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Protective Effects of Vitamin C on Nrf2 and TSPO Genes Expression in the Urinary System of Ovariectomized Rabbits

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Abstract | Ascorbic acid, also known as vitamin C, is a water-soluble vitamin essential for growth, development, and tissue repair. This study aimed to assess the impact of vitamin C on the urinary systems of ovariectomized rabbits. Twenty rabbits, aged 8–9 weeks, were divided into four groups: intact rabbits receiving distilled water (Group 1), intact rabbits receiving oral vitamin C (10.166 mg/kg/B.W) (Group 2), ovariectomized rabbits receiving distilled water (Group 3), and ovariectomized rabbits receiving oral vitamin C (Group 4). All treatments were administered orally daily. At the end of the experiment, the animals were anesthetized, and kidney tissues were collected for RNA isolation. The expression of genes encoding translocator protein (TSPO) and nuclear factor erythroid 2 related factor 2 (Nrf2) was analyzed in the kidney tissues. Vitamin C significantly reduced blood levels of urea, creatinine, salt, potassium, and calcium in both intact and ovariectomized groups ($p \leq 0.05$). Additionally, gene expression decreased significantly in the ovariectomized group ($p \leq 0.05$). These findings underscore the crucial role of vitamin C in renal function. Upregulation of Nrf2 and TSPO gene expression in ovariectomized rabbits suggests an adaptive response to mitigate ovariectomy-induced kidney tissue damage. Overall, these results highlight the potential therapeutic benefits of vitamin C in preserving renal function and warrant further investigation into its mechanisms of action.

Keywords | Ascorbic acid, Ovariectomy, Renal system, ANP, Nrf2, Rabbits

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INTRODUCTION

Most animals, including dogs and cats, synthesize vitamin C in their livers, which is then broadly dispersed throughout their bodily tissues. Vitamin C plays a vital physiological role in many metabolic processes, such as immunological control, oxidative stress reduction, and tissue development and maintenance (Gordon et. al., 2020). Additionally, it is a cofactor in the synthesis of significant compounds including vasopressin and catecholamines. As a powerful antioxidant and radical scavenger, vitamin C protects cell constituents against oxidative stress, which mediated by reactive oxygen species (ROS) and free radicals. Wyckelsma et al. (2020) report that most vertebrates can synthesize vitamin C that increases during stress. Decreased vitamin C levels have been documented in a wide variety of diseases, and in critically ill human patients, may be associated with increased severity of disease and decreased survival (Gordon et. al., 2020). But humans can no longer make vitamin C on their own. In addition to being an essential antioxidant, vitamin C is an enzyme cofactor in several critical biological processes. Vitamin C insufficiency has been alleviated by pharmacokinetic modifications, such as increased renal reuptake, recycling, and absorption of vitamin C in comparison to species that synthesize the vitamin (Paul, 2022; Frikke-Schmidt et al., 2016). Renal damage was prevented and treated by vitamin C in these studies. The markers of renal functioning and oxidative stressors assessed included blood creatinine, urea, malondialdehyde, and reduced glutathione levels (Offor et.

al., 2017). The findings demonstrated that vitamin C both prevented damage to the kidneys from occurring from outside sources and aided in their healing after harm had already occurred. Treatment with vitamin C reduces the amount of time the kidneys require to heal. Additionally, studies on reduced glutathione and malondialdehyde have shown that vitamin C therapy may successfully increase blood antioxidant capacity, preventing kidney impairment (Offor et al., 2017). One possible mechanism by which vitamin C protects kidney tissue from oxidative stress is by neutralizing free radicals with reactive oxygen. Evidenced by significant decreases in kidney tissue malondialdehyde, plasma urea, and creatinine levels, as well as estrogen and ERs' involvement in various physiological processes in the kidney, pretreatment with vitamin C has been shown to substantially enhance renal functioning in certain investigations (Feng et al., 2022). For example, it plays a critical role in regulating the kidney's endothelin-1 (ET-1) system and preserving mitochondrial homeostasis. Through its receptors, estrogen contributes to kidney regeneration and repair. Because of its receptors in the proximal tubule, estrogen also plays a role in maintaining the body's phosphorus balance. The susceptibilities and outcomes of many renal illnesses have been linked to ERα polymorphisms (Yang et al., 2023). While it is acknowledged that estrogens may protect against chronic glomerular disorders, the role of estrogens during acute kidney injury (AKI) is still debated. Endogenous estrogen in ovariectomized mice caused epithelial cell injury and subsequent renal fibrosis, but by day 21, there was no discernible decline in GFR (Buléon et al., 2020). This study aimed to assess the impact of vitamin C on the urinary systems of ovariectomized rabbits.

MATERIALS AND METHODS

Ethical approval

The ethical approval of this study was obtained from the Ethics Committee of College of Veterinary Medicine, University of Baghdad.

Management of experimental animals

This experiment was conducted from July 2023 to October 2023 at the animal house at the University of Baghdad's College of Veterinary Medicine. In this investigation, twenty female rabbits weighing between 840 and 1150 grams were used; their ages varied from 8 to 9 weeks. These animals were housed in an air-conditioned room with a temperature range of 20 to 25 degrees Celsius and a 12-hour photoperiod every day (Bodera et al., 2019). Feeding regime, was ad libitum access to fresh water and a pellet feed.

Technique of ovariectomy operation

Ten rabbits underwent ovariectomy for both ovaries in accordance with the protocol of Kenkre and Bassett (2018). The ovaries of the remaining 10 rabbits remained intact. After surgery, each animal received personalized care, and for five days, penicillin and streptomycin was administered intramuscularly.

Supplemental dosage of vitamin C

The dosage for this experiment was the 1-3 tablet/day human recommended dose supplied by MEDCELLPHARMA® CO. Netherland. Three hundred and five milligrams (or 300 mg of vitamin C) were found in each tablet. Next, via stomach intubation, each female rabbit in the two groups received 1ml/1kg/B.W. (10.166 mg/kg B.W) (Al-Azawi and Alkenany, 2017). This dosage was given to each rabbit every day for thirty days.

Collection of blood samples

Each rabbit's jugular vein was used to extract fasting blood at the end of the 60-day of the experiment. The samples were separated into two sections: the first was collected using an anticoagulant tube for blood parameters, and the second was separated after centrifugation for 20 minutes at a speed of 3000 revolutions per minute (rpm) for the purpose of collecting serum. Before being used, serum samples were kept in a freezer at -18 °C (Sareen and Dutt, 2018).

Experimental design

The rabbits were evenly divided into four groups after two weeks of acclimatization: two groups (G1, G2) remained intact, while the other two groups (G3, G4) underwent ovariectomy. Daily doses of vitamin C were administered to groups two and four (Al-Azawi and Alkenany 2017). Distilled water was given to the other two groups (G1, G3) on a daily.

Parameters determination

When creatinine and alkaline picrate react, they produce a coloration known as Jaffe's reaction, which is orange-yellow. There was a clear correlation between the absorbance of the orange-yellow color and the creatinine concentration of the sample. According to Patil et al., (2014), the absorbance was measured at around 510 nm. Milligrams per deciliter was the unit of measurement for creatinine. The reaction between urea, water, and urease produces ammonia and carbon dioxide as waste products. The result of the process was measured by the reduction in absorbance at 340 nm, which occurs when NADH is transformed to NAD. This technique measures NADH (which converts to NAD+) at 340 nm and relies on a linked enzyme process involving urease and glutamate dehydrogenase. When the patient was being evaluated for pre-renal hyperuremia, renal hyperuremia, glomerulonephritis, chronic nephritis, polycystic kidney, nephrosclerosis, tubular necrosis, or post-renal hyperuremia, the usual laboratory tests to rule

out these conditions include creatinine and urea (Roche, 2011).

Serum Na+ levels measured to diagnose imbalances in fluid and electrolyte levels, abnormalities in the acid-base balance, or overconsumption of sodium. Moreover, elevated sodium levels might be a sign of renal illness, hypothyroidism, edema, and the syndrome of improper secretion of antidiuretic hormone (SIADH) (Delaney et al., 2006).

The approach uses an indirect (specimen was diluted by the instrument prior to analysis) ion-selective electrode (ISE) method for detection of the serum electrolyte concentrations and activity. Potassium (K) was the primary intracellular cation and was vital to brain and muscle cells (Roche, 2016).

Using this technique, calcium forms a compound with 5-nitro-5'methyl-BAPTA (NM-BAPTA) in an alkaline environment. Following this complex's reaction with EDTA, a colorful product was produced, the intensity of which was directly correlated with the specimen's calcium content. At 340 nm, it was measured photometrically (Roche, 2013).

Ammonium molybdate was used in the procedure as the color-forming reagent. The final product was measured at 340 nm, with a secondary wavelength of 700 nm. When sulfuric acid was present, ammonium molybdate and inorganic phosphate combine to create an ammonium phosphomolybdate complex with the formula (NH4)3[PO4(MoO3)12]. The content of inorganic phosphate was closely correlated with the concentration of phosphomolybdate that forms in the reaction (Roche, 2011).

Analysis of gene expression

Total RNA isolation was performed using the RealBest Extraction 100 magnetic nonabsorbent kit from Vector-Best in Novosibirsk, Russia, according to the manufacturer's instructions. The stability and quality of the RNA were assessed using a spectrophotometer. Subsequently, the extracted RNA was converted into cDNA using M-MLV Reverse Transcriptase (Synthol, Russia), following the manufacturer's instructions, to serve as a template for specific primer-based real-time polymerase chain reaction (EvaGreenRT-PCR kit; Synthol, Russia).

Table 1 displays the primer sequences, all supplied by Bioneer (Korea). GAPDH was used as the reference housekeeping gene when examining the expression of these genes of interest. Initiate the RT-PCR process using the ExicyclerTM 96 Real-Time Quantitative Thermal Block software. After an hour of reverse transcription at 50 °C, PCR activation begins with five minutes at 95 °C, followed by two cycles of cycling at 95 °C for 20 seconds and 60 °C for 45 seconds, respectively. This procedure is repeated 45 times. To ensure specificity of the PCR products, melting curve analysis was performed. The delta-delta CT method was used to relatively quantify the expression of a particular gene (Livak and Schmittgen, 2001).

Statistical analysis

Using SAS software (SAS Institute Inc., 2010), all data were subjected to a one-way ANOVA and a least significant differences (LSD) analysis to determine if there were any significant differences in the means.

RESULTS

As shown in Table 2, the serum urea values for different groups indicate a significant rise in serum urea in G3 compared to other groups ($p<0.05$). Additionally, rabbits subjected to ovariectomy (G3) showed a substantial increase in serum creatinine (CREA) compared to G1 and G4 ($p<0.05$). After 60 days of treatment, ovariectomy rabbits (G3) displayed significantly higher blood potassium (K) and sodium (Na) levels compared to other groups ($p<0.05$).

The findings also revealed that rabbits administered with vitamin C (G2) had considerably lower blood calcium and phosphate levels compared to other groups ($p<0.05$). Furthermore, the G2 group exhibited a significantly reduced blood total protein concentration compared to G1, G3, and G4 ($p<0.05$; Table 2).

In Table 3, the analysis of Nrf2 levels showed that the G2 group had significantly higher levels compared to G1 and G3 ($p\leq0.05$). Conversely, ovariectomy rabbits receiving D.W. (G3) demonstrated a significant decrease in Nrf2 gene expression compared to control and other groups ($p\leq0.05$). Moreover, the G2 group exhibited substantially elevated Nrf2 gene expression ($p\leq0.05$), while lower levels were observed in G4 rabbits compared to G3 and G1 group.

Regarding TSPO gene expression, the G2 group displayed a statistically significant increase compared to control and other groups ($p\leq0.05$). Additionally, the G4 group exhibited significantly ($p\leq0.05$) greater expression compared to control groups (G3; Table 3).

Table 1: Primer and their sequences used in the study.

Primer		Sequence	Product Size
Nrf2 (Nuclear factor erythroid 2 related factor 2)	F	CCCACACAAGGTTCGGCATCAC	43bp
	R	TGGCGATTCCTCTGGCGTCT	
TSPO (Translocater protein)	F	GTGGACCTCCTGCTCCTCAC	
	R	ACGCCATGTAAGGGTAGAGC	100 bp
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	F	ATGCCCCCATGTTTGTGATG	52 bp

Table 2: Effect of vitamin C on kidney function biomarkers in both intact and ovariectomized rabbits.

Parameter	G1 (Intact rabbits received D.W)	G2 (Intact rabbits received vit. C)	G3 (Ovariectomize rabbits received D.W)	G4 (Ovariectomize rabbits received vit. C)	LSD
Serum Urea (mg/dL)	52.8 ± 0.32^{C}	48.8 ± 0.31^{D}	67.1 ± 0.66^{A}	56.5 ± 0.72^{B}	2.7
Serum Creatinine(mg/dL)	1.72 ± 0.008^{B}	1.0 ± 0.004^{D}	3.7 ± 0.01^{A}	1.6 ± 0.009^{C}	0.04
Serum Na (mEq/L)	135.3 ± 0.72^{C}	97.2 ± 0.52^{D}	198.7 ± 0.68^{A}	172.5 ± 1.0^{B}	3.6
Serum K (mEq/L)	2.644 ± 8.78^{C}	1.060 ± 8.89^{D}	3.544 ± 11.88^{A}	2.938 ± 8.64^{B}	0.14
Serum Ca (mEq/L)	13.26 ± 0.92^{B}	10.56 ± 0.53^{C}	43.70 ± 2.41^{A}	14.58 ± 0.85^{B}	3.58

$^{A-D}$ Different superscript letters on the means in a row indicate significant difference ($p<0.05$).

Table 3: Relative gene expression analysis of the TSPO and Nrf2 across several groups.

Parameter	G1 (Intact rabbits received D.W)	G2 (Intact rabbits received vit. C)	G3 (Ovariectomize rabbits received D.W)	G4 (Ovariectomize rabbits received vit. C)	LSD
Nrf2	1.00 ± 0.00^{D}	11.40 ± 0.500^{A}	2.62 ± 0.476^{C}	5.14 ± 0.279^{B}	1.5
TSPO	1.00 ± 0.00^{D}	11.05 ± 0.335^{A}	3.92 ± 0.438^{C}	6.99 ± 0.500^{B}	1.9

Values are presented as Means ± SE (n = 5 rabbits /group). The different capital letters refer significant differences between groups within each row ($P\leq0.05$).

DISCUSSION

Reduced levels of ovarian hormones may significantly influence the pathophysiology of renal disorders, according to clinical and experimental research (Bairey et al., 2019). Estrogen and estrogen receptors (ERs) play critical roles in numerous physiological processes in the kidney. For instance, they are crucial for preserving mitochondrial homeostasis and regulating the kidney's endothelin-1 (ET-1) system. Additionally, they may offer protection against certain renal illnesses via the estrogen/ERs signaling pathways. Evidence suggests that estrogens have a protective influence on AKI, and ovarian hormones may modify it, particularly in ischemic AKI. Vitamin C is an effective antioxidant that prevents reactive vitamin E, and other research has shown that ovariectomy raises serum creatinine and blood urea nitrogen levels, as well as tubular lesions in ischemia/reperfusion (Thuillier and Hauet, 2012). Vitamin C plays a role in preventing oxidative stressors, reducing reactive oxygen production, and preventing the accumulation of oxidation products harmful to the kidneys (Bairey et al., 2019).

According to some research, vit. C enhances renal function by lowering levels of malondialdehyde, creatinine, and urea. Vit. C can support immunity by enhancing immune system cell activities. Effective therapies include angiotensin II (Ang II) antagonists, which work by blocking the angiotensin AT1 receptor (AT1R). Sodium and water reabsorption, potassium excretion, and acid-base homeostasis are all aided by aldosterone's action on the kidney's collecting duct of nephrons and the late distal tubule. According to Begum et al. (2012), it helps cells deal with harmful substances and increases their ability to scavenge oxidative stress. In order to do these tasks, it influences the following enzymes: epithelial sodium channels, bicarbonate-chloride antiporters, and sodium-potassium exchange pumps (Wagner, 2014).

In the clinical setting, blood urea and serum creatinine measurements are very helpful in assessing renal function (Bandebuche et. al., 2017). According to the study's findings, ovariectomized rabbits had mean blood creatinine levels that were higher. Since ovariectomized rabbits have higher renin levels, it is also likely that the decrease in estrogen levels caused kidney injury by altering the ren-

nin-angiotensin pathway (Ikegwuonu et. al., 2020). Serum creatinine and urea nitrogen concentrations are conventional screening tests used to assess renal function. Findings demonstrated that the elevated blood serum creatinine level in the experimental rabbits was caused by their increased salt levels. On the 28th day, the greatest level of creatinine was detected. Nephrotoxicity was caused by an increased level of creatinine in blood serum (Saleh et. al., 2015). Because creatinine was biologically inert, it offers benefits over urea as a renal function test. Serum creatinine levels increase when renal function declines (Krishnaswamy and Lukose, 2015).

The distal nephron's epithelial sodium channel (ENaC) in the kidney is primarily responsible for blood pressure stability and the management of sodium and water balance. In ovariectomized (OVX) rats, the absence of estrogen reduction increases systolic blood pressure. Additionally, estrogen has critical impacts on salt and water balance, leading to higher expression of α-ENaC in the kidneys of OVX rabbits (Xue Zhang et. al., 2019). Additionally, a drop in estrogen levels led to renal damage via the rennin-angiotensin pathway, whereby ovariectomized rabbits had greater renin levels (Ikegwuonu et. al., 2020). Rennin-induced release of aldosterone from the adrenal zona glomerulosa: Aldosterone is a mineralocorticoid hormone that regulates salt and water in the body, and generated in the adrenal cortex's zona glomerulosa (Zheng and Bollag, 2003). Low estrogen levels have been associated with pulmonary fibrosis. Furthermore, it could cause the renin-angiotensin system (RAS) to become overactive (Shawky et al., 2022).

Menopause-related estrogen insufficiency appears to increase renal oxidative stress, potentially putting individuals at risk for renal malfunctions. To elucidate the mechanism(s) by which these treatments may exert reno-protective effects on oxidative stress and inflammatory changes in rat models of menopause, the reno-protective effects of estradiol and Moringa oleifera supplementation were assessed in a menopausal rat model (Donia et al., 2023). It has been shown that estrogen promotes the storage of vitamin D and increases the expression of the vitamin D receptor, resulting in females having a stronger anti-inflammatory response. Estrogens are important regulators of bone turnover in both sexes. During puberty, these hormones are crucial for both longitudinal and width development, as well as for controlling bone turnover. Postmenopausal osteoporosis in women is mostly caused by low levels of estrogen (Noirrit-Esclassan et al., 2021). According to Li et al (2020), estrogen is essential for maintaining calcium homeostasis.

According to the findings of AL-bdeery et al. (2018), a drop in estrogen levels causes a decrease in calcium levels, which in turn causes a rise in PTH levels. Van Abel et al. (2002) found that estrogen increases the effectiveness of 1α-hydroxylase. An essential step in the process of calcium absorption from the gut and reabsorption by the kidneys, this enzyme converts 25-hydroxyvitamin D3 into 1,25-dihydroxyvitamin D3. The quantity of calcium affects the PTH level in the blood. Results are in agreement with those of Xi et al. (2020) and de Barboza et al. (2015), who found that estrogen increases kidney calcium reabsorption and that low estrogen levels affect colon calcium absorption.

A member of the cap 'n' collar family of transcription factors, NRF2 (or nuclear factor erythroid 2-related factor-2) is ubiquitously and minutely generated (Ibrahim et al., 2020, Buelna-Chontal et al., 2014). According to Wu et. al. (2019), NRF2 controls genes that have "antioxidant and cytoprotective" qualities. According to Panieri et al. (2023), the physiological function of active NRF2 is to shield the cell from disproportionate deficiency caused "by metabolic, xenobiotic, and oxidative stress." A gene-modified mouse line in which the Keap1 gene was deleted exclusively in adult renal tubular cells nicely recapitulates the renoprotective effect of Nrf2 activation in systemic Keap1-KD mice (Nezu and Suzuki, 2020). On the other hand, Keap1 deletion in mouse myeloid cells, which accumulate in the tubular interstitium of injured kidneys, shows no protective effect on kidney damage (Jin et. al., 2023). Thus, Nrf2-mediated renoprotection against oxidative stress carried out by tubular epithelial cells rather than myeloid cells. In addition, T cells contribute to renal inflammation and gather in the interstitia of damaged kidneys in response to renal oxidative stress (Guerrero-Hue et. al., 2020). It's interesting to note that T lymphocytes may be involved in Nrf2-mediated renoprotection from oxidative stress because deletion of the Keap1 gene in mouse T lymphocytes reduces kidney damage caused by IRI (Noel et al., 2015). Estrogen participates in kidney repair and regeneration through its receptors. Through its receptors in the proximal tubule, estrogen also has a role in maintaining the balance of phosphorus (Ma et al., 2021). Numerous renal illnesses have been linked to the ERα polymorphisms and their susceptibilities and outcomes. Consequently, a number of kidney illnesses, such as AKI generated by different causes, DKD, lupus nephritis (LN), IgA nephropathy (IgAN), and consequences from CKD may be influenced by the altered or dysregulated estrogen/ERs signaling pathways (Ma et al., 2021).

One of the key components of steroidogenesis, translocator protein (TSPO), originally known as peripheral-type benzodiazepine receptor (PBR), has been identified and described in a number of tissues. However, TSPO was also implicated in other processes and cell activities, including

the control of oxygen homeostasis, protein import, membrane biogenesis, apoptosis, and mitochondrial membrane fluidity. TSPO often found in the distal portions of the nephron in the kidney, extending from the medullary collecting ducts to the thick ascending limb of the loop of Henle. On the other hand, TSPO expression clearly shifts toward more proximal regions of the nephron in response to stressors such as ischemia reperfusion damage, and the protein discovered as high as proximal tubular cells and the Bowman Capsule. As the injury worsens, TSPO also seen in invasive mononucleated cells, where it produced by both smooth muscle and endothelial cells in a manner that mimics the invasion by CD4+ helper T cells. In this article, examine the possible use of TSPO-directed therapy for ischemia reperfusion damage, with a focus on organ preconditioning. Also go into depth on how the degree of the damage correlates with proximal TSPO staining, especially as it relates to monomeric (18 kDa) TSPO and its function in preventing apoptosis and hypoxia-reoxygenation. There is discussion of the protein's possible relationships to pathways involved in embryogenesis and regeneration processes that are triggered in response to damage (Thuillier and Hauet, 2012).

CONCLUSION

The study revealed that ovariectomy in rabbits led to reduced kidney function, as indicated by decreased serum urea, creatinine, and electrolyte levels. Vitamin C supplementation was associated with increased Nrf2 levels, suggesting its potential in mitigating oxidative stress and preserving renal function. These findings underscore the need for further mechanistic investigations.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

NOVELTY STATEMENT

This study investigated the impact of vitamin C on ovariectomized rabbits' urinary systems and unveiled novel insights into renal function and gene expression patterns.

AUTHORS CONTRIBUTION

MHA created and designed the study. **MHA** sorted and compiled the references. **MHA** examined and evaluated the information. **NSM** prepared the first draft of the text, and **ZSZ** gave insightful feedback that helped to mold the work into its present state. The final manuscript was critically examined and approved by all writers. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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