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The Involvement of Progesterone in Glial Cell Viability

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ABSTRACT

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We studied the effects of ethynodiol diacetate on the viability of hydrogen peroxide stressed glial cells. We wanted to determine the extent in which varying concentration of ethynodiol diacetate affect cell viability of cultured glial cells from hydrogen peroxide toxicity. Based on previous studies, we predicted that the viability of hydrogen peroxide-stressed cells will increase with the presence of the progesterone, ethynodiol diacetate, and that cell viability will increase with increasing concentration of ethynodiol diacetate. Glial cells of $1x10^6$ cells/well were introduced into a 96-well plate and allowed to grow for 24 hours in an incubator at 37°C and 5% CO_2. Cells were treated with 0 nM (control), 10 nM, 100 nM, 1 μM, 10 μM ethynodiol diacetate in doublets, with half of the cells being stressed with hydrogen peroxide. Data were analyzed using a 2-way ANOVA and a Tukey's post hoc test with a p-value of less than 0.05 being significant. Viability did not differ significantly between stressed and unstressed cells (2-way ANOVA, $F_{9,30}=1.673$, $P=0.211$) or between cells receiving varying concentrations of ethynodiol diacetate (2-way ANOVA, $F_{9,30}=0.638$ $P=0.641$). The results illustrate that ethynodiol diacetate, when under these treatment parameters, does not have protective effects on the glial cell cultures.

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Background

Hormonal birth control is a form of female contraception. Around sixty percent of women who utilize contraception currently use a non-permanent method, such as hormonal birth control (Daughery et al. 2014). Common hormonal methods include the birth control pill, patch, and vaginal ring (Daughery etal. and Jones et al., 2014, 2012) and usually contain varying amounts of steroid hormones including estrogens and progesterones. When consumed, estrogen and progesterone send a signal to the brain which causes a range of effects within the body including preventing ovulation, stopping production of the luteinizing hormone (LH), and stopping the pituitary gland from secreting the follicle-stimulating hormone (FSH) (Nichols, 2018). One role of progesterone and estrogen is assisting in the prevention of the secretion of gonadotropins (FSH and LH) through a negative feedback mechanism (Shaw et al., 2010).

Progesterone is derived from the placenta and/or the corpus luteum and has been shown to play an essential role in the stimulation of the uterus in preparation for pregnancy (Siiteri et al., 1977). It prevents ovulation and thickens the cervical mucus. Some adverse side effects caused by hormonal birth control include weight gain, changes in or the absence of a menstrual cycle, mood changes, and the worsening of migraines. With high levels of progesterone specifically, side effects include stomach problems, premenstrual syndrome (PMS)-like symptoms, irregular bleeding, breast discomfort or enlargement, and depression (Childs, 2018).

The progesterone of interest in my study is ethynodiol diacetate. This progesterone is used to help prevent pregnancy by thickening the mucus within the vagina, which in turn

prevents the sperm from penetrating the egg. Ethynodiol diacetate is a synthetic form of the naturally-occurring female sex hormone, progesterone (Briggs, 1975). Ethynodiol diacetate has a molecular weight of 384.5 g/mol and a chemical formula of $C_{24}H_{32}O_4$. During the menstrual cycle an ovum is released from the ovary through a process known as ovulation. Following ovulation, the follicle that released the ovum becomes known as the corpus luteum. The corpus luteum produces progesterone which prevents the further release of eggs. Progesterone also prepares the lining of the uterus for possible pregnancies that may occur. When pregnancy occurs the progesterone levels remain high, and if no pregnancy occurs the levels drop resulting in a menstrual period. Ethynodiol diacetate prevents the release of additional ova from the ovaries, thus preventing pregnancy (Briggs, 1975). Progesterone affects brain physiology through three principle mechanisms: regulation of gene expression, modulation of neurotransmitter systems, and activation of signaling cascades (Brinton, D.R., et al., 2008). The mechanism of ethynodiol diacetate involves binding to progesterone and estrogen receptors. Cells expressing these receptors are found in the female reproductive tract, mammary gland, the hypothalamus, and the pituitary. Upon binding to the receptor, the ethynodiol diacetate will slow the frequency of the release of gonadotropin releasing hormone (GnHR) from the hypothalamus and slow the preovulatory luteinizing hormone (LH) surge (Briggs, 1975). Ethynodiol diacetate is a first-generation progesterone of average progestational activity. It has minor estrogenic effects and little androgenic activity (Progestin, 2018). Ethynodiol diacetate is a derivative of norethindrone, another type of progesterone.

Astrocytes are a form of glial cells, which are predominantly found in the spinal cord and brain. These cells play a role in the formation of the blood-brain barrier, as well as serving as supportive cells for neurons. One function to note is that the astrocytes within the hypothalamus

play a role in the central nervous system through the regulation of hormones, particularly female hormones (Micevych, et al., 2010). When ovulation occurs, the hypothalamus releases the gonadotropin releasing hormone (GnRH); this in turn triggers an increase in luteinizing hormone (LH) which triggers ovulation (Micevych et al., 2010)

Recent studies have illustrated that progesterone plays a role in multiple non-reproductive functions in the central nervous system. These functions include the regulation of mood, cognition, mitochondrial function, neurogenesis and regeneration, myelination, and recovery from traumatic brain injury (Brinton et al., 2008). One study indicated that when progesterone was delivered to a patient after a brain injury, it caused a decrease in the cytotoxic responses as well as promoting functional recovery (Grossman, et al., 2004). Brain injuries create an inflammatory response by disturbing the blood brain barrier, which in turn causes edema and the permeation of inflammatory cells (Carlos et al., 1997). Progesterone also plays a role in diminishing neurological abnormalities after ischemia (Chen et al., 1999) and spinal cord injuries (Thomas et al., 1999), as well as the reduction of neuronal cell death (Cervantes et al., 2001).

Hydrogen peroxide is a byproduct of respiration that, when in excessive quantities, can lead to cellular damage caused by oxidative stress. One study showed that hydrogen peroxide alters astrocyte membrane and cytoskeleton through damaging lipids, proteins, DNA, and through signaling molecules which cause the intracellular pathways to cause apoptosis (Choi et al., 2007).

Despite advances in hormonal knowledge, not much is known about the effects progesterones have on the viability of astrocytes directly. Therefore, the goal of this study was to determine the effect progesterones have on the viability of hydrogen peroxide-stressed astrocytes. I hypothesized that the viability of hydrogen peroxide-stressed cells will increase with

the presence of the progesterone, ethynodiol diacetate, and that as the concentration of ethynodiol diacetate increases, so will cell viability.

METHODS

Cells

The cell line used in this study were 1321N1, which were cultured human glioma cells with astrocyte-like properties (Sigma Aldrich). The cells were plated in a T-75 flask with 10 ml media, which consisted of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The media in the well plate was replaced every two to three days. The astrocytes were stored and incubated at 37°C with 5% supplemented CO_2.

Once the cells became roughly 70-80% confluent in the flask, the existing media was removed and a 5 mL solution containing 0.53 mM EDTA and 25% trypsin was added to rinse the flask. The trypsin-EDTA solution helped remove the adherent cells from the flask's surface. Once the cells were removed from the surface, the suspension was placed into a tube and centrifuged at 3000 x rpm for 3 minutes in order to pellet the cells. The supernatant containing trypsin was then removed and discarded, leaving behind the cell pellet which was resuspended in 5 mL fresh media. This solution was then plated into a 96-well plate. Each well contained 200 µL of media with a concentration of about $1x10^6$ cells/mL (Grimes and Hughes, 2015). An automated BioRad cell counter was used in order to determine the concentration of cells in the wells. A solution of 100 µL of prepared astrocytes with an equal amount of trypan blue solution was prepared in one of the wells in the 96-well plate, which allowed for the determination of total and live cell count. A 10 µL amount of the trypan blue and glial cell solution was added to the slide using a micropipette, which was then inserted into the counter where the live and total counts were determined. The number of cells counted was used to help calculate the amount of cell suspension and media needed to dilute the concentration to $1x10^6$ cells/mL. Once the volume of cell suspension was calculated, it was then subtracted from the total amount of media that would be added into the cell suspension. A 200 µL cell and media solution was then added to

each well and then placed back into the incubator. After these steps, treatment began the following day.

Treatment of Astrocytes

To test the effects of progesterone on glial cell viability, cultures treated with varying concentrations of ethynodiol diacetate (0 nM (control), 10 nM, 100 nM, 1 μM, 10 μM) were tested for viability. The ethynodiol diacetate was diluted to the appropriate concentration using phosphate buffered saline. The independent variables measured were ethynodiol diacetate treatment (at varying concentrations) and whether or not the cells were stressed with hydrogen peroxide. The control wells received phosphate buffered saline in volumes equal to that of the treatments. The cells were incubated at 37°C for one hour. Half of the wells were then treated with 5 μL of a 40 mM hydrogen peroxide solution for one hour. Following stress treatment, media was replaced in each well with 200 μL of fresh media supplemented with the appropriate ethynodiol diacetate concentration. Viability was then measured after incubating for 24 hours. Three trials were completed with the above steps. The 96-well plate used is diagrammed below.

	1	2 +0 μM P	3	4 +10 μM P	5	6 +1 μM P	7	8 +10 nM P	9	10 +100 nM P	11	12
A +H_2O_2		+P +H_2O_2		+P +H_2O_2		+P +H_2O_2		+P +H_2O_2		+P +H_2O_2		
B	200μL media											
C -H_2O_2		+P -H_2O_2		+P -H_2O_2		+P -H_2O_2		+P -H_2O_2		+P -H_2O_2		

Figure 1. The 96-well plate treatment diagram. The cells in rows A and B were first treated with the progesterone, Ethynodiol diacetate with the concentrations, 0 μM, 10 μM, 1 μM, 1 nM, and 10 nM and 100 nM, in wells 0, 2, 4, 6, 8, and 10, respectively. All the cells in row A were stressed with 5 μL of a 40 mM hydrogen peroxide solution, with all the cells in row C receiving no hydrogen peroxide. After one hour hydrogen peroxide was added and the cells were stressed for an hour. The solutions were then changed and replaced with 200 μL of media and the respective ethynodiol diacetate concentrations. Ethynodiol diacetate (P): Hydrogen Peroxide (H_2O_2): with (-) and (+) indicating the presence or absence of the variable.

MTT Cell Proliferation Assay

Following treatment, cell viability was assessed using the MTT Cell Proliferation Assay from Sigma Aldrich. This was done by removing 100 µl of the media and adding 10 µl of the MTT reagent to each well, followed by incubation at 37°C for three hours. One 100 µl of media containing no cells was added to a well to serve as a blank for comparison. Once the precipitate formed, 100 µl of the MTT solubilization solution was then added to the wells, and the absorbance was taken at 570 nm. A high absorbance indicated a high cell proliferation.

Analysis of data

To evaluate the 3 trials performed and to determine statistical significance between the two treatments, a 2-way Analysis of Variance (ANOVA) was used with a p-value of significance being less than 0.05 along with a Tukey's post hoc test.

RESULTS

Absorbance values were obtained from the MTT Assay. From the MTT assay results, a ratio was determined between the treated cells and the control by subtracting the absorbance of the blank and then dividing by the control; the results were then averaged and plotted. Viability did not differ significantly between stressed and unstressed cells (2-way ANOVA, $F_{9,30}=1.673$, $P=0.211$) or between cells receiving varying concentrations of ethynodiol diacetate (2-way ANOVA, $F_{9,30}=0.638$ $P=0.641$). This is seen in Figure 1, which compares the results of the stressed versus unstressed astrocyte cells as well as the varying ethynodiol diacetate concentrations.

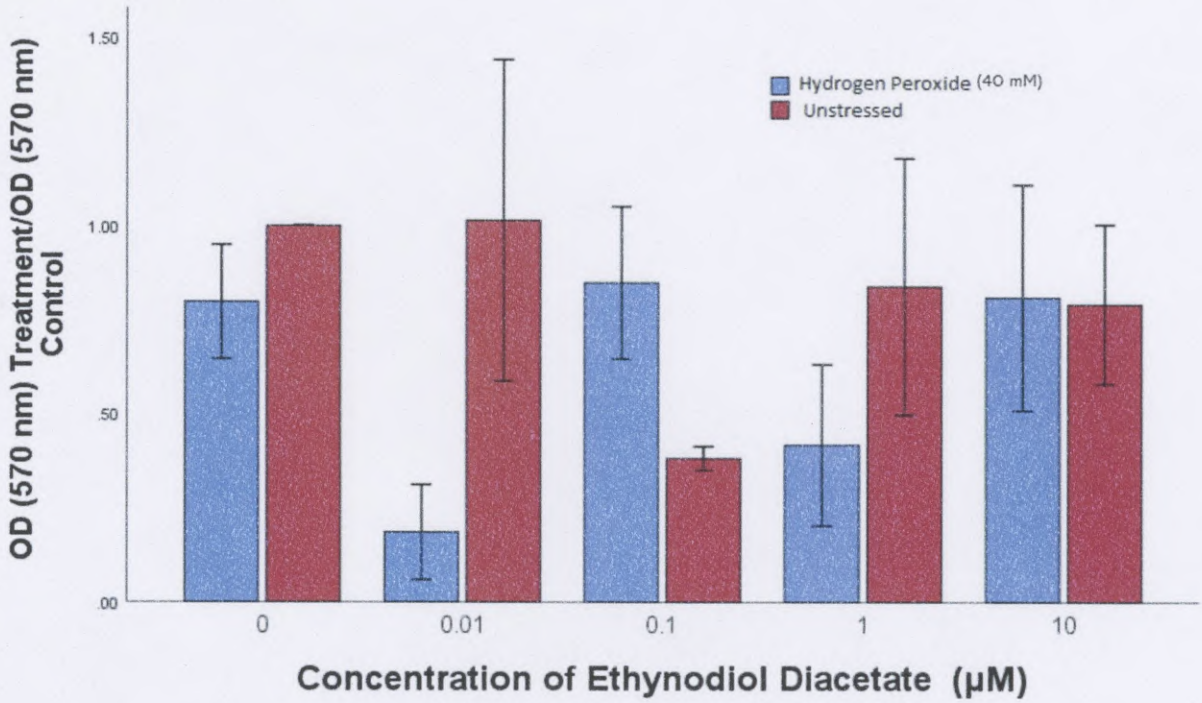

Figure 1. Comparison of glial cell viability (+/- 1 S.D) for cells treated with 0 μM, 0.01 μM, 0.1 μM, 1 μM, and 10 μM Ethynodiol diacetate that were either stressed with 5 μL of a 40mM hydrogen peroxide solution or unstressed. Results were not significant between the stressed and unstressed data (2-way ANOVA, $F_{9,30}$=1.673, P=0.211) and between the varying concentration of Ethynodiol diacetate (2-way ANOVA, $F_{9,30}$=0.638 P=0.641).

The absorbance values compared to control are reported for each concentration and between stressed and unstressed are listed as average ratios (+/- standard error). The average ratio of the cells treated with 0 μM ethynodiol diacetate and stressed with hydrogen peroxide was 0.797 (+/- 0.1517). The average absorbance ratio of the cells treated with 0.01 μM ethynodiol diacetate and stressed with hydrogen peroxide was 0.185 (+/- 0.1261) and unstressed was 1.014 (+/- 0.4270). The average absorbance ratio of the cells treated with 0.1 μM ethynodiol diacetate and stressed with hydrogen peroxide was 0.8486 (+/- 0.2025) and unstressed was 0.3818 (+/- 0.0320). The average absorbance ratio of the cells treated with 1 μM ethynodiol diacetate and

stressed with hydrogen peroxide was 0.4167 (+/- 0.2140) and unstressed was 0.8378 (+/-0.3408). The average absorbance ratio of the cells treated with 10 μM ethynodiol diacetate and stressed with hydrogen peroxide was 0.8087 (+/- 0.3002) and unstressed was 0.7907 (+/- 0.2120).

DISCUSSION

Treatment with ethynodiol diacetate and with hydrogen peroxide showed no significant difference in glial cell viability when compared to the unstressed control. The results did not support the hypothesis that the viability of hydrogen peroxide-stressed cells would increase with the pretreatment of ethynodiol diacetate. A previous study indicated that when progesterone was delivered to a patient after a brain injury, it caused a decrease in the cytotoxic responses as well as promoting functional recovery (Grossman, et al., 2004). Another study done by Cervantes et al., (2001) showed that progesterone played a role in the reduction of neuronal cell death. In my study, however, ethynodiol diacetate did not provide any protection to the glial cells against the hydrogen peroxide stressor. The concentrations used in this study, 0 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM, may have been too low to induce any protective effects towards the glial cells. Higher concentrations, though, would not be analogous to *in vivo* levels. For example, the *in vivo* blood concentration of ethynodiol diacetate from consuming oral hormonal contraceptives is 0.31 μM. In addition to concentrations, the glial cells were not properly adhering to the wells and were removed during trials, thus possibly skewing the data as both live and dead cells were being removed. This observation could be indicative of the cells either being unhealthy prior to treatment or requiring a longer incubation time to adhere to the wells.

One study showed that hydrogen peroxide alters astrocyte membrane and cytoskeleton through damaging lipids, proteins, DNA, and through signaling molecules which cause the intracellular pathways to cause apoptosis (Choi et al., 2007). The treatment of cells with

hydrogen peroxide appeared to causes a decrease in cell viability, but cannot be concluded without doing further tests to determine the oxidative stress caused by hydrogen peroxide to glial cells. Thus, future studies could look at directly measuring the oxidative stress hydrogen peroxide imposes on the cells.

This study was limited to three trials due to time constraints and contamination to the glial cells. Data was limited when concerning the effects of progesterone on glial cell viability, due to most research being centered towards the effects progesterone has on reproductive functions and not how they affect glial cells. Therefore, it became difficult to determine adequate concentrations and exposure time for ethynodiol diacetate. Thus, it is possible that ethynodiol diacetate did not have enough time to exert effects on the glial cells.

The ability to better understand the effects of ethynodiol diacetate, a main progesterone used in hormonal contraceptives, on astrocytes will aid in the understanding of its role in neurochemistry.

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