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EFFECT OF ESTROGEN PRETREATMENT ON GLIAL-LIKE CELL VIABILITY FOLLOWING STRESS RESPONSE HORMONE TREATMENT

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ABSTRACT

The role of steroid hormones is critical in cellular function. Previous studies have found a positive correlation between the endogenous estrogen 17β-estradiol and cellular protection. Conversely, exogenous conjugated equine estrogens provide less protective mechanisms than endogenous hormones, and little is known about the role of estrogens in cellular protection during a stress response. In the present study, we compare the effects of short-term estrogen pretreatments (alone and in combination) on cell viability when cells with glial cell morphology are exposed to either epinephrine or cortisol. Results showed that 1 μM 17β-estradiol resulted in decreased cell viability following the epinephrine treatment; none of the 17β-estradiol pretreatments affected viability following the cortisol treatment. The highest concentration (10 μM) of the conjugated equine estrogen equilenin, either alone or in combination with 17β-estradiol, yielded significantly lower cell viability following epinephrine and cortisol stressors. The results of this study suggest certain estrogens, especially in combination, could be detrimental to cells associated with the nervous system during a stress response.

Keywords: 17β-estradiol, equilenin, cell viability, stress response

INTRODUCTION

Women with truncated estrogen production in the perimenopausal, menopausal, and postmenopausal stages are commonly prescribed supplemental estrogens and progesterone to relieve symptoms (Brunner et al. 2010). The efficacy of this treatment depends on the type of estrogen as well as the application and duration of treatment. Overall, research suggests estrogen treatments offer cellular protection to some degree in various tissues. Findings from the Women's Health Initiative, however, show increased cardiovascular risk in certain groups taking supplemental estrogen prescriptions (Goldman 2004). Presumably, differences in chemical structure between the various estrogens used for treatment contribute to their relative degree of cellular protection. For example, in addition to 17β-estradiol, a well-known endogenous estrogen treatment, conjugated equine estrogens (CEE) are also used in some steroidal hormone replacement therapies. These include sodium equilin sulfate, sodium 17α-dihydroequilin sulfate, sodium equilenin sulfate, sodium 17β-dihydroequilin sulfate, sodium 17α-dihydroequilenin sulfate, and sodium 17β-dihydroequilenin sulfate (Zhao and Brinton

2006). Given discrepancies in the efficacy, and potential harm, of hormone treatments, there is a critical need to examine the actions of individual estrogens on specific tissues.

One area of study of growing interest is the role of these estrogens in neuroprotection. Estradiols, and specifically 17β-estradiol, contribute to the regulation of brain activity, structural proteins synthesis, and neuroprotection (Tozzi et al. 2015). Estrogen receptors in the brain include the estrogen receptor alpha, the estrogen receptor beta, and the G protein-coupled estrogen receptor (Bean et al. 2014). Estrogen pretreatment of astrocytes have been linked to ischemic nerve injury through activation of estrogen receptors in the brain (Ma, et al. 2016). Zhao and Brinton (2006) pretreated rat primary-cultured basal forebrain neurons with varying concentrations of estrogens prior to stressors and measured levels of MTT formazan, lactate dehydrogenase release, and ATP levels. Estrone, 17β-estradiol, equilin, and dehydroestrone provided notable neuronal protection, with a combination of estrogens providing the most protection of the tested hormones. Conversely, studies suggest CEEs with a longer biological half-life may be linked to further complications in menopausal women compared to endogenous estrogens (Brunner et al. 2010; Hendrix et al. 2006). Although there is limited research on CEEs being linked to decreasing neuron viability, hormone replacement therapy that includes CEEs has been linked to nervous system issues (Hendrix et al 2006). CEEs take longer to metabolize than endogenous human estrogens, and have been linked to nervous system issues including ischemic strokes and dementia (Hendrix et al. 2006; Shumaker et al. 2004).

Astroglia cells are known for their abundance in the brain, their role in supporting neurons, and their ability to regulate extracellular conditions to maintain homeostasis (Bélanger and Magistretti 2009). Studies have shown these cells to be affected by estrogens (Arevalo, et al. 2010; Grimes and Hughes, 2015). Nonetheless, Duong et al. 2020 found that estrogen's ability to offer protective mechanisms in glial cells were dependent on the environment and thus health of the cell.

The role of the type of stressors used to evaluate the effects of estrogens in the brain is not well characterized, and results suggest the effects are conditional. For example, the negative impact of beta amyloid and excitotoxic glutamate treatment in primary neuronal cultures was countered by the pretreatment of various estrogens (Zhao and Brinton 2006). The potential impacts of deleterious effects are dependent on the time of exposure. For instance, some cellular protection was associated with CEE pretreatment when cultured rat astrocytes were stressed with hydrogen peroxide for one hour (Grimes and Hughes 2015). In the same study, however, twenty-four-hour treatment with hydrogen peroxide resulted in attenuated cell viability regardless of estrogen pretreatment.

Epinephrine and cortisol are linked to stress and are widely studied in the nervous system. Epinephrine, a key effector of sympathetic nerve activation, is associated with wide-spread cellular stress and death at high concentrations (Weiming et al. 1998; Qin et al. 2015). Like epinephrine, cortisol levels rise and fluctuate during periods of internal or environmental stress. High concentrations and prolonged release of cortisol are linked to detrimental effects in astrocytes, including an increase in oxidative stress and a decrease in cellular viability (Chen et al. 2014). It is therefore critical to examine the influence of estrogens on cultured cells exposed to hormones involved in stress response.

The current research examines the effects of estrogen treatment on a human astrocytoma cell line with glial-like morphology exposed to either epinephrine or cortisol. The hormones used are the endogenous human estrogen 17β-estradiol and the conjugated equine estrogen equilenin. Since hormone therapy often includes a combination of endogenous and equine estrogens, 17β-estradiol and equilenin were selected to represent two common types of estrogens used in combination. Two-way ANOVA analyses were conducted for each of the two stress hormone treatments to determine if cell viability measurements varied among the type of estrogen used, estrogen concentration, and their interactions. We hypothesized that 17β-estradiol pretreatment would convey greater protection of cells during stress response compared to equilenin pretreatment alone or in combination.

MATERIALS & METHODS

Cell Culture

The 1321N1 cell line was purchased from Millipore Sigma. These cells originated from a human brain astrocytoma and have glial cell morphology. Generally, glial cells provide neuronal support, protection, regulate homeostasis, and repair in the central nervous system (Bélanger and Magistretti 2009). The cells were propagated in a T-75 mL flask with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) 5% antibiotic/antimycotic, and 2 mM L-glutamine. The cells were grown in an incubator at 37°C and 5% carbon dioxide (Grimes and Hughes 2015). Once approximately 80% confluency was reached, cells were subcultured using 0.25% trypsin-EDTA and plated in 96-well plates at a concentration of $1x10^6$ cells/mL. Once plated in the wells, treatments began the next day. Cells were placed in two experimental groups composed of one stress hormone each: epinephrine or cortisol. Each group was tested in seven separate trials to assess cell viability in response to the presence of stress hormone, estrogen concentration, and their potential interaction.

Estrogen Treatment

Both estrogens (17β-estradiol and equilenin, Millipore Sigma) were stored at 4°C and prepared the same day of treatment to prevent degradation. A 0.1 M 17β-estradiol in DMSO stock solution was first prepared, followed by dilution to the target concentrations in PBS. The day following subculturing, cells were treated with 17β-estradiol or equilenin, or a combination of the two, at concentrations of 0 nM (controls), 10 nM, 100 nM, 1 μM, and 10 μM. These concentrations were selected based on a similar study conducted by Grimes and Hughes (2015), which pretreated rat astrocytes with CEEs and then exposed them to hydrogen peroxide. The cultures were returned to the incubator following treatment.

Stress Response Hormone Treatment

After one hour of estrogen exposure, the cells were exposed to one of two stress response hormones (100 μM epinephrine or 1 μM cortisol, Sigma Aldrich) or PBS (control) for one hour. These stressor concentrations were selected based on preliminary studies, which included concentrations ranging from 0M to 1mM. The stressor concentrations of 100 μM epinephrine (dissolved in PBS) or 1 μM cortisol (stock solution in methanol was diluted in PBS) were selected based on personal communication and unpublished preliminary results that indicated these concentrations yielded a significant decrease in cell viability compared to the controls. Moreover, previous literature also supports the use of the chosen corticosteroid concentration (Anacker et al. 2015; Tsunashima, et al. 2011). The hormone concentrations were determined based on preliminary studies using flow cytometry to measure oxidative stress indicators. The media was then changed to remove the hormones, and the cells received post-hormone estrogen treatment (same concentration as previously delivered) for 24 hours following stress response hormone removal. The cultures were returned to the incubator during all treatments.

MTT Assay

All cells were analyzed using an MTT assay (Sigma Aldrich, TOX-1) to assess cell viability following the 24-hour incubation with the estrogen treatment. The MTT assay indicated the degree of cellular metabolic activity using a colorimetric analysis. Cells reduced MTT to formazan, which resulted in a colorimetric shift detected by the Bio-Rad Benchmark Microplate Reader measuring absorbance at 570 nm. Higher absorbance unit values indicate higher cell metabolic activity and thus viability when comparing treatments to controls.

Analysis

A two-way ANOVA was performed on the epinephrine and cortisol trials to assess absorbance values obtained from the MTT assay, which serves as an indicator of cell growth. Sources of variation in the two-way ANOVA included presence of stress response hormone, estrogen concentration, and the stress response hormone x estrogen concentration interaction. To meet the assumptions of homoscedasticity for the ANOVA, data was log transformed for normality and used in the analysis with back-transformed means reported. Tukey's Post-hoc tests were performed to assess effects of the sources of variation on cell growth. All data analysis was conducted using JMP®, Version *11.2.0*. SAS Institute Inc., Cary, NC, 1989-2019.

RESULTS

Epinephrine trials

For the 17β-estradiol treatments, the presence/absence of the stress response hormone epinephrine did not have a significant effect on absorbance readings indicative of cell growth (optical density at 570 nm) (Table 1). However, 17β-estradiol concentration did have a significant effect on the absorbance readings (Table 1); 1 μM 17β-estradiol yielded a significantly lower value compared to the control, although neither differed significantly from other concentrations (Figure 1a). For the 17β-estradiol treatments, the interaction between these two sources of variation (i.e., epinephrine presence/absence and estrogen concentration) was not significant (Table 1).

The presence/absence of epinephrine also had no significant effect on the absorbance readings for the equilenin treatments (Table 1), although the effect of equilenin concentration was significant (Table 1); 10 μM equilenin yielded a significantly lower absorbance value compared to all other treatments (Figure 1b). The interaction between epinephrine presence/absence and estrogen concentration was not significant for the equilenin treatments (Table 1).

The presence/absence of epinephrine for the 17β-estradiol+equilenin treatments had no significant effect on absorbance readings (Table 1). However, the effect of 17β-estradiol+equilenin concentration on the absorbance readings was significant (Table 1). Specifically, the control treatment yielded a significantly higher absorbance value compared to all other concentrations except for the 100 nM treatment, which also differed significantly from the 10 μM treatment (the lowest absorbance value); the remaining treatments had intermediate absorbance values and did not differ significantly from each other (Figure 1c). The interaction between epinephrine presence/absence and estrogen concentration was not significant for the 17β-estradiol+equilenin treatments (Table 1).

Cortisol trials

For the 17β-estradiol treatments, the presence/absence of the stress response hormone cortisol did not have a significant effect on absorbance readings indicative of cell growth (optical density at 570 nm) (Table 1). Absorbance readings did not vary significantly among estrogen concentrations (Figure 2a), and the interaction (cortisol presence/absence and estrogen concentration) was not significant (Table 1).

The presence/absence of cortisol also had no significant effect on absorbance readings for the equilenin treatments (Table 1), although significant differences were detected among equilin concentrations (Table 1). Specifically, the 10 μM treatment yielded a significantly lower absorbance value compared to the control, 10 nM, and 100 nM concentrations; an intermediate absorbance value was obtained for the 1 μM equilenin treatment, which did not differ significantly from those of all other concentrations (Figure 2b). The interaction between cortisol presence/absence and estrogen concentration was also not significant (Table 1).

For the 17β-estradiol+equilenin treatments, the effect of cortisol presence/absence on the absorbance readings was not significant (Table 1). However, the effect of 17β-estradiol+equilenin concentration on the absorbance readings was significant (Table 1), where the 10 μM treatment had a significantly lower absorbance value compared to all other treatments (Figure 2c). There was no significant interaction between cortisol presence/absence and estrogen concentration for the 17β-estradiol+equilenin treatments (Table 1).

Table 1. Results from a two-way ANOVA comparing cells treated with estradiol, equilenin, and estradiol+equilenin for one hour followed by stress response hormone (100 μM epinephrine or 1μM cortisol) exposure for one hour. Table includes degrees of freedom (d.f.) and the F value (F) results for each analysis. ***P-values < 0.05**.

Stress response hormone	Estrogen Treatment	Source of Variation	d.f.	F
Epinephrine	17β-Estradiol	Epinephrine presence/absence	1	0.53
		Estrogen conc.*	**4**	**3.02**
		Epinephrine presence/absence X Estrogen conc.	4	0.09
	Equilenin	Epinephrine presence/absence	1	0.33
		Estrogen conc.*	**4**	**13.89**
		Epinephrine presence/absence X Estrogen conc.	4	0.7
	17β-Estradiol + Equilenin	Epinephrine presence/absence	1	0.02
		Estrogen conc.*	**4**	**7.66**
		Epinephrine presence/absence X Estrogen conc.	4	0.41
Cortisol	17β-Estradiol	Cortisol presence/absence	1	1.53
		Estrogen conc.	4	1.29
		Cortisol presence/absence X Estrogen conc.	4	0.74
	Equilenin	Cortisol presence/absence	1	0.08
		Estrogen conc.*	**4**	**4.01**
		Cortisol presence/absence X Estrogen conc.	4	1.37
	17β-Estradiol + Equilenin	Cortisol presence/absence	1	0.17
		Estrogen conc.*	**4**	**9.89**
		Cortisol presence/absence X X Estrogen conc.	4	0.61

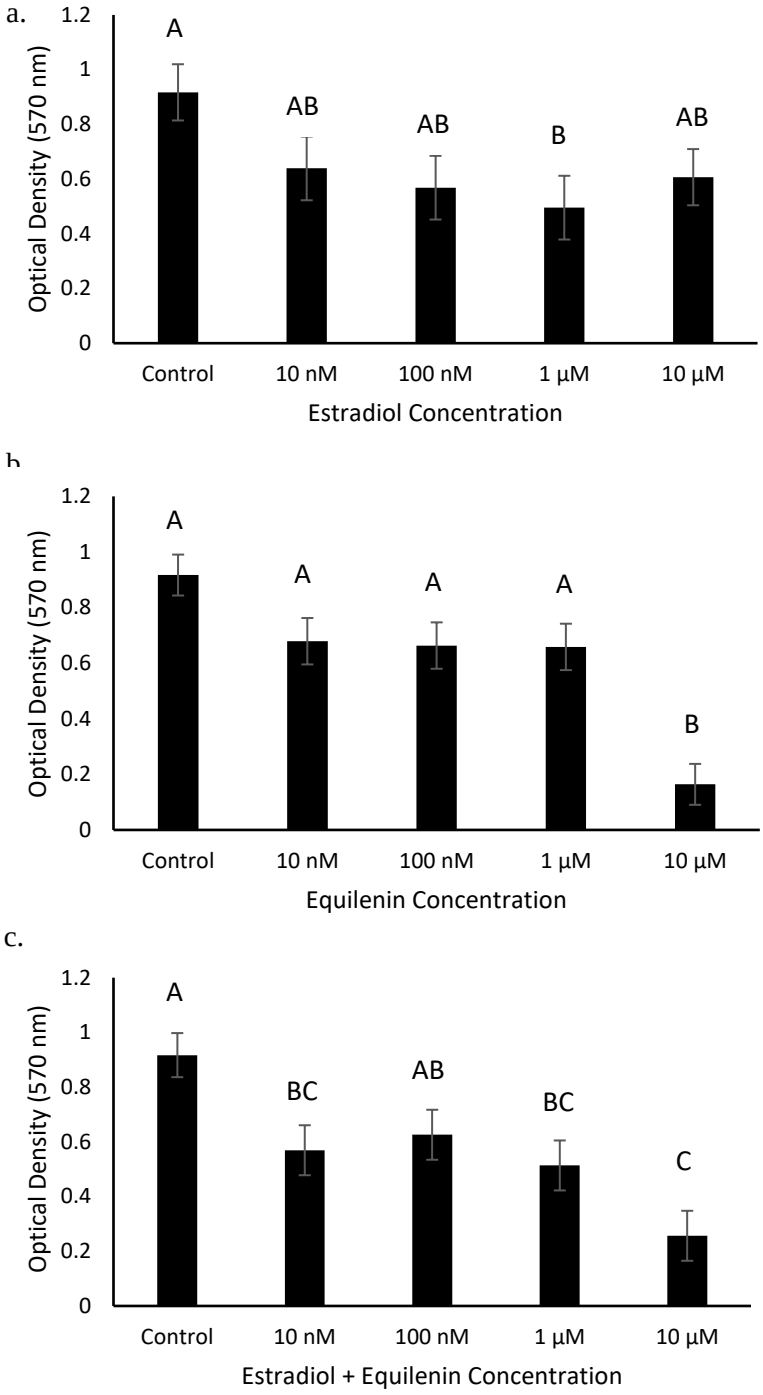

Figure 1. Absorbance values from MTT assay following treatment with estrogen and subsequent epinephrine. Cultured cells were treated with 17β-estradiol (a), equilenin (b), or a combination of the two (c) at concentrations of 10 nM, 100 nM, 1 µM, or 10 µM for one hour *in vitro* before being treated with 100 µM epinephrine. Letters indicate results of Tukey's post-hoc comparison of means over seven trials.

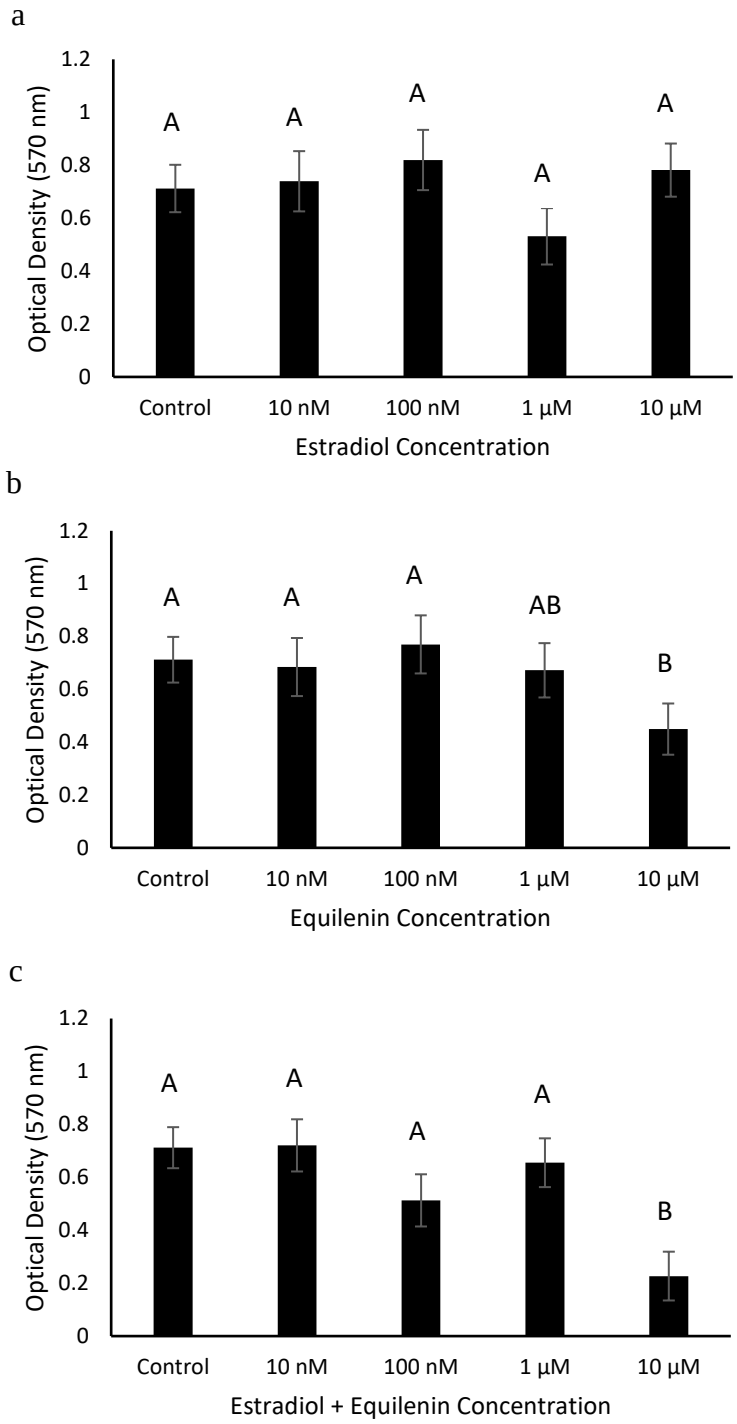

Figure 2. Absorbance values from MTT assay following treatment with estrogen and subsequent cortisol. Cultured cells were treated with 17β-estradiol (a), equilenin (b), or a combination of the two (c) at concentrations of 10 nM, 100 nM, 1 µM, or 10 µM for one hour *in vitro* before being treated with 1 µM cortisol. Letters indicate results of Tukey's post-hoc comparison of means over seven trials.

DISCUSSION

Overall, 17β-estradiol pretreatments did not protect the cells that were treated with epinephrine. Contrary to our hypothesis, pretreatment with the second highest estradiol concentration (1 μM) was associated with reduced viability compared to control. This result differs from previous studies that show higher concentrations of 17β-estradiol provide significant protection in rat neuronal cultures (Zhao and Brinton 2006). Our study also included an analysis of cell viability when the stress response hormone cortisol was applied to the cell cultures. However, pretreatment with 17β-estradiol offered no significant protection to the cortisol stressor, regardless of the concentration. Collectively, our results, and those of previous studies, indicate that cell type, species, and type of stressor may play a key role in determining the protective ability of endogenous estrogen on cell viability in response to cellular stress.

The decrease in cell viability in the present study was also measured following 10 μM equilenin pretreatment and either stressor (epinephrine or cortisol), as well as for the combination treatment (17β-estradiol+equilenin). These results appear to support prior studies, which found that CEEs, including equilenin, offer less protection to cells compared to 17β-estradiol and may in fact be deleterious (Brunner et al. 2010). The results of the Women's Health Initiative initially identified an increased risk of cardiovascular events associated with combined estrogen treatments (Goldman 2004). The extent to which negative effects occur depend on a multitude of factors including, but not limited to, the timeline of treatment after menopause (Shumaker et al. 2004). The results of our *in vitro* study indicate human cultured cells with glial-like properties do not respond well to higher concentrations of combined estrogens.

It is important to note a human astrocytoma cell line was used in the present study. Although these cells have glial cell morphology, a cancerous cell line with altered propagation pathways may have stark differences in response to hormone treatments compared to noncancerous cells. Notably, previous studies using other cell types and species yielded different outcomes. For example, a study involving rat astrocytes showed a significant protection in combined estrogen treatment compared to single estrogen treatment (Zhao and Brinton 2006). Our previous research indicated CEE protection of murine astrocytes when exposed to one hour of a hydrogen peroxide stressor (Grimes and Hughes 2015). Thus, the type and origin of the cell nervous system-associated cell likely has a significant impact on the collective outcomes of these studies.

Aside from cell type and species of origin, the applied stress response hormone is an important consideration. Our current study used epinephrine and cortisol, whereas previous studies used stressors such as hydrogen peroxide or glutamate. In using the MTT assay as an indicator of viability, it is difficult to identify the optimal concentration. If the concentration of the stress response hormone is too high, for example, the cells may die regardless of estrogen hormone pretreatment. Conversely, a suboptimal concentration will not adequately weaken the cells, resulting in an unstressed condition. Given the precarious balance of this design, it is advantageous to consider other means to measure cell stress rather than only cell survival. Oxidative stress assays would help to clarify whether estrogen pretreatment protects cells under these conditions. (Doung et al. 2020).

It is important to emphasize that the cells had only one hour of estrogen pretreatment before stress hormone exposure, and the same estrogen concentration was applied to the wells for twenty-four hours following stress response hormone removal. Consequently, this was a short-term exposure study, which suggests cellular harm with the higher concentrations of equilenin or 17β-estradiol + equilenin was not through transcriptional changes. For example, the estrogens could be acting through plasma membrane receptor signaling (Yu et al. 2017). These receptors elicit faster responses compared to changes in gene expression. It is known that equilenin can bind to ERα and ERβ receptors like estradiol. However, there may be differences in binding affinities that contribute to varying results (e.g., Luo et al. 2017; Mosquera et al. 2014). Studying the receptor binding and receptor signaling would give further insight to the influence of plasma membrane receptor signaling versus transcriptional regulation.

Taken together, the impact of stress response hormones may increase the detrimental effects on cell viability when estrogens are administered at higher concentrations. Combination of estrogens, including CEEs, may exacerbate cell damage and further decrease viability. To fully examine the neurological effects of CEEs, primary cultures of both neurons and astrocytes should be examined and compared to elucidate the particular link to brain function. Future studies should also include stressor optimization and oxidative stress direct measurements to further elucidate the protective role of these hormones on the cell stress response. Examining other steroidal hormones including equilin, estrone, and progestins would be helpful to examine the structural impacts of these hormones and their physiological effects. In conclusion, this study found decreased viability in short-term pretreatment of higher concentrations of estradiol and equilenin associated with either epinephrine or cortisol exposure.

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