

## ORIGINAL ARTICLE

## LOWER LEVEL OF eNOS AND C-TYPE NATRIURETIC PEPTIDE IN PATIENTS WITH ISOLATED SYSTOLIC HYPERTENSION

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**Background:** Isolated systolic hypertension (ISH) is defined as systolic blood pressure (SBP)  $\geq 140$  mmHg and diastolic blood pressure (DBP)  $< 90$  mmHg. ISH occurs as a result of arterial stiffness with major health consequences if uncontrolled. Abnormal level of endothelial nitric oxide synthase (eNOS), C-type natriuretic peptide (CNP), Brain natriuretic peptide (BNP) and cyclic guanosine monophosphate (cGMP) are expected to be associated with arterial stiffness. However, direct associations of these factors with ISH are still unclear. Changes of plasma level of these factors in ISH and hypertensive patients (both SBP and DBP are high, reflecting the level of their expression and production, therefore contributing to the pathophysiology of these diseases. **Methods:** Samples were collected from patients in Alnoor Specialist Hospital, Makkah, Saudi Arabia. ELISA was used to measure serum level of these parameters in three groups: (I) ISH patients (n=26); (II) hypertensive patients (n=12) and (III) normal subjects (n=12). **Results:** In ISH, there is decrease in serum level of eNOS ( $0.06 \pm 0.006$ ; n=32) and CNP ( $0.08 \pm 0.01$ ; n=26) as compared to normal subjects ( $p < 0.05$ ). CNP is lower in ISH as compared to hypertensive ( $0.16 \pm 0.03$ ; n=12;  $p < 0.05$ ). No changes in NT-proBNP ( $0.13 \pm 0.02$ ; n=35) as compared to normal and hypertensive groups ( $p > 0.05$ ). In hypertensives, low serum level of eNOS ( $p < 0.05$ ) were found, but not CNP ( $0.16 \pm 0.03$ ; n=12) or NT-proBNP ( $0.13 \pm 0.02$ ; n=15) as compared to normal subjects ( $p > 0.05$ ). **Conclusion:** Down-regulation of eNOS and CNP in ISH suggest their possible involvement in the pathophysiology of ISH. These findings open a new understanding of the pathophysiology of ISH and hypertension and bring hope for better treatment of these diseases.

**Keywords:** Isolated systolic hypertension, Arterial stiffness, eNOS, CNP

## INTRODUCTION

The prevalence of hypertension is increasing in Saudi Arabia and currently it affects more than one fourth of the adult Saudi population.<sup>1</sup> Isolated systolic hypertension (ISH) is the dominant form of hypertension, especially among patients 50 years or older.<sup>2</sup> It is characterised by systolic blood pressure (SBP) of  $\geq 140$  mmHg and diastolic blood pressure (DBP) of  $\leq 90$  mmHg. Recent research revealed that elevated systolic pressure is an important predictor than elevated diastolic blood pressure for cardiovascular diseases such as coronary artery disease, congestive heart failure, and stroke.<sup>3</sup>

ISH is characterised by increased arterial stiffness.<sup>4</sup> The mechanisms underlying arterial stiffness and subsequently ISH in human are not fully understood. Although, some factors have been demonstrated to be involved in arterial stiffness such as; endothelial nitric oxide synthase (eNOS),<sup>5</sup> but, the involvement of factors such as C-type natriuretic peptide (CNP),<sup>6</sup> and Brain natriuretic peptide (BNP) still need exploration to understand the pathophysiology of ISH. It is presumed that changes in the blood level of these parameters in ISH and hypertensive patients (both SBP and DBP are high) might reflect the level of their expression and production, therefore contributing to the pathophysiology of these disease statuses, which is still not clear.

The major problem nowadays is to find specific antihypertensive drugs that can be used for a selective reduction of SBP, may be because these antihypertensive medications are not targeting the actual causative factors leading to arterial stiffness and subsequently elevation in SBP.

The purpose of this study was to examine the hypothesis that the cause of ISH that occur as a result of arterial stiffness may be down-regulation in the expression and production of vasoactive factors such as eNOS, CNP, BNP and cGMP. Additionally, this study will also help to provide important link with the underlying mechanisms leading to ISH and subsequently open windows for better treatment of this common type of hypertension.

## MATERIAL AND METHODS

This study was carried out at the Department of Physiology, Faculty of Medicine, Umm Alqura University, Makkah, Saudi Arabia. Patients (aged 40–80 years) with ISH, hypertension (HTN) and normal were selected and data including age, gender, life style such as smoking, physical activity, salt intake habits and medications were obtained. The informed consent was obtained from each patient, after approval of the experimental protocol by a local institutional human research ethics committee.

Patients with diabetes, chronic inflammatory arthritis, sickle-cell anaemia and  $\beta$ -thalassemia were

excluded to avoid interference. For every patient, 3 subsequent readings were obtained for taking the average to ensure correct record of data. The informed consent was obtained from each patient, after approval of the experimental protocol by a local institutional human research ethics committee.

Blood samples were collected from Alnoor Specialist Hospital, Makkah, Saudi Arabia, and evacuated in serum separated tube (SST). The samples were kept in ice, light-protected and transported immediately to be centrifuged at 1000 rpm for 15 min. The serum was removed and immediately stored in eppendorf tubes (-86 °C) until they were tested.

The equipments used were Centrifuge (Labtech, Korea), multi-channel pipette (Nish), auto washer, micro plate reader (Biotech), vortex mixer (DNE, Taiwan), plate mixer (Kantan, Korea, -86 °C Deep freezer (-90 °C; Labtech, Korea), and 37 °C incubator (Labtech, Korea). ELISA kits for eNOS, CNP, cyclic Guanosine Monophosphate (cGMP) and N-Terminal pro-BNP were obtained from USCNLIFE, China.

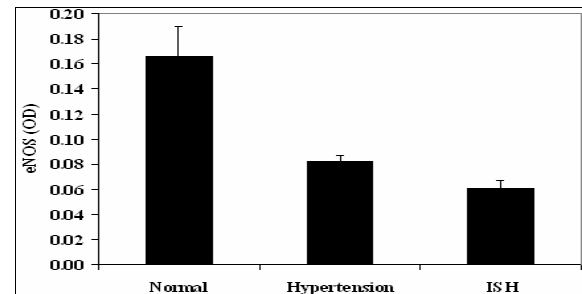
The assay procedure followed for measurement of eNOS, CNP, cGMP and NT-proBNP was this that 100 µL each of standard, control and sample were added per well in a microplate and covered with adhesive strip then incubated for 2 hours at 37 °C. After that, the liquid of each well was removed without washing and added 100 µL of detection reagent-A to each well and incubated for 1 hour at 37 °C. Then, each well was aspirated and washed. The washing process was done by filling each well with wash buffer (350 µL) using an autowasher. These aspiration and wash processes were repeated thrice. After the last wash, any remaining wash buffer was removed by aspirating or decanting through inverting the plate and blotting it against clean paper towels. This step was followed by adding 100 µL of detection reagent-B to each well and again covered with a new adhesive strip then incubated for 1 hour at 37 °C. And then, 90 µL of substrate solution was added to each well and incubated for 30 min. at room temperature and protected from light. Finally, 50 µL of stop solution was added to each well. If colour change does not appear uniformly the plate was gently taped to ensure complete mixing. The optical density (OD) of each well were then determined within 30 min. by using a microplate reader set to 450 nm.

The serum level of all the measured parameters is expressed as Mean±SEM. Comparisons between groups was made by using *t*-test, where statistical significant differences were considered when  $p < 0.05$ .

## RESULTS

### Level of Endothelial Nitric Oxide Synthase (eNOS):

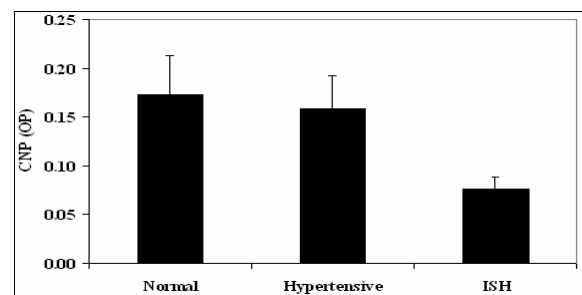
As shown in Figure-1, serum level of eNOS was 65%, significantly lower in patients with ISH ( $0.06 \pm 0.006$ ;  $n=32$ ) compared to normal subjects ( $0.17 \pm 0.02$ ;  $n=20$ ;  $p < 0.05$ ). Although, eNOS is also lower in patients with ISH as compared to hypertensive patients ( $0.08 \pm 0.004$ ;  $n=20$ ), but this difference is just significant, i.e.,  $p$  value is only slightly high (0.08). On the other hand, eNOS is 53% lower in hypertensive patients as compared to normal subjects, which is statistically significant ( $p < 0.05$ ).



**Figure-1: Comparison between the serum levels of eNOS in three groups of patients; Isolated Systolic Hypertension, Hypertensive and Normal subjects, (n=20)**

### Level of C-type Natriuretic Peptide (CNP):

As shown in Figure-2, serum level of the CNP was 53%, significantly lower in patients with ISH ( $0.08 \pm 0.01$ ;  $n=26$ ) compared to normal subjects ( $0.17 \pm 0.04$ ;  $n=12$ ;  $p < 0.05$ ). Similarly, CNP was also 50%, significantly lower in patients with ISH as compared to hypertensive patient ( $0.16 \pm 0.03$ ;  $n=12$ ;  $p < 0.05$ ). However, there is no statistical significant difference between hypertensive patients and normal subjects ( $p > 0.05$ ).



**Figure-2: Comparison between the serum levels of C-type natriuretic peptide in three groups of patients; Isolated Systolic Hypertension, Hypertensive and Normal subjects, (n=14)**

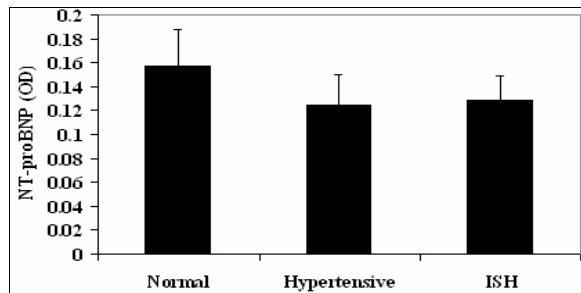
### Level of N-Terminal Pro-Brain Natriuretic Peptide (NT-proBNP):

As shown in Figure-3, serum level of NT-proBNP was non-significantly different ( $p > 0.05$ ) when compared statistically between all the three groups, i.e., ISH

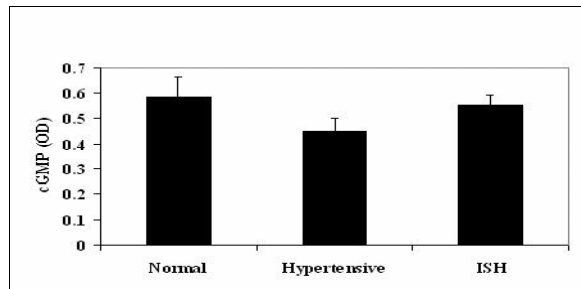
patients ( $0.13 \pm 0.02$ ;  $n=35$ ), normal subjects ( $0.16 \pm 0.03$ ;  $n=16$ ) and hypertensive patients ( $0.13 \pm 0.02$ ;  $n=15$ ).

#### Level of Cyclic Guanidine Monophosphate (cGMP):

As shown in Figure-4, serum level of cGMP is non-significantly ( $p > 0.05$ ) different statistically, when compared between all the 3 groups, i.e., ISH patients ( $0.55 \pm 0.04$ ;  $n=40$ ) normal subjects ( $0.58 \pm 0.07$ ;  $n=20$ ) and hypertensive patients ( $0.45 \pm 0.05$ ;  $n=20$ )



**Figure-3: Comparison between the serum levels of NT-proBNP in three groups of patients; Isolated Systolic Hypertension, Hypertensive and Normal subjects, (n=15)**



**Figure-4: Comparison between the serum levels of cGMP in three groups of patients; Isolated Systolic Hypertension, Hypertensive and Normal subjects, (n≥20)**

## DISCUSSION

### eNOS

Reduced levels of eNOS suggest that the synthesis of the anti-hypertensive and anti-atherogenic substance, nitric oxide (NO) is reduced. NO with its vasodilative, anti-aggregative, anti-proliferative, and anti-inflammatory action plays a crucial role in the maintenance of the physiological conditions within the cardiovascular system through cGMP-dependent and independent mechanism.

It is generally accepted that ISH is mainly due to arterial stiffness. Acute inhibition of eNOS causes aortic stiffness independent of the concomitant increase in blood pressure indicating that NO plays a role in modulating aortic compliance. In my study the level of eNOS is reduced in HTN patients and further reduced in ISH. This study also demonstrates that cGMP level is preserved in both the ISH and HTN groups like controls. However, since NO play other roles through

cGMP-independent manner, physiological role such as vascular smooth muscle hyperpolarisation<sup>7</sup> might also be affected. In this connection, further studies are required to explore this role of eNOS.

There exist important interactions between endothelin factor (ET-1) and NO pathway. ET-1 impairs NO production and down-regulates the expression of eNOS in endothelial cells.<sup>8</sup> Another data (Unpublished) demonstrates that ET-1 is up-regulated in hypertensive patients but not in ISH, this suggests that this endothelium-derived vasoconstrictor might be involved in the pathophysiological reduction of the level of eNOS in this group of patients, while in ISH, the mechanism of reduced level of eNOS in this group of patients is unlikely to involve ET-1.

Another mechanism linking ET-1 to NO pathway may be via formation of reactive oxygen species (ROS), which will result in decreased bioactivity of NO by virtue of formation of peroxynitrite. The level of eNOS is reduced in both hypertensive and even more in ISH, suggesting a reduction in the synthesis of NO. Having its important antihypertensive and anti-atherogenic effects, involvement of NO in the therapy of these cardiovascular diseases should be considered.

### CNP

The present study demonstrates that serum level of CNP is reduced in patient with ISH as compared to hypertensive patients and normal subjects. To the best of my knowledge this is the first report that shows the specific reduction of the level of CNP in this group of patients. Our results in hypertensive patients in agreement with an earlier reported study in which they found that the plasma level of CNP did not change in hypertensive patient as compared to control.<sup>9</sup>

Reduced level of CNP could contribute to various pathophysiological processes in ISH patients as several studies have shown many important physiological roles of this endothelial-derived natriuretic peptide. In vitro, CNP have been shown to inhibit the proliferation of vascular smooth muscle cells (VSMC) and endothelial cells (EC).<sup>10</sup> CNP also maintain an anti-atherogenic influence on the blood vessel wall through inhibition of leukocyte recruitment and platelet-leukocyte interactions via suppression of P-selectin expression.<sup>11</sup> The observation made in the present study, in which CNP is reduced in ISH patients suggests that ISH patient is subjected to abnormal vascular homeostasis.

In cultured rat and human aortic smooth muscle cells CNP inhibit plasminogen activator inhibitor-1 mRNA expression, in response to angiotensin-II or platelet derived growth factor (both of which induce its expression under normal circumstances).<sup>12</sup> This suggests that CNP play an important anti-atherogenic role and those patients with reduced level of CNP as observed in ISH patients are

more subject to atherosclerosis than other groups. CNP work as an endogenous inhibitor of vascular angiotensin-converting enzyme activity.<sup>13</sup> Angiotensin-II induces collagen production in culture cells suggesting enhanced vascular stiffness.<sup>14</sup> Angiotensin-II increases pulse wave velocity in healthy human indicating an increase in arterial stiffness.<sup>15,16</sup> These studies along with the findings of the present study suggest that the reduced level of CNP (as seen in ISH) might lead to over-activity of ACE and subsequently abnormal arterial wall stiffness leading to increased systolic hypertension.

CNP appears to be more rapidly hydrolysed by neutral endopeptidase than the other natriuretic peptides,<sup>17</sup> thus, endopeptidase inhibition may be a potential therapeutic intervention by enabling beneficial manipulation of natriuretic peptide levels thereby preserving the physiological role of CNP in ISH patients. In summary, our study shows that serum level of CNP is reduced in ISH patient. Considering its physiological importance in the regulation of vascular tone and inhibition of atherogenesis, further studies considering these effects should be made to explore its therapeutic benefits.

#### **BNP**

BNP can be measured either directly by measuring its level in the plasma or by measuring its N-terminal peptide, NT-proBNP, which has longer half life giving better indication of the level of BNP in the plasma. It is not known whether the level of BNP is specifically reduced or increased in ISH patients. However, as represented by its longer half life NT-proBNP, BNP appears to be unchanged in all three groups. This indicates that the synthesis of this hormone is not altered. Its normal level suggest that ISH is either not associated with changes in the level of BNP or may be the current stage of the disease in our patients did not reach the pathophysiological condition that affect its level. Several studies have indicated that advanced stages of certain cardiovascular diseases such as hypertension, congestive heart failure and myocardial ischemia are associated with pathophysiologically high level of BNP.<sup>18-21</sup> However, as the level of BNP in both groups of hypertensive patients (in the present study) is still normal, indicating that reduced level of CNP and eNOS could appear earlier in the pathophysiological stages of ISH.

#### **cGMP**

Surprisingly, although the level of eNOS is reduced, in the present study demonstrating that the level of the second messenger, cGMP, which is produced following the binding of NO to soluble guanylate cyclase (sGC), is preserved in both the HTN and ISH groups, like controls. The mechanism explaining this paradox is unclear. However, it is known that cGMP is produced

via two pathways: NO-cGMP pathway; through conversion of guanine triphosphate (GTP) into cGMP by the sGC.<sup>22,23</sup> The second pathway is through natriuretic peptides (NP)-cGMP pathway; through the conversion of GTP into cGMP by the particulate form of guanylate cyclase (pGC). The later pathway is mediated mainly by three natriuretic peptides namely, ANP, BNP and CNP.<sup>23</sup>

Several studies have found strong relationship between sGC and pGC in the regulation of the level of cGMP and its physiological roles. sGC and pGC cooperatively regulate cGMP-mediated relaxation in human and murine vascular tissues. In human vessels, the potency of ANP was increased after inhibition of endogenous NO synthesis. Aortas from eNOS knockout (KO) mice were more sensitive to ANP than tissues from wild-type (WT) animals. In aortas from WT mice, the potency of ANP was increased after pre-treatment with eNOS or sGC inhibitor.<sup>24</sup> ANP caused vasodilatation of the forearm resistance vasculature that was significantly greater in individuals lacking NO. It was concluded in another study<sup>25</sup> that a crosstalk occurs between the NO-sGC-cGMP and ANP-pGC-cGMP pathways occurs to regulate cGMP-dependent vasodilatation *in vivo* in both mice and humans. These studies suggest that when the level of cGMP is reduced through one pathway the other pathway will work to compensate for the down-regulation of the other pathway. In the present study, although eNOS is reduced, it is possible that other natriuretic peptide such as ANP and BNP acted to increase the production of cGMP to compensate for reduced synthesis of cGMP by NO-sGC-cGMP pathway.

It is unknown if this compensatory mechanism also exists within pGC pathway itself. Since CNP is also reduced and cGMP is preserved, then it is possible that the sensitivity of pGC to ANP and BNP is increased to compensate for the reduced level of CNP. It is therefore deduced from the results obtained in the present study that although the level of eNOS and CNP is reduced in ISH patients, the level of cGMP is preserved, suggesting the presence of a compensatory physiological mechanism(s) that works to normalize the level of cGMP. However, further studies are required to proof this suggestion.

#### **CONCLUSION**

Down-regulation of eNOS and CNP in ISH suggest their possible involvement in the pathophysiology of ISH. Further, the presence of compensatory mechanism(s) in the cGMP pathway does not rule out the possible defects in the cGMP-independent roles of these factors. These findings open a new window in the understanding of the pathophysiology of ISH and hypertension and bring hope for better treatment of

these diseases. Large trial studies based on medication and regulating these factors are required.

## ACKNOWLEDGEMENTS

We gratefully acknowledge that this project was supported and funded by the Institute of Research and Revival of Islamic Heritage in Umm Alqura University, Makkah, Kingdom of Saudi Arabia. Gratitude should also go to Alnoor Specialist Hospital for facilitation of this project.

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