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The interaction of dietary isoflavones and estradiol replacement on behavior and brain-derived neurotrophic factor in the ovariectomized rat

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Abstract

Phytoestrogens are plant derived, non-steroidal compounds naturally found in rodent chows that potentially have endocrine-disrupting effects. Isoflavones, the most common phytoestrogens, have a similar structure and molecular weight to 17 β -estradiol (E2) and have the ability to bind and activate both isoforms of the estrogen receptor (ER). Most isoflavones have a higher affinity for ER β , which is involved in sexually dimorphic behavioral regulation. The goal of this study was to examine the interaction of isoflavones and E2 presence in the OVX rat on anxiety- and depressive-like behavior and the related BDNF pathophysiology. E2 administration resulted in anxiogenic behaviors when isoflavones were present in the diet ($p < 0.05$), but anxiolytic behaviors when isoflavones were not present ($p < 0.05$). E2 resulted in antidepressive-like behaviors in animals fed an isoflavone-rich diet ($p < 0.05$), with no effect when isoflavones were removed. Increased hippocampal BDNF expression was observed in animals fed an isoflavone-rich diet after E2 administration ($p < 0.05$). BDNF expression in the amygdala and hypothalamus was increased after E2 treatment in animals fed an isoflavone-rich diet. Overall, these results demonstrate that the presence of dietary isoflavones can differentially regulate the effect of E2 replacement on behavior and BDNF expression.

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Conflict of interests

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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Keywords

Isoflavones; 17 β -estradiol; Anxiety; Depression; BDNF

1. Introduction

Numerous studies show that non-steroidal, plant derived phytoestrogens, found in standard rodent chows, are endocrine-disrupting compounds resulting in altered metabolic and behavioral regulation [1–5]. Soy-derived isoflavones are the most common phytoestrogen studied due to their high abundance in the rodent diet [3,6]. Isoflavones have a similar molecular weight and structure to 17- β estradiol (E2), therefore the effects on rodent physiology and behavior may be a result of estrogen-dependent signaling pathways due to interaction with estrogen receptors (ERs) α and β [7,8]. Isoflavone derivatives have preferential binding to ER β ; however, to induce transcriptional activity, isoflavones are needed at high concentrations [7,9]. Depending on tissue, concentration, duration of exposure, and surrounding hormonal milieu, isoflavones can elicit either estrogenic or anti-estrogenic responses [8,10,11].

ER α and ER β have differential functions, physiology and expression profiles [12–14]. Utilizing ER knockout (ERKO) mice, the roles of the ER isoforms has begun to be elucidated. Male and female ER α KO mice exhibit infertility attributed to abnormal female uterine maturation and ovarian development and decreased male testes size and sperm count. ER β KO males have normal fertility; however, females have variable fertility as a result of abnormal ovarian development [12,15,16]. ER β , on the other hand, may play a functional role in nonreproductive, emotional behavior regulation. ER β KO, but not ER α KO mice, exhibit heightened anxiety-like behaviors in open field and elevated plus maze (EPM) testing [17]. Likewise, administration of diarylpropionitrile (DPN), an ER β agonist, but not propyl-pyrazole-triol (PPT), an ER α agonist, resulted in anxiolytic phenotypes in both male and female animals [18]. ER α and ER β have substantial overlap in expression; however there is a unique distribution of these receptors within the central nervous system [14,19]. Both isoforms are found in high expression in the amygdala and hypothalamus. The hippocampus and cortex have a greater abundance of ER β than ER α [13,14].

Isoflavone-dependent behavioral alterations have been reported in both male and female animals [3,20–24]. These behavioral responses may be due, in part, to the abundance and affinity of isoflavones for ER β in limbic regions [7,14]. Current literature is at conflict with reports of isoflavones resulting in an increase, decrease or no effect on behavioral response [1,3,4,21]. Intact male and female animals that received life-long dietary isoflavone exposure exhibit anxiolytic behaviors [4]. Acute genistein infusion to ovariectomized (OVX) rats results in anxiolytic behaviors in the black and white anxiety assay. In this model, animals infused with genistein spent more time exploring the white compartment (illuminated/light compartment) [25]. Conversely, long-term isoflavone exposure (14 days) resulted in anxiogenic behaviors in intact male rats [21]. This increase in anxiety-like behavior is complemented by an enhanced stress-induced increase in the stress hormones, corticosterone and vasopressin [21]. In addition to anxiety-related behaviors, isoflavones

have also been reported to regulate a multitude of other behaviors including spatial memory [2], visual spatial accuracy [26], learning and memory [3,4,20], as well as, depressive-like behaviors [24]. Chronic treatment of genistein to intact female rats resulted in antidepressant-like behaviors in the Porsolt forced swim test (FST), regardless of estrous stage [24]. Equol administration, when isoflavones were not present in diet, resulted in a decrease in depressive-like behaviors and increased serotonin in the OVX female rat [27].

Therefore, with this conflict in the role isoflavones play in regulating anxiety- and depressive-like behaviors and related mechanisms, we sought to examine the interaction of diet and E2 in the OVX rat. Briefly, OVX females were exposed to a 2 (isoflavone rich or isoflavone free chow) X 2 (VEH or E2) paradigm. The interaction of dietary isoflavones and E2 on behavior was studied using the EPM and FST. In addition, brain-derived neurotrophic factor (BDNF) expression was measured in emotion and stress relevant brain structures such as the hippocampus, amygdala, prefrontal cortex (PFC) and hypothalamus. Our results suggest that isoflavones may modulate behavioral response dependent on the availability of circulating hormones.

2. Material and methods

2.1. Subjects

Forty ovariectomized (OVX) female Sprague Dawley rats, weighing between 200–225 g, were purchased from an approved vendor (Sprague Dawley Outbred Rat[®] SD[®], Harlan, Indianapolis, IN). Upon arrival, rats were given 7 days for acclimation and individual handling. Animals were pair-housed and placed on a reverse light/dark cycle (12:12 light:dark with lights out at 1300 h). The room was temperature (22–25 C) and humidity (50%) controlled. Rats had *ad libitum* access to food and water throughout the experimental timeline. After acclimation, rats were maintained on either standard rodent chow (2018 Teklad Global 18% Protein Rodent Diet; Harlan, Madison, WI) or placed on an isoflavone-free rodent chow (Modified AIN-93G Purified Rodent Diet with Corn Oil Replacing Soybean Oil; Dyets, Inc., Bethlehem, PA). Dietary isoflavone content has previously been characterized and reveals a total of 199.4 µg/g of isoflavone equivalents in standard rodent chow and 0 µg/g of isoflavone equivalents in the IF chow [28,29].

All handling and care of animals was conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences, Bethesda, Maryland.

2.2. Experimental design

The goal of this study was to address how dietary isoflavones can interact with E2 administration to regulate behavioral responses, specifically examining anxiety- and depressive-like behaviors. Animals were randomly assigned to one of four treatment groups, each with n = 10. This is a 2 × 2 factorial design examining the effect of dietary isoflavone (standard chow versus IF chow) and E2 presence (vehicle (VEH) versus E2). Starting at day 0, animals were exposed to either standard chow or IF chow and continued on the designated

diet for the remainder of the 28 days. On day 13, animals received either VEH or E2 treatment for the remainder 14 days. Behavior testing was completed on experimental days 21–25 (Fig. 1).

2.3. E2 administration

E2 administration followed previously established protocol [30]. OVX animals received either vehicle (27% hydroxypropyl- β -cyclodextrin in sterile water, Acros Organics, Fair Lawn, NJ) or E2 (Sigma Chemical Co., St. Louis, MO) dissolved in VEH over 14 days. Animals were anesthetized (Isoflurane, USP, Phoenix Pharmaceuticals Inc., St. Joseph, MO) and an osmotic mini-pump (Alzet Model 2002 0.5ML, Curoc Co., Cupertino, CA) was implanted subcutaneously between the shoulder blades. E2 was released at a continuous rate of 0.05 μ l/h and administered at 0.25 mg/kg body weight which reflects the physiological E2 levels during rat proestrous [30,31].

2.4. Anxiety-like behavior assay (Elevated plus maze)

Testing was between 0900 and 1100 h (lights on) on experimental days 21–23 (Fig. 1). The EPM consists of four arms (45 $\times$ 10 cm). The plus-shaped maze has two open arms and two arms enclosed by a 50 cm high opaque black Plexiglas. Animals were placed individually on a 10 $\times$ 10 cm square platform located in the center of the four arms. Rats were allowed to freely explore for 5 min, during which behavior was video recorded. Time spent in open arms/closed arms and number of open arm/closed arm entries were blindly scored by two observers.

2.5. Depressive-like behavior assay (Porsolt forced swim test)

All testing was completed between 0900 and 1100 h between days 23–25. The FST was conducted 48 h after completion of EPM using a randomized block design (Fig. 1). The FST utilizes transparent cylindrical Plexiglas tanks (60 $\times$ 20 cm) filled with 30 cm tap water (22–25 C). After being placed in the water tank, rats were allowed to freely behave for 4 min and was video recorded. Blind scoring of time spent swimming (struggling) and time floating (immobile) was performed. Between each animal, tanks were cleaned with 70% ethanol and replaced with clean water.

2.6. Brain collection and dissection

All tissue was collected on experimental day 27 (Fig. 1). Rats were euthanized using carbon dioxide inhalation overdose between 0900 and 1100 h (lights on). Animals were immediately decapitated and the brain was removed and flash frozen in 2-methyl-butane placed on dry ice. Brains were stored at -80°C until use.

The following crude dissections of the amygdala, hippocampus, PFC and hypothalamus were based on previously established protocols [32,33]. Frozen brains were placed ventrally on a crude cutting block and rapidly dissected on ice into 1 mm sections using a sterile razor blade. A section was made posterior to the optic chiasm and anterior to the mammillary bodies. This section was placed anterior facing and the MBH was collected at the ventral third of the third ventricle, using the hypothalamic sulci as lateral boundaries. A bilateral section of the amygdala was dissected between the stria terminalis and external capsule on

the medial edge and the amygdala capsule of the lateral edge. The PFC (infralimbic cortex) was dissected throughout the rostral-caudal plane of the forceps minor of the corpus callosum. Hippocampal dissections were made throughout the rostral-caudal span of the median eminence along the ventral floor of the lateral ventricle and ventral to the corpus callosum. Brain regions were collected and stored at -80°C .

2.7. BDNF enzyme-linked immunosorbent assay (ELISA)

Dissected brain regions were individually homogenized in RIPA buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% NP40, 10% glycerol, 1 mM PMSF, 10 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ leupeptin, 0.4 mM sodium orthovanadate) for ~ 15 – 20 s based on established protocols [30]. In brief, homogenates were centrifuged at $20,000 \times g$ at 4°C for 20 min and supernatant was removed. Total protein concentrations were determined using BCA protein assay (Pierce, Rockford, IL).

Total free BDNF was measured using the BDNF E_{max} ImmunoAssay System kit (Promega, Madison, WI) following manufacturer's protocol. Intra-assay coefficient of variance is 8.8%.

2.8. Statistical analysis

The interactions of dietary isoflavones and estradiol administration were analyzed using IBM SPSS Statistics 22 (Armonk, NY). Graphs were generated using GraphPad Prism 7 (La Jolla, CA). EPM and FST data were analyzed using a two-way analysis of variance (ANOVA) followed by a Fisher's least significant different (LSD) *post hoc*. Due to the nature of the block design of the experiment, we analyzed for effect of day in both EPM and FST. There was no effect of day, therefore all data was grouped regardless of day tested. BDNF concentrations were normalized to standard chow, VEH treated animals. A two-way ANOVA followed by a Fisher's LSD *post hoc* was performed for BDNF data analysis. A two-tailed value of $p < 0.05$ was considered significant.

3. Results

3.1. Anxiety-like behaviors

To determine the effect of dietary isoflavone presence and E2 replacement on anxiety-like behaviors we utilized the EPM assay between experimental days 21–23. A two-way ANOVA of the number of open arm entries revealed no significant effect of food, no significant effect of hormone, but a significant interaction [$F(1, 36) = 21.11$, $p < 0.05$]. A two-way ANOVA examining time spent in the open arms revealed no significant effect of food, no significant effect of hormone, but a significant interaction [$F(1, 36) = 14.76$, $p < 0.05$]. Overall, E2 replacement resulted in anxiogenic behaviors in OVX animals fed regular, isoflavone-rich chow ($p < 0.05$) and conversely anxiolytic behaviors in animals fed isoflavone free chow ($p < 0.05$). VEH treated, isoflavone-free animals had decreased time spent in open arms and decreased open arm entry ($p < 0.05$). E2 treated animals fed an isoflavone-free diet had increased time spent in open arms and increased open arm entry ($p < 0.05$) (Fig. 2).

3.2. Depressive-like behaviors

Depressive-like behaviors were measured using the FST between days 23–25. A two-way ANOVA of time spent struggling revealed no significant effect of hormone, no significant effect of food, and no significant interaction. A two-way ANOVA examining time spent immobile (floating) revealed no significant effect of hormone, no significant effect of food, and no significant interaction. Multiple comparison analysis demonstrated that E2 replacement in standard chow increased time spent struggling ($p < 0.05$) and decreased time spent immobile ($p < 0.05$). Removal of isoflavones from diet in VEH treated animals resulted in increased time spent struggling ($p < 0.05$) and decreased time spent immobile ($p < 0.05$) (Fig. 3).

3.3. BDNF

Brain regions involved in behavioral regulation were dissected to determine the effect of dietary isoflavones and E2 replacement on total free BDNF expression in individual regions. All BDNF data was normalized to standard chow, VEH treated animals. A two-way ANOVA examining hippocampal BDNF revealed no significant effect of food, a significant effect of hormone [$F(1, 22) = 6.543, p < 0.05$], and no significant interaction. In animals fed a regular isoflavone-rich diet, E2 increased BDNF expression ($p < 0.05$). A two-way ANOVA analyzing the amygdala revealed a significant effect of food [$F(1, 27) = 7.121, p < 0.05$], no significant effect of hormone and no significant interaction. E2 treated animals fed an isoflavone free diet had decreased BDNF expression compared to E2 treated animals fed an isoflavone-rich diet ($p < 0.05$). A two-way ANOVA of PFC BDNF expression revealed no significant effect of food, no significant effect of hormone, and no significant interaction. A two-way ANOVA examining the hypothalamus revealed a significant effect of food [$F(1, 14) = 7.009, p < 0.05$], no significant effect of hormone, and no significant interaction. Isoflavone free chow fed animals had decreased BDNF expression after E2 treatment compared to animals fed standard chow ($p < 0.05$) (Fig. 4).

4. Discussion

The interaction of dietary isoflavone exposure and E2 replacement in the adult rat was studied to examine the resultant behavioral phenotypes. In this study, we demonstrate that the presence of isoflavones in diet can alter an OVX animal's anxiety- and depressive-like behavioral response to E2 administration. In addition, the interaction of isoflavones and E2 altered BDNF, which is implicated in the pathophysiology of anxiety and depression [34–37].

Consistent with the conflicting literature [38–40], E2 replacement resulted in increased or decreased anxiety-like behaviors dependent on the presence of dietary isoflavones. Utilizing the EPM, we observed that in the presence of dietary isoflavones, E2 replacement in OVX animals resulted in anxiogenic behaviors. Conversely, when diet was void of isoflavones, E2 replacement results in anxiolytic behaviors. OVX is shown to result in depressive-like behaviors and E2 replacement reverses this phenotype [41,42]. In animals fed an isoflavone-rich diet, E2 replacement, as expected, resulted in anti-depressive like behaviors in the FST; however, there was no effect of E2 in animals fed an isoflavone-free diet.

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Isoflavones have the ability to cross the blood brain barrier, although concentrations are much less than those found in plasma [43]. However, OVX in female rats increases permeability of the blood brain barrier and E2 replacement reverses this permeability back to that of control, non-OVX animals [44]. Due to our experimental timeline, OVX animals were fed either an isoflavone-rich or isoflavone-free diet for 14 days prior to receiving E2 treatment. With this, isoflavones may have increased permeability through the blood brain barrier to interact with ERs, specifically ER β , within limbic structures.

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The amygdala, hippocampus, and PFC play a specific function in behavioral regulation. These sexually dimorphic structures are reciprocally interconnected with the hypothalamus and activation of this circuitry provides a neuroendocrine and behavioral output [45]. Specifically, the amygdala is involved in fear, anxiety and reward emotional processing and the hippocampus primarily controls learning and memory [46–49]. The PFC is an associative structure involved in executive processing of a multitude of sensory inputs [50]. These limbic structures contain relatively high expression of both ER α and ER β [13,14,51].

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The availability and expression abundance of ERs, as a result of phytoestrogen exposure, may play a role in the behavioral regulation we observed. The presence of dietary isoflavones, or lack thereof, has previously been reported to alter ER α and ER β expression throughout key behavior-dependent limbic structures [52]. Male animals exposed to isoflavones in diet displayed down regulation of ERs. The removal of dietary isoflavones resulted in ER expression increases in the hypothalamus, amygdala, cortex and hippocampus [52]. Of importance for our study is the observed alteration of ER β in these structures as a result of phytoestrogens. Therefore, the presence of isoflavones in our OVX female rat model, may alter the expression of the available pool of ERs within the limbic neurocircuitry. Altered ER β expression in these regions, as a result of isoflavones, could explain the observed increase/decrease in anxiety- and depressive-like behaviors. Therefore, E2 replacement may have an enhanced anxiolytic effect in cases where isoflavones are not present and ER expression is up-regulated. Conversely, the availability of isoflavones and the resultant ER expression, may desensitize the anxiolytic effect of E2 [5]. Further research is required to determine the interaction of endogenous E2 levels and isoflavone presence in altering receptor expression, ratio and/or sensitivity in the limbic regions regulating anxiety- and depressive-like behaviors.

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With the evidence that BDNF is implicated in the pathophysiology of anxiety and depressive behaviors [34,35,37], we examined how dietary isoflavone and E2 interact to regulate brain BDNF. We observed increased BDNF expression in the hippocampus after E2 replacement only when animals were fed a diet rich in isoflavones. This same trend is apparent in the amygdala, but significance was not reached. In the amygdala and hypothalamus, E2 replacement decreased total BDNF expression in animals fed an isoflavone-free diet compared to animals fed an isoflavone-rich diet

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The neurotrophin BDNF is involved in neuronal survival, growth, and differentiation is found in high abundance throughout the limbic structures [53,54]. Due to the complexity of depression and anxiety models, BDNF levels are altered, often, but not always, decreased [55–57]. Studies examining BDNF in relation to anxiety models are more conflicting, with

the expression being dependent on the specific anxiety disorder diagnosis [57]. Animal models of various stressors show decreased BDNF in the hippocampus with chronic antidepressant treatment increasing BDNF levels throughout brain regions [58,59]. Recently, a variant in the BDNF protein (Val66Met polymorphism) is shown to increase susceptibility to neuropsychiatric disorders [60,61]. Val66Met knock-in mice display increased anxiety, which is reversed by antidepressant treatment [37]. Therefore, our data reflects what is observed in the literature. E2 replacement acted as an antidepressant in animals fed standard chow, and in turn, increased hippocampal BDNF. No depressant effect of E2 replacement was observed behaviorally or in BDNF expression in animals exposed to an isoflavone-free diet. Contrary to what we expected, we observed that animals displaying anxiogenic behaviors had increased levels of BDNF in the amygdala and hypothalamus.

The differential behavior phenotypes may be a result of steroid-specific regulation of BDNF. Previous studies show that BDNF and its receptor are affected by natural hormonal fluctuations across the female estrous cycle [62]. Proestrus levels of estrogen and chronic E2 treatment decrease BDNF in the amygdala and hippocampus [62,63]. However, in OVX animals, E2 administration results in increased BDNF in the hippocampus and cortex [63,64]. BDNF colocalizes with ERs, specifically ER α [65] and has an estrogen response element involved in regulation [66,67]. Therefore, just as E2 has the ability to regulate BDNF, it is not unreasonable that the presence phytoestrogens may also impose a level of regulation. Current studies have shown that phytoestrogens can up-regulate BDNF in female rats [68], but future work needs to investigate the mechanistic of this regulation.

Overall, we demonstrate the E2 replacement after OVX can result in conflicting anxiety- and depressive-like behavioral outcomes dependent on whether isoflavones are present in diet. Likewise, due to E2's ability to regulate BDNF expression, it is likely that phytoestrogens can exert this same effect. We show that depending on the availability of dietary isoflavones, E2 replacement can have differential effects on total BDNF expression. Therefore, when examining behavioral responses and related mechanisms, it is important to consider dietary components and their potential interaction with endogenous levels of gonadal hormones.

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HIGHLIGHTS

- E2 administration resulted in anxiogenic behaviors when dietary isoflavones were present.
- E2 administration resulted in anxiolytic and anti-depressive like behaviors when dietary isoflavones were not present.
- BDNF expression increased after E2 administration when animals were fed an isoflavone-rich diet.
- BDNF expression in the amygdala and hypothalamus was increased after E2 treatment in animals fed an isoflavone-rich diet.

Fig. 1.

Experimental timeline. Adult female rats were purchased OVX. Animals were given 7 days to acclimate upon arriving at the facility. After acclimation, (designated day 0), animals were given either standard rodent chow or an isocaloric isoflavone-free chow. On day 13 OVX rats were subcutaneously infused with either VEH or E2 for the remainder 14 days of the study. Between days 21–25, behavioral testing was conducted. Tissue was collected on day 27.

Fig. 2.

Interaction of dietary isoflavones and hormone replacement on anxiety-like behaviors in the EPM. In animals fed a standard, isoflavone-rich diet, E2 increased anxiety-like behaviors as observed by **A)** decreased number of open arm entries and **B)** decreased time in open arms. In contrast, in animals fed an isoflavone-free diet, E2 decreased anxiety-like behaviors as shown by **A)** decreased number of open arm entries and **B)** decreased time in open arms. VEH treated animals had increased anxiety-like behaviors when exposed to isoflavone-free chow compared to standard chow. E2 treated animals had decreased anxiety-like behaviors when exposed to isoflavone-free chow compared to standard chow. Bars represent mean values $\pm$ SEM, $n = 10$ per treatment group. *E2 compared to VEH ($p < 0.05$).

Fig. 3.

Effect of isoflavones and E2 on depressive-like behaviors in the Porsolt FST. E2 replacement decreased depressive-like behaviors only in animals fed a standard diet as shown by **A**) increased time spent struggling and **B**) decreased time spent immobile. VEH treated animals displayed **A**) increased time spent struggling and **B**) decreased time spent immobile when exposed to an isoflavone-free diet compared to a standard diet indicative of a decrease in depressive-like behaviors. Bars represent mean values $\pm$ SEM, $n = 6-8$ per treatment group. *E2 compared to VEH ($p < 0.05$).

Fig. 4.

Total free BDNF expression in specific brain regions as a result of the interaction of dietary isoflavones and E2 treatment in the OVX rat. **A)** In the hippocampus, E2 treatment increased BDNF expression compared to VEH treatment only when animals were fed a diet rich in isoflavones. **B)** E2 replacement resulted in decreased BDNF expression in the amygdala when animals were fed an isoflavone-free diet compared to a standard diet. **C)** There was no effect of diet or hormone on BDNF expression in the PFC. **D)** In the hypothalamus, E2 treatment decreased BDNF expression in animals fed an isoflavone-free diet. Bars represent mean values $\pm$ SEM. Hippocampus and Amygdala $n = 6-8$, PFC and Hypothalamus $n = 4-6$. *E2 compared to VEH ($p < 0.05$).