Renal Actions of Neutral Endopeptidase Inhibition and Its Effects on Gene Expression of Atrial Natriuretic Peptide and Neutral Endopeptidase in Rats with Chronic Heart Failure

Ayman Z. Elsamanoudy¹ & Amr M Abbas²
Departments of Medical Biochemistry¹ and Medical Physiology², Faculty of Medicine, Mansoura University, Egypt

ABSTRACT

Background and Aim of work: The aim of the current study is to evaluate the effects of acute and chronic inhibition of NEP, by ONO-9902, on ANP, and NEP gene expressions, hemodynamic and renal parameters in rats with chronic heart failure (CHF) following left coronary artery ligation (CAL). Methods: The study comprised 48 male Sprague-Dawley rats (220–240 g) which were divided into sham and CAL groups. Myocardial infarction was induced by left CAL. All rats were divided into untreated and orally treated with ONO-9902 (300 mg/kg/day) from the 1st to 6th week after the operation. At the 1st and 6th weeks after the operation, gene expression of ANP and NEP, plasma ANP, cGMP, and aldosterone concentrations, urine volume, Na and ANP excretion, creatinine clearance, renal cGMP generation, body and organ weight were measured. Results: CAL led to sodium and water retention, increased plasma level of ANP and aldosterone, in addition to increase in ANP gene expression as well as decrease in renal generation of cGMP. Acute treatment of rats with CAL by NEPI, at the first week after the operation, inhibited the NEP gene expression with increased plasma ANP concentration and gene expression, which caused diuresis and natriuresis and increased renal cGMP generation. Moreover, chronic treatment by NEPI caused significant decrease in lung weight, lung body weight ratio, NEP gene expression and PAC, non significant increase in plasma concentration and gene expression of ANP, diuresis and natriuresis with increased renal cGMP generation. GFR is not significantly changed either before or after treatment. Conclusions: It is concluded that gene expression and plasma level of ANP increased in CHF. Also, chronic treatment with NEP inhibitor improves pulmonary edema and decreases Na and water retention in rats with CHF by decreasing degradative effect of NEP on ANP which leads to prolongation of its bioactivity. So, ONO-9902 may offer a new therapeutic approach in patients with CHF. Keywords: heart failure; kidney; atrial natriuretic peptide; neutral endopeptidase; aldosterone; rats.

INTRODUCTION

Chronic heart failure (CHF) is a pathophysiological condition characterized by avid sodium retention with increased cardiac volume and pressure overload, peripheral edema, activation of the renin-angiotensin-aldosterone system (RAAS), adrenergic systems, and
reduced renal function despite elevation of endogenous natriuretic peptides (atrial and brain natriuretic peptides: ANP and BNP) (1). Indeed, increased sodium retention with edema formation is a hallmark of CHF underscoring a key role of the kidney in that disorder (2).

The natriuretic peptides are a family of three genetically distinct peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (3). Although BNP was first described in the mammalian brain, ANP and BNP are mainly synthesized in the heart (4). The discovery of atrial natriuretic peptide (ANP) and subsequent studies showing its production, storage and secretion from cardiac tissues in response to increased intra-cardiac pressure have established the heart as a bonafide endocrine organ with an important role in body fluid and blood pressure homeostasis (5).

Under physiological conditions, ANP is synthesized in the atria, whereas BNP is produced by atrial and ventricular cardiomyocytes (6). The main actions of ANP and BNP include natriuresis, diuresis, and inhibition of the renin-angiotensin-aldosterone system (7). In heart failure, plasma concentrations of ANP and BNP is elevated (8). Moreover, previous studies demonstrated that cardiac ANP and BNP mRNA were induced in heart failure. In terminal human heart failure, BNP and ANP mRNA in cardiac tissue from explanted hearts were elevated in parallel (9). Both peptides become important to counteract the water and sodium retention and to decrease the peripheral vasoconstriction, which are induced in heart failure by an activated renin-angiotensin-aldosterone system and by vasopressin (10).

It has been reported that infusion of ANP or BNP decreased both preload and after load and increased cardiac output in patients with congestive heart failure whose plasma concentrations of ANP and BNP had already been high as compared with those of normal subjects (11). These findings imply that only a high level of endogenous ANP and BNP would not improve the pathophysiology of heart failure and that an additional increase in these peptides may be necessary to elicit the improvement. However, the therapeutic potential of ANP and BNP themselves is limited because these peptides are inactive in an oral administration and are short-acting after intravenous administration. Thus, an orally active analogue of drugs that can inhibit ANP degradation would be expected to be useful for the therapy of CHF (12).

ANP is inactivated rapidly in vivo. One important metabolic pathway of its inactivation involves enzymatic degradation by neutral endopeptidase (NEP); also called enkephalinase, (EC3.4.24.11) (13). NEP is a zinc-containing membrane-bound enzyme that is widely distributed in organs, particularly in the kidney and lungs (14). Since NEP plays a major role in the clearance of ANP under pathological conditions where there is a higher plasma concentration as in CHF (15), NEP inhibition is regarded as a process of maintaining the biological activity of endogenous ANP by preventing its degradation.
Therefore, inhibition of the ANP degradation by treatment with NEP inhibitor would be expected to exert beneficial effects on CHF because of amplification of the ANP action.

Endogenous opioid such as methionine-enkephalin may play an important role in regulating the processing of nociceptive information. These agents are mainly inactivated by metalloendopeptidase, enkephalinase (neutral endopeptidase). Inhibition of enkephalinase is an area of great interest for the development of new analgesics, because enkephalinase inhibitors increase the levels of endogenous enkephalins in the central nervous system. The orally administered ONO-9902 is one member of these enkephalinase inhibitors (16).

The aim of the current work was to investigate the effect of acute and long term (chronic) inhibition of NEP with the orally administered ONO-9902, an anti-nociceptive agent, on ANP, and NEP gene expressions, hemodynamic and renal parameters in rats with CHF following left coronary artery ligation (CAL).

MATERIALS & METHODS

Substances
ONO-9902,(4S)-4-{(2S)-benzyl-3-[(1RS)-1,3-dihydro-3-isobenzofuranyl-1-thio]propionylamino-4-(N-phenylcarbamoyl)-butyric acid (Sigma, St. Louis, MO, USA).

Animals
Sixty male Sprague Dawley rats weighing 220–240 g were used in the present study. They were purchased from Vaccine and Immunization Authority (Helwan, Cairo, Egypt) and housed (Animal House, Medical Physiology department, Faculty of Medicine, Mansoura University, Egypt) in standard cages in groups of four to six animals per cage under controlled conditions (temperature 23±1°C, constant humidity of 55±5%, and a 12:12 light/dark cycle). The animals were fed ad libitum and tap water. All experimental procedures of the present study were approved by the Medical Research Ethics Committee of Mansoura University, Egypt.

Heart failure following left coronary artery ligation
Myocardial infarction was produced by coronary artery ligation (CAL) according to the method described previously by Sanbe et al. (17). Briefly, the animals were anaesthetized with pentobarbital sodium (45 mg/kg, i.p.), intubated and artificially ventilated with air. The skin was incised along the left sternal border, and the fourth rib was cut proximal to the sternum. The pericardial sac was perforated, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated approximately 2 mm from its origin with a suture of 5-0 silk string. The heart was repositioned in the chest and the wound was then sutured with strings. Among the 36 animals that had undergone the operation, 8 died within 24 h and 4 within 1 week of the operation. The remaining 24 rats were used for the subsequent studies. A sham operation was also performed without CAL in 24 rats. At 1 week after the operation, both CAL rats and
sham-operated (Sham) rats were divided into NEP inhibitor (ONO-9902) -treated and untreated groups.

**Experimental protocols**

**In the first series of experiments,** acute effects of oral ONO-9902 treatment on rats with CAL or on those of sham-operated rats (Sham) at the 1\textsuperscript{st} week after the operation were examined.

**Group I: (12 rats):** included sham operated rats at the 1\textsuperscript{st} week after the operation. Those rats were further classified into:

- **Ia (6 rats):** included sham operated rats without treatment at the 1\textsuperscript{st} week after the operation.
- **Ib (6 rats):** included sham operated rats in which acute effects of ONO-9902 (single oral dose of 300 mg/kg body weight) \cite{12}, at the 1\textsuperscript{st} week after the operation, were examined.

**Group II: (12 rats):** included rats with CAL at the 1\textsuperscript{st} week after the operation. Those rats were further classified into:

- **IIa (6 rats):** included rats with CAL without treatment at the 1\textsuperscript{st} week after the operation.
- **IIb (6 rats):** included rats with CAL in which acute effects of ONO-9902 (single oral dose of 300 mg/kg body weight) at the 1\textsuperscript{st} week after the operation, were examined.

**In the second series of experiments,** chronic effects of ONO-9902 treatment on rats with CAL or Sham rats at the 6\textsuperscript{th} week after the operation were examined.

**Group III: (12 rats):** included sham operated rats at the 6\textsuperscript{th} week after the operation. Those rats were further classified into:

- **IIIa (6 rats):** included sham operated rats without treatment at the 6\textsuperscript{th} week after the operation.
- **IIIb (6 rats):** included sham operated rats in which chronic effects of ONO-9902 (300 mg/kg body weight/day from the 1\textsuperscript{st} week, orally) \cite{12}, at the 6\textsuperscript{th} week after the operation, were examined.

**Group IV: (12 rats):** included rats with CAL at the 6\textsuperscript{th} week after the operation. Those rats were further classified into:

- **IVa (6 rats):** included rats with CAL without treatment at the 6\textsuperscript{th} week after the operation.
- **IVb (6 rats):** included rats with CAL in which chronic effects of ONO-9902 (300 mg/kg body weight/day from the 1\textsuperscript{st} week, orally), at the 6\textsuperscript{th} week after the operation, were examined.

**In rats from all groups,** NEP and ANP genes expression were assayed. Also, plasma level of cGMP, ANP and aldosterone, renal parameters (GFR, urinary sodium, ANP and cGMP excretion), mean arterial pressure (MAP) and heart rate were measured.

**Sampling**

Blood samples were obtained from rat tail vein under anesthesia with diethylether and divided into two tubes: the first one containing K\textsubscript{2}EDTA, mixed well and utilized directly for RNA extraction. The second tube contains K\textsubscript{2}EDTA, mixed well, centrifuged at 7000rpm for 10 minutes to obtain plasma which was stored at -30 \textdegree C until assay of plasma ANP, cGMP and aldosterone. Another venous blood samples were withdrawn and collected into tubes containing heparin, centrifuged at 7000 rpm for 10 minutes to obtain...
heparinized plasma for plasma Na and creatinine. Urine samples were obtained in a metabolic cage in which rats were housed for 24 hours which permitted urine collection. Urine samples were collected and centrifuged for 10 minutes at 7000 rpm then refrigerated until analysis of urinary ANP, cGMP, Na and creatinine.

Biochemical parameters

Total RNA extraction from the whole blood of rats:

Total RNA extraction was carried out from rat whole blood using E.Z.N.A ® Blood RNA kit (product # R6614) provided from Omega Bio-Tek USA., Inc. following the manufacturer’s instructions. The concentration of isolated RNA was determined spectrophotometrically by measuring the optical density (OD) at 260 nm (Jenway, Genova Model, UK). 10µl of each sample was added to 990ul of DEPC treated water and quantified by measuring the absorbance at 260nm as RNA yield (µg/ml) = A260 X 40 X 100 (dilution factor) (18). The purity of RNA was determined by gel electrophoresis through agarose gel electrophoresis and ethidium bromide staining to show 2 sharp purified bands, these two bands represented 28S and 18S ribosomal RNA.

RT-PCR for extracted RNA:

RT-PCR was performed using Ready-to-Go RT-PCR beads for first cDNA synthesis and PCR reaction provided by Amersham Biosciences, England. Cat. No. 27-9266-01, according to the method of Berchtold(19). Ready-to-Go RT-PCR beads utilize Moloney Murine leukemia virus (M-MuLV) reverse transcriptase and Taq polymerase to generate PCR product from RNA template. Each bead is optimized to allow the first strand cDNA synthesis and PCR reaction to proceed sequentially as a single tube, single step reaction. The reaction passed as follow:

A) Synthesis of cDNA:

The followings were added to each tube containing the beads:
2 µl of first strand primer, provided by the kit, 3 µl containing 30 pmol of PCR gene-specific primer (sense), 3µl containing 30 pmol of PCR gene-specific primer (anti-sense), 25 µl of total template RNA containing 1ug and 17µl of DEPC-treated water to obtain a total volume of 50 µl. One tube was prepared as a negative control reaction to test for DNA contamination.

The dehydrated bead (without template and primers) was incubated at 95°C for 10 minutes to inactivate the M-MuLV reverse transcriptase. 50 µl mineral oil were added to overlay the reaction. The reactions were transferred to the thermal cycler and incubated at 40°C for 30 minutes for synthesis of cDNA followed by incubation at 95°C for 5 minutes to inactivate the reverse transcriptase and completely denature the template.

Gene specific primers used were:

Gene specific primers were purchased from Biolegio. BV, PO Box 91, 5600 AB Nijmegen, Netherlands.
B) Amplification of cDNA by PCR:
Thermal cycling reaction was performed using thermal cycler (Minicycler PTC-150 with the following program: 35 cycles consisting of three steps; Denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. An additional final extension at 72°C for 10 minutes.

C) Detection of amplified RT-PCR products:
The products was subjected to agarose gel electrophoresis using 2% agarose stained with ethidium bromide and visualized via light UV Transilluminator (Model TUV-20, OWI. Scientific, Inc. 800  242-5560) and photographed under fixed conditions (the distance, the light and the zoom).

The results photos were analyzed with scion image ® release Alpha 4.0.3.2. software for windows ® which performs bands detection and conversion to peaks. Area under each peak were calculated in square pixels and used for quantification. Gene expression levels were determined by calculating the ratio between the square pixel value of the target gene in relation to the control gene (house keeping gene).

Minus RT controls permitted to rule out genomic contamination. Similarly, no products were detected when the RT-PCR step was carried out with no added RNA, indicating that all reagents were free target sequence contamination.

Estimation of plasma and urinary ANP: ANP was measured in rat plasma and urine using AssayMax-ANP EILSA Kit (Cat No.# ER A7010-1) which employs a quantitative sandwich enzyme immune assay technique, the measurement protocol was done according to the manufacturer instruction, reading at 450 nm wave length using plate reader (Tecan, SunRise Absorbance reader).

Determination of other biochemical parameters: Sodium is measured in heparinized plasma and urine by commercial kits according to Trinder. Creatinine in plasma and urine were assayed with colorimetric kits (Spinreact, Spain; Ref: 1001111).

Glomerular filtration rate (GFR) was determined by creatinine clearance (CrCl). Plasma aldosterone concentration (PAC) was measured by an enzyme labeled immunometric assay using Immulite 2000 Kit (Diagnostic Products Corp.)

Separation of free cGMP by high-performance liquid chromatography (HPLC): Plasma and urine were extracted for 30 minutes on ice with 2.5 volumes of...
working perchloric acid (PCA) (8 ml of stock PCA 70 % solution were completed to 100 ml distilled water). The insoluble material was removed by centrifugation at 4°C and 1300 g for 10 minutes. The supernatant was collected and neutralized with 5% KOH, then centrifuged and the resulting supernatant is analyzed directly. Hewlett Packard HPLC model 1984 B equipped with variable UV detector (Hewlett Packard, 1050 series, USA) adjusted at wavelength 254 nm was used. The separation was done on reversed phase (RP 18 C Lichrosorb, Hibar, Merck, Darmstadt, Germany) column and the mobile phase was (0.1 M K₂HPO₄ /KH₂PO₄) buffer adjusted at pH 6.0 with flow rate 1 ml / minute. Standard of cGMP (Sigma, St Louis, USA) was prepared to get a final concentration of about 18 nmol/injection (20 µl) in (0.1 M K₂HPO₄ /KH₂PO₄) buffer. The standard was injected in the HPLC instrument individually to identify the retention time of each one, and then a standard mixture was injected to construct a calibration curve for all standards. An external standard calibration method was used to calculate the peaks of each nucleotide using the formula: Absolute amount of Y = area Y × response Y × DF.

Net renal generation of cGMP was determined using the formula: Net renal generation of cGMP = (urinary cGMP X urine flow rate) - (plasma cGMP X creatinine clearance) (27).

Fraction excretion of Na⁺ (FE Na⁺):

\( FE_{Na} = \frac{\text{Mass of Na excreted}}{\text{Mass of Na filtered}} \)

The fraction excretion of Na⁺ can be defined as the fraction of the total amount of Na⁺ in the glomerular filtrate that appears in the final urine.

Thus:

\( \text{Mass of Na excreted} = U_{Na} \times V \), where \( U_{Na} = \text{concentration of Na⁺ in urine (mg/ml)} \) and \( V = \text{volume of urine (ml/min)} \)

\( \text{Mass of Na filtered} = \text{GFR} \times P_{Na} \), where \( P_{Na} = \text{concentration of Na⁺ or K⁺ in plasma (mg/ml)} \)

Therefore,

\( FE_{Na} = \frac{V \times U_{Na}}{P_{Na} \times \text{GFR}} = \frac{U_{Na} \times P_{Cr}}{U_{Cr} \times P_{Na}} \)

Measurements of hemodynamic parameters

Heart rate (HR), mean arterial pressure (MAP) were measured by non-invasive method of rat’s tail cuff plethysmography using LE 5001 pressure meter (LETICA Scientific Instrument, Cornell, Barcelona, Spain) (29).

Statistical analysis

The data were expressed as mean ± standard error of mean (Mean ± SEM). Data were processed and analyzed using the Statistical Package of Social Science version 10.0 (SPSS, version 10.0). ANOVA was done followed by Fisher’s PLSD method. A minimum level of significance is considered if P is ≤0.05.
## RESULTS

Table 1: Effect of a single administration (acute treatment) of ONO-9902 on plasma ANP, NEP gene expressions, hormonal hemodynamic and renal parameters in rats with left coronary artery ligation (CAL) and of sham-operated rats (Sham) at the 1st week after the operation

<table>
<thead>
<tr>
<th></th>
<th>Sham (Untreated)</th>
<th>Sham (Treated)</th>
<th>CAL (Untreated)</th>
<th>CAL (Treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(1) plasma ANP and NEP gene expressions:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEP / GAPDH mRNA</td>
<td>0.45±0.08</td>
<td>0.27±0.04(^a)</td>
<td>0.29±0.03(^b)</td>
<td>0.14±0.01(^ab)</td>
</tr>
<tr>
<td>ANP / GAPDH mRNA</td>
<td>0.8±0.1</td>
<td>1.0±0.2</td>
<td>3.4±0.1(^b)</td>
<td>6.8±0.2(^ab)</td>
</tr>
<tr>
<td><strong>(2) Plasma concentration of ANP, PAC and cGMP:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ANP concentration (pg/ml)</td>
<td>43.7±3.1</td>
<td>47.2±4.3</td>
<td>110±5.7(^b)</td>
<td>155.8±4.8(^ab)</td>
</tr>
<tr>
<td>Plasma cGMP, pmol/mL</td>
<td>4.6±0.6</td>
<td>4.9±0.6</td>
<td>10.2±0.9(^b)</td>
<td>15.9±0.7(^ab)</td>
</tr>
<tr>
<td>PAC (pg/ml)</td>
<td>150.4±5.1</td>
<td>156.4±5.6</td>
<td>304.2±7.1(^b)</td>
<td>296.6±8.5(^b)</td>
</tr>
<tr>
<td><strong>(3) Hemodynamic parameters:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>95±7</td>
<td>91±10</td>
<td>87±5</td>
<td>82±8</td>
</tr>
<tr>
<td>HR</td>
<td>387±14</td>
<td>408±10</td>
<td>369±13</td>
<td>385±12</td>
</tr>
<tr>
<td><strong>(4) Renal parameters:</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Urine flow (µl/min)</td>
<td>25.5±4.2</td>
<td>30.2±3.9</td>
<td>8.4±1.1(^b)</td>
<td>19.4±2.4(^ab)</td>
</tr>
<tr>
<td>CrCl (mL/min)</td>
<td>1.25±0.2</td>
<td>1.2±0.2</td>
<td>0.9±0.2</td>
<td>0.95±0.2</td>
</tr>
<tr>
<td>U(_Na).(\mu)Eq/min</td>
<td>3.7±0.3</td>
<td>4.1±0.3</td>
<td>2.1±0.1(^b)</td>
<td>3.5±0.1(^a)</td>
</tr>
<tr>
<td>FE(_Na)(%)</td>
<td>0.021±0.002</td>
<td>0.025±0.003</td>
<td>0.009±0.001(^b)</td>
<td>0.018±0.002(^a)</td>
</tr>
<tr>
<td>U(_ANP).(\mu)Eq/24hours</td>
<td>88±5.2</td>
<td>95±6.1</td>
<td>97±8.7</td>
<td>193±9.1(^ab)</td>
</tr>
<tr>
<td>Renal cGMP, pmol/min</td>
<td>25.7±3.5</td>
<td>29.6±3.9</td>
<td>9.7±0.9(^b)</td>
<td>32.8±3.3(^a)</td>
</tr>
</tbody>
</table>

\(^a\): \(p<0.05\) compared to the corresponding untreated group.

\(^b\): \(p<0.05\) compared to the corresponding sham group.
Table 2: Effects of long-term treatment with ONO-9902 on changes in body and organ weights, hormonal, hemodynamic and renal parameters, in rats with left coronary artery ligation (CAL) and in sham-operated rats (Sham) at the 6th week after the operation

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>CAL</th>
<th>Sham</th>
<th>CAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>(1) Body and organ weights</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body (g)</td>
<td>329±4</td>
<td>323±4</td>
<td>286±4b</td>
<td>285±4</td>
</tr>
<tr>
<td>Lung (mg)</td>
<td>995±12</td>
<td>963±16</td>
<td>2076±65b</td>
<td>1768±96ab</td>
</tr>
<tr>
<td>Lung (mg)/body wt. (g)</td>
<td>3.50±0.68</td>
<td>2.98±0.44</td>
<td>7.28±0.1b</td>
<td>6.23±0.2ab</td>
</tr>
<tr>
<td>Heart wt (mg)</td>
<td>687.31±10</td>
<td>681.48±9</td>
<td>765.6±21b</td>
<td>740.45±23</td>
</tr>
<tr>
<td>Heart wt (mg)/body wt(g)</td>
<td>2.08 ± 0.10</td>
<td>2.1 ± 0.4</td>
<td>2.67 ± 0.14b</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>(2) Plasma NEP, ANP and BNP gene expressions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEP /GAPDH mRNA</td>
<td>0.51±0.06</td>
<td>0.25±0.04a</td>
<td>0.24±0.02b</td>
<td>0.11±0.03ab</td>
</tr>
<tr>
<td>ANP/GAPDH mRNA</td>
<td>0.65±0.1</td>
<td>0.85±0.2</td>
<td>6.5±0.4b</td>
<td>6.9±0.4b</td>
</tr>
<tr>
<td>(3) Plasma concentration of ANP, PAC and cGMP:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ANP concentration (pg/ml)</td>
<td>47.1±7.7</td>
<td>53.3±8.7</td>
<td>171.8±12.5b</td>
<td>179.8±11.8b</td>
</tr>
<tr>
<td>Plasma cGMP, pmol/ml.</td>
<td>5.1±0.6</td>
<td>5.5±0.6</td>
<td>14.2±0.9b</td>
<td>24.9±0.7ab</td>
</tr>
<tr>
<td>PAC (pg/ml)</td>
<td>156.6±5.3</td>
<td>161.2±5.8</td>
<td>390.2±8.6b</td>
<td>195.8±8.5ab</td>
</tr>
<tr>
<td>(4) Hemodynamic and cardiac parameters</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HR (beats /min)</td>
<td>385±9</td>
<td>371±6</td>
<td>377±7</td>
<td>386±7</td>
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<tr>
<td>MAP (mmHg)</td>
<td>99±4</td>
<td>93±3</td>
<td>92±4</td>
<td>100±4</td>
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<tr>
<td>(5) Renal parameters</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Urine flow (μl/min)</td>
<td>27.5±4.6</td>
<td>33.2±3.9</td>
<td>5.5±1.2b</td>
<td>22.5±2.5ab</td>
</tr>
<tr>
<td>CrCl (ml/min)</td>
<td>1.3±0.3</td>
<td>1.2±0.2</td>
<td>0.8±0.3</td>
<td>0.9±0.3</td>
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<tr>
<td>UNa.V μEq/min</td>
<td>3.8±0.3</td>
<td>4.3±0.4</td>
<td>1.0±0.1b</td>
<td>2.9±0.1ab</td>
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<tr>
<td>FENa,%</td>
<td>0.022±0.002</td>
<td>0.026±0.003</td>
<td>0.004±0.001b</td>
<td>0.02±0.002a</td>
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<tr>
<td>UANP.V (pg/24hours)</td>
<td>93±5.4</td>
<td>101±6.1</td>
<td>105±8.7</td>
<td>233±8.4a</td>
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<tr>
<td>Renal cGMP, pmol/min</td>
<td>28.8±2.1</td>
<td>32.2±2.4</td>
<td>8.5±0.7b</td>
<td>34.8±2.3a</td>
</tr>
</tbody>
</table>

a: p<0.05 compared to the corresponding untreated group.
b: p<0.05 compared to the corresponding sham group.
Fig (1) RT-PCR product of neutral endopeptidase gene expression in rats of the first series of experiments: Lane 1: The DNA marker, Lane 2: RT-PCR product of the internal control (housekeeping) gene (GAPDH gene expression), Lane 3: RT-PCR product of the NEP gene expression of Ia, Lane 4: RT-PCR product of the NEP gene expression of Ib, Lane 5: RT-PCR product of the NEP gene expression of IIa, Lane 6: RT-PCR product of the NEP gene expression of II b, Lane 7: negative control.

Fig (2) RT-PCR product of neutral endopeptidase gene expression in rats of the second series of experiments: Lane 1: The DNA marker, Lane 2: RT-PCR product of the internal control (housekeeping) gene (GAPDH gene expression), Lane 3: RT-PCR product of the NEP gene expression of IIIa, Lane 4: RT-PCR product of the NEP gene expression of III b, Lane 5: RT-PCR product of the NEP gene expression of VI a, Lane 6: RT-PCR product of the NEP gene expression of VI b, Lane 7: negative control.

Fig (3) RT-PCR product of atrial natriuretic peptide gene expression in rats of the first series of experiments: Lane 1: The DNA marker, Lane 2: RT-PCR product of the internal control (housekeeping) gene (GAPDH gene expression), Lane 3: RT-PCR product of the ANP gene expression of Ia, Lane 4: RT-PCR product of the ANP gene expression of Ib, Lane 5: RT-PCR product of the ANP gene expression of IIa, Lane 6: RT-PCR product of the ANP gene expression of II b, Lane 7: negative control.

Fig (4) RT-PCR product of atrial natriuretic peptide gene expression in rats of the second series of experiments: Lane 1: The DNA marker, Lane 2: RT-PCR product of the internal control (housekeeping) gene (GAPDH gene expression), Lane 3: RT-PCR product of the ANP gene expression of IIIa, Lane 4: RT-PCR product of the ANP gene expression of III b, Lane 5: RT-PCR product of the ANP gene expression of VI a, Lane 6: RT-PCR product of the ANP gene expression of VI b, Lane 7: negative control.

ΦX174 DNA/HindIII Marker was purchased from Fermentas Life Science, 830 Harrington Court, Ontario, Canada. The products of RT-PCR of the genes expression was detected as follow: internal control (GAPDH) produced a single band at 700 bp product, NEP produced a single band at 116 pb product and ANP produced a single band at 326 bp product.
Effects of acute treatment with NEPI on ANP, and NEP gene expressions, plasma concentration of ANP, PAC and cGMP, hemodynamic and renal parameters (table 1):

With the exception of significant reduction in NEP gene expression in sham rats received acute treatment with NEPI, all other measured parameters (ANP gene expressions, plasma level of ANP, cGMP and aldosterone levels) were non significantly changed. NEP gene expression decreased, plasma level of ANP, cGMP, and aldosterone and ANP gene expression increased significantly while heart rate and MAP were non significantly changed in CAL rats without treatment after one week relative to the corresponding sham rats. Acute treatment of CAL rats with NEPI significantly decreased NEP and increased ANP plasma level and gene expression and cGMP whereas it caused non significant change in heart rate, MAP and PAC.

In rats with CAL after one week, despite the non significant decrease in GFR, reflected by creatinine clearance, urine volume, amount and fraction of sodium excreted significantly decreased which confirmed avid sodium retention. Moreover, urinary ANP was non significantly changed whilst renal cGMP generation decreased significantly. Acute treatment of these rats with NEPI caused significant increase in urine volume, amount and fraction of sodium excreted, urinary ANP and renal cGMP generation whereas non significant change in GFR was reported.

Effects of long term treatment with NEPI on body and organ weights, ANP and NEP gene expressions, plasma concentration of ANP, PAC and cGMP, hemodynamic and renal parameters (table 2):

The body weight was decreased whereas the lung weight, lung weight/body weight, heart weight, heart weight/body weight ratio were increased by CAL. These findings suggest that the rats with CAL exhibited pulmonary edema and cardiac hypertrophy. Chronic treatment with ONO-9902 significantly attenuated the increases in lung weight and lung weight/body weight, whereas it did not affect the increase in heart weight of the rats with CAL.

All measured parameters in sham rats received long term treatment with NEPI, (ANP gene expression, plasma level of ANP, cGMP and aldosterone levels, heart rate and MAP) were non significantly changed. While plasma level and gene expressions of ANP, plasma cGMP, and PAC in CAL rats were significantly increased relative to sham rats, NEP gene expression was decreased and heart rate and MAP were non significantly changed. Long term treatment with NEPI caused a significant increase in cGMP, and decrease in PAC but non significant change in NEP mRNA, plasma level and gene expression of ANP, heart rate and MAP.

In CAL rats after 6 weeks, there was significant decrease in urine volume, amount and fraction excretion of sodium, and renal cGMP generation but non significant change in urinary ANP excretion. Despite the non significant change in GFR with
long term treatment by NEPI, urine volume, amount and fraction excretion of sodium, urinary ANP excretion, and renal cGMP generation were significantly increased.

**DISCUSSION**

The current study was designed to evaluate the effect of acute and long term (chronic) inhibition of NEP with ONO-9902 on ANP, and NEP gene expressions, hemodynamic and renal parameters in rats with CHF following left coronary artery ligation (CAL). ONO-9902 was recognized as an anti-nociceptive agent due to its ability to inhibit enkephalinase. It is an oral prodrug, which is hydrolyzed to release the active form, ONO-BB-039-02. It was used in the current study as NEP inhibitor drug.

In the present study, there is a significant decrease in NEP and an increase in ANP gene expressions in chronic heart failure (CHF) induced by CAL when compared to the sham group. Following processing from pre-prohormone to prohormone (the storage form), cleavage and secretion of mature ANP is predominantly in response to increased transmural atrial pressure or stretch, which in an intact physiological organism is mainly a consequence of volume expansion. However, several other stimuli are capable of inducing ANP release as vasopressin-, adrenomedullin-, endothelin (ET)-, angiotensin II (Ang II)-, enkephalin- and morphine-receptor stimulation as well as tumor necrosis factor-α and other cytokines. Moreover, our results demonstrated sodium and water retention with elevated plasma ANP and aldosterone levels in rats with CHF.

In the present study, a single administration of ONO-9902 resulted in the inhibition of the NEP gene expression, which was accompanied by a significant elevation of the plasma concentration and gene expression of ANP in rats with CAL. This is consistent with the effects of the NEP inhibitor ecadotril (sinorphan), a NEP inhibitor that is an orally active prodrug of (S)-thiorphan, on the plasma NEP activity and the ANP concentration in rats with heart failure produced by aortovenocaval (AV) fistula as reported by Wegner et al. In the sham rats, in vivo treatment with ONO-9902 also diminished the NEP gene expression but the plasma concentration of ANP was not increased. It is considered that the inactivation of circulating ANP is attributed to clearance receptor-mediated internalization and/or enzymatic degradation of these peptides and that NEP plays a major role in the clearance of ANP when its plasma concentration of the animals with heart failure is high. Therefore, it is conceivable that NEP inhibitors may enhance the biological activity of endogenous ANP and thereby produce the favorable effect only when the plasma ANP level is high. In the current study, the significant reduction of NEP mRNA of sham or CAL rats on treatment with NEPI, ONO-9902, indicated that ONO-9902 inhibited the NEP activity irrespective of the presence or absence of CAL.

An important finding in the present study was that long-term treatment with ONO-9902
significantly attenuated the CHF-induced increases in lung weight and lung/body weight ratio. Therefore, ONO-9902 improved the pulmonary edema of the rat with CAL. **Maki et al.**\(^{(12)}\) reported that long term treatment of CAL rats with NEPI significantly attenuated the CHF-induced increase in left ventricular end diastolic pressure (LVEDP), lung weight, lung/body weight ratio, systemic vascular resistance (SVR) and the decrease in aortic flow (AF), stroke volume index (SVI), and cardiac output index (COI). Their results suggest that chronic treatment with NEP inhibitor may prevent the CHF-induced increases in both left ventricular pre- and after-load. Since this drug did not affect MAP, the reduction in SVR may secondarily improve AF, SVI and COI in rats with CAL. These changes may be attributable, at least in part, to the NP system activation caused by the NEPI. Moreover, **Yoshida et al.**\(^{(33)}\) reported that long-term treatment with the NEP inhibitor candoxatril had no effect on the cardiac contractile function and plasma ANP level, though the renal NEP activity was inhibited by the treatment. Also, our results demonstrate that the elevation in plasma concentrations and gene expression of ANP, in rats with CAL, was further increased but non significantly by chronic treatment with NEPI. **Wegner et al.**\(^{(31)}\) reported that in rats with heart failure produced by AV fistula, long-term treatment with ecadotril, NEP inhibitor, elevated the plasma ANP concentration and improved the diminished renal function. The latter finding suggests that an increase in the plasma concentration of ANP is a prerequisite for long-lasting improvement of hemodynamic function by NEP inhibitor. AV fistular model exhibited greater preload pressure, a strong stimulant for ANP synthesis, and thereby caused a larger increase in the plasma ANP \((31)\) unlike CHF model following myocardial infarction. Thus, in the AV fistular model, the elevated preload pressure may be enough to augment ANP biosynthesis in the myocardium. In contrast, **Maki et al.**\(^{(12)}\) observed reduction in the plasma ANP concentration following chronic treatment of rats with CHF by NEPI. Moreover, as the plasma concentrations of ANP and BNP are sensitive indices for the severity of heart failure \((34)\), therefore, they consider the reduction in the plasma ANP and BNP concentrations following chronic treatment with NEPI to be as a result of the improvement of the pathophysiology of CHF.

The present study showed a significant increase in plasma aldosterone concentration (PAC), and decrease in urinary sodium excretion and urine volume in rats with CAL despite the non significant change in GFR. The marked increase in PAC, and decrease in fractional excretion of sodium with preserved GFR in untreated CHF indicates that increasing tubular sodium reabsorption was responsible for that avid sodium retention in CHF. The tubular segment primarily responsible for sodium retention in advanced CHF remains unclear, but a previous study by **Margulies and Burnett**\(^{(35)}\) suggested enhanced distal nephron sodium reabsorption in advanced
Moreover, the non significant change in urinary ANP excretion, observed in the current study, with the significant decrease in urinary cGMP, the second messenger of ANP, excretion in rats with untreated CHF suggests that renal resistance to the natriuretic action of ANP was developed in CHF. These results were in accord with previous reports (36,37). Therefore, the current study also importantly extends our understanding of the renal hyporesponsiveness to elevated concentrations of endogenous ANP in CHF. Previous studies have suggested that the attenuated natriuretic response to elevated plasma ANP in CHF may be related to ANP receptor down-regulation, enhanced degradation and clearance of ANP, altered postreceptor signal transduction, and activation of counter-regulatory neurohumoral systems such as renal nerves and the intrarenal renin angiotensin system as well as decreased renal perfusion pressure (38,39). The increase in sodium excretion in the present study in response to NEP-I, suggests that enhanced renal degradation of endogenous ANP in CHF may also contribute to the attenuated renal natriuretic response in CHF.

Previous investigations have established that NEP inhibition in experimental and human CHF results in an increase in sodium excretion, although the magnitude of natriuresis to NEP inhibition varies among reports (40). Investigations in animal models of CHF in the rat and cardiomyopathic hamster have reported that NEP inhibition may result in an exaggerated natriuresis compared with control animals (41). Indeed, such an exaggerated natriuresis to NEP inhibition has also been reported in humans with chronic renal failure (42). Moreover, other reports have shown that NEP inhibition in control animals and in severe CHF is not associated with an increase in GFR despite significant natriuretic actions (43). The current study showed that acute treatment of CAL rats with NEPI caused diuresis and natriuresis, increased plasma and urinary ANP, and renal cGMP generation while GFR was preserved. Moreover, chronic treatment of rats with CAL by NEPI significantly increased urine volume, sodium excretion, urinary ANP level, renal cGMP generation in spite of the non significant change in plasma ANP level and GFR. The mechanism of these renal diuretic and natriuretic actions most likely are linked to enhanced renal action of filtered ANP at the level of the renal tubule as urinary cGMP was significantly higher in NEPI treated CHF as compared to the untreated group in the absence of any higher level of GFR in the NEPI group. Therefore, increased urinary excretion of ANP, secondary to inhibition of renal ANP degradation by NEPI, with increased renal cGMP generation in CAL treated rats suggests that NEPI potentiates the attenuated local renal responses to ANP action which leads to a decrease in sodium reabsorption at nephron sites known to be responsive to ANP. Such local potentiation of ANP action by NEPI-I in the present study is consistent with previous studies (27, 43).

The renal tubular mechanism(s) of action of NEPI remain unclear but
is suggested by the localization of markedly high concentrations of NEP within proximal tubule brush border vesicles of the kidney\textsuperscript{(44)}. Studies have reported that the metabolic degradation of ANP by proximal tubule vesicles may be nonsaturable, thus serving to markedly limit, beyond the proximal tubule, the availability of ANP\textsuperscript{(44)}. In the absence of a significant increase in GFR, the principal renal mechanism of action of NEP-I in CHF may therefore be to decrease the degradation of ANP within proximal tubule brush border potentiating the renal action of ANP within the nephron at or beyond the proximal tubule\textsuperscript{(43)}. The natriuretic action of NEP-I includes a decrease in proximal tubule reabsorption, perhaps by inhibiting intrarenal angiotensin II concentrations in the pathophysiological state of CHF associated with activation of the renin-angiotensin-aldosterone system\textsuperscript{(45,46)}. Wong et al.\textsuperscript{(47)} have demonstrated that an increase in urinary cGMP is a marker for the renal biological action of ANP. Thus, the increase in urinary cGMP in the current study with NEP-I further supports an action of NEP-I to potentiate the renal tubular action of endogenous ANP. Kenny and Stephenson\textsuperscript{(48)} reported that NEP-I may permit, by inhibiting the degradation of ANP in the proximal tubule, pharmacological rather than physiological concentrations of intact ANP to reach more distal segments of the nephron specifically the inner medullary collecting duct\textsuperscript{(48)} where ANP receptors are abundant. Therefore, exaggerated decrease in distal fractional reabsorption was predicted based on the known presence of ANP receptors at the level of the terminal nephron\textsuperscript{(49)}. Sonnenberg et al.\textsuperscript{(50)} demonstrated that intra-luminal ANP modulates distal nephron sodium transport, thus providing further support to this concept.

In the present study, NEP-I did not cause significant changes in heart rate and arterial pressure. This attenuated vasoactive action on arterial pressure has been previously documented in both animals and humans with CHF\textsuperscript{(46)}. Thus, NEP-I in CHF has a selective renal action independent of changes in arterial pressure.

Potentiation of renal responses to ANP by chronic NEP-I does not exclude a role for other peptide modulators of renal function. Mechanisms through which NEP-I potentiates renal ANP action might also involve a contribution by other factors, notably kinins, which are degraded by NEP\textsuperscript{(51)}. In humans with advanced CHF, Munzel et al.\textsuperscript{(52)} observed marked increases in urinary excretion of the prostacyclin metabolite 6-keto-PGF-1α during natriuretic responses to NEP-I, further implicating kinins as a cofactor for ANF potentiation by NEP-I. Therefore, the altered pattern of sodium excretion produced by chronic NEP-I in the present study may be a consequence of synergistic local potentiation of ANP, kinins, and prostaglandins occurring without increases in circulating ANP.

An additional important finding is that chronic NEP-I reduced markedly the plasma aldosterone concentration confirming the results reported by
Martin et al. (53) who found, in severe experimental chronic heart failure, a marked decrease in the plasma aldosterone level with chronic oral NEPI treatment. Rademaker et al. (54), in a model of ovine pacing induced heart failure, showed a decrease in plasma aldosterone with a short term 4 day treatment period with a NEPI infused intravenously. Based upon the known high expression of the natriuretic peptide A receptor in the zona glomerulosa of the adrenal gland, one could speculate the mechanism of aldosterone suppression with chronic NEPI may involve ANP (53). This is also supported by the findings of Rebuffat et al. (55) who reported that a 7-day infusion of ANP in rats induced atrophy of the zona glomerulosa cells and also inhibited their secretory activity. It is well established that aldosterone increases tubular sodium reabsorption in inner medullary collecting duct cells which is also the site of action of ANP. Therefore, enhancing ANP presence by inhibiting its degradation in the proximal nephron where NEP is abundant may result in greater delivery to the terminal nephron, thus antagonizing the actions of aldosterone while also decreasing aldosterone release. This should result in improved handling of sodium by the kidney (53). Indeed, our studies support such a conclusion. In conclusion, there is an increase in the gene expression and plasma concentration of ANP in case of CHF. Long-term NEP inhibition (NEP-I) by ONO-9902 improves pulmonary edema and decreases Na and water retention in rats with CHF probably by deceasing degradative effect of NEP on ANP with the consequence of increased gene expression and plasma level of ANP and inhibiting aldosterone secretion. Accordingly, NEP-I in CHF acts to decrease tubular reabsorption of sodium in this sodium-retaining state, thus inducing diuresis and natriuresis. This action appears independent of changes in systemic or renal hemodynamics. Therefore, NEP-I may serve as a new therapeutic approach to enhance the renal natriuretic action of elevated endogenous ANP in patients with CHF.

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The aim of this study was to assess the effect of neutral endopeptidase (NEP) inhibition and furosemide in an ovine model of heart failure.
و قد أظهر هذا البحث أن الفشل القلبي المزمن يسبب في زيادة التعبير الجيني لجين ANP و نقص التعبير الجيني لجين NEP و زيادة مستوى cGMP في البلازما و نقص حجم البول و انزيمات الالدسترون و cGMP و زيادة وزن الرئتين بدون حدوث تغيير في حجم الرئتين و ضغط الدم و معدل ضربات القلب.

البحث يوضح أيضا أن العلاج بواسطة ONO-9902 مرة واحدة بعد أسبوع يتسبب في زيادة التعبير الجيني لجين ANP و نقص التعبير الجيني لجين NEP و زيادة مستوى cGMP في البلازما و انزيمات الالدسترون و cGMP في البول و زيادة إنتاج ANP. بالإضافة إلى ذلك، حدوث تغيير في حجم الرئتين و ضغط الدم و معدل ضربات القلب.

كما تظهر نتائج البحث أيضا أن العلاج بواسطة ONO-9902-8702 كل يوم من نهاية الأسبوع الأول حتى نهاية الأسبوع السادس يسبب في نقص وزن الرئتين و نقص التعبير الجيني لجين ANP و نقص انزيمات الالدسترون في البلازما و انزيمات الالدسترون و cGMP في البول و زيادة حجم البول و انزيمات الالدسترون و cGMP أثناء العلاج بدون حدوث تغيير في حجم الرئتين و ضغط الدم و معدل ضربات القلب.

البحث يوضح أيضا أن العلاج يزيد من زيادة دقات القلب و حجم البول في مجموعة الفئران المصابين بفشل قلبي مزمن بدون علاج.

و من نتائج هذا البحث نستنتج أن هناك زيادة في التعبير الجيني لجين ANP في حالات الفشل القلبي المزمن. كما نستنتج أن العلاج يمكن تحسين نتائج ANP و cGMP و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون و انزيمات الالدسترون...