VIA MEDICA
ORIGINAL ARTICLE
2011, Vol. 18, No. 5, pp. 532–537
10.5603/CJ.2011.0009

ISSN 1897–5593

Effects of levosimendan without loading dose on systolic and diastolic function in patients with end-stage heart failure

Stefano Lunghetti[1], Elisabetta Palmerini[1], Rossella Urselli[1], Silvia Maffei[1], Elisa Guarino[2], Marta Focardi[1], Sergio Mondillo[1], Roberto Favilli[1]

[1]Department of Cardiology, University of Siena, Italy
[2]Department Internal Medicine, University of Siena, Italy

Abstract

Background: *Levosimendan (L) is used in clinical practice for the treatment of severe heart failure (HF); it has inotropic and vasodilatory effects, without increasing myocardial oxygen consumption. In acute HF, levosimendan improves hemodynamic parameters; previous studies have demonstrated that it has favorable effects on left ventricular (LV) diastolic function. The aim of our study was to evaluate the effect of on LV long-axis function that represents the earlier marker of diastolic dysfunction.*

Methods: *We enrolled 41 patients (age 62 ± 12 years) admitted to our Department for acute HF, NYHA class IV and severe LV dysfunction. Twenty-six patients were treated with L (0.1 μg/kg/min ev for 24 h without loading dose) and 15 patients were treated with standard therapy (C). We evaluated clinical, blood exams and echocardiographic parameters at baseline and one week after L or C treatment.*

Results: *Baseline demographic, clinical and biochemical data were similar in both groups. After one week, the L group had shown a significant improvement in NYHA class and a reduction of pro-B-type natriuretic peptide (pro-BNP). In echocardiographic study, we observed an improvement in LV longitudinal function ($p < 0.05$) and LV ejection fraction ($p < 0.05$) with a reduction of E/E' ($p < 0.05$) in the L group. We divided the L group into ischemic and non-ischemic patients and we demonstrated a significant increase in systolic function in the former. No differences were found between subgroups in diastolic function.*

Conclusions: *L therapy, without loading dose, improves NYHA class and ventricular function in patients with acute HF; we believe that these prolonged hemodynamic effects are due to active metabolities of L.* (Cardiol J 2011; 18, 5: 532–537)

Key words: levosimendan, heart failure

Introduction

Heart failure (HF) is a public health problem with increasing incidence, poor prognosis and frequent need for re-hospitalization [1, 2]. Intravenous positive inotropic agents play an important role in treating acute decompensation of chronic heart failure (CHF) [3–5]. Levosimendan (L) is a calcium sensitizer with positive inotropic properties that works on the sensibilization of Ca^{2+} channels of myocites increasing intracellular Ca^{2+} concentration [6, 7]. The intracellular Ca^{2+} binds troponin C, re-

Address for correspondence: Stefano Lunghetti, MD, Department of Cardiology, Azienda Ospedaliera Universitaria Senese, Viale Bracci no. 2, 53100 Siena, Italy, e-mail: stefano.lunghetti@libero.it

Received: 11.03.2011 Accepted: 20.06.2011

sulting in a stabilization of the link between actin and myosin, improving the contractility during systole [8]. As an additional effect, L opens the K^+-ATP dependent channels on cytosolic and mitochondrial membranes, increases K^+ concentration in the mytocondrial cytosol, and thus may delay apoptosis of myocites in HF patients [9, 10].

Left ventricular (LV) long-axis function represents the expression of subendocardial twitch; it has been demonstrated that it is an earlier marker, even more than ejection fraction (EF), of LV dysfunction. Little data exists on the effects of L on echocardiographic parameters of LV systolic and diastolic function in patients with advanced HF. Parissis et al. [11] demonstrated an improvement of LV diastolic function evaluated with echocardiographic parameters such as mitral E/A and E/E' ratio after L therapy; in addition, the authors showed an increasing LV deceleration time and isovolumic relaxation time after L therapy [12].

Heart failure guidelines suggest the use of L with a loading dose if systolic blood pressure (SBP) is over 100 mm Hg [3]. The pharmacokinetics of L are linear and the plasma concentration of the drug increases in a dose-proportional manner following single dose i.v. administration and infusion of the drug [13, 14]. Data from non-invasive studies has shown that the maximal hemodynamic response to L is 24–48 h after stopping infusion [15, 16]. The prolonged hemodynamic action of L is due to the formation of active metabolities; for this reason, patients with HF can have hypotensive episodes even after stopping the drug. No data exists about the administration of L intravenously without a loading dose in patients with acute HF. The aim of our study was to investigate the use of L without a loading dose in a cohort of end-stage CHF patients admitted to our institution for acute decompensation HF compared to standard therapy, and its effect on clinical, blood exams and echocardiographic parameters of diastolic and systolic function.

Methods

Study population

We evaluated 41 patients (age 62 ± 12 years) with acute NYHA class IV HF and severe LV dysfunction (LVEF < 30%). Informed consent was obtained from all subjects.

Exclusion criteria were: severe LV outflow obstruction; SBP < 80 mm Hg; heart rate > 130/min; recent myocardial infarction (< eight weeks) or active myocardial ischemia; serum creatinine level higher than 2.5 mg/dL or dialysis; hepatic failure; acute or chronic infectious or inflammatory diseases. All patients had not received digoxin or other parenteral positive inotropics but only HF standard therapy, as shown in Table 1. Twenty-six patients were treated with L therapy, administered as continuous 24-h infusion at 0.1 μg/kg/min, without an intravenous loading dose, and 15 patients, the control group (C), were treated with standard therapy only. The decision to administer L without a loading dose was based on clinical assessment of the patients.

In the L group, 16 and ten patients had post-ischemic and idiopathic cardiomyopathy respectively; in the C group, ten and five patients had post-ischemic and idiopathic cardiomiopathy, respectively.

Ultrasound measurements

Standard echocardiography performed in all patients included the evaluation of mitral annular motion by tissue Doppler imaging (TDI) and M-mode, using SONOS 5500 equipment (Hewlett Packard, Andover, MA, USA) with a phases array transducer of 2.5 MHz and TDI technology. Echocardiographic exams were performed by an experienced echocardiographer physician blinded to all data. Recordings were acquired with subjects in the left lateral decubitus during shallow respiration or end expiratory apnea.

Echocardiography was performed at baseline and one week after L or standard treatment. LV systolic function was estimated by LVEF using the biplane modified Simpson's method [17] and the long-axis function by TDI S' wave [18] and M-mode mitral annular plane systolic excursion (MAPSE) [19], averaging excursion amplitudes recorded at the four mitral annular sites. Tricuspid annular plane systolic excursion (TAPSE) was also measured using two-dimensionally guided M-mode imaging from the apical four-chamber view.

LV diastolic function was estimated by pulsed wave (PW) Doppler on transmitral flow assessing peak velocities in early (E) and late diastole (A), E/A ratio, deceleration time of E wave (DT) and TDI E' (early) and A' (late) waves [19]. The E/E' ratio was also calculated and used as an index of LV filling pressures [20, 21].

Blood exams

Blood exams, included B-type natriuretic peptide (pro-BNP) levels, were performed at baseline and one week after L or standard treatment.

Statistical analysis

Statistical analysis was performed using STATVIEW 5.0. Quantitative values were expressed as mean ± standard deviation and qualitative values as %. The χ^2 test or Fisher's exact test was used for categorical values between two groups, the Student's t test or Mann-Whitney U test was utilized to compare the continuous variables between groups. The paired t test or Wilcoxon's paired test was used to compare values before and after drug administration. Correlation analysis was performed using the Pearson's correlation coefficients. A p value of < 0.05 was considered statistically significant.

Results

There were no differences in baseline demographic, clinical or blood exams between the two groups (Table 1). During L infusion, we did not observe severe hypotensive episodes compared to standard therapy. Baseline echocardiographic parameters did not differ, except for MAPSE which was higher in the C group than the L group (9 ± 3 mm *vs* 6 ± 1 mm, p = 0.03) as shown in Table 2.

After one week, both the L group and the C group showed a significant improvement in NYHA functional class (L IV *vs* III, p = 0.01); in the L group we obtained a significant reduction of pro-BNP levels (8,174 ± 9,226 *vs* 4,335 ± 7,947, p = 0.02) compared to the C group (5,713 ± 3,124 pg/mL *vs* 3,191 ± ± 1,936 pg/mL, p = NS).

Echocardiographic evaluation of the L group demonstrated a significant increase in MAPSE (6 ± ± 1 mm *vs* 9 ± 2 mm, p = 0.01; Fig. 1), LVEF (24.6 ± ± 4.8% *vs* 27.6 ± 4.6%, p = 0.02) and an E/E' ratio reduction (14.66 ± 4.31 *vs* 8.34 ± 3.6, p = 0.02; Fig. 2). TAPSE didn't improve significantly (14 ± 6 mm *vs* 16 ± 0.4 mm, p = NS). In the C group, after one week, LVEF (25.6 ± 4.4% *vs* 29 ± 6.4%, p = NS), MAPSE (9 ± 3 mm *vs* 9 ± 2 mm, p = NS), TAPSE (15 ± 6 mm *vs* 15 ± 6 mm, p = NS) and E/E' ratio (14.5 ± 0.6 *vs* 13.7 ± 0.8, p = NS) had improved, but not significantly.

We divided the L group into ischemic and non-ischemic cardiomyopathy and we demonstrated a significant increase in MAPSE (6 ± 1 mm *vs* 8 ± ± 3 mm, p = 0.02) in the ischemic *vs* the non-ischemic subgroup (8 ± 2 mm *vs* 9 ± 3 mm, p = NS). No significant differences appeared between the ischemic/non-ischemic subgroups in diastolic myocardial function or LV filling pressure.

Discussion

In our study, we evaluated the efficacy of L, administered without loading dose, independently

Table 1. Baseline characteristics of levosimendan (L) and standard therapy (C) groups.

	Levosimendan (n = 26)	Standard therapy (n = 15)
Age (years)	64 ± 7	68 ± 9
Sex, male	15 (58%)	9 (60%)
Body mass index [kg/m^2]	26 ± 1.1	28 ± 1.8
Ischemic/non-ischemic patients	16/10	10/5
New York Heart Association	IV	IV
Pro-BNP [pg/mL]	8,174 ± 9,226	5,713 ± 3,124
Systolic blood pressure [mm Hg]	98 ± 6	102 ± 4
Diastolic blood pressure [mm Hg]	64 ± 6	70 ± 12
Heart rate [bpm]	70 ± 11	80 ± 8
Medicaments:		
Diuretics (furosemide)	26 (100%)	15 (100%)
Angiotensin converting enzyme inhibitors	23 (88%)	14 (93%)
Angiotensin receptor blockers	5 (19%)	3 (20%)
Beta-blocker	20 (77%)	9 (60%)
Spironolactone	12 (46%)	8 (53%)
Aspirin	21 (81%)	11 (73%)
Amiodarone	10 (38%)	6 (40%)
Anticoagulants	6 (23%)	4 (27%)
Statin	20 (77%)	12 (80%)

Data is expressed as mean ± standard deviation or percentage

Table 2. Echocardiographic parameters before and after treatment in the levosimendan (L) and control groups (C).

	Levosimendan (n = 26)		Standard therapy (n = 15)	
	Time 0	1 week	Time 0	1 week
LVEDD [mm]	71 ± 9	70 ± 9†	69 ± 11	69 ± 8†
LVESD [mm]	60 ± 10	59 ± 11†	58 ± 11	51 ± 8†
LVEF [%]	24 ± 5	27 ± 4*	25 ± 4	29 ± 6†
E/A	1.6 ± 0.5	1.1 ± 0.8†	2.4 ± 0.4	2.3 ± 0.6†
E/E′	14.6 ± 4.3	8.3 ± 3.6*	14.5 ± 0.6	13.7 ± 0.8†
IVRT	74 ± 24	88 ± 40†	51 ± 42	56 ± 12†
DT	133 ± 54	154 ± 44†	140 ± 18	139 ± 32†
MAPSE [mm]	6 ± 1	8 ± 2*	9 ± 3	9 ± 2†
TAPSE [mm]	14 ± 5	16 ± 4†	14 ± 6	15 ± 6†
SBP [mm Hg]	111 ± 6	107 ± 10†	105 ± 10	110 ± 10†
DBP [mm Hg]	64 ± 6	60 ± 6†	70 ± 12	68 ± 10†
Heart rate [bpm]	74 ± 12	71 ± 8†	80 ± 8	73 ± 9†
New York Heart Association	IV	III	IV	III
Pro-BNP [pg/mL]	8,174 ± 9,226	4,335 ± 7,947*	5,713 ± 3,124	3,191 ± 1,936†

Data is expressed as mean ± standard deviation; LVEDD — left ventricular end-diastolic diameter; LVESD — left ventricular end-systolic diameter; LVEF — left ventricular ejection fraction; IVRT — isovolumetric relaxation time; DT — deceleration time; MAPSE — mitral annular plane systolic excursion; TAPSE — tricuspid annular plane systolic excursion; SBP — systolic blood pressure; DBP — diastolic blood pressure; *p < 0.05; †p = NS

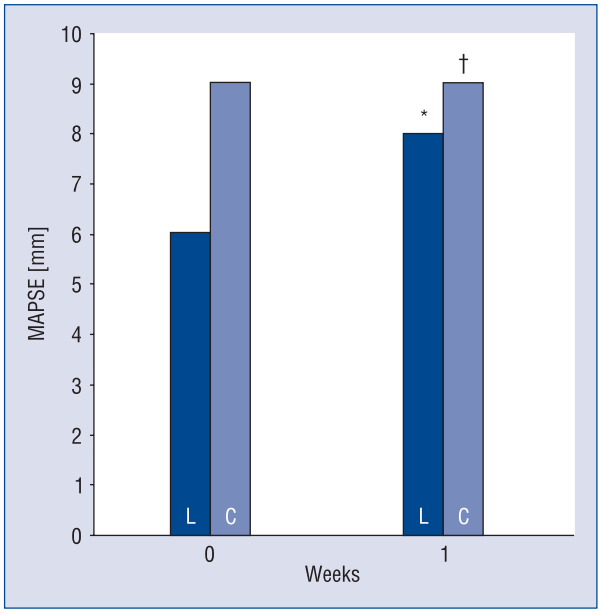

Figure 1. MAPSE variations in levosimendan (L) and standard therapy (C) groups after one week of treatment; *p < 0.05; †p = NS

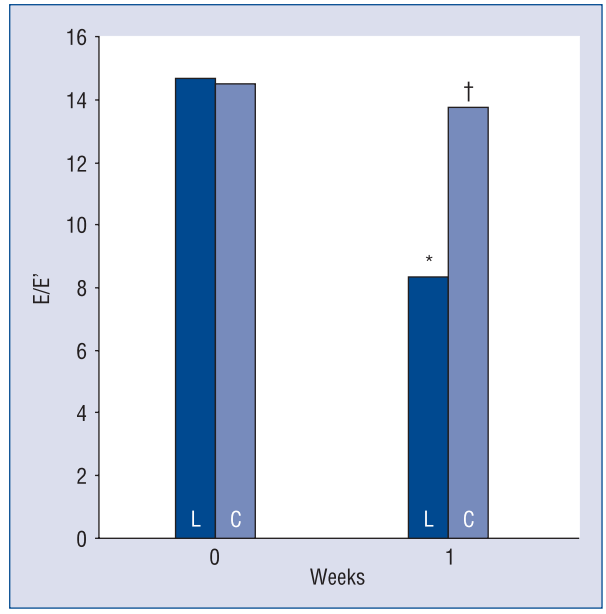

Figure 2. E/E′ variations in levosimendan (L) and standard therapy (C) groups after one week of treatment; *p = 0.02; †p = NS

of values of baseline blood pressure, compared to standard therapy (C) in patients with acute decompensated HF. We analysed changes in clinical, biochemical and echocardiographic parameters.

It is well demonstrated that L improves symptoms [22] and reduces mortality [23] during both acute HF and CHF if administered with a loading dose. Our study demonstrated that continuous i.v.

infusion of L leads to an improvement in clinical NYHA class, pro-BNP blood levels and echocardiographic indices of ventricular function after seven days. Unlike other studies performed with a loading dose of L, we had these effects without an increase in hypotension episodes [24].

Several studies have suggested that BNP or pro-BNP levels at the time of hospitalization have a prognostic value in patients with HF [25–27]; on the other hand, a decrease in BNP levels during hospitalization is associated with a reduction in deaths and rehospitalizations after 30 days [28]. The increase of blood pro-BNP levels was due to myocardial wall stress condition and directly related to LV filling pressures [29, 30]. Our data demonstrated a significant decrease of pro-BNP levels seven days after L therapy compared to C, underscoring the favorable effects of L in acute HF. The decrease of pro-BNP levels is associated with an increase in echocardiographic parameters such as EF, MAPSE and E/E'.

Few echocardiographic studies have analyzed the effect of L on LV function, in particular its effects on LV longitudinal function. LV systolic contraction is a complex phenomenon resulting from interaction among differently arranged myocardial layers, leading to simultaneous longitudinal and circumferential shortening, radial thickening, and twisting [31]. Because of the high vulnerability of longitudinal subendocardial fibers to several injury mechanisms, assessment of the longitudinal component of LV shortening is an important parameter for the early detection of LV contractile impairment [32]. Using TDI and M-mode analysis, we can evaluate accurately LV longitudinal systolic and diastolic performance. In our study, after L treatment we obtained an improvement of LVEF and MAPSE and a reduction of E/E' ratio.

These results are related to the effect of L on LV filling pressures: cardiac output increases while pulmonary wedge pressure decreases, with a reduction of both pre-load and post-load forces [33].

After dividing the L group into ischemic and non-ischemic cardiomyopathy, we found a higher increase of MAPSE in the ischemic *vs* the non-ischemic subgroup, with no changes in diastolic function parameters or LV filling pressure.

An ischemic heart contains several types of cells: healthy, stunned and hibernated. Hibernated cells are missing calcium-related proteins, so the calcium release is altered [34]. Levosimendan works in healthy muscle cells improving the intracellular calcium concentration so it increases muscle contraction without increasing oxygen consumption. A previous study demonstrated that L can also reduce apoptosis by activating mitochondrial K_{ATP} channels [35] and this suggests a mechanism of cardiac myocytes protection. This can explain our results in ischemic compared to non-ischemic patients.

Limitations of the study

Our results indicate that L may have positive effects in HF without a loading dose in terms of safety and efficacy. The present study, however, has some limitations due to the small number of patients, especially the subgroups. Our study shows however that L can be safely administered in patients with end-stage HF. Further studies enrolling more patients are needed to validate these results.

Conclusions

Heart failure guidelines suggest the use of L with loading dose if SBP > 100 mm Hg; we demonstrated that continuous i.v. L administration without loading dose can improve NYHA class, EF and diastolic function compared to standard therapy with a decrease in the number of hypotension episodes. In particular, we showed that LV longitudinal function increases and LV filling pressure reduces significantly. These longitudinal function improvements were greater in patients with post-ischemic rather than idiopathic cardiomyopathies. We believe that these prolonged hemodynamic effects are due to active metabolities of levosimendan.

Acknowledgements

All authors declare that no potential, perceived, or real conflict of interests exists in connection with this manuscript.

References

1. McMurray JJV, Stewart S. The burden of heart failure. Eur Heart J, 2002; 4 (suppl. D): D50–D58.
2. Bundkirchen A, Schwinger RHG. Epidemiology and economic burden of chronic heart failure. Eur Heart J, 2004; 6 (suppl. D): D57–D60.
3. Dickstein K, Cohen-Solal A, Filippatos G et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008. The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Eur Heart J, 2008; 29: 2388–2442.
4. Innes CA, Wagstaff AJ. Levosimendan: A review of its use in the management of acute decompensated heart failure. Drugs, 2003; 63: 2651–2671.
5. Mebazaa A, Nieminen MS, Packer M et al. Levosimendan vs dobutamine for patients with acute decompensated heart failure: The SURVIVE Randomized Trial. JAMA, 2007; 297: 1883–1891.

6. Givertz MM, Andreou C, Conrad CH. Direct myocardial effects of levosimendan, a novel calcium sensitizer, in humans with left ventricular dysfunction. Circulation, 1998; 98 (suppl. 17): 1–579.
7. Hasenfuss G, Pieske B, Castell M, Kretschmann B, Maier LS, Just H. Influence of the novel inotropic agent levosimendan on isometric tension and calcium cycling in failing human myocardium. Circulation, 1998; 98: 2141–2147.
8. Haikala H, Kaivola J, Nissinen E, Wall P, Levijoki J, Lindén IB. Cardiac troponin C as a target protein for a novel calcium sensitizing drug, levosimendan. J Mol Cell Cardiol, 1995; 27: 1859–1866.
9. Kopustinskiene DM, Pollesello P, Saris NE. Levosimendan is a mitochondrial KATP channel opener. Eur J Pharmacol, 2001; 428: 311–314.
10. Yildiz O. Vasodilating mechanisms of levosimendan: Involvement of K+ channels. J Pharmacol Sci, 2007; 104: 1–5.
11. Parissis JT, Panou F, Farmakis D et al. Effects of levosimendan on markers of left ventricular diastolic function and neurohormonal activation in patients with advanced heart failure. Am J Cardiol, 2005; 96: 423–426.
12. Hasenfuss G, Pieske B, Castell M, Kretschmann B, Maier LS, Just H. Influence of the novel inotropic agent levosimendan on isometric tension and calcium cycling in failing human myocardium. Circulation, 1998; 98: 2141–2147.
13. Lilleberg J, Sundberg S, Häyhä M, Akkila J, Nieminen MS. Haemodynamic dose-efficacy of levosimendan in healthy volunteers. Eur J Clin Pharmacol, 1994; 47: 267–274.
14. Lilleberg J, Sundberg S, Nieminen MS. Dose-range study of a new calcium sensitizer, levosimendan, in patients with left ventricular dysfunction. J Cardiovasc Pharmacol, 1995; 26 (suppl. 1): S63–S69.
15. Kivikko M, Antila S, Eha J, Lehtonen L, Pentikäinen PJ. Pharmacodynamics and safety of a new calcium sensitizer, levosimendan, and its metabolites during an extended infusion in patients with severe heart failure. J Clin Pharmacol, 2002; 42: 43–51.
16. Kivikko M, Antila S, Eha J, Lehtonen L, Pentikäinen PJ. Pharmacokinetics of levosimendan and its metabolites during and after a 24-hour continuous infusion in patients with severe heart failure. Int J Clin Pharmacol Ther, 2002; 40): 465–471.
17. Lang RM, Bierig M, Devereux RB et al. Recommendations for Chamber Quantification: A report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, Developed in Conjunction with the European Association of Echocardiography, a Branch of the European Society of Cardiology. J Am Soc Echocardiogr, 2005; 18: 1440–1463.
18. Nagueh SF, Appleton CP, Gillebert TC et al. Recommendations for the evaluation of left ventricular diastolic function by echocardiography. J Am Soc Echocardiogr, 2009; 22: 107–133.
19. Nikitin NP, Witte KK, Thackray SD, de Silva R, Clark AL, Cleland JG. Longitudinal ventricular function: Normal values of atrioventricular annular and myocardial velocities measured with quantitative two-dimensional color Doppler tissue imaging. J Am Soc Echocardiogr, 2003; 16: 906–921.
20. Ommen SR, Nishimura RA, Appleton CP et al. Clinical utility of Doppler echocardiography and tissue Doppler imaging in the estimation of left ventricular filling pressures: A comparative simultaneous Doppler-catheterization study. Circulation, 2000; 102: 1788–1794.
21. Kazik A, Wilczek K, Poloński L. Management of diastolic heart failure. Cardiol J, 2010; 17: 558–565.
22. Follath F, Cleland JG, Just H et al. Efficacy and safety of intravenous levosimendan compared with dobutamine in severe low-output heart failure (the LIDO study): A randomised double-blind trial. Lancet, 2002; 360; 196–202.
23. Moiseyev VS, Poder P, Andrejevs N et al. Safety and efficacy of a novel calcium sensitizer, levosimendan, in patients with left ventricular failure due to an acute myocardial infarction. A randomized, placebo-controlled, double-blind study (RUSSLAN). Eur Heart J, 2002; 23: 1422–1432.
24. Parle NM, Thomas MD, Dembo L, Best M, Driscoll GO. Repeated infusions of levosimendan: Well tolerated and improves functional capacity in decompensated heart failure. A single-centre experience. Heart Lung Circ, 2008; 17: 206–210.
25. Yu CM, Sanderson JE. Plasma brain natriuretic peptide: An independent predictor of cardiovascular mortality in acute heart failure. Eur J Heart Fail, 1999; 1: 59–65.
26. Burger MR, Burger AJ. BNP in decompensated heart failure: Diagnostic, prognostic and therapeutic potential. Curr Opin Investig Drugs, 2001; 2: 929–935.
27. Di Somma S, Magrini L, Tabacco F et al. Brain natriuretic peptide and N-terminal pro-B-type natriuretic peptide show a different profile in response to acute decompensated heart failure treatment. Congest Heart Fail, 2008; 14: 245–250.
28. Berger R, Moertl D, Peter S et al. N-terminal pro-B-type natriuretic peptide-guided, intensive patient management in addition to multidisciplinary care in chronic heart failure: A 3-arm, prospective, randomized pilot study. J Am Coll Cardiol, 2010; 55: 645–653.
29. Parekh N, Maisel AS. Utility of B-natriuretic peptide in the evaluation of left ventricular diastolic function and diastolic heart failure. Curr Opin Cardiol, 2009; 24: 155–160.
30. Lubien E, DeMaria A, Krishnaswamy P et al. Utility of B-natriuretic peptide in detecting diastolic dysfunction: Comparison with Doppler velocity recordings. Circulation, 2002; 105: 595–601.
31. Mizuguchi Y, Oishi Y, Miyoshi H, Iuchi A, Nagase N, Oki T. The functional role of longitudinal, circumferential, and radial myocardial deformation for regulating the early impairment of left ventricular contraction and relaxation in patients with cardiovascular risk factors: A study with two-dimensional strain imaging. J Am Soc Echocardiogr, 2008; 21: 1138–1144.
32. Marwick TH. Clinical applications of tissue Doppler imaging: A promise fulfilled. Heart, 2003; 89: 1377–1378.
33. Antoniades C, Tousoulis D, Koumallos N, Marinou K, Stefanadis C. Levosimendan: Beyond its simple inotropic effect in heart failure. Pharmacol Ther, 2007; 114: 184–197.
34. Wang SQ, Lakatta EG, Cheng H, Zhou ZQ. Adaptive mechanisms of intracellular calcium homeostasis in mammalian hibernators. J Exp Biol, 2002; 205: 2957–2962
35. Yokoshiki H, Katsube Y, Sunagawa M, Sperelakis N. The novel calcium sensitizer levosimendan activates the ATP-sensitive K1 channel in rat ventricular cells. J Pharmacol Exp Ther, 1997; 283: 375–383.