Rosmarinic Acid Protects the Testes of Rats against Cell Phone and Ultra-high Frequency Waves Induced Toxicity

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Introduction
Because of the incessant evolution of new technologies, many people are exposed to various frequencies of electromagnetic fields and wireless devices such as cell phones and Ultra-high Frequency (UHF) waves at home or work. Wireless devices

What's Known
- Cell phone and Ultra-High Frequency (UHF) waves cause oxidative stress, contributing to the generation of Reactive Oxygen Species (ROS), which results in aging and dysfunctions, such as multiple sclerosis, Parkinson's, and Alzheimer's diseases.
- Rosmarinic Acid (RA), as a polyphenol, has anti-inflammatory, antitumor, and anti-hepatitis characteristics.

What's New
- RA as an anti-oxidant could save the testes and diminish the induction of ROS by increasing antioxidant enzymes and decreasing the lipid peroxidation and oxidative stress indicators in rats.
- RA as a food supplement can be useful for protecting humans exposed to cell phones and UHF environmental contamination.

Abstract
Background: Cell phone and Ultra-High Frequency (UHF) waves produce oxidative stress and cause testicular toxicity. This investigation was directed to evaluate the effectiveness of Rosmarinic Acid (RA) against oxidative stress caused by UHF radiation in rats.

Methods: Forty-two male Wistar rats were divided into six groups. The control received 5 mL normal saline (0.9% NaCl) by gavage, the cell phone group received 915 MHz, the UHF waves group just received 2450 MHz, the RA/cell phone group received RA plus 915 MHz, RA/UHF waves group received RA plus 2450 MHz, and RA just received RA (20 mg/kg). After 30 days of consecutive radiation, the biochemical and histopathological parameters of their testes were measured. Statistical comparison was made using one-way ANOVA followed by Tukey’s post hoc test.

Results: Cell phone and UHF wave radiation significantly diminished the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and glutathione content (P<0.001). On the opposite, UHF significantly increased oxidative stress indices including malondialdehyde level, nitric oxide level, and protein carbonyl content (P<0.001). UHF also significantly reduced the number of Sertoli cells, spermatogonia, primary spermatocyte, epithelial height, and seminiferous tubular and luminal diameters (P<0.001). RA, as an effective antioxidant, reverses the above-mentioned harms and moderates the adverse effects of UHF on the testes of rats by significantly diminishing the oxidative stress indices and antioxidant enzyme rise and improving the histological parameters (P<0.001).

Conclusion: RA can protect the testes of rats from UHF-induced toxicity by reducing oxidative stress. RA as a food supplement might be useful for protecting humans exposed to UHF environmental contamination.


Keywords: ● Rosmarinic acid ● Testes, Oxidative stress ● UHF waves, cell phone
irradiated UHF, and the memory of animal and human models was influenced by it. Free radical and oxidative stress were produced, which have a decisive role in modulating redox responses in vitro and in vivo and assist in the production of reactive oxygen species (ROS), the principal delinquent in the degeneration of neurons. Moreover, oxidative stress can regulate the biochemical alterations leading to elderly and dysfunctions such as Parkinson's, Alzheimer's, multiple sclerosis, and amyotrophic lateral sclerosis.³

Antioxidants can be both endogenous and exogenous and may prevent complications caused by high-frequency electromagnetic radiation by performing mechanisms such as suppressing oxidative stress via neutralizing ROS and free radicals.² Several endogenous antioxidants can moderate the harmful effects of radiation, including Superoxide Dismutase (SOD), Glutathione (GSH), Glutathione Peroxidase (GPx), Malondialdehyde (MDA), Nitric Oxide (NO), Protein Carbonyl (PC), and Total Antioxidant Capacity (TAC). Sometimes first-line defense antioxidants such as SOD, CAT, GSH, and GPx cannot alone protect the body from damage caused by ROS-induced oxidative stress. Therefore, natural antioxidants as exogenous molecules, can diminish free radicals.³ Rosmarinic Acid (RA), as a polyphenol, has anti-inflammatory, anti-hepatitis, and antitumor characteristics. RA is found in rosemary, lemon balm, savory, peppermint, oregano, thyme, and sage.

RA has been identified as a strong antioxidant in some studies, besides its anti-inflammatory and anti-tumor effects.⁴ The principal goal of this project was to evaluate the radio-protective effects of RA on oxidative stress by measuring the changes in TAC level, GSH content, and the activities of antioxidant enzymes, such as SOD, CAT, and GPx, and the values of stress oxidative indicators such as MDA, NO level, and PC content in the testes of Wistar rats in the presence of 915 and 2450 MHz radiofrequency radiations.

**Materials and Methods**

**Chemicals**

RA, Bovine Serum Albumin (BSA), Phosphate Buffer Saline (PBS), GSH, 2,4-dinitrophenylhydrazine (DNPH), tri-2-pyridyls-triazine (TPTZ), trichloroacetic acid (TCA), N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD), 1,1,3,3-tetraethoxypropane (TEP), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), and the Bradford reagent were bought from Sigma Chemicals (St. Louis, Mo., USA).

**Animals**

A total of 42 male adult Wistar rats from the animal laboratory of Baqiyatallah University with weights between 200 and 250 g were prepared (seven per cage) and fed in the form of water and granules. Temperature (22±1 °C), humidity (35 to 60%), and light (12 h light/dark cycle) were set. Rats were randomly divided into six groups (table 1). Radiation groups were irradiated 60 min/day for 30 consecutive days.⁵ In this study, the dose of RA was chosen similar to other publications that have worked in this field.⁶ The code of ethics assigned to this project by Ahvaz Jundishapur University of Medical Science (AJUMS) is IR.AJUMS.ABHC.REC.1397.013. The time of administration of Normal Saline (NS) or RA was one hour before the rats were exposed to radiation.

**UHF-producing System**

The incident electromagnetic field from the Gigahertz Transversal Electromagnetic Mode (GTEM) cell was uniform over the entire biological object. The power density was 0.98 mW/cm² for 915 MHz and 0.79 mW/cm² for 2450 MHz in this project.

**Sample Collection**

One day after the last treatment, rats intraperitoneally were anesthetized with ketamine (80 mg/Kg) and xylazine (8 mg/Kg) combination.⁷ First, the testicles of the rats were separated and washed with NS, and then the rats were cut off. To perform the tissue identification analysis, the left testis tissues were fixed in Bouin’s solution and then embedded in paraffin. To perform biochemical evaluations, the right testis was used. An ice-cold Tris-HCl buffer with a concentration of 0.1 molar was used for the preparation of tissue samples.

<table>
<thead>
<tr>
<th>Table 1: Classification of the studied groups</th>
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<tbody>
<tr>
<td><strong>Groups</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Cell phone</td>
</tr>
<tr>
<td>UHF waves</td>
</tr>
<tr>
<td>RA/Cell phone</td>
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<tr>
<td>RA/UHF waves</td>
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<tr>
<td>RA</td>
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M (pH 4.7) was used to homogenize the testes (1/10 w/v) using the German device (WiseTis HG-150D, PMI-Labortechnik GmbH Company). Bradford’s method was used to measure protein homogenates and crystalline BSA.

**GSH Assay**

As previously described, the GSH content was measured. The homogenate and 100 μL of TCA (25%) were combined as well as 0.1 mL of the supernatant with 2 mL of DTNB. At the wavelength of 412 nm, the comparison was made between the standard curve and its absorbance. To express the obtained results, nmol/mg protein was reported.

**Activity of the Antioxidant Enzymes**

To determine SOD, CAT, and GPx activities (units/mg protein), a commercial kit from Zell Bio GmbH (Germany) was used.

**MDA Assay**

A composite material (0.5 mL of tissue homogenate plus 2.5 mL of 10% (w/v) TCA) was made, and the mixture was centrifuged for 10 min at 1000 g. Afterward, 2 mL of each supernatant sample was mixed with 1 mL of TBA solution (0.67%, w/v). Keeping the sample in boiling water for 30 min and cooling it at room temperature, led to the pink color of the solution. A spectrometer (Shimadzu UV-VIS 160A, Japan) was used to measure the absorbance of the sample at 532 nm. A standard curve was used to determine the tissue level of MDA (nmol/mg protein).

**NO Assay**

To determine the level of NO in the testicles, the Griess reaction was used, which included the measurement of its nitro-nitrite products. To deproteinize the homogenates, 40 μL of zinc sulfate (30% (w/v)) was mixed with 800 μL of the desired sample, and after 10 min of incubation, it was centrifuged (1000 × g, 10 min).

To measure the nitrate, 2.5 g of cadmium granules were added to the tubes, and then the tubes were incubated for 2 hours at room temperature. Then, 50 μL of Griess reagent (equal volumes of sulfanilamide 2% in H3PO4 5% and 0.2% NEDD in deionized water) was combined with 50 μL supernatant, and the mixture was incubated for 10 min at room temperature. A spectrometer was used to measure absorption at a wavelength of 540 nm.

**PC Assay**

Homogenate (0.5 mL) plus an equivalent volume of 0.1% DNPH (w/v) in 2 N hydrogen chloride were incubated at room temperature for one hour. TCA 20% was added to the output, and the supernatant was removed after centrifugation. Ethyl acetate-ethanol (1:1 volume; 0.5 mL) was used to wash the pellets and then resuspended in 1000 μL of Tris buffer as well as guanidinium chloride (8.0 M). The dissolved hydrazones were analyzed at a wavelength of 370 nm, and the concentration of protein carbonyl derivatized with 2-4-DNPH was determined using the extinction coefficient of 22000 M⁻¹cm⁻¹. It was used to express the obtained carbonyl values in nanomoles per milligram of protein.

**TAC Assay**

A cheap colorimetric way for TAC assay was the Ferric-Reducing Antioxidant Power (FRAP) method. 50 μL of the supernatant was blended with 1.5 mL of fresh FRAP reagent (25 mL of 0.3 M sodium acetate buffer, pH 3.6; 2.5 mL of 0.01 M TPTZ in 0.04 M HCl; 2.5 mL of 0.02 M FeCl3·6H2O). Then, it was incubated for five min at a temperature of 37 °C, and the absorbance was assessed at 593 nm.

**Protein Determination**

The protein of rat testis was determined by the Bradford method using bovine serum albumin as a standard solution.

**Histopathological Studies**

Testes were fixed in paraffin and cut into pieces. The pieces were stained with hematoxylin and eosin (H&E). Finally, 12 seminiferous tubule parts of stage VII-VIII for each rat were evaluated under a light microscope. With the use of Motic software, tubule diameter (from the basal lamina to the basal lamina of the other side), epithelial height, and ductal diameter were analyzed.

**Statistical Analysis**

Data were shown as mean±SD, and statistical analyses were done by version 8 of GraphPad Prism software. The Kolmogorov-Smirnov test showed that the data distribution was normal. Statistical evaluation was done by one-way ANOVA along with Tukey’s post hoc test. A P<0.05 was considered significant.

**Results**

**Reaction of RA on Antioxidant Enzymes and GSH**

The GSH and the activity of CAT, SOD, and GPx were decreased notably in the cell phone and UHF waves groups compared to the control (P<0.001). RA remarkably raised the GSH
content in RA/cell phone and RA/UHF waves compared to the cell phone and UHF waves groups, respectively (P<0.001).

RA remarkably raised the GPx in RA/cell phone and RA/UHF waves compared to the cell phone and UHF waves groups (P<0.001 and P=0.03). Treatment with RA notably elevated the SOD activity in RA/cell phone and RA/UHF waves groups compared to the cell phone and UHF waves groups (P<0.001 and P=0.02). RA notably increased the CAT activity in RA/UHF waves compared to the UHF waves group (P=0.01). Furthermore, RA alone did not change the amount of GSH and the function of CAT, SOD, and GPx compared with the control (figures 1 and 2).

**Reaction of RA on MDA and NO**

Despite significant increases in MDA and NO (P<0.001) in rats’ testes exposed to cell phone and UHF waves compared to the control, the RA/cell phone and RA/UHF waves groups showed significant decreases in MDA and NO when compared to the cell phone and UHF waves (P=0.01 and P=0.02). The levels of MDA and NO were not changed by treatment with RA alone compared to the control. Moreover, the MDA level in the UHF waves was increased compared to the cell phone (P=0.02) (figure 3).

**Reaction of RA on TAC and PC**

PC was remarkably raised and TAC was notably diminished in the cell phone and UHF waves groups compared to the controls (P<0.001). RA notably decreased PC in RA/cell phone and RA/UHF waves compared to the cell phone and UHF waves groups (P<0.001). RA administration notably reversed the TAC level. The administration of RA alone to intact rats did not alter TAC and PC compared to the control group. Besides, there was a further PC increase in the UHF waves compared to the cell phone group (P=0.02) (figure 4).

**Reaction of RA on the Light Microscopic Detections**

The histological examination of the testicular tissue indicated that the epithelial structure of the seminiferous tubules in the RA and control groups was normal. A decrease in germinal...
epithelial cells, including primary spermatocytes, spermatogonia, and Sertoli cells ($P<0.001$), and disorder in the positioning of the epithelium, were revealed in the cell phone and UHF in the seminiferous tubules compared to the control. Moreover, the epithelial height and the diameter of the duct and spermatogenic tubules in the cell phone and UHF diminished notably compared to the control ($P<0.001$).

The epithelium was eradicated, and the empty space between the cells was shown (black arrow) (figure 5). The epithelium in some tubules is separated from its main position and moves into the lumen of the spermatogenic tubules (white arrow). More desolation was observed in the UHF wave compared to the cell phone in the tissue images. In fact, the sperm tubules lost their regular shapes, the number of epithelial cells decreased drastically, and spaces between the germinal epithelial cells (black arrow) and epithelium fall into the lumen (white arrow) were indicated. Treatment with RA remarkably improved seminiferous tubular diameter ($P<0.001$), seminiferous luminal diameter ($P<0.001$), seminiferous epithelial height ($P<0.001$), number of spermatogonia ($P<0.001$), and primary spermatocyte ($P=0.03$) in the RA/cell phone and RA/UHF waves groups compared to the cell phone and UHF waves groups. In the RA/UHF group, histological images indicated that the form of the tubules was distinctly arranged, and the epithelium was largely returned to normal, but the number of epithelial cells was qualitatively diminished, and the intraepithelial spaces were evident in some places (black arrow).

Moreover, administration of RA alone to normal rats did not significantly change these indices in the testes of the control (figure 5). The evaluation data of the number of germinal epithelium cells, the diameter of the tube, as well as the height of the epithelium, are summed up in table 2.

**Discussion**

Chronic exposure of rats’ testes to cell phone and UHF waves caused a notable diminishing
Previous studies showed GPx, CAT, and SOD as antioxidants were reduced after facing mice to 2.45 GHz microwave radiation, and GSH was significantly decreased following UHF. Salah and colleagues showed that SOD was significantly reduced following exposure to UHF. Ozguner and others indicated that GPx, SOD, and CAT activities were decreased with 30 min/day rats exposure to cell phones for 90 consecutive days. It was shown that GPx and SOD levels were decreased by chronic electromagnetic field exposure. Amara and colleagues reported a decrease in the GPx, CAT, and SOD levels of testis after exposure to UHF.

RA treatment, as an exogenous antioxidant, reversed the side effects of UHF in this study, which is consistent with previous studies. Zhang and others showed that GPx, SOD, and CAT activity were increased after RA administration in the liver and kidney of rats. Fernando and colleagues showed that RA increased SOD and CAT activities by scavenging intracellular ROS induced by UVB.

Our results showed that UHF radiation
caused the enhancement of oxidative stress indices including NO and MDA levels in the testes of rats, consistent with previous research. Additionally, 945 MHz radiation (power density, 3.67 W/m²) enhanced the MDA level in the blood sample of rats. Salah and colleagues revealed that 2.45 GHz radiation, 1 hour per day, for 21 consecutive days increased the MDA level in the liver and kidney of rats. Amara and others reported an enhancement in the MDA levels of testis after exposure to UHF. On the opposite side, RA treatment of mice caused a reduction of MDA levels in the liver and kidney. RA protects dopaminergic neurons by inhibiting NO production. Moreover, the administration of 5 mg RA per day for each rat as a compensator decreased the MDA level after exposure to a 50 Hz electromagnetic field (EMF). Our results authenticated these studies that NO and MDA in rats’ testes were significantly decreased in RA/cell phone and RA/UHF waves groups in comparison with cell phone and UHF waves groups, respectively.

Under normal conditions, testes have a high TAC level due to their enzymatic and/or non-enzymatic antioxidant capacity. Thus, by decreasing the amount of TAC level in the testes of male rats infertility occurred. We showed that TAC was decreased basically in the testes of rats in confronting with cell phone and UHF radiations, and RA could compensate for this complication by increasing the amount of TAC level. Notably, MDA level and PC contents in the NS/UHF waves group were more increased than the NS/cell phone group. This means that UHF radiation is likely to be more powerful and therefore more harmful than cell phone radiation.

Our results showed that UHF causes dysfunction of the testis, which leads to impaired spermatogenic activity. Additionally, UHF decreased the number of cells of the spermatogenic lineage, the height of germinal epithelium, spermatogonia, primary spermatocytes, and Sertoli cells in male rats confronted with cell phone radiation and UHF waves for 30 days. These results are consistent with prior data that UHF radiation caused a decrease in testis weight and serious testicular emaciation with a decline of germ cells in seminiferous tubules. The tubules were condensed and mainly evacuated from germ cells. Moreover, it was shown that microwave radiation caused a decrease in the diameters of seminiferous tubules and sperm count and destruction in spermatogenesis. It has been shown that UHF decreases the size of the testicular organs and the diameter of the seminiferous tubules. A significant protective effect of RA on tissue structures of germinal epithelium, seminiferous tubules, and ductal part of tubules was observed by histopathological results.

Improving the diameter of the spermatogenic tubes may cause an elevation in the action of spermatogenesis. Khaki and others showed that low-frequency magnetic fields had negative effects on testicular histology, and these harmful effects of radiation were less in the groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Cell phone</th>
<th>P value vs. control</th>
<th>UHF waves</th>
<th>P value vs. control</th>
<th>RA/cell phone</th>
<th>P value vs. cell phone</th>
<th>RA/UHF waves</th>
<th>P value vs. UHF waves</th>
<th>RA</th>
<th>P value vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seminiferous tubular diameter (µm)</td>
<td>334±18</td>
<td>224±14*</td>
<td>&lt;0.001</td>
<td>210±11*</td>
<td>0.001</td>
<td>262±16*</td>
<td>&lt;0.001</td>
<td>231±19</td>
<td>0.07</td>
<td>340±22</td>
<td>0.1</td>
</tr>
<tr>
<td>Seminiferous luminal diameter (µm)</td>
<td>83±5</td>
<td>61±4*</td>
<td>&lt;0.001</td>
<td>53±3*</td>
<td>&lt;0.001</td>
<td>72±6*</td>
<td>&lt;0.001</td>
<td>62±4*</td>
<td>0.02</td>
<td>86±7</td>
<td>0.2</td>
</tr>
<tr>
<td>Seminiferous epithelial height (µm)</td>
<td>135±12</td>
<td>81±7*</td>
<td>&lt;0.001</td>
<td>76±5*</td>
<td>&lt;0.001</td>
<td>103±9*</td>
<td>&lt;0.001</td>
<td>98±8*</td>
<td>&lt;0.001</td>
<td>138±13</td>
<td>0.1</td>
</tr>
<tr>
<td>No. of spermatogonia / tubule</td>
<td>53±5</td>
<td>27±3*</td>
<td>&lt;0.001</td>
<td>23±2*</td>
<td>&lt;0.001</td>
<td>39±4*</td>
<td>&lt;0.001</td>
<td>36±4*</td>
<td>&lt;0.001</td>
<td>51±6</td>
<td>0.3</td>
</tr>
<tr>
<td>No. of primary spermatocyte / tubule</td>
<td>40±6</td>
<td>20±3*</td>
<td>&lt;0.001</td>
<td>16±2*</td>
<td>&lt;0.001</td>
<td>28±5*</td>
<td>0.03</td>
<td>23±4</td>
<td>0.08</td>
<td>43±6</td>
<td>0.2</td>
</tr>
<tr>
<td>No. of sertoli cell/tubule</td>
<td>13±2</td>
<td>6±1*</td>
<td>&lt;0.001</td>
<td>5±1*</td>
<td>&lt;0.001</td>
<td>9±2</td>
<td>0.06</td>
<td>8±2</td>
<td>0.1</td>
<td>14±3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The evaluation data of the number of germinal epithelium cells, the diameter of the tube, as well as the height of the epithelium, were summed up. Statistical evaluation was done by one-way ANOVA along with Tukey’s post hoc test. A P<0.05 was significant. Values are means±SD, (n=7). *P<0.05 indicates a significant difference in comparison with the control group. #P<0.05 indicates a significant difference compared with the cell phone group. †P<0.05 indicates a significant difference in comparison with the ultra-high frequency group. RA: Rosmarinic acid; UHF: Ultra-high frequency.
that received RA.\textsuperscript{30} Raisi and colleagues also showed that RA improved sperm parameters, increased antioxidant activity, and reduced histopathological damage.\textsuperscript{31} In diabetic rats, RA could treat the complication by preventing lipid peroxidation, showing that RA could inhibit damage oxidative and balance cholinergic neurotransmission in the diabetic state.\textsuperscript{6} RA as an exogenous natural antioxidant could protect the tissue damage induced by free radicals by improving the proceedings of endogenous antioxidant enzymes and also scavenging the superoxide radicals in the testes of rats. The formation of ROS is followed by the rise of lipid peroxidation. It has been shown that the MDA was significantly lower in RA-treated groups and controls, which means lipid peroxidation can be suppressed by RA via the scavenging free radicals mechanism in rats.\textsuperscript{32} Because of the incorporation of conjugated structures in the polyphenol frameworks, especially the dihydroxyphenol or catechol structure, as well as the presence of a carboxylic group, RA could prevent hydroxyl radicals.\textsuperscript{33} Besides, it was shown that in aqueous media, conjugation of a carboxylic acid group with two catechol construction caused enhancement of the antioxidant activity of RA.\textsuperscript{34} Moreover, it was shown that the antioxidant activity of RA is principal because of redox properties, which have a major responsibility in deactivating free radicals, quelling singlet and triplet oxygen, or disintegrating peroxides.\textsuperscript{35}

Attah and colleagues declared through a literature review in 2022 that exposure to high radiofrequency radiation (RFR) (≥2.45 GHz) could result in numerous health impairments. They indicated that oxidative stress and cellular damage were induced by high RFR in Wistar rats compared to unexposed groups. In this review study, no reports indicated non-harmful effects of high radiofrequency radiation on the health of rats.\textsuperscript{36} On the other hand, there has been no evidence that rosemary acid has no effect on oxidative stress parameters and antioxidant enzymes. On the contrary, Nadeem and others reported the therapeutic potential of rosemary acid on a wide range of diseases in a comprehensive review.\textsuperscript{37}

In this project, one of our limitations was not investigating the pathways of inflammation and apoptosis. Besides, one of the reasons for not evaluating other signaling pathways was financial issues.

**Conclusion**

UHF may destroy the morphological and operational properties of testes. Male infertility might be caused by exposure of rats to UHF waves and cell phone radiation via decreasing testicular antioxidant enzymes as well as increasing testicular oxidative stress indices. It was found that RA as an antioxidant could save the testes and diminish the induction of ROS by increasing antioxidant enzymes and decreasing lipid peroxidation and oxidative stress indicators. Because of the notable protective effect of RA, it can be considered a promising cure to compensate for the harmful effect of UHF on the testes of rats and improve the histological parameters.

It seems that the measurement of inflammatory factors and cell death pathways, including apoptosis, can be useful to explain the mechanism of the effect of RA on the testicles of rats against cell phones and UHF waves.

**Acknowledgment**

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**Authors’ Contribution**

J.F.A, M.G, A.M, and H.Sh: Conception and design; performing the experiments, analysis, and interpretation of data for the work, drafting and reviewing. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of Interest:** None declared.

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