available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Effects of V_2-receptor antagonist tolvaptan and the loop diuretic furosemide in rats with heart failure

Punniyakoti T. Veeraveedu [a], *Kenichi Watanabe* [a,*], *Meilei Ma* [a], *Suresh S. Palaniyandi* [a,d], *Ken'ichi Yamaguchi* [b], *Makoto Kodama* [c], *Yoshifusa Aizawa* [c]

[a] *Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima Akiha-ku, Niigata City, Niigata 956-8603, Japan*

[b] *Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

[c] *First Department of Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

[d] *Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305, USA*

ARTICLE INFO

Article history:
Received 25 September 2007
Accepted 26 November 2007

Keywords:
Chronic heart failure
V_2-receptor antagonism
Aquaretics
Vasopressin
Hyponatremia

ABSTRACT

Diuretics are frequently required to treat fluid retention in patients with chronic heart failure (CHF). Unfortunately, they can lead to a decline in renal function, electrolyte depletion, and neurohormonal activation. Arginine vasopressin (AVP) promotes renal water reabsorption via the V_2 receptor (V_2R) and its levels are increased in CHF. This study was conducted to characterize the diuretic effect of tolvaptan, a non-peptide AVP V_2R antagonist, and furosemide, a loop diuretic in a rat model of CHF after experimental autoimmune myocarditis. CHF was elicited in Lewis rats by immunization with porcine cardiac myosin, and 28 days after immunization rats were treated for 28 days with oral tolvaptan, and furosemide. CHF was characterized by left ventricular remodeling and impaired systolic and diastolic function. Tolvaptan produces a diuresis comparable to furosemide. Unlike tolvaptan, furosemide significantly increased urinary sodium and potassium excretion. Tolvaptan markedly elevated electrolyte-free water clearance (E-CH_2O) or aquaresis to a positive value and increased urinary AVP excretion. In contrast to tolvaptan, furosemide elevated only electrolyte clearance (E-Cosm) but not E-CH_2O. The differences in diuretic profile reflected the changes in plasma sodium and hormone levels. Tolvaptan dose dependently elevated plasma sodium concentration, but furosemide tended to decrease it. Furosemide significantly elevated plasma renin activity and aldosterone concentration. On the other hand, tolvaptan did not affect these parameters. Our results suggest that, tolvaptan have a potential medical benefit for the treatment of edematous conditions in CHF by removing excess water from the body without activating the RAAS or causing serum electrolyte imbalances.

1. Introduction

Chronic heart failure (CHF) is a clinical syndrome caused by heart disease that is characterized by abnormal sodium and water retention resulting in edema [1]. In CHF patients, diminished cardiac output activates the sympathetic nervous system and renin–angiotensin–aldosterone system (RAAS) and the non-osmotic release of arginine vasopressin (AVP).

* *Corresponding author*. Tel.: +81 250 25 5267; fax: +81 250 25 5021.
E-mail address: watanabe@nupals.ac.jp (K. Watanabe).

0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.11.011

This causes a decrease in renal blood flow and an increase in the filtration fraction resulting in an increase in water and sodium retention and consequently edema [2]. Loop diuretics are frequently required to treat fluid retention in patients with CHF. Unfortunately, they can lead to a decline in renal function, electrolyte depletion (loss of sodium and other essential electrolytes, which may exacerbate the hyponatremia), and neurohormonal activation [3]. Furthermore, despite the widespread and long history of use of diuretics in CHF, some detrimental actions of diuretics continue to emerge. Importantly, by increasing distal tubular delivery of sodium, loop diuretics activate the tubuloglomerular feedback mechanism, which causes vasoconstriction of the afferent arteriole and reduction in renal blood flow [4–6]. Thus loop diuretics may compromise renal function, which has been identified in retrospective analyses as a powerful predictor for CHF mortality [7]. Potassium wasting diuretics, which have been associated with increased mortality, can also lead to serum potassium depletion, which in turn, can promote arrhythmias [8,9]. Furthermore, serum sodium concentration is one of the best predictors of cardiovascular mortality, with hyponatremia patients showing substantially shorter survival than patients with a normal sodium concentration [10]. It is not clear whether neurohormonal activation and decreased serum sodium level are the result of more advanced heart failure or whether they contribute to the progression of mortality. This has prompted efforts to develop more physiological strategies to treat volume overload. Thus, solute-free water diuretics (aquaretics) are preferable for the treatment of edematous state associated with CHF.

Similar to other neurohormones that are activated in CHF, circulating AVP is elevated in patients with CHF [11]. The precise role of AVP in the pathophysiology of cardiovascular disease is controversial. AVP acts via three receptor types: V_{1a}, V_{1b} (V_3), and V_2. AVP regulates various physiological processes including vascular tone regulation, cardiovascular contractility and body fluid regulation through activation of V_{1a} and V_2 receptors, respectively [11]. The recent development of non-peptide orally active AVP-receptor antagonists has allowed reevaluation of the precise role of AVP in experimental animal models of hypertension [12,13] and heart failure [14,15]. Tolvaptan is a modified benzazepine derivative that was selected as a potent human V_2-receptor (V_2R) antagonist through a series of structural conversions of mozavaptan. The potent aquaretic properties of tolvaptan in rats and its pharmacological profile were reported by Yamamura et al. [16]. Tolvaptan exerts an aquaretic effect by blocking the V_2 receptors at the renal collecting ducts and thereby inhibiting water reabsorption. AVP binding studies of this agent reported a 29:1 (V_2:V_{1a}) receptor selectivity in cloned human AVP receptors and 250 times more potent to rat V_2R than to rat V_1R and produced aquaresis after single and multiple dosing in rats [16]. The first human study using this V_2R antagonist (tolvaptan) in patients with CHF was performed by Gheorghiade et al. [17]. They found that, patients with blockade of V_2 receptors had an increase in urine volume and a decrease in body weight that were maintained throughout the study. Recently several investigators have demonstrated that addition of tolvaptan to the standard therapy in patients with heart failure decreased body weight and edema, corrected hyponatremia and appeared to be well-tolerated with no adverse effects on heart rate (HR), blood pressure, electrolytes, neurohormonal activation or renal function, despite its potent aquaretic effect [18–20].

Though, the differences between aquaresis and natriuresis have been investigated acutely in normal rats and human CHF [3,21], but not been explored chronically with the same experimental procedures. Therefore, we compared the diuretic effects of tolvaptan, a non-peptide AVP V_2R antagonist and furosemide, a most commonly used loop diuretic, in rat model of myosin-induced CHF after experimental autoimmune myocarditis (EAM).

2. Materials and methods

2.1. Materials

Tolvaptan (7-chloro-5-hydroxy-1-[2-methyl-4-(2-methylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1*H*-1-benzazepine) was a gift from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), and furosemide was purchased from Wako (Osaka, Japan). Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc., Kanagawa, Japan.

2.2. Experimental design

All experiments were carried out using 8-week-old male Lewis rats and were performed in accordance with the guidelines of our institute [22]. Lewis rats were injected in the footpads with antigen-adjuvant emulsion according to the procedure described previously [22,23]. In brief, porcine cardiac myosin was dissolved in phosphate-buffered saline at 5 mg/ml and emulsified with an equal volume of complete freund's adjuvant with 11 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Lab., Detroit, MI, USA). CHF in rats was induced by immunization with 0.1 ml of emulsion once by subcutaneous injection into their rear footpads (0.1 ml to each footpad). The morbidity of EAM was 100% in rats immunized by this procedure [22,23]. Rats immunized with myosin became ill and immobile on day 14, and their activity gradually recovered beginning at the fourth week. Nine (23%) out of the 39 rats died between days 14 and 28 after immunization. All hearts from the dead rats showed extensive myocardial necrosis and pericardial effusion. Twenty-eight days after immunization, the postmyocarditis dilated cardiomyopathy (DCM) develops in the rats. The surviving 30 rats were divided into five groups and received oral administration (p.o.) of tolvaptan (3 mg/(kg day), group T3, $n = 6$; 10 mg/(kg day), group T10, $n = 6$), furosemide (30 mg/(kg day), group F30, $n = 6$; 100 mg/(kg day), group F100, $n = 6$) or vehicle (1% hydroxypropyl methyl cellulose, group V, $n = 6$) for 28 days. Age matched Lewis rats without immunization was used as normal controls. Doses of tolvaptan (3 and 10 mg/kg) and furosemide (30 and 100 mg/kg) were selected on the basis of aquaretic properties demonstrated in an earlier reports [15,21,24]. Hirano et al. [21] reported that aquaretic properties of tolvaptan at 1 and 10 mg/kg are equal to furosemide at 10 and 100 mg/kg, respectively. Moreover, very recently we have reported aquaretic properties of tolvaptan at a dose of 3 and 10 mg/kg [15]. Hence we selected

30 and 100 mg/kg of furosemide, which is equal to 3 and 10 mg/kg of tolvaptan.

2.3. Treatments and measurements

Tolvaptan and furosemide were administered orally to the groups as indicated in Table 1. Immediately after administration, the rats from groups V, T3, T10, F30 and F100 were placed individually in metabolic cages and urine samples were collected for 4 h after drug administration on days 1 and 28. The urinary volume (UV) and body weight (BW) were noted. The collected urine was used for measurement of urinary parameters (urine osmolality, sodium, potassium, creatinine and AVP). Urinary AVP excretion was determined by radioimmunoassay (RIA) using the method reported previously [25]. Urinary creatinine excretion was determined after enzymatic conversion to creatine with creatinine amidohydrolase (SRL, Tokyo, Japan). Except metabolic caging study, all the following protocol were carried out using six groups of rats indicated in Table 1.

2.4. Hemodynamic and echocardiographic studies

Rats were anesthetized with 2% halothane in O_2 and subjected to surgical procedures to measure hemodynamic parameters on day 56. After the instrumentation, the concentration of halothane was reduced to 0.5% to record steady-state hemodynamic data. Hemodynamic parameters such as mean blood pressure (MBP), peak left ventricular (LV) pressure (LVP), central venous pressure (CVP), LV end-diastolic pressure (LVEDP) and the rate of intra-ventricular pressure rise and decline (±dP/dt) were recorded as described previously [22]. Two-dimensional echocardiographic studies were performed under 0.5% halothane using an echocardiographic machine equipped with a 7.5 MHz transducer (SSD-5500, Aloka, Tokyo, Japan). M-mode tracings were recorded from the epicardial surface of the right ventricle, and the short axis view of the left ventricle was recorded to measure the LV dimension in diastole (LVDd) and LV dimension in systole (LVDs). LV fractional shortening (FS) was calculated using the following formula: (LVDd − LVDs)/LVDd × 100 (%). The study was performed in a blinded manner.

2.5. Analytical methods

After the measurement of myocardial functional analysis, blood was withdrawn for measurement of plasma osmolality, sodium, potassium, renin and aldosterone. Urine and plasma electrolytes (sodium and potassium) were measured using an electrolyte autoanalyzer (ATWill EA-06, Yokohama, Japan). Plasma and urine osmolality were determined by freezing point depression with an osmometer (Dai-ichi Kagaku OM-6040, Kyoto, Japan). Plasma renin and aldosterone were determined by standardized RIA. Plasma creatinine concentration was measured by autoanalyzer (CX-3, Beckman) using modified Jaffe method.

2.6. Histopathological analysis

After the performance of myocardial functional analyses, rats were sacrificed and the hearts were excised. The heart weight

Table 1 – Changes in hemodynamic, echocardiographic and histopathological parameters after 4 weeks of treatment with tolvaptan and furosemide in rats with heart failure

	Group N (n = 5)	Group V (n = 5)	Group T3 (n = 6)	Group T10 (n = 6)	Group F30 (n = 5)	Group F100 (n = 5)
Histopathology						
Body weight (g)	411 ± 6.9	327 ± 6.8**	324 ± 4.5**	322 ± 10.4**	328 ± 11.4**	274 ± 7.8**,##
Heart weight (g)	0.994 ± 0.01	1.234 ± 0.06*	1.331 ± 0.04**	1.259 ± 0.06**	1.285 ± 0.03**	0.977 ± 0.04##
H/B (g/kg)	2.418 ± 0.03	3.783 ± 0.23**	4.112 ± 0.16**	3.916 ± 0.13**	3.931 ± 0.14**	3.567 ± 0.11**
Area of fibrosis (%)	3.6 ± 0.6	29 ± 3.3**	20.8 ± 4.5*	20 ± 2.2*	28.8 ± 5.9**	26 ± 4.8**
Hemodynamic data						
CVP (mmHg)	1.6 ± 0.8	6.5 ± 1.4*	5.8 ± 1.0*	4.5 ± 0.6	3.7 ± 0.5	3.5 ± 0.7
MBP (mmHg)	107 ± 6.8	69.2 ± 7.6**	72.2 ± 3.4**	79 ± 4.5**	75 ± 5.2**	78 ± 2.6**
LVP (mmHg)	126 ± 6.4	81.6 ± 8.1**	85 ± 4.4**	93 ± 3.9*	92 ± 7.5*	100 ± 10.5
LVEDP (mmHg)	5.6 ± 1.3	14 ± 1.1**	12.1 ± 0.6**	10.8 ± 0.7**	12 ± 0.7**	11.5 ± 0.7**
+dP/dt (mmHg/s)	6037 ± 239	3902 ± 569**	4646 ± 316*	6004 ± 1100	5493 ± 792	6259 ± 1010
−dP/dt (mmHg/s)	4986 ± 419	2775 ± 369**	3544 ± 305*	4269 ± 894	4082 ± 695	4719 ± 773
HR (beats/min)	407 ± 18	353 ± 30	330 ± 13	350 ± 13	340 ± 20	352 ± 21
Echocardiographic data						
LVDd (mm)	6.4 ± 0.3	9.0 ± 0.8**	8.7 ± 0.3**	8.4 ± 0.3*	8.5 ± 0.2**	7.7 ± 0.3
LVDs (mm)	3.0 ± 0.3	8.0 ± 0.6**	7.4 ± 0.4**	6.5 ± 0.3**	6.9 ± 0.5**	6.1 ± 0.4**
FS (%)	55 ± 2.9	11 ± 2.7**	16 ± 3.2**	22 ± 2.1**	18 ± 4.2**	21 ± 2.5**
EF (%)	89 ± 1.8	27 ± 6.0**	38 ± 7.0**	50 ± 3.5**	41 ± 8.8**	47 ± 4.7**

Results are presented as the mean ± S.E. n, no. of rats. BW, body weight; HW, heart weight; HW/BW, ratio of heart weight to body weight; CVP, central venous pressure; MBP, mean blood pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; ±dP/dt, rate of intra-ventricular pressure rise and decline; HR, heart rate; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction; group N, aged matched untreated rats; group V, rats with heart failure treated with vehicle; groups T3 and T10, rats with heart failure treated with tolvaptan 3 and 10 mg/(kg day), respectively; groups F30 and F100, rats with heart failure treated with furosemide 30 and 100 mg/(kg day), respectively. *$P < 0.05$ and **$P < 0.01$ vs. group N; ##$P < 0.01$ vs. group V.

(HW) was measured immediately, and their ratio to BW was calculated. The excised hearts were cut into about 2 mm transverse slices and fixed in 10% formalin. After being embedded in paraffin, several transverse sections were obtained from the ventricle, and stained with the Azan-Mallory staining. The area of myocardial fibrosis was measured quantitatively using a color image analyzer (CIA-102, Olympus, Tokyo, Japan), making use of the differences in the Azan-Mallory stained color (blue fibrotic area opposed to red myocardium). The results were presented as the ratio of the fibrotic area to the whole area of myocardium [22].

2.7. Calculations

To clarify the effects of tolvaptan and furosemide, electrolyte-free water clearance (E-CH_2O), free water clearance (classic CH_2O), electrolyte clearance (E-Cosm) and osmolar clearance (classic Cosm) were calculated as described previously [21]. The formula used were

$$\text{E-CH}_2\text{O} = \text{UV} - \text{E-Cosm}$$
$$\text{E-Cosm} = \frac{(U_{\text{Na}} + U_{\text{K}})\text{UV}}{P_{\text{Na}}}$$
$$\text{classic Cosm} = \frac{\text{Uosm}}{\text{Posm}}\text{UV}$$
$$\text{classic CH}_2\text{O} = \text{UV}\left(1 - \frac{\text{Uosm}}{\text{Posm}}\right)$$

where U_{Na} is the urinary sodium concentration, U_K is the urinary potassium concentration, P_{Na} is the plasma sodium concentration, Uosm is the urinary osmolality and Posm is the plasma osmolality. We calculated E-Cosm except the values for plasma potassium concentration, because plasma potassium concentration is low enough to be negligible compared with plasma sodium concentration.

2.8. Statistical analysis

All values are expressed as means ± S.E. Statistical analysis of differences between the groups was performed by one-way ANOVA, followed by Tukey's or Bonferroni's method and two-tailed *t*-test when appropriate. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of tolvaptan and furosemide on survival rate and body weight

Tolvaptan and furosemide was orally administered once daily for 28 days. One rat died from vehicle and each of the furosemide-treated group between days 28 and 56 but none of the rats died in the tolvaptan groups. On day 0, the BW did not differ among the five groups. However, mean BW was significantly reduced ($P < 0.05$) at day 1 in rats treated with tolvaptan (10 mg/kg) in comparison to vehicle-treated CHF rats. BW was significantly decreased ($P < 0.01$) among five groups treated with tolvaptan, furosemide or vehicle in comparison to normal rats. No differences in BW gain were seen between the CHF group with or without tolvaptan treatment (Table 1) at the end of study period. However, BW was significantly decreased in group F100 in comparison to that in group V.

3.2. Aquaretic effect of multiple dosing of tolvaptan and furosemide in CHF rats

Fig. 1 shows the metabolic caging parameters such as urine volume, sodium and potassium excretion and osmolality in CHF rats. Both tolvaptan and furosemide significantly increased urine volume on days 1 and 28 in comparison to group V (Fig. 1). On chronic treatment, there were no differences in urine volume between tolvaptan- and furosemide-treated groups, at both low and high doses. However, on day 1 urine volume was significantly increased at low dose of tolvaptan in comparison to that of low dose of furosemide (Fig. 1). Furosemide significantly increased urinary sodium and potassium excretion compared with vehicle- and tolvaptan-treated rats (Fig. 1). Tolvaptan at 10 mg/kg significantly increased urinary sodium excretion, but the increase was very less compared to that in the furosemide group at the high dose, whereas there were no differences in the net urinary excretion of potassium between group V- and tolvaptan-treated rats. Tolvaptan significantly decreased urine osmolality than that in vehicle- and furosemide-treated rats. Furosemide also decreased urine osmolality compared to that of group V rats (Fig. 1). On day 28 tolvaptan treatment significantly increased urinary AVP excretion in comparison to that in groups V, F30 and F100. However, on day 1 urinary AVP excretion was also increased in group F100 compared to group V (Fig. 2). It is important to note that, in addition to the constant change in urine osmolality, the significant AVP excretion during the study period further supports the notion that repeated administration did not alter the aquaretic effect of tolvaptan (Figs. 1 and 2). There were no differences in the net urinary excretion of creatinine between the tolvaptan, furosemide and vehicle group (group T3: 4.3 ± 0.4 mg/(kg 4 h), group T10: 4.6 ± 0.2 mg/(kg 4 h), group F30: 5.2 ± 0.2 mg/(kg 4 h), group F100: 5.9 ± 0.3 mg/(kg 4 h), and group V: 4.9 ± 0.7 mg/(kg 4 h), respectively) at day 28. On day 28, high dose of tolvaptan markedly elevated E-CH_2O, classic CH_2O to a positive value (Fig. 3). In contrast, furosemide elevated E-Cosm, classic Cosm but not E-CH_2O or classic CH_2O (Fig. 3).

3.3. Histopathology

The HW and HW/BW were significantly larger in group V than in group N rats (Table 1). Chronic V_2R blockade by tolvaptan and low dose of furosemide had no effect on these parameters. High dose of furosemide reduced HW and HW/BW but the effect was significant only with HW (Table 1). The area of fibrosis was significantly larger in vehicle-treated CHF rats compared to normal rats. Both tolvaptan and furosemide tended to decrease the fibrotic area compared with that in group V rats, but the effect did not reach statistical significance. There was little or no evidence of fibrosis in the normal rats (Table 1).

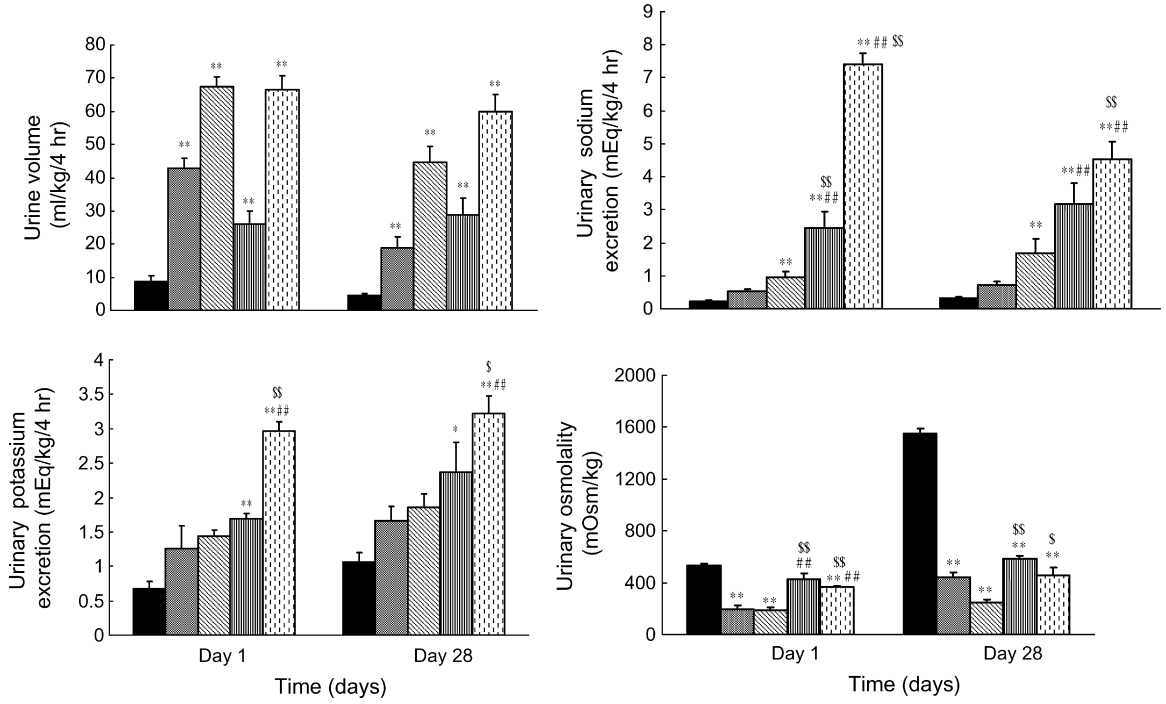

Fig. 1 – Effects of tolvaptan and furosemide on urinary parameters in CHF rats after 1 and 28 days treatment. Rats from groups V, T3, T10, F30 and F100 were placed into individual metabolic cages, and urine volume, sodium and potassium excretion and osmolality were assessed. Values are expressed as means ± S.E. (■) Vehicle-treated CHF rats; (▨) rats treated with tolvaptan 3 mg/(kg day); (▧), rats treated with tolvaptan 10 mg/(kg day); (▩) rats treated with furosemide 30 mg/(kg day); (▣) rats treated with furosemide 100 mg/(kg day). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle; $^{\#\#}P < 0.01$ vs. group T3; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ vs. group T10.

3.4. *Hemodynamic measurements*

Although HR was not different among the six groups of rats, CVP and LVEDP were significantly higher, and MBP and LVP were significantly lower in group V in comparison to group N, indicating systolic and diastolic dysfunction in vehicle-treated CHF rats. Myocardial functional parameters tended to be improved by both pharmacological interventions in a dose-dependent manner, but the effect did not attained statistical significance when compared to group V. In particular, both tolvaptan and furosemide had no significant adverse or beneficial effect on cardiac function in rats with CHF (Table 1).

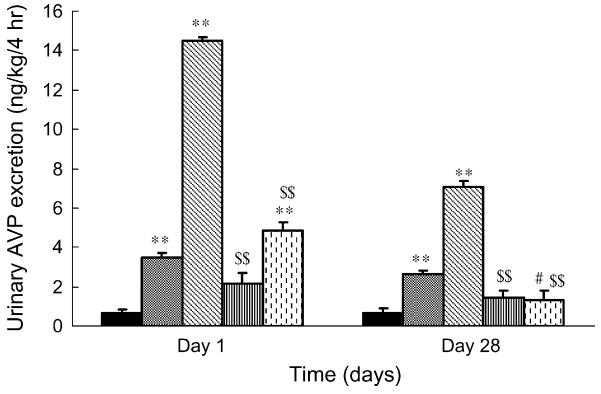

Fig. 2 – Effects of tolvaptan and furosemide on urinary AVP in CHF rats after 1 and 28 days treatment. Values are expressed as means ± S.E. (■) Vehicle-treated CHF rats; (▨) rats treated with tolvaptan 3 mg/(kg day); (▧) rats treated with tolvaptan 10 mg/(kg day); (▩) rats treated with furosemide 30 mg/(kg day); (▣) rats treated with furosemide 100 mg/(kg day). $^{**}P < 0.01$ vs. vehicle; $^{\#}P < 0.05$ vs. group T3; $^{\$\$}P < 0.01$ vs. group T10.

3.5. *Echocardiographic assessments*

Echocardiographic studies in vehicle-treated CHF rats showed evidence of left ventricular remodeling, with increased LVDd and LVDs ($P < 0.01$) and reduced FS ($P < 0.01$), indicating impaired systolic function compared with that in group N rats (Table 1). Treatment with tolvaptan and furosemide tended to increase FS and ejection fraction (EF) compared with those in CHF rats, but the effect was not significant. Moreover, treatment with tolvaptan and furosemide in CHF rats had no significant effect on cardiac geometry or function (Table 1).

3.6. *Biochemical and hormonal data*

The plasma sodium concentration and osmolality was significantly decreased in group V compared to those in group N (Table 2). The plasma sodium concentration was significantly elevated by tolvaptan in a dose-dependent manner. On the other hand, furosemide tended to decrease plasma sodium concentration. The plasma osmolality was significantly

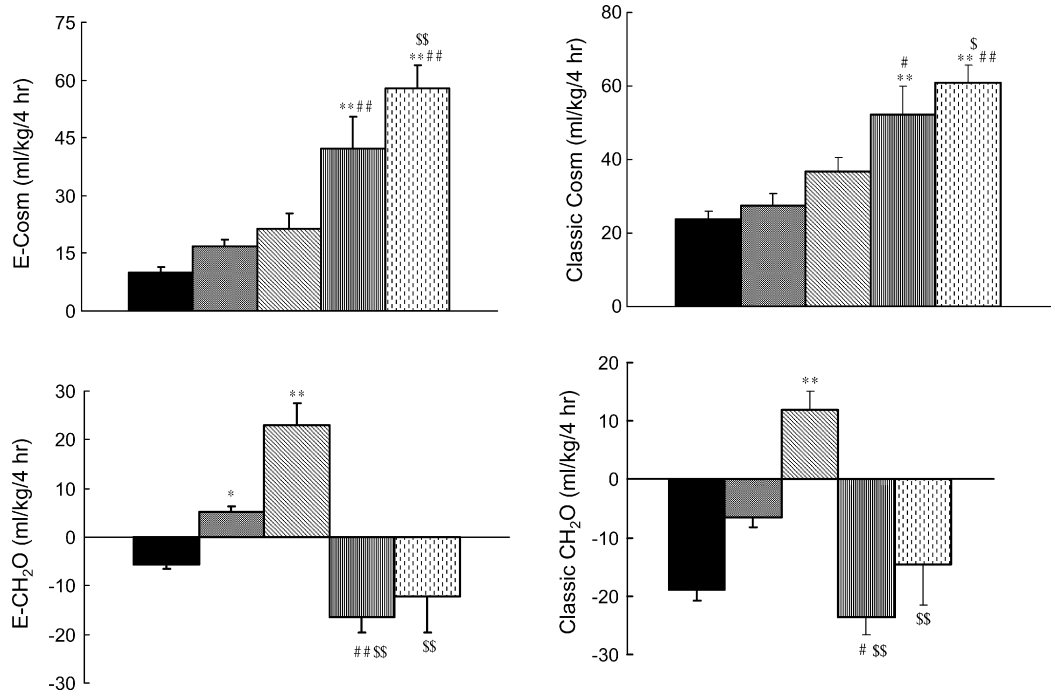

Fig. 3 – Effects of tolvaptan and furosemide on electrolyte clearance (E-Cosm), osmolar clearance (classic Cosm), electrolyte-free water clearance (E-CH_2O), and free water clearance (classic CH_2O) in CHF rats after 4 weeks treatment. Data are presented as means ± S.E. (■) Vehicle-treated CHF rats; (▨) rats treated with tolvaptan 3 mg/(kg day); (▧) rats treated with tolvaptan 10 mg/(kg day); (▩) rats treated with furosemide 30 mg/(kg day); (▣) rats treated with furosemide 100 mg/(kg day). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. group T3; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ vs. group T10.

elevated by tolvaptan and furosemide. However, there were no significant differences in plasma potassium concentration among the groups (Table 2). Furosemide significantly elevated plasma renin activity and aldosterone concentration in comparison to groups V, T3 and T10, but tolvaptan did not affect these parameters. Furosemide significantly increased plasma creatinine concentration, but tolvaptan did not (Table 2).

4. Discussion

In this study, we report the chronic effects of tolvaptan (aquaretic), and furosemide (natriuretic) on plasma parameters, such as sodium and hormone levels, in rats with CHF after EAM. These parameters are well known to be affected by extracellular fluid volume. Chronic V_2R blockade by tolvaptan increased urine volume and decreased urine osmolality in a dose-dependent manner. Tolvaptan produced a diuresis equivalent to furosemide (Fig. 1), the diuretics effects of tolvaptan at 3 and 10 mg/kg were almost equipotent to those of furosemide at 30 and 100 mg/kg, respectively, indicating that tolvaptan shows almost 10 times more potent than furosemide in CHF rats. Furthermore, whereas, furosemide was associated with an increase in potassium excretion, tolvaptan did not increase urinary potassium excretion compared with vehicle-treated CHF rats. Conversely, furosemide led to a significant natriuresis

Table 2 – Biochemical and hormonal data measured after 4 weeks of treatment with tolvaptan and furosemide in rats with heart failure

	Group N ($n = 5$)	Group V ($n = 5$)	Group T3 ($n = 6$)	Group T10 ($n = 6$)	Group F30 ($n = 5$)	Group F100 ($n = 5$)
Plasma sodium (mequiv./l)	146 ± 1.7	137 ± 1.0**	141 ± 1.1*,#	144 ± 1.1##	130 ± 0.7**,##,$$,¶¶	133 ± 1.1**,#,$$,¶¶
Plasma potassium (mequiv./l)	6.7 ± 0.5	6.9 ± 0.4	6.7 ± 0.1	6.3 ± 0.2	6.4 ± 0.4	6.0 ± 0.2
Plasma osmolality (mosmol/kg)	315 ± 3	304 ± 1.0*	322 ± 3.9##	327 ± 2.5*,##	313 ± 1.1#,¶¶	323 ± 2.1##
Plasma renin activity (ng/(ml h))	0.36 ± 0.04	0.46 ± 0.05	0.38 ± 0.08	0.37 ± 0.05	1.1 ± 0.2**,##,$$,¶¶	0.9 ± 0.1**,##,$$,¶¶
Plasma aldosterone (pg/ml)	305 ± 8	348 ± 19	363 ± 24	307 ± 16	461 ± 8**,##,$$,¶¶	495 ± 15**,##,$$,¶¶
Plasma creatinine (mg/dl)	0.09 ± 0.01	0.13 ± 0.03	0.15 ± 0.03	0.16 ± 0.04	0.26 ± 0.02**,#	0.30 ± 0.01**,##,$$,¶

Results are presented as the mean ± S.E. n, no. of rats. Group N, aged matched untreated rats; group V, rats with heart failure treated with vehicle; groups T3 and T10, rats with heart failure treated with tolvaptan 3 and 10 mg/(kg day), respectively; groups F30 and F100, rats with heart failure treated with furosemide 30 and 100 mg/(kg day), respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. group N; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. group V; $^{\$\$}P < 0.01$ vs. group T3; $^{¶}P < 0.05$ and $^{¶¶}P < 0.01$ vs. group T10.

and also increased urinary potassium excretion compared with vehicle-treated CHF rats (Fig. 1). This aquaretic versus natriuretic response was further confirmed by calculating E-CH_2O and E-Cosm. It has been suggested that E-CH_2O analysis is superior to the calculation of free water clearance (classic CH_2O) to document the role of the kidney in generating the dysnatremias, since the E-CH_2O takes into consideration the fact that urea is an ineffective osmoles [26,27] and E-CH_2O is a more correct parameter than classic CH_2O with regard to regulation of both plasma sodium and plasma osmolality, thus E-CH_2O reflected tonicity balance better than the classic CH_2O in the treatment of electrolyte-abnormal patients [26,27]. Electrolyte clearance (E-Cosm) is defined as the osmolar clearance of only effective osmoles (sodium, potassium, and their accompanying anions), except permeable osmoles that do not alter tonicity (e.g., urea). That is, E-CH_2O excludes the spurious effects of the ineffective osmoles on the measurement of free water excretion [21]. In agreement with study reported by Hirano et al. [21], tolvaptan markedly elevated E-CH_2O to a positive value in a dose-dependent manner. In contrast, furosemide elevated only E-Cosm but not E-CH_2O (Fig. 3). These results suggest that tolvaptan and furosemide exert an aquaretic and a natriuretic effect, respectively. Furthermore, urine osmolality and urinary AVP excretion (Figs. 1 and 2) showed significant changes throughout the study period with tolvaptan in comparison with vehicle-treated group, indicating that the aquaretic effect of tolvaptan was unchanged during 28 days of repeated administration [15].

The increase in electrolyte excretion induced by the high dose of tolvaptan was less than that induced by furosemide but was statistically significant. It was previously demonstrated that V_2R antagonists increased sodium excretion in rats [16,28] but not in dogs or monkeys. These results are consistent with some reports in vitro that rats and mice possess AVP-sensitive adenylate cyclase activity in the thick ascending limb of Henle's loop, but dogs and humans do not [29]. Therefore, our results suggest that tolvaptan inhibits sodium reabsorption at the AVP-sensitive segment in the thick ascending limb of Henle's loop.

The differences in the mode of diuretic actions between the aquaresis and natriuresis reflected the changes in plasma electrolytes and hormone levels (Table 2). Tolvaptan dose dependently elevated plasma sodium level. This effect is clinically important for the treatment of water-retaining diseases, such as congestive heart failure and liver cirrhosis, because dilutional hyponatremia and hypokalemia frequently develop secondary to these diseases. Conventional natriuretic can correct the sodium-retaining states but may exacerbate hyponatremia and hypokalemia. In fact, furosemide tended to decrease plasma sodium in this study. However, the plasma potassium was not changed in the furosemide-treated group. It is clinically well known that furosemide is natriuretic and can also cause potassium loss. Indeed, potassium loss is more prominent in furosemide groups than in group V. Recently, it has been reported that the use of loop diuretic might explain the poor outcomes of patients treated with non-potassium sparing diuretic described in the previously described retrospective studies [8,9]. In the present study, chronic use of a V_2R antagonist not only decreases edema but also decreases mortality rate. The impact of chronic oral use of tolvaptan on mortality is currently being tested in the Efficacy of Vasopressin Antagonism in Heart Failure: Outcome Study with Tolvaptan (EVEREST) trail [19,20].

Furosemide significantly elevated the plasma renin activity and the aldosterone level (Table 2). The activation of the RAAS induced by furosemide is consistent with previous observations in humans [3,30,31], and the mechanism has been explained by several investigators as described below. First, furosemide interacts with the Na^+–K^+–Cl^- co-transporters in the thick ascending limb of Henle's loop. Lorenz et al. [32] demonstrated that the initiating signal for the macula densa to the control of renin secretion is an inverse change in the transport rate via the luminal Na^+–K^+–Cl^- co-transporters in perfused, superfused preparations of the isolated rabbit juxtaglomerular apparatus. Thus, furosemide is believed to have accelerated renin secretion by interaction with this segment in our study. Second, furosemide stimulates renal production of prostaglandins, such as prostaglandin E_2 [33,34]. It is known that the prostaglandins are one of the modulators of renin secretion [35–37]. Furosemide, therefore, may accelerate renin secretion in part via stimulation of renal prostaglandin production. In fact, Bailie et al. [38] demonstrated that indomethacin or meclofenamate prevented rise in renin release by furosemide in dogs. Tolvaptan did not affect plasma renin activity or the aldosterone level in our study. Tolvaptan was effective in CHF rats in comparison to normal rats (data not shown), its water diuretic (aquaretic) effects were maintained over a long period, although its effects were more pronounced on the first day of treatment and the renal effects were accompanied by increases in plasma sodium and osmolality without significant changes in HR, blood pressure or plasma potassium.

In the present study, hemodynamic and echocardiographic analyses demonstrated LV remodeling with increased LVEDP, $\pm dP/dt$, LVDs, and reduced FS in vehicle-treated CHF rats in comparison to control rats, indicating impaired systolic and diastolic function of the myocardium (Table 1). It has been reported that LV dilatation plays a role in the development of CHF and that the degree of LV enlargement is adversely related to survival in post-infarction patients [39]. This is the first study to demonstrate the comparative effects of a tolvaptan and furosemide on cardiac function and survival in a rat model of CHF after EAM. Although both pharmacological interventions had no significant beneficial effects on cardiac geometry or function in CHF, it is important to note that it had no adverse effects on these parameters (Table 1). One rat died from vehicle and each of furosemide group between days 28 and 56 but none of the rats died in the tolvaptan-treated groups. Moreover, number of rats used in this study were not large enough ($n = 6$ per group). Further investigation is necessary to compare beneficial effects of tolvaptan and furosemide on survival rate.

Plasma creatinine concentration was increased significantly with furosemide (Table 2), suggesting that furosemide decreased glomerular filtration rate (GFR). GFR is traditionally considered the best overall index of renal function in health and disease [40]. Because GFR is difficult to measure in clinical practice, most clinicians estimate the GFR from serum creatinine concentration. However, the accuracy of this estimate is limited because serum creatinine concentration

is affected by factors other than creatinine filtration. To circumvent these limitations, GFR can be measured directly by clearance studies of exogenous markers, such as inulin, iohexol, iothalamate, urea and creatinine. In clinical practice, however creatinine clearance is used to measure GFR. Creatinine is an endogenous molecule, synthesized in the body, which is freely filtered by the glomerulus (but also secreted by the renal tubules in very small amounts). Creatinine clearance is therefore a close approximation of the GFR [40]. Creatinine clearance was significantly decreased in the furosemide-treated rats in comparison to group V ($P < 0.05$) (data not shown). Moreover, reduction of GFR by furosemide has been demonstrated by several investigators [4,41,42]. On the other hand, tolvaptan treatment did not affect plasma creatinine concentration in our study, but it increased urinary AVP excretion in CHF rats, the effect that was not caused by furosemide treatment. Although we did not measure plasma AVP in these animals, V_2R blockade by tolvaptan has been reported to enhance its level in patients with CHF [18,43]. As AVP is removed from the plasma mainly through renal clearance [44], our observation might have reflected the phenomena occurring in its plasma level. It could also be possible to hypothesize that tolvaptan binding to the V_2 receptors in the kidney might contribute to increase renal clearance of AVP.

Of note, CHF is a syndrome that is characterized not only by fluid retention but also by avid sodium retention. The balance of sodium and water excretion is important for the treatment of edematous states. That is, sodium excretion is surely necessary to ameliorate ascites and edema, but excessive sodium loss might exacerbate pre-existing hyponatremia, which frequently occurs in patients with heart failure or liver cirrhosis. Therefore, tolvaptan as an aquaretic likely will have to be combined with a natriuretic drug. From a clinical point of view, combination therapy is significant because a balanced excretion of sodium and water is the desirable in the treatment of volume overload. It will be especially interesting to see whether combination with aldosterone antagonists might be efficacious, as both available aldosterone antagonists have been shown to decrease mortality in clinical trials [45,46].

In conclusion, in rats with CHF, V_2R blockade by tolvaptan enhanced aquaresis, plasma osmolality and sodium without adversely affecting renal hemodynamics, urinary sodium or potassium excretion or neurohormonal systems. In contrast, furosemide markedly increased urinary electrolyte excretion, decreased plasma sodium level and increased plasma renin activity and aldosterone levels. The beneficial results of tolvaptan demonstrated in this report indicates that aquaretics may emerge as an important therapeutic option for the edematous conditions in CHF, due to its ability to remove excess water from the body without activating the RAAS or causing serum electrolyte imbalances.

Acknowledgments

We thank Gurusamy Narasimman, Rajarajan A.T., Sayaka Mito, Kunisaki Megumi and Hiroko Tanaka for their assistance in this research work. We express our sincere thank to Dr. Hitoshi Hama and Mr. Mamoru Koike (Kido Hospital, Niigata, Japan) for providing facility and helping in the determination of electrolytes and to Otsuka Pharmaceutical Co. (Tokushima, Japan) for measuring plasma aldosterone and creatinine. This research was supported by a Yujin Memorial Grant; The Ministry of Education, Science, Sports and Culture, Japan and by a grant from the Promotion and Mutual Aid Corporation for Private Schools, Japan.

REFERENCES

[1] Haslet C, Chilvers ER, Hunter JAA, Boon NA. Davidson's principles and practice of medicine, 18th ed., London: Churchill Livingstone; 1999.
[2] Abraham WT, Schrier RW. Body fluid volume regulation in health and disease. Adv Intern Med 1994;39:23–47.
[3] Costello-Boerrigter LC, Smith WB, Boerrigter G, Ouyang J, Zimmer CA, Orlandi C, et al. Vasopressin-2-receptor antagonism augments water excretion without changes in renal hemodynamics or sodium and potassium excretion in human heart failure. Am J Physiol Renal Physiol 2006;290:F273–8.
[4] Chen HH, Redfield MM, Nordstrom LJ, Cataliotti A, Burnett Jr JC. Angiotensin II AT1 receptor antagonism prevents detrimental renal actions of acute diuretic therapy in human heart failure. Am J Physiol Renal Physiol 2003;284:F1115–9.
[5] Gottlieb SS, Brater DC, Thomas I, Havranek E, Bourge R, Goldman S, et al. BG9719 (CVT-124), an A1 adenosine receptor antagonist, protects against the decline in renal function observed with diuretic therapy. Circulation 2002;105:1348–53.
[6] Vallon V. Tubuloglomerular feedback and the control of glomerular filtration rate. News Physiol Sci 2003;18:169–74.
[7] Dries DL, Exner DV, Domanski MJ, Greenberg B, Stevenson LW. The prognostic implications of renal insufficiency in asymptomatic and symptomatic patients with left ventricular systolic dysfunction. J Am Coll Cardiol 2000;35:681–9.
[8] Cooper HA, Dries DL, Davis CE, Shen YL, Domanski MJ. Diuretics and risk of arrhythmic death in patients with left ventricular dysfunction. Circulation 1999;100:1311–5.
[9] Domanski M, Norman J, Pitt B, Haigney M, Hanlon S, Peyster E. Diuretic use, progressive heart failure, and death in patients in the Studies of Left Ventricular Dysfunction (SOLVD). J Am Coll Cardiol 2003;42:705–8.
[10] Lee WH, Packer M. Prognostic importance of serum sodium concentration and its modification by converting-enzyme inhibition in patients with severe chronic heart failure. Circulation 1986;73:257–67.
[11] Lee CR, Watkins ML, Patterson JH, Gattis W, O'connor CM, Gheorghiade M, et al. Vasopressin: a new target for the treatment of heart failure. Am Heart J 2003;146:9–18.
[12] Burrell LM, Phillips PA, Risvanis J, Aldred KL, Hutchins AM, Johnston CI. Attenuation of genetic hypertension after short-term vasopressin V1A receptor antagonism. Hypertension 1995;26:828–34.
[13] Burrell LM, Phillips PA, Stephenson JM, Risvanis J, Rolls KA, Johnston CI. Blood pressure-lowering effect of an orally active vasopressin V1 receptor antagonist in mineralocorticoid hypertension in the rat. Hypertension 1994;23:737–43.
[14] Naitoh M, Suzuki H, Murakami M, Matsumoto A, Arakawa K, Ichihara A, et al. Effects of oral AVP receptor antagonists OPC-21268 and OPC-31260 on congestive heart failure in conscious dogs. Am J Physiol 1994;267:H2245–54.

[15] Veeraveedu PT, Watanabe K, Ma M, Palaniyandi SS, Yamaguchi K, Suzuki K, et al. Effects of nonpeptide vasopressin V_2 antagonist tolvaptan in rats with heart failure. Biochem Pharmacol 2007;74:1466–75.
[16] Yamamura Y, Nakamura S, Itoh S, Hirano T, Onogawa T, Yamashita T, et al. OPC-41061, a highly potent human vasopressin V2-receptor antagonist: pharmacological profile and aquaretic effect by single and multiple oral dosing in rats. J Pharmacol Exp Ther 1998;287:860–7.
[17] Gheorghiade M, Niazi I, Ouyang J, Czerwiec F, Kambayashi J, Zampino M, et al. Tolvaptan investigators. Vasopressin V2-receptor blockade with tolvaptan in patients with chronic heart failure: results from a double-blind, randomized trial. Circulation 2003;107:2690–6.
[18] Udelson JE, McGrew FA, Flores E, Ibrahim H, Katz S, Koshkarian G, et al. Multicenter, randomized, double-blind, placebo-controlled study on the effect of oral tolvaptan on left ventricular dilation and function in patients with heart failure and systolic dysfunction. J Am Coll Cardiol 2007;49:2151–9.
[19] Konstam MA, Gheorghiade M, Burnett JC, Grinfeld L, Maggioni AP, Swedberg K, et al. Effects of oral tolvaptan in patients hospitalized for worsening heart failure: the EVEREST Outcome Trial. J Am Med Assoc 2007;297:1319–31.
[20] Gheorghiade M, Konstam MA, Burnett Jr JC, Grinfeld L, Maggioni AP, Swedberg K, et al. Short-term clinical effects of tolvaptan, an oral vasopressin antagonist, in patients hospitalized for heart failure: the EVEREST Clinical Status Trials. J Am Med Assoc 2007;297:1332–43.
[21] Hirano T, Yamamura Y, Nakamura S, Onogawa T, Mori T. Effects of the V_2-receptor antagonist OPC-41061 and the loop diuretic furosemide alone and in combination in rats. J Pharmacol Exp Ther 2000;292:288–94.
[22] Watanabe K, Ohta Y, Nakazawa M, Higuchi H, Hasegawa G, Naito M, et al. Low dose carvedilol inhibits progression of heart failure in rats with dilated cardiomyopathy. Br J Pharmacol 2000;130:1489–95.
[23] Kodama M, Matsumoto Y, Fujiwara M, Masani F, Izumi T, Shibata A. A novel experimental model of giant cell myocarditis induced in rats by immunization with cardiac myosin fraction. Clin Immunol Immunopathol 1990;57: 250–62.
[24] Miyazaki T, Yamamura Y, Onogawa T, Nakamura S, Kinoshita S, Nakayama S, et al. Therapeutic effects of tolvaptan, a potent, selective nonpeptide vasopressin V_2 receptor antagonist, in rats with acute and chronic severe hyponatremia. Endocrinology 2005;146:3037–43.
[25] Yamaguchi K, Koike M, Hama H. Plasma vasopressin response to peripheral administration of angiotensin in conscious rats. Am J Physiol 1985;248:R249–56.
[26] Shoker AS. Application of the clearance concept to hyponatremic and hypernatremic disorders: a phenomenological analysis. Clin Chem 1994;40:1220–7.
[27] Mallie JP, Bichet DG, Halperin ML. Effective water clearance and tonicity balance: the excretion of water revisited. Clin Invest Med 1997;20:16–24.
[28] Kinter LB, Dytko G, Ashton D, McDonald J, Huffman W, Stassen F. Discovery and therapeutic utility of vasopressin antagonists in rats. J Cardiovasc Pharmacol 1986;8(Suppl 7):S36–43.
[29] Morel F. Sites of hormone action in the mammalian nephron. Am J Physiol 1981;240:F159–64.
[30] Francis GS, Siegel RM, Goldsmith SR, Olivari MT, Levine TB, Cohn JN. Acute vasoconstrictor response to intravenous furosemide in patients with chronic congestive heart failure. Activation of the neurohumoral axis. Ann Intern Med 1985;103:1–6.
[31] Anand IS, Kalra GS, Harris P, Poole-Wilson PA, Panzali A, De Giuli F, et al. Diuretics as initial and sole treatment in chronic cardiac failure. Cardioscience 1991;2:273–8.
[32] Lorenz JN, Weihprecht H, Schnermann J, Skott O, Briggs JP. Renin release from isolated juxtaglomerular apparatus depends on macula densa chloride transport. Am J Physiol 1991;260:F486–93.
[33] Williamson HE, Bourland WA, Marchand GR, Farley DB, Van Orden DE. Furosemide induced release of prostaglandin E to increase renal blood flow. Proc Soc Exp Biol Med 1975;150:104–6.
[34] Abe K, Yasujima M, Chiba S, Irokawa N, Ito T, Yoshinaga K, et al. Effect of furosemide on urinary excretion of prostaglandin E in normal volunteers and patients with essential hypertension. Prostaglandins 1977;14: 513–21.
[35] Whorton AR, Lazar JD, Smigel MD, Oates JA. Prostaglandin-mediated renin release from renal cortical slices. Adv Prostagl Thromb Res 1980;7:1123–9.
[36] Beierwaltes WH, Schryver S, Sanders E, Strand J, Romero JC. Renin release selectively stimulated by prostaglandin I2 in isolated rat glomeruli. Am J Physiol 1982;243: F276–83.
[37] Hayashi Y, Hisa H, Satoh S. Role of prostaglandin in norepinephrine and renin release in canine kidney. Am J Physiol 1987;253:F929–34.
[38] Bailie MD, Crosslan K, Hook JB. Natriuretic effect of furosemide after inhibition of prostaglandin synthetase. J Pharmacol Exp Ther 1976;199:469–76.
[39] Pfeffer MA, Braunwald E, Moye LA, Basta L, Brown Jr EJ, Cuddy TE, et al. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators. N Engl J Med 1992;327:669–77.
[40] Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 1999;130:461–70.
[41] Romano G, Favret G, Bartoli E. Micropuncture study of the effect of furosemide on proximal and distal tubules of the rat nephron. Ren Physiol Biochem 1995;18:209–18.
[42] Tenstad O, Williamson HE. Effect of furosemide on local and zonal glomerular filtration rate in the rat kidney. Acta Physiol Scand 1995;155:99–107.
[43] Thibonnier M. Vasopressin receptor antagonists in heart failure. Curr Opin Pharmacol 2003;3:683–7.
[44] Lauson HD. Metabolism of antidiuretic hormones. Am J Med 1967;42:713–44.
[45] Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med 1999;341:709–17.
[46] Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, et al. Eplerenone post-acute myocardial infarction heart failure efficacy and survival study investigators. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med 2003;348:1309–21.