They occur in eight different forms, including four tocopherols (with a saturated phytyl tail) (Figure 1A) and four tocotrienols (with an unsaturated isoprenoid side chain). Depending on the number and position of methyl groups on the chromanol ring, they are designated as α -, β -, γ - and δ forms [11]. Dietary intake of tocopherols is suggested to reduce cancer risk due to their strong antioxidant properties [12, 13].

α -Tocopherol (α -T) is known as the classic vitamin E as it is the major tocopherol found in plasma and tissues [14]. However, some large-scale human trials with α -T have provided inconsistent conclusions [15–18]. The outcome of these trials reflects our incomplete understanding of the biological activities of different forms of tocopherols and emphasizes the need to investigate their role in cancer. γ -T is the most common form of vitamin E in the U.S. diet, present in vegetable oils such as soybean, corn and cottonseed [19]. It is reported to have stronger anti-inflammatory and anti-cancer activities compared to α -T [19]. γ -TmT, a naturally occurring tocopherol mixture rich in γ -T, is a byproduct of refined vegetable oil [20]. We have recently shown the inhibition of lung, colon, prostate and breast carcinogenesis by γ -TmT [21–23].

In earlier studies, we have reported that γ -TmT suppressed mammary tumor growth in *N*-methyl-*N*-nitrosourea (NMU) induced Sprague Dawley rats [22, 24]. Since NMU is a synthetic carcinogen, we utilized the August-Copenhagen Irish (ACI) rats which develop mammary tumors in response to estrogen treatment, and therefore, provide a more physiologically relevant model for studying mammary tumorigenesis. Being highly sensitive to estrogen, they exhibit 80–100% tumor incidence after chronic estrogen exposure [25]. Mammary tumorigenesis in ACI rats is remarkably similar to that of humans, with respect to aneuploidy, genomic instability and histopathology [26–29]. Prolonged exposure of ACI rats to high doses of estrogen (27 mg) can induce pituitary tumors along with mammary tumors [30], making breast cancer prevention studies difficult in this model. To overcome this problem, we used a lower dose of estrogen (9 mg) in silastic tubing in our study to induce

mammary hyperplasia while avoiding excessive pituitary gland enlargement. Previously, γ -TmT was reported to suppress ER-mediated signaling at intermediate stages of estrogen-induced mammary hyperplasia in ACI rats [31]. However, the effect of γ -TmT at earlier stages of mammary hyperplasia is not understood. In the present study, the ACI rat model was used to investigate the biological activity of γ -TmT at early stages of mammary hyperplasia induced by estrogen. We demonstrate that dietary supplementation of γ -TmT reduces estrogen-induced oxidative/nitrosative stress and stimulates nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-dependent antioxidant response in early mammary hyperplasia. Thus, γ -TmT could be an effective agent for the prevention of estrogen induced breast cancer.

Materials and Methods

Animals and Experimental Procedures

Female ACI rats were purchased from Harlan Laboratories (Indianapolis, IN) at 6–7 weeks of age. Silastic tubings of 1.3 cm length were packed with 9 mg of 17 β -estradiol (E2) (Steraloids, Newport, RI). After two weeks of acclimatization, the rats were subcutaneously implanted with control (sham) or 9 mg E2-filled silastic tubings. The implantation procedure was adapted from a previously described method [32]. Rats receiving sham implants were fed with the control diet, while those receiving E2 implants were fed with control or 0.3% γ -TmT diet. Diets were administered from the day of implantation. Rats were sacrificed at four time points - 1, 3, 7 and 14 days following implantation. For each time point, the negative control group included four animals and the treatment groups included six animals. Blood was collected at autopsy and serum stored at -80°C . Mammary glands were collected and frozen or fixed in formalin for further analysis.

Diets

Naturally occurring γ -TmT preparation was obtained from BASF Corporation (Kankakee, Illinois; Covi-ox T-90, Batch number 0008778732). It contained 11.5% α -T, 1.2% β -T, 22.3% δ -T and 56.1% γ -T. Semi-purified AIN-93M obtained from Research Diets, Inc. (New Brunswick, NJ) was used as the control diet. Test diets were prepared by adding required percentages of γ -TmT to the AIN-93M diet. The diets were stored at 4°C in sealed containers and food cups were replenished with fresh pellets twice a week.

Analysis of Tocopherol Levels in the Serum and Mammary Glands

The levels of tocopherols (α -, γ -, δ -) and their metabolites in the rat serum and mammary glands were analyzed by high performance liquid chromatography using previously described methods [20, 33].

Enzyme Immunoassay for 8-isoprostane

EIA kit from Cayman Chemicals (Ann Arbor, MI) was used to determine 8-isoprostane levels. For the extraction of 8-isoprostane, 100 μl of serum samples were mixed with 1 ml ethyl acetate, vortexed for 30 minutes and centrifuged at $10,000\times g$ for 20 minutes. The organic layer was collected and dried using a speed vacuum evaporator. The dried samples

were reconstituted in EIA buffer and the assay was performed following the manufacturer's protocol.

Histopathological Analysis and Immunohistochemistry

Mammary glands were collected at autopsy and fixed in 10% formalin for 24 hours. Subsequently, they were embedded in paraffin and sectioned at 4 μ m thickness. Tissue sections were stained with haematoxylin and eosin (H & E) for histopathological analysis. For immunohistochemistry, slides were incubated overnight at 4°C with antibodies to 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) (1:100, N45.1; JaiCA/GENOX Corporation, Baltimore, MD) or nitrotyrosine (1:250, MAB5404; Millipore, Billerica, MA). This was followed by incubation with biotinylated secondary antibody and avidin/biotin peroxidase complex for 30 minutes each at room temperature. The sections were then stained with 3'-diaminobenzamine substrate and counterstained with Modified Harris Haematoxylin. Mammary gland sections from all animals per treatment group were stained for the above listed markers. Representative images were taken randomly with Nikon Eclipse E800 fitted to Nikon Digital Sight Ri 1. The staining was quantified using an Aperio ScanScope (Vista, CA). Quantification was performed by randomly selecting five sections and counting over 10,000 cells per slide. Positive staining for nitrotyrosine was found in the cell cytosol. For 8-oxo-dG, positive staining was localized in the nuclei of cells.

mRNA Expression Analysis using Quantitative PCR

RNA extraction from the frozen mammary glands, reverse transcription and quantitative PCR were carried out as previously reported [34]. Labeled primers for Nrf2, catalase, glutathione peroxidase (GPx), superoxide dismutase 1 (SOD1) and GAPDH were obtained from Applied Biosystems (Carlsbad, CA).

Statistical Analysis

The data are represented as mean $\pm$ SE. Statistical significance was evaluated by two-tailed Student's *t*-test. Within each experimental time point, the E2 group was compared to negative control (represented as 'a') and γ -TmT treated group compared to E2 group (represented as 'b'). *P*-values < 0.05 were considered significant.

Results

Estrogen Increases Liver and Pituitary Gland Weights in ACI Rats

As shown in Table 1, no difference in average body weight was observed among the control, E2 and γ -TmT treated groups at 1, 3, 7 and 14 days. E2 treatment is known to increase liver and pituitary gland weight in ACI and other rodent models [35]. In this study, E2 treatment did not change liver and pituitary gland weight at 1 and 3 days. However, at 7 and 14 days, E2 caused significant increase of liver and pituitary gland weight of ACI rats compared to the control. At 14 days, liver weight of the 0.3% γ -TmT treated group was lower than that of the E2 group (*P* < 0.05). γ -TmT did not alter the E2-induced pituitary gland weight of the rats at any of the time points.

E2 Treatment Induces Hyperplasia in the Mammary Glands of ACI Rats

Mammary tissue sections of rats were stained with H & E (Figure 1B). At all four time points, the mammary glands of negative control groups showed normal lobular architecture and distribution of fat tissue. Mild lobular hyperplasia was evident as early as 3 days of E2 treatment. At 7 and 14 days, the mammary tissue of E2 treated groups fed with both control or γ -TmT diets were highly proliferated with dilated ducts. Increased proliferation of the terminal lobular units was evident along with expansion into the surrounding fat tissue. Treatment with 0.3% γ -TmT had no effect on E2-induced mammary hyperplasia. Further analysis was carried out to determine molecular changes induced by γ -TmT in the hyperplastic mammary tissues of ACI rats.

Administration of Dietary γ -TmT Increases the Levels of Tocopherols and their Metabolites in the Serum and Mammary Glands of ACI Rats

To determine the bioavailability of dietary tocopherols in the experimental animals, the levels of δ -, γ -, α -T and their respective short chain metabolites, carboxyethyl hydroxychromans (CEHCs) and carboxymethylbutyl hydroxychromans (CMBHCs) (Figure 1A), in the serum and mammary glands of the rats were measured (Figures 2 and 3). As shown in Figure 2, increased levels of δ - and γ -T were detected in the rat serum from day 1 of γ -TmT administration, while α -T levels remained fairly constant. Serum levels of α -, δ - and γ -CEHC metabolites also increased from day 1, with δ - and γ -CEHC levels being much higher compared to α -CEHC. The concentration of CMBHC metabolites also increased in the γ -TmT fed groups. Analysis of tocopherols levels in the rat mammary glands (Figure 3) demonstrated significantly increased levels of δ - and γ -T from day 7 of γ -TmT supplementation. This was accompanied by increased concentrations of α -, δ - and γ -CEHC as well as CMBHC metabolites in the mammary tissues from day 7. The concentration of tocopherols, which are hydrophobic compounds, were several fold higher in the adipose-rich mammary glands compared to their serum levels at the same time point. The hydrophilic metabolites, δ -, γ - and α -CEHC and CMBHC, were more abundant in the serum compared to the mammary glands.

Treatment with γ -TmT Reduces Estrogen-Induced Nitrosative Stress in the Mammary Glands of ACI Rats

Nitrotyrosine is a well-known marker of nitrosative stress [36]. Its levels in rat mammary glands were analyzed by immunohistochemistry (Figure 4A). E2 treatment increased nitrotyrosine levels in the mammary glands from day 1 of treatment. At 1 and 3 days, γ -TmT did not change the levels E2-induced nitrotyrosine. However, at 7 and 14 days, a significant reduction of nitrotyrosine levels was observed in response to γ -TmT administration: nitrotyrosine was reduced by 70% ($P < 0.01$) and 90% ($P < 0.001$) at days 7 and 14, respectively (Figure 4B).

Treatment with γ -TmT Reduces Oxidative Stress Markers in the Mammary Glands and Serum of ACI Rats

Oxidative stress plays a key role in E2-induced mammary carcinogenesis [6]. 8-oxo-dG is a marker of oxidative DNA damage [37]. To evaluate oxidative stress in the mammary glands

of ACI rats, immunohistochemical analysis of 8-oxo-dG was performed (Figure 4C). E2 treatment increased 8-oxo-dG levels from day 1. At 1 and 3 days, γ -TmT did not change the levels of 8-oxo-dG in mammary tissues. However, γ -TmT supplementation significantly reduced E2-induced 8-oxo-dG levels by 72% ($P < 0.001$) and 67% ($P < 0.05$) at 7 and 14 days, respectively (Figure 4D). 8-Isoprostane is a biomarker of oxidative stress. It is produced *in vivo* by the free radical catalyzed peroxidation of arachidonic acid [38]. The serum levels of 8-isoprostane were quantified by EIA (Figure 5). Increased levels of 8-isoprostane were detected in the E2 treated groups from day 3. γ -TmT reduced the E2-induced 8-isoprostane levels by 80% ($P < 0.001$), 44% ($P < 0.05$) and 46% ($P < 0.05$) at the 3-, 7- and 14 day time points, respectively.

Administration of γ -TmT Results in Increased mRNA Expression of Nrf2 and its Downstream Antioxidant Enzymes

Cells exposed to oxidative stress must augment their antioxidant capacity to maintain redox homeostasis [39]. Nrf2 is a transcription factor that is activated by elevated levels of oxidative stress marker [39]. It enhances the expression of a multitude of antioxidant and phase II detoxifying enzymes that restore redox homeostasis in cells [39]. To elucidate the mechanism underlying the inhibition of oxidative stress by γ -TmT, mRNA expression of Nrf2 and some of its downstream antioxidant enzymes in rat mammary glands was analyzed by q-PCR. As shown in Figure 6, 9 mg E2 treatment alone did not change the mRNA levels of Nrf2. However, administration of γ -TmT resulted in a significant induction of Nrf2 as early as day 1. In addition, γ -TmT induced mRNA expression of the Nrf2 regulated antioxidant enzymes, superoxide dismutase1 (SOD1), glutathione peroxidase (GPx) and catalase. SOD1 and Gpx levels were induced from day 1 while catalase was induced from day 3 of γ -TmT supplementation.

Discussion

ER positive breast cancer accounts for two thirds of all breast cancers [40]. Understanding the molecular events underlying the transition of mammary glands from normal to hyperplastic and neoplastic stage is essential to find safe agents capable of preventing the onset and progression of breast cancer. Tocopherols are suggested to have anti-cancerous activity [13], δ -T and γ -T being more potent [19, 20]. Very limited information is currently available on the effect of δ -T and γ -T at different stages of breast cancer. Moreover, our knowledge is mostly based on late stage or mammary tumor studies [22, 24]. The role of δ -T and γ -T in early mammary hyperplasia is largely unexplored.

In this study, we examined the effect of a naturally occurring tocopherol mixture rich in γ -T (γ -TmT) in the early stages (1, 3, 7 and 14 days) of E2-induced mammary hyperplasia using the ACI rat model. It is known that ACI rats exposed to high doses of E2 (27 mg) over a long period develop pituitary tumors which can interfere with breast cancer prevention or treatment studies [30]. Therefore, our study utilized a lower dose of E2 (9 mg) in silastic tubing to achieve a controlled induction of mammary hyperplasia without significant pituitary gland enlargement. We intend to use the same E2 dose in future studies for investigating the role of γ -TmT in the intermediate and late stages of mammary

carcinogenesis in ACI rats. With 9 mg of E2, hyperplasia of mammary glands was evident starting from 3 days of E2 implantation. Dietary supplementation with 0.3% γ -TmT had no effect on hyperplasia or cell proliferation (Supplemental Figure 1) at these early time points. We previously reported that the levels of the cell proliferation marker, proliferating cell nuclear antigen (PCNA), in hyperplasia of mammary glands did not change at two weeks but were significantly decreased at ten weeks by 0.3% γ -TmT treatment [31]. Thus the influence of γ -TmT on cell proliferation is a later event in estrogen-induced mammary hyperplasia.

Reactive oxygen and nitrogen species (RONS) can promote the initiation and progression of tumor by causing genomic instability, mutations, deregulated cell proliferation [41]. Metabolites of estrogen are implicated in oxidative stress and DNA damage [42]. Proliferation of genetically damaged cells may lead to tumor development [42]. 8-oxo-dG is an oxidized derivative of deoxyguanosine, being a frequent product of DNA oxidation and a mutagenic lesion in DNA. During DNA replication, unrepaired 8-oxo-dG lesions induce G-T transversions, which may contribute to carcinogenesis [43]. 8-Isoprostane is a prostaglandin-like compound produced by the free radical catalyzed peroxidation of arachidonic acid [38]. Quantification of 8-isoprostane is recognized as a reliable method of assessing oxidative stress *in vivo* [10]. Increasing number of evidences suggests that reactive nitrogen species can affect the redox status of cells to modify cellular macromolecules and cause cancer [10, 43]. Elevated levels of nitrotyrosine, a biomarker of nitrosative stress has been shown to induce DNA damage [10, 43]. Previously γ -T, δ -T and γ -TmT have been reported to reduce nitrosative stress and induce antioxidant response in the NMU-model of breast cancer [22]. However, tocopherols had no inhibitory effect on the levels of oxidative stress markers in these late stages of mammary cancer [22]. Therefore, in the current study, we investigated the effect of γ -TmT on oxidative and nitrosative stress markers at early stages of E2-induced mammary hyperplasia. ACI rats administered with γ -TmT exhibited lower levels of 8-oxo-dG, 8-isoprostane and nitrotyrosine compared to the E2 control rats as early as day 7. γ -TmT is capable of trapping RONS and reducing oxidative lesions that may lead to mutations and increase the risk of mammary carcinogenesis. Therefore, inhibition of E2-induced oxidative/nitrosative stress by γ -TmT treatment in early mammary hyperplasia may contribute to lowering the risk of mammary carcinogenesis.

The Nrf2 antioxidant pathway is an important cellular defense mechanism against oxidative stress, genotoxicity and tumor formation [44, 45]. Upon activation, the transcription factor Nrf2 binds to anti-oxidant responsive elements to induce the expression of antioxidant and phase II detoxification enzymes [39]. These enzymes protect cells from neoplastic transformation by maintaining redox homeostasis [39, 46]. Furthermore, mammary carcinogenesis in response to estrogen has been associated with decreased Nrf2 expression [47]. In the present study, γ -TmT treatment induced mRNA levels of Nrf2 and its downstream antioxidant enzymes SOD1, catalase and GPx. SOD1 protects cells from superoxide radical by catalyzing its dismutation to hydrogen peroxide, while catalase decomposes hydrogen peroxide to hydrogen and water, neutralizing its toxicity [48]. GPx eliminates both hydrogen peroxide and organic peroxides by way of reduced glutathione [48]. SOD, catalase and GPx are considered as primary antioxidant enzymes directly

involved in the elimination of ROS [43]. Thus, induction of these enzymes by γ -TmT may protect cells from the oxidative damage caused by E2 and prevent their malignant transformation.

In conclusion, treatment of ACI rats with E2 induces oxidative and nitrosative stress markers in their tissues while supplementation with 0.3% γ -TmT diet reduces them. Thus, inhibition of oxidative/nitrosative stress via induction of Nrf2-mediated antioxidant response is the key influence of γ -TmT administration in estrogen-induced early mammary hyperplasia. This may contribute to the chemopreventive effect of γ -TmT in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACI	August Irish Copenhagen
CEHC	carboxyethyl hydroxychroman
CMBHC	carboxymethylbutyl hydroxychroman
EIA	enzyme immunoassay
E2	17 β -estradiol
ER	estrogen receptor
GPx	glutathione peroxidase
H & E	haematoxylin and eosin
8-oxo-dG	8-hydroxy-2'-deoxyguanosine
NMU	<i>N</i> -methyl- <i>N</i> -nitrosourea
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PCNA	proliferating cell nuclear antigen
q-PCR	quantitative polymerase chain reaction
RONS	reactive oxygen and nitrogen species
SOD1	superoxide dismutase1
T	tocopherol

γ -TmT γ -tocopherol-rich mixture of tocopherols.

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A

B

Figure 1.

(A) Structures of tocopherols and their key metabolites, carboxymethylbutyl hydroxychroman (CMBHC) and carboxyethyl hydroxychroman (CEHC). (B) H & E staining in the mammary glands of ACI rats. Mammary gland sections from each experimental group were stained with H & E. Representative sections from the negative control, E2 and 0.3% γ -TmT treated groups are shown (200X).

Figure 2. Analysis of α -, δ -, γ -T and their respective short chain metabolites, carboxyethyl hydroxychroman (CEHC) and carboxymethylbutyl hydroxychroman (CMBHC), in the serum of ACI rats. The data are presented as mean $\pm$ SE (n=4 negative control, n=6 treatment groups). P-values are compared to the E2 controls of the respective time points (b). Statistical significance, * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Figure 3.

Analysis of α-, δ-, γ-T and their respective short chain metabolites, carboxyethyl hydroxychroman (CEHC) and carboxymethylbutyl hydroxychroman (CMBHC), in the mammary glands of ACI rats. The data are presented as mean ± SE (n=4 negative control, n=6 treatment groups). *P*-values are compared to the E2 controls of the respective time points (b). Statistical significance, **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

Figure 4.

Effect of γ -TmT supplementation on nitrosative and oxidative stress in ACI rats. **(A)** Representative images of nitrotyrosine immunostaining in the mammary glands of ACI rats (400X). **(B)** Quantification of nitrotyrosine staining in the mammary glands. The data are expressed as mean $\pm$ SE (n=4 negative control, n=6 treatment groups). **(C)** Representative images of 8-oxo-dG immunostaining in the mammary glands of ACI rats (400X). **(D)** Quantification of 8-oxo-dG staining in the mammary glands. *P*-values are compared to the negative (a) or E2 (b) control of the respective time points. Statistical significance, **P* < 0.05, [†]*P* < 0.01, [‡]*P* < 0.001.

Figure 5.

Effect of γ -TmT supplementation on serum 8-isoprostane levels in ACI rats. Serum samples from control, E2 and 0.3% γ -TmT treated groups were analyzed for 8-isoprostane. The data are expressed as mean $\pm$ SE (n=4 negative control, n=6 treatment groups). *P*- values are compared to the negative (a) or E2 (b) control of the respective time points. Statistical significance, **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

Figure 6.

Effect of γ -TmT supplementation on antioxidant response genes in ACI rats. mRNA expression levels of Nrf2, SOD1, catalase and GPx1 in the mammary glands of rats were determined by q-PCR and normalized to GAPDH. The data are expressed as mean $\pm$ SE (n=4 negative control, n=6 treatment groups). *P*- values are compared to the negative (a) or E2 (b) control of the respective time points. Statistical significance, **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

Table 1

Body, Liver and Pituitary Gland Weights of Female ACI Rats at 1, 3, 7 and 14 days

Treatment Group	Treatment Period (days)	No of Rats per group	Body Weight (g)	Liver Weight (g)	Liver/Body Weight ratio	Pituitary Gland Weight (mg)
Control	1	4	132.7 ± 3.8	5.7 ± 0.4	4.3 ± 0.2	5.2 ± 1.4
E2	1	6	132.3 ± 2.7	5.9 ± 0.1	4.4 ± 0.1	7.6 ± 1.2
E2 + 0.3% γ -TmT	1	6	131.5 ± 2.1	5.5 ± 0.4	4.2 ± 0.1	7.5 ± 0.2
Control	3	4	138.7 ± 6.3	5.6 ± 0.3	4.0 ± 0.1	6.2 ± 0.5
E2	3	6	134.2 ± 4.7	5.5 ± 0.3	4.1 ± 0.1	8.8 ± 1.0
E2 + 0.3% γ -TmT	3	6	134.6 ± 2.6	5.9 ± 0.1	4.4 ± 0.1	8.5 ± 1.0
Control	7	4	135.5 ± 3.5	5.1 ± 0.1	3.8 ± 0.2	6.5 ± 0.2
E2	7	6	135.7 ± 5.2	6.2 ± 0.3 ^a	4.6 ± 0.2 ^a	10.8 ± 0.6 [†] a
E2 + 0.3% γ -TmT	7	6	139.8 ± 5.7	6.4 ± 0.3	4.6 ± 0.1	13.0 ± 0.4
Control	14	4	129.6 ± 11.4	4.2 ± 0.4	3.3 ± 0.2	7.2 ± 0.4
E2	14	6	148.6 ± 2.2	7.4 ± 0.2 [‡] a	5.1 ± 0.1 [‡] a	13.6 ± 1.4 [‡] a
E2 + 0.3% γ -TmT	14	6	150.1 ± 4.3	6.8 ± 0.2	4.5 ± 0.1 [*] b	16.1 ± 2.4

ACI rats with sham implants were fed with control diet. Those with E2 were fed with control or 0.3% γ -TmT diet for 1, 3, 7 and 14 days. Values are presented as mean ± SE (n=4 negative control, n=6 treatment groups). *P*-values are compared to the negative (a) or E2 (b) controls of the respective time points. Statistical significance,

^{*} *P* < 0.05,

[†] *P* < 0.01,

[‡] *P* < 0.001.