Different effects of long- and short-acting loop diuretics on survival rate in Dahl high-salt heart failure model rats

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Abstract

Objectives: We compared therapeutic effects of furosemide, a short-acting loop diuretic, and azosemide, a long-acting one, in hypertensive heart failure rats to test the hypothesis that long-acting diuretics are superior to short-acting types in heart failure.

Methods: Dahl salt-sensitive rats fed an 8% NaCl diet from age 8 weeks were divided at age 21 weeks (compensated hypertrophic stage) into three groups: rats treated with furosemide (40 mg/kg/day), those treated with azosemide (80 mg/kg/day) and untreated rats. Rats fed a 0.3% NaCl diet served as controls.

Results: Both medications prevented left ventricular systolic dysfunction and enlargement at age 31 weeks, and attenuated macrophage infiltration, reactive oxygen species generation, and gelatinolytic activity to the same degree. Azosemide suppressed left ventricular fibrosis to the control level, but furosemide did not. Azosemide ameliorated myocardial catecholamine depletion and improved survival rate. Furosemide increased plasma norepinephrine levels and did not exert such beneficial effects.

Conclusions: Azosemide provided better prognosis in heart failure rats compared with furosemide, partly through attenuation of the reflex increase in cardiac sympathetic neuronal activity caused by the development of heart failure. The current findings suggest a need for clinical trials examining whether long- and short-acting diuretics provide a different prognosis in patients with heart failure.

Keywords: Antihypertensive/diuretic agents; Heart failure; Hormones

1. Introduction

Clinical trials have demonstrated beneficial effects of angiotensin converting enzyme inhibitor, β-blocker, angiotensin II type 1 receptor blocker and aldosterone receptor antagonist in patients with heart failure, and therefore, these medicines are recommended as effective therapeutic strategies in the treatment of heart failure [1,2]. Although diuretics are also recommended as essential medicines in patients with heart failure symptoms and/or fluid retention, there are no controlled or randomized trials that have adequately assessed the effect of a long-term administration of diuretics on morbidity and mortality [1,2].

Loop diuretics, widely used for the treatment of heart failure, can be divided into two classes as vasodilators: short- and long-acting ones. Previous clinical studies demonstrated that short-acting vasodilators increased mortality in patients with coronary artery disease in spite of their antihypertensive effects [3,4]. A probable mechanism is a reflex increase in sympathetic activity, because such adverse effects are not observed in long-acting vasodilators. Thus, short-acting vasodilators are not prescribed for the long-term treatment of patients with cardiovascular diseases. In clinical practice, furosemide, a short-acting...
loop diuretic, is most commonly used in the treatment of heart failure [1]. Short-acting diuretics are presumed to provide the same adverse outcomes as short-acting vasodilators, and McCurley et al. recently demonstrated adverse effects of furosemide in a tachycardia-induced porcine model of heart failure [5].

To test our hypothesis that long-acting diuretics provide better prognosis than short-acting ones in heart failure, we compared effects of furosemide, a short-acting loop diuretic, and azosemide, a long-acting one, on survival rate in an animal model of heart failure. We used hypertensive Dahl salt-sensitive rats fed high-salt diet as a heart failure model, because hypertension is a major underlying cardiovascular disease of heart failure [6]. In clinical practice, with increasing age and greater obesity, there is a greater propensity for salt sensitivity that is commonly observed in hypertensive patients [7] and is an independent predictor of poor prognosis [8]. Thus, findings in this study may be extrapolated to no small part of heart failure patients.

2. Methods

2.1. Animal models

Male Dahl salt-sensitive rats fed 8% NaCl from age 8 weeks were used as a hypertensive systolic heart failure model, and those continuously fed 0.3% NaCl were normotensive and served as age-matched control [9,10]. In each of the following four studies, different rats were used. The study schedule was based upon our previous study [9]. This investigation conforms with the guiding principles of Osaka University Graduate School of Medicine with regard to animal care and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1.1. A study to determine doses of furosemide and azosemide

To determine the dose of furosemide and azosemide, the Dahl high-salt heart failure model rats were randomly assigned at age 21 weeks, a compensatory hypertrophic stage [9], into six groups: groups treated with furosemide at 40 mg/kg/day (n = 8), furosemide at 80 mg/kg/day (n = 8), azosemide at 40 mg/kg/day (n = 8), azosemide at 80 mg/kg/day (n = 8) and azosemide at 120 mg/kg/day (n = 8) and an untreated group (n = 8). Furosemide, azosemide or placebo was given every morning by gastric gavages for 7 days. Systolic blood pressure was measured with a tail cuff system (BP-98A, Softron, Tokyo, Japan) early in the morning before giving the drug, and Fig. 1 shows the data 1, 2 and 7 days after the initiation of the drug administration. Only a comparison of systolic blood pressure between the rats treated with furosemide at 40 mg/kg/day and azosemide at 80 mg/kg/day showed no significant difference. In the following protocols, four groups, i.e., the age-matched Dahl low-salt control rats, the Dahl high-salt heart failure model rats treated with furosemide at 40 mg/kg/day, and azosemide at 80 mg/kg/day showed no significant difference. In the following protocols, four groups, i.e., the age-matched Dahl low-salt control rats, the Dahl high-salt heart failure model rats treated with furosemide at 40 mg/kg/day from age 21 weeks, the Dahl high-salt heart failure model rats treated with azosemide at 80 mg/kg/day from

![Fig. 1. Changes in systolic blood pressure 1, 2 and 7 days after the initiation of the drug administration in the dose-finding study. A p value for each comparison is reported in the right upper panel. Systolic blood pressure was not different between the rats treated with furosemide at 40 mg/kg/day and azosemide at 80 mg/kg/day, and there was a significant difference between the furosemide- and azosemide-treated rats at the other comparisons. Values are means±S.E.M.](https://academic.oup.com/cardiovascres/article-abstract/68/1/118/287547/fig1)
2.1.2.1. Quantification of gene expression.

We previously reported that Dahl salt-sensitive rats fed 8% NaCl from age 8 weeks showed a gradual rise in blood pressure and fell into heart failure around 26 weeks old, and the mean ± 2S.D. of the age when the rats fell into heart failure was 31 weeks old [9]. Thus, following the measurement of systolic blood pressure at age 31 weeks, the rats from the four groups (n = 6, respectively) were anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and transthoracic echocardiography was conducted to measure left ventricular (LV) cavity size and wall thickness and to calculate LV endocardial and mid-wall fractional shortenings in a fashion previously described [9,10]. After the echocardiographic study and blood sampling to measure plasma aldosterone concentration, the heart and the lung were harvested, and the LV and lung weights were recorded and corrected for tibial length [11]. An apical part of LV specimen used for measurement of mRNA levels was immediately placed in liquid nitrogen and stored at −80 °C. An upper-middle part of LV specimen for in situ zymography and immunohistochemistry was immersed in OCT compound and frozen at −80 °C. The specimen at the papillary muscle level was immersed in a cold 4% paraformaldehyde solution for 16 to 24 h.

2.1.2.2. Immunohistochemistry.

Cryostat transverse sections were stained using mouse anti-rat macrophages monoclonal antibody (1 : 50 dilution, Ki-M2R, BMA Biomedicals Ltd., Augst, Switzerland) or mouse monoclonal anti-4-hydroxy-2-nonenal (HNE) antibody (1 : 50 dilution, NOF Medical Department, Tokyo, Japan) as previously described [13]. The data were obtained from 6 rats of each group.

2.1.2.3. Pathological study.

The specimen was immersed in 4% paraformaldehyde solution and embedded in paraffin. Transverse section (2-μm thick) of the LV free wall at the papillary muscle level was microscopically examined with Azan Mallory stain at 100× magnification, and the percent area of fibrosis was determined as previously described [13]. Sections were also stained with picrosirius red and were assessed with a polarization microscopy [11]. The data were obtained from 6 rats of each group.

2.1.2.4. In situ gelatin zymography.

The film in situ gelatin zymography that allows identification of net functional gelatinolytic activity in tissues [14] was conducted using a commercially available kit (MMP in situ ZymoFilm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) as previously described using 6 rats of each group [10,14]. Our recent study has demonstrated that the film in situ gelatin zymography allows the quantitative assessment of gelatinolysis, and that the gelatinolysis in the film in situ gelatin zymography is principally attributed to matrix metalloproteinase activity [10].

2.1.3. Cardiac and plasma norepinephrine assay study

Cardiac and plasma concentrations of norepinephrine were determined in another group of rats at age 31 weeks (n = 5, respectively). Following the decapitation and blood sampling, the hearts were immediately excised, and LV samples were homogenized with a Polytron in 10% TCA. After centrifugation of blood (1500 g, 10 min) and LV myocardial homogenate (10,000 g, 30 min), norepinephrine was determined in plasma and supernatants of LV myocardial homogenates by high-performance liquid chromatography as previously described [15,16].
2.1.4. Effects of furosemide and azosemide on survival rate

Survival rate was compared using untreated (n = 16), and azosemide- (n = 23) and furosemide- (n = 23) treated Dahl high-salt rats. Deaths were recorded daily. Survival was depicted graphically.

2.2. Statistical analysis

Results were expressed as mean values ± S.E.M. Data were analyzed using statistical software (STATVIEW version 5.0, SAS Institute Inc.). Differences among groups were assessed using one-factor analysis of variance and Fisher’s protected least significant difference test. The serial data were assessed by analysis of variance for repeated measures followed by Fisher’s test. Survival was depicted graphically using Kaplan–Meier survival curves and compared using log-rank test. In all tests, p < 0.05 for two-sided comparisons was considered statistically significant.

3. Results

3.1. Effects of diuretics assessed at age 31 weeks

Echocardiographic and hemodynamic data at age 31 weeks are summarized in Table 1. The untreated Dahl rats on high-salt diet exhibited an elevation of systolic blood pressure, reduced LV endocardial and mid-wall fractional shortenings, and increased LV end-diastolic diameter, LV weight/tibial length and area of fibrosis compared with the age-matched Dahl low-salt control. These hemodynamic and structural alterations were associated with an increase in lung weight/tibial length, indicating the presence of pulmonary congestion due to increased LV filling pressure [9].

Administration of furosemide and azosemide significantly and equally lowered blood pressure compared to untreated Dahl high-salt rats, but there was no significant difference between these groups (Table 1). Both pharmacological interventions prevented decreases in LV endocardial and mid-wall fractional shortenings and increases in LV end-diastolic diameter and lung weight/tibial length (Table 1). Azosemide was effective in ameliorating LV mass/tibial length in the high-salt subjects, although furosemide was not according to statistics comparing furosemide and azosemide groups to the untreated group. The percent area of fibrosis and the collagen type I mRNA level were significantly lower in the furosemide-treated Dahl high-salt rats than in the untreated Dahl high-salt group, but these were still significantly higher than those in the Dahl low-salt control rats (Table 1, Fig. 2). Azosemide decreased the area of fibrosis and the collagen type I mRNA level to the control level. However, there was no statistical difference in LV mass/tibial length, the area of fibrosis and the collagen type I mRNA level between furosemide and azosemide groups. It could be that there were not enough subjects to guarantee a statistical difference when one was present and that a type II statistical error has occurred. Gene expression of collagen type III was not significantly changed following either pharmacological treatment.

The administration of furosemide and azosemide suppressed macrophage infiltration and reactive oxygen species (ROS) generation as assessed with the HNE staining [13] to a similar degree (Fig. 3). The TGF-β1 mRNA level was significantly lower in the furosemide-treated Dahl high-salt rats than in the untreated Dahl high-salt rats, but was still higher than that in the Dahl low-salt control group (Fig. 2). Azosemide decreased the TGF-β1 mRNA expression to the level of the Dahl low-salt control rats. The interleukin-1β mRNA level was lower in the azosemide-treated rats but not in the furosemide-treated rats compared to the untreated Dahl high-salt rats (Fig. 4). Gene expression of ACE, ECE and preproendothelin-1 was suppressed in the furosemide- and azosemide-treated rats to a similar degree (Fig. 4). The

<table>
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<th>Hemodynamic, LV structural and pathological parameter at 31 weeks</th>
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<td>Dahl low-salt</td>
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<td>Control</td>
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<td>6</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>136±1</td>
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<td>Heart rate (bpm)</td>
<td>365±9</td>
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<tr>
<td>Body weight (g)</td>
<td>481±7</td>
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<td>LV end-diastolic dimension (mm)</td>
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<td>LV posterior wall thickness at end-diastole (mm)</td>
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<td>LV endocardial fractional shortening (%)</td>
<td>35.9±0.6</td>
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<td>LV mid-wall fractional shortening (%)</td>
<td>18.7±0.5</td>
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<td>LV mass/tibial length (mg/mm)</td>
<td>16.3±3.9</td>
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<td>Lung weight/tibial length (mg/mm)</td>
<td>27.8±4.9</td>
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<td>Area of fibrosis (Azan Mallory stain) (%)</td>
<td>2.1±0.1</td>
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<tr>
<td>Area of fibrosis (picrosirius red stain) (%)</td>
<td>1.4±0.2</td>
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Values are expressed as mean±S.E.M. *p<0.05 vs. control group, †p<0.05 vs. untreated.
net functional gelatinolytic activity as assessed by the in situ zymography increased in the untreated rats, and both furosemide and azosemide equally attenuated the gelatinolytic activity (Fig. 5). Plasma aldosterone level was not altered by furosemide (0.16±0.01 pmol/ml) or azosemide (0.21±0.02 pmol/ml) compared with the untreated rats (0.21±0.02 pmol/ml).

3.2. Effects of diuretics on cardiac and plasma norepinephrine level

Cardiac norepinephrine levels were significantly reduced in the untreated Dahl high-salt groups compared to the Dahl low-salt control group (Fig. 6). Furosemide did not change the cardiac norepinephrine level compared with the untreated rats, but azosemide significantly increased it. In contrast to cardiac norepinephrine levels, plasma norepinephrine levels of the furosemide-treated rats (3.82±0.71 ng/ml) were significantly higher than that of the untreated (2.13±0.14 ng/ml, p<0.05) and the azosemide-treated rats (2.35±0.38 ng/ml, p<0.05). There were no significant difference in the plasma norepinephrine levels of the untreated and the azosemide-treated rats.

Systolic blood pressure was measured before and 2, 4 and 8 h after giving the drug in the Dahl high-salt rats aged 21 weeks. There was no difference in blood pressure before and 2 h after giving the drug between the furosemide- and azosemide-treated rats; however, blood pressure was sig-

Fig. 2. (A) Ventricular tissue sections stained with Azan Mallory (upper) and picrosirius red (lower) stainings in a rat of each group. Furosemide attenuated ventricular fibrosis slightly, but not to the level of a Dahl low-salt control rat. Azosemide attenuated it to the level of a control rat. (B) The mRNA level of type I and type III collagen. Furosemide decreased the type I collagen mRNA level, but not to the level of a control rat. Azosemide decreased it to the level of a control rat. *p<0.05 vs. control group, super †p<0.05 vs. untreated group. Values are means±S.E.M.
nificantly lower 4 and 8 h after giving the drug in the furosemide-treated rats (4 h 185±8 mmHg, 8 h 161±10 mmHg) than in the azosemide-treated rats (4 h 211±11 mmHg, 8 h 188±4 mmHg, p<0.05 respectively) or in the untreated rats (4 h 211±9 mmHg, 8 h 198±11 mmHg, p<0.05 respectively).

3.3. Survival rate

The effects of furosemide and azosemide on survival rate are shown in Fig. 7. Furosemide tended to improve the survival rate, but there was no statistically significant difference between the untreated and the furosemide-treated...
Dahl high-salt rats. In contrast, the survival rate significantly improved in the azosemide-treated rats compared with the untreated and the furosemide-treated rats.

4. Discussion

Myocardial catecholamine was depleted in association with LV enlargement and systolic dysfunction at age 31 weeks in the Dahl high-salt heart failure model rat. Azosemide, a long-acting diuretic, and furosemide, a short-acting diuretic, similarly improved LV geometrical, functional and hemodynamic parameters and also inflammatory changes at this stage. However, azosemide, not furosemide, ameliorated myocardial catecholamine depletion and improved survival rate. The current results support our hypothesis that long-acting diuretics provide better prognosis than short-acting ones in heart failure.

Current guidelines recommend diuretics for treating heart failure [1,2], because diuretics are clearly effective in relieving heart failure symptoms. Thus, loop diuretics are widely used in the treatment of heart failure; however, retrospective studies reported increased risks in patients with heart failure of the use of non-potassium-sparing diuretics including loop diuretics [17,18]. Loop diuretics consist of short- and long-acting types. Several recent experimental studies reported lack of benefits and presence of adverse effects for furosemide, a short-acting loop diuretic, in heart failure models [5,19,20]. Thus, we hypothesized that the use of short-acting loop diuretics might explain the poor outcome of patients treated with non-potassium-sparing diuretics described in the previously described retrospective studies [17,18]. The current results support our hypothesis and extend recent experimental findings by demonstrating that a long-acting loop diuretic, azosemide, was superior to a short-acting one, furosemide, in the treatment of heart failure in Dahl rats. The current findings are partly compatible with clinical studies that show superiority of torasemide, a long-acting loop diuretic, to furosemide [21,22]. Since torasemide inhibits aldosterone binding to its receptor [23,24] and several clinical studies demonstrated usefulness of aldosterone blockers in patients with heart failure [17,18,25,26], it is unclear whether the benefits of torasemide were provided through its longer half-life and longer duration of action or through aldosterone blockade. The current and previous studies suggest that long-acting loop diuretics rather than short-acting types should be chosen for long-term administration of loop diuretics for chronic heart failure treatment.

The reduction in cardiac norepinephrine stores in heart failure patients is considered to result from the increased cardiac adrenergic drive and is closely related to poor prognosis [27–30]. The current study showed lower cardiac and higher plasma norepinephrine levels in the rats treated with furosemide compared to those treated with azosemide. Clinical studies with a small number of patients reported a higher level of plasma norepinephrine and a greater influence on heart rate variability after the administration of furosemide compared to azosemide [31,32]. The previous and current results suggest that azosemide attenuates sympathetic activity compared to furosemide, and that the difference in effects on sympathetic activity may at least partly explain lower mortality in the azosemide-treated rats. Both heart failure and arrhythmia may have caused death in this study; however, these could not be discriminated. Clinical studies reported an increase in mortality in association with the prescription of short-acting vasodilators in patients with coronary artery disease [3,4] as well as a
superiority of long- to short-acting vasodilators in patients with heart failure [33]. The adverse effects of short-acting vasodilators may be attributed to the increase in the reflex in sympathetic activity at least partly derived from an acute decrease in blood pressure. The current study showed that furosemide induced a greater drop in blood pressure 4 and 8 h after administration than azosemide. Such an acute change in blood pressure may be partly responsible for the reflex increase in sympathetic activity associated with the furosemide administration.

There was a slight, although not statistically significant, difference in blood pressure between the rats treated with 40 mg/kg/day of furosemide and those with 80 mg/kg/day of azosemide (Fig. 1 and Table 1). Systolic blood pressure was slightly higher in the azosemide-treated rats compared with the furosemide-treated rats in Fig. 1, and vice versa in Table 1. There is no clear tendency in the difference in blood pressure between the treatment regimens. The difference in blood pressure between the untreated and the furosemide-treated Dahl high-salt rats was much greater than that between the furosemide- and azosemide-treated rats (Table 1), but the treatment with furosemide did not improve the survival rate (Fig. 7). Thus, such a small difference in blood pressure between the furosemide- and azosemide-treated rats is unlikely to explain the difference in the survival rate. One may still argue that different results might be derived from administration of other doses. However, this study demonstrated that administration of furosemide at 80 mg/kg/day or azosemide at 120 mg/kg/day decreased blood pressure much more (Fig. 1). There was no significant difference in body and lung weights at age 31 weeks between the furosemide- and azosemide-treated rats, suggesting the similar effect on fluid status (Table 1). Thus, to compare their effects under similar depressor effects, the used doses were likely appropriate.

LV fibrosis in association with LV hypertrophy is well known to play a crucial role in the development of heart failure, particularly through the promotion of myocardial stiffening [34]. Azosemide tended to attenuate LV hypertrophy, LV fibrosis and collagen type I mRNA level at an age of 31 weeks compared with furosemide in this study. Expression of TGF-β1 and interleukin-1β decreases in association with beneficial effects of pharmacological interventions on LV structure in heart failure [13], and their gene expression tended to be suppressed by azosemide rather than furosemide in this study. Norepinephrine enhances cell proliferation and collagen production of fibroblasts, and its effects are facilitated by TGF-β1 [35]. Thus, the attenuation of sympathetic activity in association with the administration of azosemide compared with furosemide might provide better effects on LV structural remodeling, particularly LV fibrosis, and result in a better survival rate.

Macrophage infiltration, ROS generation, LV enlargement and the gelatinolytic activation that is considered to promote LV remodeling [36] were ameliorated to the same degree. Cardiac renin–angiotensin and endothelin systems are upstream triggers of the deleterious cascades leading to cardiac remodeling and dysfunction [37–39]; however, both of the pharmacological interventions similarly suppressed gene expression of ACE, ECE and preproendothelin-1 in the myocardium in this study. McCurley et al. proposed that a furosemide-induced increase in blood aldosterone level was implicated in the promotion of systolic dysfunction in a
tachycardia-induced porcine model of heart failure [5]. However, furosemide did not increase plasma aldosterone level compared with azosemide in this study. The assessment of the parameters was conducted at age 31 weeks in this study, and the survival rate was not different at this point between the two treated groups (Fig. 7). The current results suggest that sympathetic activation preceded deleterious pathways in association with the treatment with short-acting loop diuretics, resulting in poor outcomes. However, we assessed only a part of many parameters that affect pathogenesis of heart failure, and a lack of serial assessment of the parameters made it difficult to clarify what contributed to increased mortality in the furosemide-treated rats. In addition, we assessed only gene expression, not protein levels, of several neurohumoral parameters. Thus, to clarify the mechanisms of the different outcomes of two diuretics, further studies are necessary.

Seeland et al. also reported that the treatment with furosemide alone did not improve the survival rate in spite of a reduction of ACE expression and activity in spontaneously hypertensive rats with myocardial infarction [20]. They found that heart rate tended to increase with the furosemide treatment, and speculated that the associated sympathetic activation contributed to the lack of benefits of furosemide. Our findings supported their conclusion by the direct measurement of cardiac and circulating norepinephrine levels and have expanded their study by demonstrating the superiority of azosemide. Seeland et al. reported that a 6-week administration of furosemide did not affect gelatinolytic activity and collagen accumulation in the same myocardial infarction model of spontaneously hypertensive rats [19]. This is different from our result, and the discrepancy may be at least partly explained by the difference in the animal models and in the duration of the furosemide administration. We used a heart failure model based on salt-sensitive hypertension, and the current findings may not be necessarily extrapolated to other types of heart failure. However, one of our results that a short-acting loop diuretic, furosemide, provided less benefits in heart failure is compatible with previous experimental and clinical studies [5, 19–22].

5. Conclusions

Azosemide, a long-acting loop diuretic, is likely favorable for the treatment of heart failure compared with furosemide, a short-acting one. The benefit of azosemide may be at least in part attributed to attenuation of the reflex increase in sympathetic activity. Currently, diuretics are widely used in the treatment of heart failure, but little attention is paid about the duration of their action. These findings will propose a need of a randomized, controlled clinical trial to examine whether long- and short-acting diuretics provide different prognosis in patients with heart failure.

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