

# Central Nucleus of Amygdala Mediate Pressor Response Elicited by Microinjection of Angiotensin II into the Parvocellular Paraventricular Nucleus in Rats

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Received: 07 February 2021  
Revised: 17 April 2021  
Accepted: 05 June 2021

## What's Known

- The Parvocellular portion of the hypothalamic paraventricular nucleus plays a critical role in cardiovascular control. Angiotensin II acts as a neurotransmitter playing a role in arterial pressure regulation.
- The parvocellular portion paraventricular nucleus has a bilateral connection to the central nucleus of the amygdala.
- Both of the above-mentioned areas are involved in cardiovascular control.

## What's New

- In animal models, microinjection of angiotensin II into the parvocellular portion paraventricular nucleus produced pressor response with no significant changes in the heart rate.
- Blockade of neuronal transmission of the central nucleus of the amygdala by local injection of cobalt chloride markedly attenuated pressor response of angiotensin II microinjected in the parvocellular portion paraventricular nucleus, suggesting that these two nuclei cooperate to perform their cardiovascular functions.

## Abstract

**Background:** The Paraventricular Hypothalamic Nucleus (PVN) coordinates autonomic and neuroendocrine systems to maintain homeostasis. Microinjection of angiotensin II (AngII) into the PVN has been previously shown to produce pressor and bradycardia responses. Anatomical evidence has indicated that a substantial proportion of PVN neurons is connected with the neurons in the central amygdala (CeA). The present study aimed to examine the possible contribution of the CeA in cardiovascular responses evoked by microinjection of AngII into the parvocellular portion of PVN (PVNp) before and after microinjection of cobalt chloride (CoCl<sub>2</sub>) into the CeA.

**Methods:** The experiments were conducted at the Department of Physiology of Shiraz University of Medical Sciences, from April 2019 to November 2019. There were two groups of 21 eight-week-old urethane anesthetized male rats, namely saline (n=9 rats) and AngII (n=12 rats) groups. Drugs (100 nL) were microinjected via a single-glass micropipette into the PVNp and CeA. Their blood pressure (BP) and heart rate (HR) were recorded throughout the experiments. The mean arterial pressure (MAP) and heart rate (HR) were compared to the pre-injection values using paired *t* test, and to those of the saline group using independent *t* test.

**Results:** Microinjection of AngII into the PVNp produced pressor response ( $P < 0.0001$ ) with no significant changes in HR ( $P = 0.70$ ). Blockade of CeA with CoCl<sub>2</sub> attenuated the pressor response to microinjection of AngII into the PVNp ( $P < 0.001$ ).

**Conclusion:** In the PVNp, Ang II increased the rats' blood pressure. This response was in part mediated by the CeA. Our study suggested that these two nuclei cooperate to perform their cardiovascular functions.

Please cite this article as: Rostami B, Hatam M. Central Nucleus of Amygdala Mediate Pressor Response Elicited by Microinjection of Angiotensin II into the Parvocellular Paraventricular Nucleus in Rats. *Iran J Med Sci.* 2022;47(3):272-279. doi: 10.30476/ijms.2021.90015.2080.

**Keywords** • Angiotensin II • Paraventricular hypothalamic nucleus • Amygdala • Blood pressure

## Introduction

In the brain, the Paraventricular Hypothalamic Nucleus (PVN) plays an essential role in regulating sympathetic drive, arterial blood pressure (AP), and body fluid homeostasis.<sup>1, 2</sup> The parvocellular portion of PVN (PVNp), generates an integrated

output to the rostral ventrolateral medulla (RVLM), which is the dominant brain region for tonic regulation of arterial blood pressure,<sup>3, 4</sup> and the intermediolateral (IML) cell column in the spinal cord to innervate the heart and vasculature of several organs.<sup>5</sup>

There are more than 30 neurotransmitters in the PVN.<sup>5, 6</sup> Glutamate and angiotensin II (Ang II) stimulate sympathetic outflow, whereas nitric oxide (NO) and gamma-aminobutyric acid (GABA) act as inhibitory neurotransmitters.<sup>7</sup> Experimental investigations have suggested that circulating Ang II in the bloodstream influences forebrain circumventricular organs (CVOs).<sup>8</sup> A major target of the projections arising from the CVOs neurons and activated by AngII is the PVN.<sup>9-11</sup> Additionally, neuronal cell bodies exhibiting Ang-like immunoreactivity have been observed in PVN, the nucleus of tractus solitarius (NTS), and the subfornical organ.<sup>11</sup> AT1 and AT2 receptors are expressed in parvo- and magnocellular portions of the PVN, suggesting that AngII of the PVN may act as a neurotransmitter involved in regulating arterial pressure.<sup>10, 12, 13</sup>

Microinjection of AngII into the PVN stimulates neurons resulting in an increase in blood pressure and consequently, in firing rate. These responses are mediated through AT1 and AT2 receptors of AngII.<sup>5, 10, 12, 13</sup> The amygdaloid complex has been involved in cardiovascular responses during aversive threats.<sup>4, 14, 15</sup> Anatomical study evidence has shown a substantial proportion of amygdaloid connections with paraventricular nucleus neurons.<sup>16</sup> Immunohistochemistry evidence has also implied that PVN collaterals, which project the amygdaloid complex, contain arginine-vasopressin (AVP) that establishes substantial synaptic connections with neurons in the central amygdala CeA.<sup>17</sup>

Overall, these findings have demonstrated the contribution of PVN and CeA to the cardiovascular responses and substantial connection of paraventricular nucleus neurons with the central amygdala. Amygdala possibly acts as a synaptic relay for the pressor effect produced by AngII injected into the PVN. This study was conducted to address this question through the blockade of CeA by cobalt chloride (CoCl<sub>2</sub>), while AngII was microinjected into the PVNP.

## Material and Methods

### Animals

Twenty-one male Sprague Dawley rats (eight-week-old, weighing approximately 250 g) were obtained from the animal house of Shiraz University of Medical Sciences and used in this study. The rats were housed in collective plastic

cages (four animals per cage) in temperature-controlled rooms at 24 °C with 12 hour light-dark cycles and free access to water and food. All the procedures were approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethics code for animal research: IR.SUMS.REC.1396.S1011) and conducted according to animal use and care guidelines. All the efforts were made to minimize the discomfort and the number of animals used.

### Femoral Artery and Vein Catheter Implant

General anesthesia was intraperitoneally (IP) induced with urethane (Sigma-Aldrich, United States), with an initial dose of 1.4 g/Kg. Throughout the experiment, the level of anesthesia was examined, and a supplementary dose (10% of the initial dose, IP) was injected, if it was necessary. Surgical anesthesia was affirmed, if no withdrawal reflex of a hind limb was observed. Rectal temperature was monitored and maintained at 37±1 °C with a heating pad temperature controller. A cannula was inserted into the trachea to ease the ventilation.

Polyethylene catheters (PE-50, AD instrument, Australia) filled with heparinized saline were inserted in the femoral artery for recording the blood pressure and heart rate. A second catheter was implanted into the femoral vein for drug injection. To record the arterial pressure and heart rate, the femoral artery catheter was connected to an ML T844 pressure transducer (AD instruments, Australia), coupled to a pre-amplifier (FE221 Bridge amplifier, AD instruments, Australia), and connected to a power lab 4/35 data acquisition system (model PL3504 AD instruments, Australia).

For drug microinjection into the PVNp, the head of each animal was placed in a stereotaxic frame (Stoelting, USA), and two holes were drilled above the parvocellular subdivision of the paraventricular nucleus PVNp using stereotaxic coordinates, 0.1-0.4 mm lateral to the midline, 1.4-2.0 mm caudal to bregma, and 7.9-8.6 mm ventral to the dorsal surface of the brain according to a rat brain atlas.<sup>18</sup>

### Drug Microinjections

A single glass micropipette (Stoelting, USA) was pulled to an internal diameter of approximately 35-45 µm (Narishige, Japan) for drug microinjection into the PVNp. The microinjections were performed as previously described by our lab and 100nL of the drugs was administered.<sup>19, 20</sup> The injection volume was measured by direct observation of the fluid meniscus in the micropipette utilizing an ocular micrometer that allowed a 2 nL resolution (U.W.O, Canada). All the drugs were dissolved in

saline (0.9% NaCl).

### Experimental Groups

These experiments were performed to find the neuronal connectivity between PVNp and CeA in cardiovascular responses to microinjection of AngII into the PVNp.

- The saline group: 100 nL of the saline (0.9% NaCl, n=9 rats) microinjected into the PVNp.

- The angiotensin group: AngII (200  $\mu$ M, 100 nL, Sigma, n=12 rats)<sup>21</sup> microinjected into the PVNp to find the neuronal connectivity between the PVNp and the CeA; AngII (200  $\mu$ M, 100 nL) was initially injected into the PVNp. Subsequently, after arterial pressure and HR returned to the baseline, reversible synaptic blocker CoCl<sub>2</sub> (5 mM, 100 nL, Sigma)<sup>20, 22</sup> was injected into the ipsilateral side of CeA. The PVNp was restimulated by AngII into the same site of the PVNp five minutes following the injection of CoCl<sub>2</sub> into the CeA.

### Histological Determination of the Microinjection Sites

At the end of each experiment, the micropipette was moved up and down a few times to produce a clear track. The rat was subjected to a high dose of anesthetic and perfused transcardially with 100 ml of 0.9% saline followed by 100 ml of 10% formalin. Afterward, the brain was obtained for determination of the microinjection sites, as previously described by our lab.<sup>23</sup> Frozen serial transverse sections (40  $\mu$ m) were cut and stained with cresyl violet 1% (Fulka). The injection and recording sites were verified according to a rat brain atlas Paxinos<sup>18</sup> using a light microscope.

### Statistical Analysis

The results are expressed as mean  $\pm$  SEM. Prism software, version 6.07 (Graphpad Software, La Jolla, CA, USA) was employed for statistical analysis. All the data were tested for normality using the Shapiro-Wilk test. Since all the data were normal, parametric tests were carried out. The changes in the mean arterial pressure ( $\Delta$ MAP) and in the heart rate ( $\Delta$ HR) were compared to those in the pre-injection value via paired *t* test and with saline groups using independent *t* test. A P value of less than 0.05 was considered statistically significant.

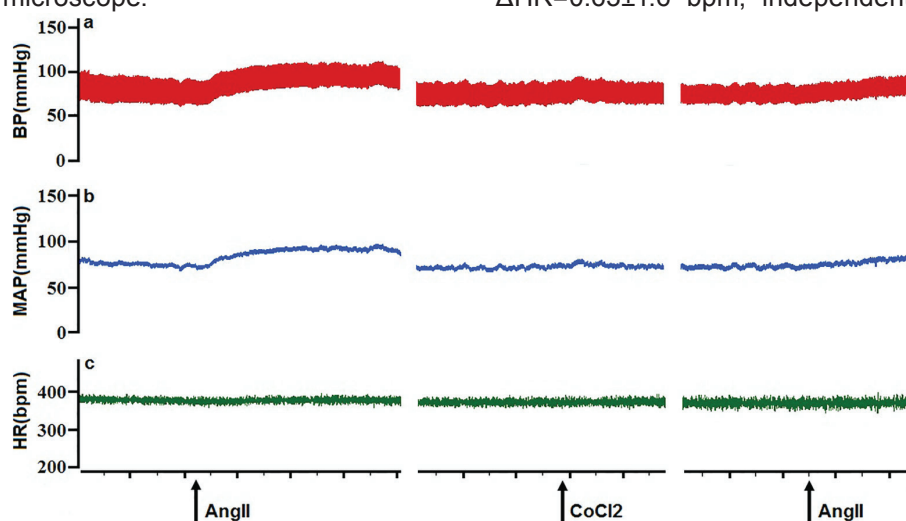
## Results

### Cardiovascular Responses to Saline Microinjection into the PVNp

In the saline group, the baseline values of MAP and HR were 91 $\pm$ 5.5 mmHg and 377 $\pm$ 4.5 beats per minute (bpm). Unilateral microinjection of 100 nL of saline did not significantly affect the MAP and HR ( $\Delta$ MAP=0.88 $\pm$ 0.4 mmHg, paired *t* test, P=0.07) or the heart rate ( $\Delta$ HR=0.5 $\pm$ 0.33 bpm, paired *t* test, P=0.17, n=9 rats).

### Cardiovascular Responses Elicited by Microinjection AngII into the PVNp before and after Microinjection of CoCl<sub>2</sub> into the CeA

In this group, the baseline values of MAP and HR were 88 $\pm$ 5.5 mmHg and 376 $\pm$ 7.5 bpm, respectively. Compared with pre-injection and the saline group, AngII (200  $\mu$ M, 100 nL) increased the baseline MAP and did not significantly affect the baseline HR ( $\Delta$ MAP=14.5 $\pm$ 1.56 mmHg, independent and paired *t* test, P<0.0001;  $\Delta$ HR=0.63 $\pm$ 1.6 bpm, independent and paired



**Figure 1:** This figure depicts the unilateral microinjection of AngII (200  $\mu$ M, 100 nL, n=12 rats) into the Parvocellular portion of PVN (PVNp) before and after blockade of the ipsilateral side of the central nucleus of the amygdala (CeA) by cobalt chloride (CoCl<sub>2</sub>, 5 mM, 100 nL). (a) AngII increased mean arterial pressure (MAP), (b) with no changes in heart rate (HR), (c) Blockade of CeA significantly attenuated pressor response to microinjection of AngII into the PVNp. The arrow shows the injection time of Ang II into the PVNp and CoCl<sub>2</sub> into CeA. a: Arterial pressure; b: Mean arterial pressure; c: Heart rate



*t* test,  $P=0.70$ ,  $n=12$  rats; figures 1 and 2).

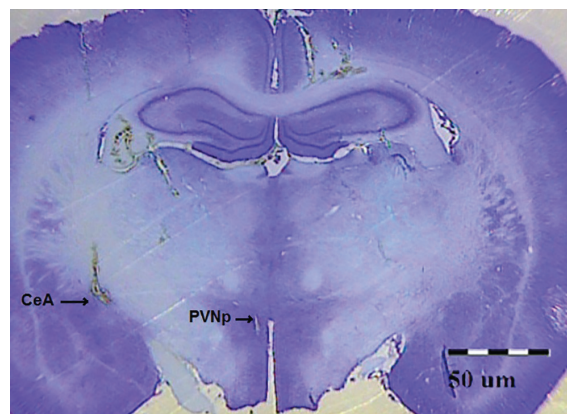
To find whether the response to AngII was mediated by CeA, AngII was initially microinjected into the PVNp. Following 30 minutes, a synaptic blocker,  $\text{CoCl}_2$  (5 mM, 100 nL), was injected into the ipsilateral side of CeA, and two to three minutes later, AngII was microinjected into the same site of the PVNp again.  $\text{CoCl}_2$  had no significant effects on the baseline MAP and HR ( $\Delta\text{MAP}=-0.4\pm0.3$  mmHg, paired *t* test,  $P=0.2$ ;  $\Delta\text{HR}=-0.6\pm0.9$  bpm, paired *t* test,  $P=0.05$ ; figures 1 and 2). Blockade of amygdala attenuated pressor response to microinjection of AngII into the PVNp and had no significant effects on HR ( $\Delta\text{MAP}$  before:  $14.5\pm1.5$  mmHg;  $\Delta\text{MAP}$  after:  $9.5\pm1.1$  mmHg, paired *t* test,  $P<0.001$ ;  $\Delta\text{HR}$  before:  $0.63\pm1.6$  bpm;  $\Delta\text{HR}$  after:  $-0.5\pm1.0$  bpm, paired *t* test,  $P=0.33$ ,  $n=12$  rats, figures 1 and 2).

In four rats, microinjection of AngII was around and outside the PVNp. We analyzed these data and none of them had a significant result (MAP before:  $75.2\pm3.4$  mmHg; MAP after:  $76\pm3.3$  mmHg, paired *t* test,  $P=0.1$ ; HR before:

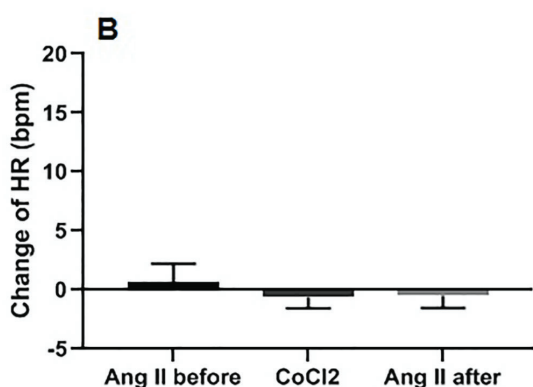
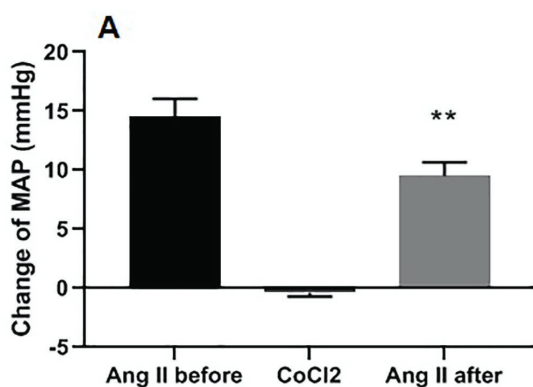
$364.2\pm12.8$  bpm; HR after:  $363.2\pm13.3$  bpm, paired *t* test,  $P=0.7$ ). These data were excluded from statistical analysis.

### Histology

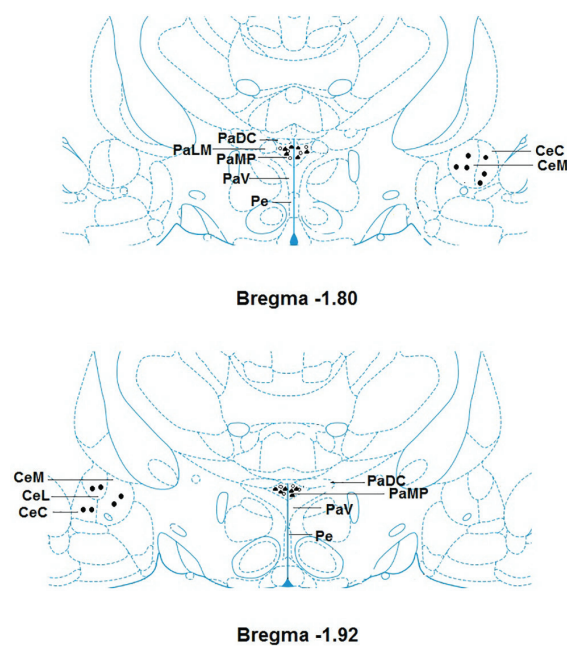
Figure 3 represents a photomicrograph with an injection site in PVNp and CeA. The distribution of the injection sites of AngII inside the PVNp and the injection sites of  $\text{CoCl}_2$  inside the central nucleus of the amygdala is illustrated in figure 4.



**Figure 3:** The injection sites are represented by arrows in the photomicrograph of the rat brain with two injection sites in parvocellular portion of PVN (PVNp) and central amygdala (CeA). The arrows show the injection sites. The line shows calibration 50 micrometer ( $\mu\text{m}$ )



**Figure 2:** This figure shows the changes in mean arterial pressure (MAP) (A) and heart rate (HR) (B) due to microinjection of AngII (200  $\mu\text{M}$ , 100 nL) into the parvocellular portion of PVN (PVNp) before and after blockade of central amygdala (CeA) by cobalt chloride ( $\text{CoCl}_2$ , 5 mM, 100 nL,  $n=12$  rats). The pressor response to AngII was significantly attenuated after microinjection of reversible synaptic blockade of CeA. Note that  $\text{CoCl}_2$  alone did not significantly affect MAP or HR (paired *t* test,  $**P<0.001$ ).



**Figure 4:** This figure demonstrates the schematic coronal section of the rat brain adapted from an atlas. The injection sites of saline (open circles) and AngII (filled triangle) in the parvocellular portion of PVN (PVNp). The injection sites of cobalt chloride ( $\text{CoCl}_2$ ) (filled squares) in the central amygdala (CeA). CeC: Central amygdaloid nucleus capsular part; CeM: Central amygdaloid nucleus, medial division; CeL: Central amygdaloid nucleus, lateral division; PaDC: PVN, dorsal cap; PaLM: PVN, lateral magnocellular part; PaMP: PVN, medial parvocellular part; PaV: Paraventricular hypothalamic ventral part; Pe: Periventricular hypothalamic nucleus

This figure, which demonstrates the schematic coronal section of the rat brain, was adapted from an atlas.<sup>18</sup> The data of the injection sites outside the PVNp or CeA were not included in the analysis.

## Discussion

In the present study, we found that microinjection of AngII into the PVNp elicited the pressor response with no significant changes in the heart rate. The pressor response was attenuated after the injection of the reversible synaptic blocker,  $\text{CoCl}_2$  into the CeA (figures 1 and 2). Anatomical evidence indicates axonal connections between the amygdala and autonomic subdivisions of the hypothalamic paraventricular nucleus, including lateral, dorsal, ventral, and medial parvocellular subnuclei.<sup>17</sup>

Microinjection of saline into the PVNp caused no significant cardiovascular responses, excluding the possibility that the responses observed after drug microinjections into the PVNp might be due to mechanical stimulation. Microinjection of AngII into the PVNp produced a pressor response and had no significant effects on the heart rate (figures 1 and 2).

This finding was in line with a previous report showing that microinjection of AngII into the PVN increases MAP with no changes in HR.<sup>24</sup> On the other hand, another study reported that AngII decreased HR, which may have been due to baroreflex responses.<sup>10</sup> We also found that the pressor response elicited by microinjection of AngII into the PVNp was attenuated after microinjection of the synaptic blocker  $\text{CoCl}_2$  into the CeA. Thus, CeA may contribute to pressor responses elicited by AngII microinjection into the PVNp.

In support of the present study, angiotensinergic receptors (for instance, AT1, AT2, and Mas receptors) and angiotensinogen and angiotensinergic terminals were identified within the medial amygdaloid nucleus (MeA),<sup>25-28</sup> and the highest level of angiotensinogen within the amygdaloid complex belonged to MeA.<sup>14</sup>

Recent evidence has reported that chronic and repeated stress increased the sympathetic tone to the heart, which was inhibited by MeA treatment with either an AT1 antagonist losartan or PD123319, an AT2 antagonist.<sup>14</sup> In addition, microinjection of PD123319 into MeA has been found to increase the reflex tachycardic response to the blood pressure decrease.<sup>14</sup> No similar studies were; so, we could not compare the results. However, the result of a paper showed that microinjection of AngII in various subnuclei of the amygdala increased the

neuronal discharge rate in rats.

The MeA and PVNp are well-known as part of the central cardiovascular control system.<sup>21</sup> Based on the results of previous studies and the results of the present study, the two possibilities could be suggested: PVN affects the amygdala through an angiotensinergic pathway between PVN and CeA. This pathway can be activated by microinjection of AngII or endogenous AngII. In support of this suggestion, it was proposed that lateral hypothalamus<sup>29, 30</sup> and central nuclei of amygdala<sup>31, 32</sup> are part of the circuitry that modulates cardiovascular responses. Anatomical studies have also shown bilateral connections between the central nucleus of the amygdala and the PVN.<sup>17</sup>

Another possibility is that AngII microinjected in PVNp increases the vasopressin release from vasopressin projecting neurons within the PVNp and affects the CeA amygdala through a vasopressinergic pathway between PVNp and CeA. Genetic, anatomic, and electrophysiological studies have shown the presence of synapses between the angiotensinergic and vasopressinergic neurons in the PVNp.<sup>33-35</sup> A previous work reported that hypothalamic vasopressinergic neurons collaterals are projecting to the amygdaloid complex and establish synaptic connections with neurons in the central amygdala CeA.<sup>16</sup> The main target for vasopressin (AVP) input to CeA are GABAergic neurons.<sup>36, 37</sup> In the CeA, V1a AVP receptor mRNA has been observed in GABAergic neurons.<sup>16</sup> Although further investigations are required for more clarification in this regard, these questions still remain unclear: which types of amygdala angiotensin receptors mediate pressor responses to microinjection of AngII in PVNp? Does endogenous PVNp angiotensin II have a similar effect?

## Conclusion

We found that AngII microinjected into the PVN produced a pressor response. These experiments revealed the first evidence concerning the contribution of CeA amygdala to microinjection of AngII pressor response into the PVNp. We suggested that these responses are mediated partly through pathways from PVNp, which end at CeA neurons, indicating that these two nuclei cooperate to perform their cardiovascular functions.

## Acknowledgment

This manuscript was derived from a part of the PhD thesis by Bahar Rostami and was sponsored

by a grant from the Vice chancellor of Research of Shiraz University of Medical Sciences (Grant number: 15236).

### Authors' Contribution

M.H: Study concept, design the study, contributed to data acquisition and analysis, interpretation of data, and critical revision of the manuscript for important intellectual content; B.R: contributed in data acquisition, data analysis and drafting the manuscript; 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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