Biology Honors Thesis

Angiotensin-Converting Enzyme 2 Expression in Ovariectomized Rat Kidney: Effects of Ethanol and Estradiol

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A thesis submitted to the Department of Biology, East Carolina University, in partial fulfillment of the requirements for Biology Honors Thesis

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I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed:_____ Date: 11/01/2021

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ABSTRACT

The COVID-19 pandemic has prompted discussion and research on angiotensin-converting enzyme 2 (ACE2) signaling. ACE2 promotes lowering blood pressure by catalyzing angiotensin II (Ang II) metabolism into angiotensin 1-7 (Ang 1-7). ACE2 is utilized as a membrane receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), also known as COVID-19. Therefore, the number of ACE2 molecules within the kidney will likely influence the severity of symptoms caused by COVID-19. Estradiol (E2), the form of estrogen produced by the ovaries, and ethanol (EtOH) have been proven to separately affect the expression of ACE2 within the kidneys of female rats. This research aimed to study the effect of EtOH, E2, and their combination on ACE2 expression in rat kidney in the absence of E2 in ovariectomized (OVX) female and male as well as OVX rats supplemented with E2 (OVXE2) using the Western blot technique. EtOH significantly increased renal ACE2 expression in OVX and male rats. By contrast, E2 reduced renal ACE expression and prevented the increase caused by EtOH in ACE2 expression in OVXE2. By researching E2 and EtOH effects on ACE2 expression, scientists can further discern how COVID-19 treatment can be tailored for alcohol-consuming females.

Acknowledgments

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Introduction

In December 2019, the first cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were recorded in Wuhan, China. This marked the beginning of an ongoing pandemic. Although researchers have been tirelessly working to learn about SARS-CoV-2, also known as COVID-19, there is still much that is unknown. The rise of this virus has caused many scientists to research the transporter that COVID-19 binds to. This transporter is known as angiotensin-converting enzyme 2 (ACE2). COVID-19 enters the respiratory tract and binds to ACE2 within the lungs via a spike protein. Once within the body, the virus can find other sites of ACE2, such as within the kidney, heart, and liver, to bind to. The greater the number of bound virions, the more severe the symptoms associated with COVID-19 are. Overexpression of ACE2 allows for more binding sites, which increases COVID-19 infection and the severity of symptoms.

The severity of symptoms is linked to a higher or lower mortality rate. Data collected by Global Health 50/50 has shown that men and women are infected with COVID-19 at the same rate, but men are more likely to die and have severe symptoms than women; the mortality ratio is about one-third females to two-thirds males (Verma, 2020). One hypothesis for the higher mortality rate of men across the globe due to COVID-19 is that men have higher concentrations of ACE2 (Capuano et al., 2020). This means that COVID-19 has more binding sites leading to higher infection potential in males than females. This is supported by the fact that males have androgens, hormones that influence male traits and reproduction, while females have estrogens, hormones that influence female traits and reproduction (Reckelhoff, 2001). The activity of plasma renin and angiotensinogen messenger RNA expression is increased when androgens are

present; meanwhile, estrogens decrease the activity of plasma renin, decrease angiotensin-converting enzyme I, and decrease angiotensin I receptor expression (McGuire et al., 2007). Therefore, ACE2 is lower in females than males.

Research conducted by Jon Lui et al. (2010) found that ACE2 activity expressed in male mice kidney was higher than in female mice kidney. Estradiol (E2) contributes to the lower ACE2 concentrations found in females (Seeland et al., 2020). E2 is a vasodilator (White, 2002), which means that it naturally counteracts high blood pressure and, as a result, the body does not need to express as much ACE2. Males do not have this biological balance, which means that ACE2 must be expressed to a higher degree to lower blood pressure. It has been found that ethanol (EtOH) increases ACE2 expression in the kidneys of males and females, specifically regarding research conducted on rats (Alawi et al., 2021).

Although ACE2 has gained interest due to the COVID-19 pandemic, its primary role is the regulation of blood pressure. ACE2 is a regulator within the renin angiotensin-aldosterone system (RAAS) and responds to an increase in blood pressure by metabolizing angiotensin II (Ang II) into angiotensin 1-7 (Ang 1-7). The RAAS involves the regulation of water and sodium absorption within the kidneys, which influences systemic blood pressure. ACE2 is found in large quantities within the kidneys because of the kidneys' importance in blood pressure regulation. As a result, COVID-19 proliferates within the kidney, which can lead to kidney failure and severe COVID-19 symptoms. Ethanol (EtOH) increases blood pressure by decreasing the nitric oxide (NO), a vasodilator, within the vascular endothelium (Husain et al, 2014). Those who frequently imbibe alcohol, ethanol (EtOH), are at increased risk of severe COVID-19 symptoms. The reason behind this is because EtOH causes an overexpression of ACE2 within the body in response to increased Ang II (Testino, 2020). When E2 and EtOH are both present it is unknown how they

will affect the expression of ACE2 in female rats. The objective of this research was to determine how E2 affects the expression of ACE2 in the presence of EtOH compared to the lone effect of EtOH on ACE2 expression in ovariectomized (OVX) rats. It is hypothesized that when E2 is present, regardless of EtOH presence, ACE2 will be lower. To achieve this objective a preliminary study involving male rats, a control group and a group treated with EtOH, was conducted. The final study consisted of four groups of OVX rats: a control group, a EtOH group, a E2 group, and a E2+EtOH group. The preliminary study results were compared with results obtained from the final study.

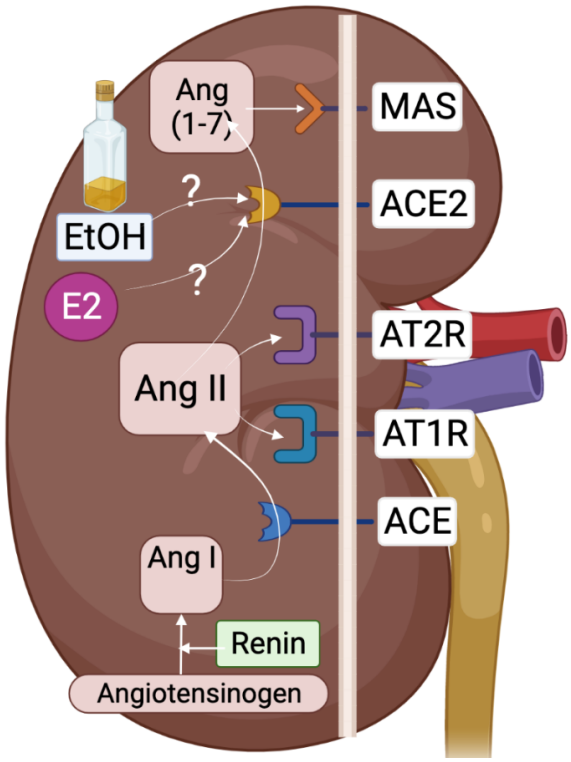

Figure 1. Renin Angiotensin-Aldosterone System (RAAS). Angiotensinogen is converted to Angiotensin II through renin. ACE2 then generates angiotensin 1-7 by removing the carboxyterminal amino acid from angiotensin II; The Mas receptor is then activated by Angiotensin 1-7. It is unknown where estradiol (E2) and ethanol (EtOH) interact with the pathway.

Methods

Phase One: Preliminary Study & Methods

The head lab technician for Dr. Abdel Abdel-Rahman's lab within the Department of Pharmacology and Toxicology at the Brody School of Medicine, where I conducted my research, harvested the organs of the male Sprague Dawley rats. The organs were kept in a minus 80°C freezer to preserve the tissue. For the preliminary study, the right kidney of each male rat was used to ensure that the same part of each kidney was cut for the tissue samples. The kidneys were relatively the same size between the control (Group 6.1; six rats) and EtOH group (Group 6.2; seven rats). The objective of the preliminary study was to determine the differences between the expression of ACE2 in EtOH and non-EtOH treated rats. After slicing the kidney tissue and placing the tissue pieces into tubes alongside 200µl of lysis buffer containing cOmplete™ Protease Inhibitor Cocktail, the samples were homogenized using an ultrasonic homogenizer and centrifuged at 4°C, 12,000 g for twelve minutes. Homogenization was performed to release the cellular contents, such as the proteins of the cell. The tissue samples were then prepared for protein qualification. BioRad color reagent, bovine serum albumin (BSA) 1.5 mg/ml, homogenized kidney tissue sample, and water were used to prepare the protein quantification sample along with six standard references.

			S0	S1	S2	S3	S4	S5
1.5 ml tube	Standard	Water (µl)	800	799	798	796	792	784
		Color Reagent (µl)	200					
		1.5 µg/µl BSA (µl)	0	1	2	4	8	16
		BSA concentration (µg/µl)	0	1.5	3	6	12	24
	Sample	Water (799 µl) + Color Reagent (200 µl) + Sample (1 µl)						

Table 1. Protein Qualification procedure. Six standard reference samples were prepared, and then each sample containing kidney tissue was prepared.

Protein qualification was conducted using i-control software and the Tecan Infinite® Series reader. After 200 µl of each protein qualification sample were loaded into individual wells in the well-plate, the absorbance of the samples was read at 595 nm. Resulting values were analyzed using a Microsoft Excel spreadsheet, and the standard curve was used to balance and calculate the protein concentration. The loading samples were prepared based on the calculated protein concentration of each tissue sample. Deionized water (16.25-x µl), the calculated amount of tissue sample (x µl), Thermo Fischer NuPAGE™ Sample Reducing Agent (10X) (2.5 µl), and NuPAGE™ LDS Sample Buffer (4X) (6.25 µl) were used in the preparation of the loading samples. The samples were vortexed and heated at 95℃ for five minutes. This was to denature the proteins. The samples were immediately cooled down on ice to stop further denaturing. The Western blot technique was used to determine the levels of ACE2 within each rat kidney. The gels that were used for electrophoresis were Thermo Fisher NuPAGE™ 4-12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 9-well. The electrophoresis chamber was filled with MOPs running buffer until full. The gels were placed in the chamber

with 500 µl of antioxidant to ensure that the proteins remain reduced. A protein marker was loaded into the first well (4 µl) followed by each of the prepared samples (20 µl). The voltage was set to 100 V for fifty minutes or until the proteins had travelled halfway down the gel. The voltage was then set to 150 V for an hour, or until the proteins had migrated down the gel. The gels were run in the same conditions. Care was taken to not run the gel for too long, otherwise the β-actin would be lost because it has a smaller molecular weight and is found farther down the gel.

Once completed, the gels were removed and placed in boxes containing transfer buffer, supported nitrocellulose membrane, and filter paper. The membrane and gel were sandwiched between two sheets of thick filter paper and placed into Trans-Blot Turbo transfer machine for thirty minutes at Standard SD (Up to 1.0 A; 25 V constant). The voltage and current running through the sandwich transferred the proteins from the gel to the membrane. After completed transfer, the membranes were blocked using blocking buffer for one hour while on a rocking platform. Blocking the membrane helps ensure that the antibody will not bind non-specifically to the membrane once added. Primary antibody made with 1:1000 Abcam 108252 Anti-ACE2 Antibody and 1:4000 Abcam 8226 Anti-β-actin Antibody was prepared and poured onto the membrane after blocking. Anti-ACE2 antibody was used because it will tag the ACE2 proteins present, which is what is being studied. Anti-β-actin antibody is the loading control. Using a loading control is important to ascertain that the samples have been properly loaded and that transfer is effective. The membrane incubated for twelve hours at 4°C. Due to the low affinity of the antibody, incubating at 4°C overnight allows the antibody to recognize and bind to the epitope tag. The primary antibody was collected, and the membrane was washed with PBST (1.0% tween) for seven minutes for four cycles to remove any low affinity antibody and provide a clearer background. The membrane

then incubated for one hour within a light blocking container at room temperature with the secondary antibody, which was made with 1:5000 IRDye® 800CW Goat anti-rabbit and 680RD Goat anti-mouse antibodies and antibody diluent. The secondary antibody binds to the primary antibody to aid in the detection of the primary antibody, which is bound to the target antigen. After collecting the secondary antibody, the membrane was washed four times for seven minutes each with PBST to improve background. The membrane(s) were placed within a PBS solution prior to being scanned at 700 (red) and 800 (green).

The membrane(s) containing the transferred proteins were analyzed using Image Studio software. The molecular weight of ACE2 antibody is 97 kilodaltons (kD) but was observed at 130 kD under reducing conditions within the electrophoresis chamber. The molecular weight of β-actin is 42 kD and was observed at approximately 43 kD under reducing conditions. To analyze the membranes, Image Studio software scanned the membranes and the 700 and 800 contrast was adjusted to better view the ACE2 and β-actin protein bands. Measurements of each ACE2 band and its corresponding β-actin band were taken using the analysis tool in Image Studio. The measurements were transferred into a Microsoft Excel spreadsheet where a linear regression of the data was used to create a bar graph to determine the relationship between ACE2 and β-actin, as well as any outliers within the data.

Phase Two: Final Study

For the final experiment, four groups of Sprague-Dawley OVX rats were used to determine the effect of E2 and EtOH together, and separately, on ACE2 expression. In congruence to the preliminary study, the organs were harvested by the head lab technician and kept in a minus 80°C freezer. The four groups used were a: control (Group 1.1; eight rats), EtOH only (Group 1.2; nine rats), E2 only (Group 2.1; eight rats), and E2+EtOH

(Group 2.2; nine rats). The same procedure as the preliminary study was followed, with slight modifications. BioRad 12% Criterion™ XT Bis-Tris Protein Gel, 18 well, 30 µl electrophoresis gels were used because of the increased number of rat kidney samples being used. As a result, BioRad buffers were used instead of Thermo Fisher buffers. The loading samples were heated at 70℃ for ten minutes instead of 95℃ for 5 minutes. Heating at a lower temperature for a longer amount of time helped the proteins not denature too quickly. Antioxidant was not needed because BioRad running buffer is sufficient in making sure the proteins remain reduced. All remaining steps were the same as in the preliminary study.

When analyzing the resulting data, for both the preliminary and final study, the p-value of the data was calculated in Excel using the t-test: paired two sample for means. The p-value (*P<0.0001), which is less than 0.05, shows the statistical significance of the difference between the groups in the final study. Note that groups 1.1, 1.2, 2.1, and 2.2 each had their own data that was compared using the bar graph, matching error bar, and p-value.

Storage of Materials

Note that the tissue samples and primary antibody were kept in a standard freezer (-20℃) to preserve their integrity. Secondary antibody was kept in a light blocking tube within a standard refrigerator (4℃) to prevent its degradation. Buffers were either stored in the refrigerator or at room temperature depending on the brand instructions.

Results

Preliminary & Final Study Comparisons

The groups containing E2 expressed the lowest levels of ACE2, with the E2+EtOH group having higher ACE2 expression than the E2 group. Analysis of the male rat control group shows that its ACE2 expression is nearly identical to the female control group. The male and OVX EtOH groups, while similar in that ACE2 was expressed to a higher degree than the control, showed that the OVX rats' ACE2 expression was higher than in the male rats. The error bars did not overlap, meaning that the ACE2 expression difference is statistically significant. Based off these results, and those found in literature, EtOH upregulates ACE2 expression in males and OVX rats. When compared to the E2 containing groups, the difference in expression is significant. The E2 group showed the lowest ACE2 expression, which corresponds to what is found in literature. E2 lowers ACE2 expression because of its ability to decrease the activity of plasma renin, decrease angiotensin-converting enzyme I, and decrease angiotensin I receptor expression (McGuire et al., 2007). The EtOH+E2 group had slightly higher ACE2 expression than the E2 group, but lower expression than the control and EtOH group. Even in the presence of EtOH, E2 lowers ACE2 expression by a significant degree.

ACE2 & β-actin Band Analysis

Looking at the protein bands in Figure 2 the control group ACE2 band is weaker than all other bands. This was not consistent across the Western blots completed during this study. Most of the blots had darker ACE2 control bands than the one in Figure 2. The weaker bands indicates that the antibody had a low affinity for the protein within the control, or that there was less protein for the antibody to bind to. It is most likely that there was less protein present, as other Western blots showed a darker band for the control. The EtOH ACE2 band was the strongest,

which is analogous to its higher ACE2 expression. The darker band indicates that more ACE2 is present in the EtOH group. The E2 and EtOH+E2 group have similarly shaded ACE2 bands. However, the EtOH+E2 ACE2 band is slightly darker than the E2 band, which agrees with the EtOH+E2 group having marginally higher ACE2 levels. While the β-actin bands were equally strong across all groups, it is worth noting that the EtOH+E2 group had a double expressed β-actin band. This was not consistent across the Western blots and was seen infrequently. This anomaly could have been due to the quality of the Anti-β-actin Antibody or non-specific binding. The control and EtOH bands for the male rats were equally dark and similar in size. However, the p-value indicates that there is still a statistically significant difference in ACE2 expression.

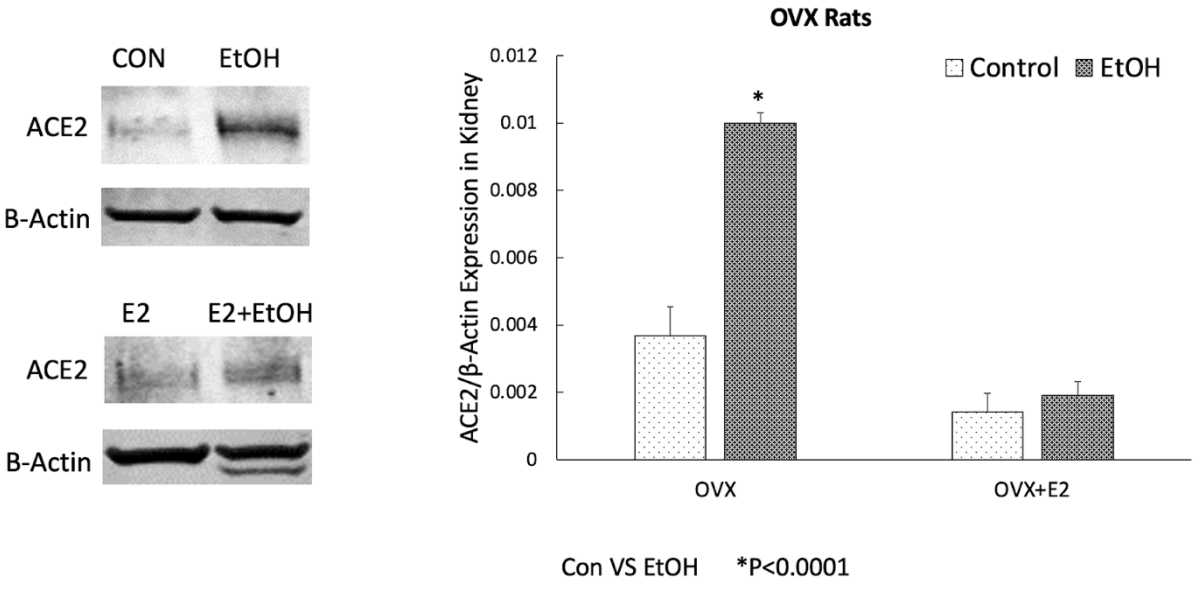

Figure 2. The E2 only group (N=8) expressed the lowest levels of ACE2, with the E2+EtOH group (N=9) having the second lowest ACE2 expression. It is worth noting that the control group (N=8) had more ACE2 expression than the E2+EtOH group, which provides additional support for the hypothesis that E2 lowers ACE2 levels. The β-actin protein band for the E2+EtOH group

was doubly expressed, which was an anomaly. To determine the p-value the function t-test: paired two sample for means was used in Excel.

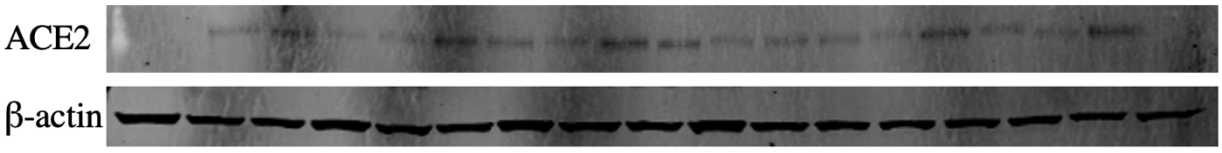

Figure 3. Western blot of OVX rat groups showing ACE2 and β-actin bands. Order that the samples were loaded: control, EtOH, E2, E2+EtOH; order was repeated until all samples had been loaded. The consistency of the β-actin bands show that the samples were properly loaded.

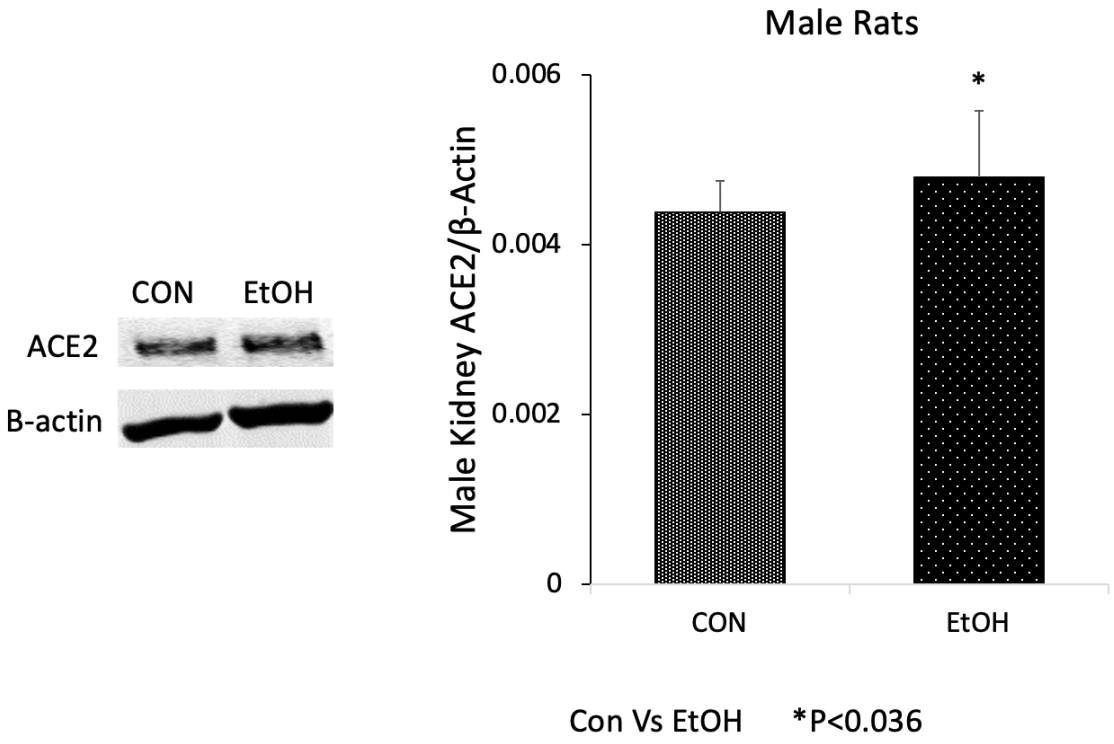

Figure 4. The control group (N=6) shows a lower expression of ACE2 than the EtOH group (N=7). To determine the p-value the function t-test: paired two sample for means was used in

Excel. The p-value shoes a significant difference between ACE2 expression in the control and EtOH groups since it is less than 0.5.

P-value Significance

The asterisk above the EtOH group in Figure 2 is the p-value of the control versus the EtOH group. The p-value is less than 0.05 (*P<0.0001), meaning that the ACE2 expression difference between the groups is statistically significant. The p-value for the male rats, as seen in Figure 4, was P<0.036, which indicates a statistically significant ACE2 expression difference between the control and EtOH group.

Discussion

Results Discussion

The final study shows that E2 decreases ACE2 expression in OVX rats. When comparing the four OVX groups, the data proves that in the presence of EtOH and E2, E2 lowers the ACE2 expression by a significant degree. E2 prompts the synthesis of Ang 1-7, which is a smooth muscle cell growth inhibitor and vasodilator (Dubey et al., 2002). This lowers blood pressure, and results in a decreased need for higher ACE2 expression. Studies show that after EtOH consumption in rats, there is a significant increase in blood angiotensin II (Ang II) levels (Wright et al., 1985). An increase in Ang II levels causes an upregulation of ACE2, thereby modulating the increase in blood pressure from the higher levels of Ang II (Bosso et al., 2020). The results of the study support the hypothesis that E2 lowers ACE2 expression regardless of the presence of EtOH.

Limitations

There were few limitations within the study. One limitation was that there is not any current research on how ACE2 expression within the kidneys is affected by the presence of both EtOH and E2. Therefore, there were no current studies to compare the results to. However, the effects of E2 and EtOH separately is known, which was the foundational knowledge for the study.

Conclusion: Significance & Importance

Knowledge on ACE2 has become increasingly important due to the COVID-19 pandemic. Understanding how E2 impacts ACE2 levels alongside EtOH within the kidney will enable COVID-19 researchers to have a broader perception of how at-risk female alcohol consumers are

for severe symptoms compared to their male counterparts and females who have had a hysterectomy. By researching the expression of ACE2 alongside E2 and EtOH within the kidney, other researchers may be able to determine courses of treatment, such as ACE2 inhibiting drugs or hormone therapy, for females that frequently consume alcohol. Current research into how estradiol treatment for women infected with COVID-19 has shown that when treated with E2, the fatality risk for women under fifty decreases more than 50% (Seeland et al., 2020).

This research could also be applied to research outside of the COVID-19 investigative field. This may include long term management of hypertension in alcoholic females with low E2. Scientists could use the results of this analysis to conduct research on how contraceptives affect ACE2 expression in alcoholic females. Hypertension is prevalent in postmenopausal women due to lack of endogenous estrogens. This puts postmenopausal women at risk of cardiovascular disease and cerebrovascular accidents (CVAs) (Barton and Meyer, 2009). Knowing that EtOH increases ACE2 in OVX rats and that E2 lowers ACE2, further research can be conducted on the effects of hormone therapy in postmenopausal alcoholic women. Overall, knowledge associated with E2 and EtOH and how it affects hypertensive agents such as ACE2 has many applications.

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