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Original Article

Furosemide and Potassium Chloride-induced Alteration in Protein Profile of Left Ventricle and its Associated Risk for Sudden Cardiac Death

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ABSTRACT

Background: Potassium ion (K^+) plays an essential role in maintaining the electrical potential across the plasma membrane of cells. An abnormal serum K^+ level is associated with increased risk of ventricular arrhythmia and sudden cardiac death (SCD) and these patients are generally prescribed with furosemide and potassium chloride (KCl). We explored the association between the use of these drugs and the risk of SCD by analyzing biochemical parameters and proteomic changes. **Materials and Methods:** The rats were administered with furosemide and KCl and their effect was analyzed by studying cardiac and oxidative markers, electrolyte content and histopathology. Two-dimensional gel electrophoresis (2-DE) and electrospray ionization-mass spectrometry were performed to investigate the LV proteomic changes. **Results:** Furosemide and KCl treatments showed significant effect on physiological and biochemical parameters, and LV histopathology of experimental rats. Proteomic analysis indicated 17 differentially expressed proteins. Among them, eight protein spots were identified using peptide mass fingerprinting. In furosemide-treated group, four proteins were upregulated and two proteins were downregulated when compared to 2-DE proteomic profile of control. While in KCl-treated rats, seven proteins were found downregulated. **Conclusion:** The present study revealed the differential expression of proteins by furosemide and KCl treatment. Thus, the results suggest that the use of these drugs leads to proteomic alteration, which involve in cardiac conductivity that might increase the risk of SCD.

Key words: Cardiac conductivity, proteomic alteration, sudden cardiac death

INTRODUCTION

Sudden cardiac death (SCD) due to ventricular abnormality is an important cause of mortality worldwide.[1] Despite the advancement in medical sciences, the magnitude of SCD in general population is unknown.[2] The current scenario of cardiovascular treatment and prevention of SCD cases involves the management of serum electrolyte. It is well recognized that potassium, an abundant intracellular cation plays a major electrophysiological role in the cardiocytes and physiological processes including the maintenance of intracellular tonicity and renal function. An abnormal serum potassium level is commonly perceived in patients with cardiac and renal diseases. The use of furosemide and potassium chloride (KCl) is well recognized in the homeostasis of serum potassium level. Even though these drugs have its beneficial and preventive effect against cardiovascular disease,[3] they also known to induce deadly cardiac arrhythmias.[4,5]

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Furosemide, a short-acting diuretic is commonly recommended as an essential drug in patients with heart failure and fluid retention. A recent study has shown that furosemide administration increases mortality in heart failure rat model. However, it was prevented

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with additional administration of ACE inhibitor.[6] The adverse effects of furosemide have also been studied in tachycardia-induced porcine model.[7] Even though, diuretics are recommended as essential medication for patients with symptoms of heart failure and/or fluid retention, no controlled or randomized trials have adequately assessed the effect of a long-term administration of diuretics on morbidity and mortality.[8]

KCl, an electrolyte supplement is used to treat the patients with hypokalemia and cardiovascular diseases. However, the study demonstrated that an increased potassium intake had beneficial effects on the cardiovascular system, the inability of potassium supplementation in prevention of myocardial infarction has also been reported.[9,10] An earlier report suggested that during an increased serum potassium concentration, the conduction in all parts of the heart might be suppressed by decreasing the rate of rise of the action potential, which leads to decrease in the resting membrane potential and shift in the level of threshold potential.[11]

In order to understand the complexity in the cardiomyocyte, several genetic, pharmacological and proteomic studies have been carried out with the help of cardiac disease induced animal models.[12-14] Despite, the medical advancement in electrocardiogram (ECG) recording and the development of two-dimensional gel electrophoresis (2-DE) protein database for rat model is well established[13] but the link between furosemide/KCl and SCD is poorly understood. Thus, the present study is aimed to analyze the LV proteomic alterations in cardiac myocytes due to the administration of furosemide and KCl. General physiological parameters, cardiac markers and electrolyte content were monitored. A comparative proteomic analysis of LV was studied using 2-DE and electrospray ionization mass spectrometry (ESI-MS) techniques.

MATERIALS AND METHODS

Animals

Female Wistar rats (180-210 g) were fed with standard diet and water *ad libitum*. Rats were housed individually in metabolic cages under standard conditions (temperature 28 ± 2°C, relative humidity 55 ± 5%, 12-h light/dark cycle). All experiments were performed under the guidelines of institutional ethical committee. Rats were divided into three groups comprising six animals each. Group 1: Control- received Tris buffer (pH 8.8) orally; Group 2: Furosemide (furosenex)-treated – received furosemide (10 mg/kg) dissolved in Tris buffer (pH 8.8); Group 3: Potassium chloride (KCl) treated – received KCl (10 mEq/kg) dissolved in Tris buffer (pH 8.8). The administration was carried out intragastrically at 12-h interval for 15 days.

Physiological parameters, electrolytes and cardiac markers

The body weight and urine output were recorded at 24-h interval throughout the experimental period. Serum and urine samples were collected and subjected to various biochemical analyses. Potassium ions were estimated using ST-100 Na/K/Cl electrolyte analyzer (Sensa core medical instrumentation Pvt. Ltd., India). Sodium, magnesium and calcium ions were estimated using the kits procured from Agappe diagnostics (India). Serum cardiac markers such as Troponin I was estimated using Elecys Troponin T STAT immunoassay in Elecys system 1010 immunoassay analyzer. CK-MB with kit purchased from Calkine kit (Crest Biosystems, India) and C-Reactive protein (CRP) with CRP turbilatex test kit (Labkit, Spain).

Protein extraction

The LV heart tissue homogenate was prepared according to modified method of Zhu and Deng.[14] One hundred milligrams of heart tissue was chopped into small pieces and homogenized in 1 ml of 9 M urea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% Dithiothreitol (DTT) and 0.14% phenylmethylsulfonyl fluoride (PMSF) on ice. The resulting homogenate was centrifuged at 12,000 g for 30 min, supernatant was collected and used for 2-DE analysis.

Two-dimensional gel electrophoresis

Four-hundred and fifty micrograms of extracted protein was mixed with 250 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.5% ampholytes and 0.004% bromophenol blue as tracking dye). The sample was loaded onto a 13-cm IPG strip (pH 4-7, GE Healthcare, Sweden) for passive rehydration overnight and focused in an IPGphor 3 (GE Healthcare, Sweden) according to the modified manufacturer's protocol (GE Healthcare, Sweden). After isoelectric focusing, consecutive reduction and alkylation of the IPG strips were carried out using 20 mg/ml DTT and 25 mg/ml iodoacetamide (IAA), respectively, in an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS). Then, the second dimension was performed in 12.5% polyacrylamide gel in SE 600 Ruby (GE Healthcare, Sweden) at 50 V followed by Coomassie staining.[15] The stained gels were scanned using 2D platinum image scanner (Amersham, Germany) and gel images were analyzed using ImageMaster Platinum software version 7.0 (IMP 7).

ESI-MS analysis

The in-gel tryptic digestion was performed[16] and the resulting peptides were separated by ESI-MS (LCQ Fleet, Thermo Fisher Instruments Limited, US) in negative ion mode. The scan range of mass spectrum was 300–2000 m/z. After data acquisition, the generated XML files were used

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to perform database searches using the MASCOT software v. 2.2 (Matrix Science, London, UK). Search was carried out in NCBInr, MSDB, and the Swiss-Prot databases with the following parameters such as species homo sapiens; peptide tolerance, 0.2 Da; MS/MS ion mass tolerance, 0.1 Da; allowed up to one missed cleavage: Fixed modification, cysteine carboxyamidomethylation; variable modification, oxidation of methionine and propionamide (for cysteine modification by acrylamide). The MASCOT score was considered significant if $P < 0.05$.

Statistical analysis

The significance in spot expression and the statistical analysis were performed using ImageMaster Platinum software version 7.0 (IMP7) (GE Healthcare, Hong Kong, China). The experimental group gels were matched with reference gel (control) for analyzing the level of expression. One-way ANOVA test with a confidence interval at 95% and $P < 0.05$ was considered to be significant by the software. For analyzing other physiological and biochemical parameters one-way ANOVA followed by *post hoc* least significant difference (*post hoc*-LSD) test was carried out using SPSS software (version 16.0, SPSS inc. Chicago, IL, USA). All the physiological parameters were expressed as mean ± SD, while the estimated electrolytes were expressed as mean ± SEM for six rats in each group.

RESULTS

Effect on physiological parameters

The effect of furosemide and KCl on change in body weight, urine output and water intake was monitored throughout the experimental period. A statistically significant ($P < 0.05$) decrease in body weight gain and an increase in urinary output were observed in experimental groups, while the water consumption showed a significant increase ($P < 0.05$) in KCl-treated rats when compared to control rats. The blood glucose level, LV and whole heart weight showed no remarkable changes in experimental rats when compared to control.

Effect on cardiac markers and electrolyte content

The analyzed cardiac markers (CRP and CK-MB) and serum electrolytes (Na^+, Mg^{2+} and Ca^{2+}) levels did not show any significant change in experimental rats compared to control [Table 1]. However, a significant increase in troponin and serum K^+ level was observed in KCl-treated rats and a significant increase ($P < 0.05$) in urinary K^+ level was observed in furosemide-treated rats. The urinary sodium electrolyte level showed a significant decrease in KCl-treated group when compared to control. While, no significant changes was found in other analyzed parameters.

Table 1: Physiological parameters, cardiac markers and electrolyte levels of furosemide and potassium chloride treated rats

Characteristics	Control ($n=6$)	Furosemide administered ($n=6$)	Potassium chloride administered ($n=6$)
Body weight gain (g/15 days)	18.67±4.04	10.88±2.23*	4.50±1.32*
Urine output (ml)	2.86±1.09	5.45±0.99*	5.87±1.52*
Water intake (ml)	11.42±0.95	12.31±1.66	16.73±1.34*
Glucose (mg/dl)	98.00±6.08	107.67±9.61	114.00±5.13
Left ventricle weight (g)	0.45±0.01	0.49±0.07	0.44±0.07
Whole heart weight (g)	0.70±0.02	0.70±0.06	0.71±0.09
Cardiac markers			
CRP (mg/L)	1.00±0.00	1.35±0.15	0.95±0.25
CK-MB (U/L)	190.00±37.60	159.85±5.35	166.70±9.70
Troponin I (ng/ml)	0.015±0.001	0.016±0.001	0.027±0.004*
Serum electrolytes			
K^+ (mmol/L)	4.50±0.10	4.75±0.05	5.00±0.10*
Na^+ (mmol/L)	136.50±0.50	138.50±0.50	135.50±0.50
Mg^{2+} (mg/dL)	1.90±0.10	2.25±0.15	2.00±0.20
Ca^{2+} (mg/dL)	7.05±0.45	7.30±0.10	8.45±0.85
Urinary electrolytes			
K^+ (mmol/L)	8.60±0.55	12.03±1.05*	10.73±0.52
Na^+ (mmol/L)	69.67±2.60	64.33±2.03	55.33±1.86*
Mg^{2+} (mg/dL)	6.33±0.38	5.03±0.90	3.83±0.84

Values of physiological parameters are expressed as mean±SD; Values of cardiac markers and electrolyte content are expressed as mean±SEM; n=6. One way ANOVA followed by Post hoc test LSD test. Comparison: Group II and Group III vs Group I. Values are statistically significant at *P<0.05

Histopathological findings

LV histopathological sections showed no remarkable change in control and furosemide-treated rats, while the KCl-treated rats showed heart muscle fibers with focal mild mononuclear inflammatory cellular infiltrate [Figure 1a-c]. In control and furosemide-treated rats, LV sections, there was normal architecture with no pathological changes. The kidney sections of the furosemide-treated rats showed tubules with swelling of epithelial cells and interstitial edema among the congested blood vessels, while the KCl-treated rats showed hydropic swelling of tubular epithelial cells [Figure 1d-f].

Left ventricular proteomic alteration

A comparative protein analysis was performed to understand the effect of furosemide and KCl on LV and its significance on the cardiac conductivity. The proteins extracted from LV tissue homogenate were resolved in 2-DE gel using narrow range (pI 4-7, linear) IPG strips. Figure 2a shows the representative 2-DE gel proteomic profile of control rats. From the gels, the detected spots were selected and compared using the ImageMaster 2D Platinum Software version 7.0 (GE Healthcare, Hong Kong, China) and quantified. A total number of 232, 240 and 200 spots could be detected by the software in the control, furosemide- and

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KCl-treated groups, respectively [Figure 2b]. Of the 17 protein spots showing differential expression, 8 protein spots were identified [Table 2]. In furosemide-treated group, 4 spots were found to be upregulated and 2 spots were downregulated, whereas in KCl-treated group 7 spots were found to be downregulated in comparison with 2-DE gels of control group.

DISCUSSION

Furosemide and KCl have long been used in the prevention and treatment of hypertension and cardiovascular diseases. Case reports have also suggested that furosemide and KCl medication is associated with an increased risk of SCD.[17,18] Thus, its involvement in the electrolyte imbalance induced cardiac arrhythmia still remains controversial in the medical world.[19] The present study was carried out to understand the furosemide and KCl-induced conduction abnormality, leading to SCD in Wistar rats by analyzing various parameters [Figure 3].

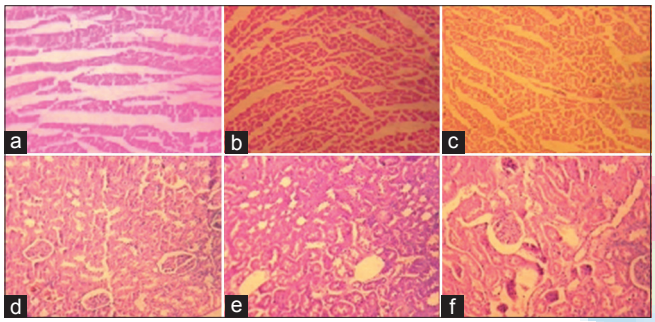


Figure 1: Histopathology of LV heart tissue of experimental groups; (a) control, (b) furosemide and (c) potassium chloride treated rats. Histopathology of kidney; (d) control, (e) furosemide and (f) potassium chloride treated rats (H and E, ×100)

Furosemide is known to induce diuresis[20] and KCl for polyuria and polydypsia,[21] which is evident from the present study. A decrease in body weight gain observed in the experimental groups might be due to the induction of polyuria. Furosemide and KCl is known to play a role in maintaining serum potassium level. Although, an elevation of serum K^+ level in KCl-treated

Table 2: Magnified 2DE map of differentially expressed LV proteome in furosemide and potassium chloride treated rats

Spot ID	Identified protein	Group 1	Group 2	Group 3
1	Potassium–gated channel subfamily G member 2			
2	Glyceraldehyde-3-phospate dehydrogenase			
3	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial			
4	Inward rectifier potassium channel 4			
7	Na(+)/H(+) exchange regulatory cofactor			
7b	Alpha-2-macroglobulin receptor-associated protein			
8	Tropomyosin beta chain			
12	Protein lin7 homolog B			

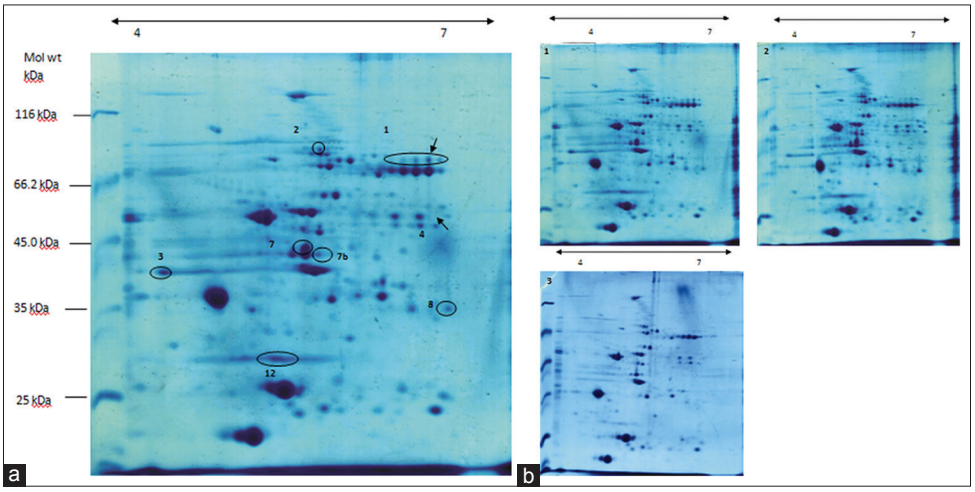


Figure 2: (a) Two dimensional proteomic profile of LV tissue homogenate of control rat. Aliquots containing 450 µg of proteins from control rat were subjected to IEF and separated by mass via SDS-PAGE (Coomassie stained). Protein spots (circled and numbered) were identified using mass spectrometry [Table 3]. (b) Representative image of 2DE gels of experimental groups. LV protein profile analyzed by 2DE gel of (1) control, (2) furosemide treated and (3) potassium treated rats. First dimension performed using immobilized pH 4-7 gradient strips, followed by SDS-PAGE in 12.5% polyacrylamide gels

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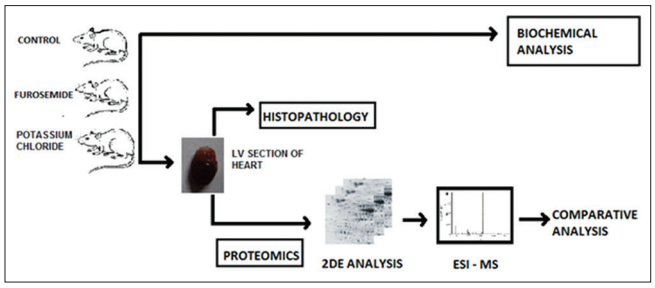


Figure 3: Graphical abstract

rats was observed, the induction of hyperkalemia was not established. The increased K^+ level probably reflects loss of K^+ from injured muscle and the release of cardiac Troponin I (cTnI)[22] is evident from the increased troponin level in KCl-treated group found in our study. Furosemide is mainly used to treat hyperkalemia, which brings about its desired effect by removing the excess serum potassium through its action on loop of Henle.[23] This property of furosemide resulted in an increased urinary potassium levels in experimental rats. The urinary sodium levels decreased in KCl-treated group might be due to the retention of these ions by the kidney, brought about by the action of the drug. The histopathology of LV and kidney sections of furosemide-treated rat with normal architecture and no sign of inflammation indicated that it has no cytopathological effect. While, KCl-treated group, the sign of inflammation in the muscle fiber has lead to the myocardial tissue damage, which is evident from the elevated troponin level. However, the hydropic swelling observed in kidney sections in experimental groups might be due to the possibility of hydropic regeneration caused by the inability of injured cells to maintain electrolyte balance through the sodium potassium pump. This is brought about by a fall in ATP causing efflux of K^+ with influx of Na^+ and thereby increasing the osmotic pressure in the cytoplasm that attracts water molecules. The resultant swelling of cells was clearly seen in experimental groups.

In this study female rats were chosen to keep uniformity among all experimental groups as well as to avail the protection against myocardial ailments. It is a well-known fact that the females are protected from coronary heart disease due to hormonal influence, whereas males are prone to development of coronary manifestation.[24] The present work was mainly focused on the effect of drug-induced proteomic changes in the experimental rats. Out of 17 differentially expressed proteins between the experimental and control group myocardium, only 8 proteins could be identified. This might be due to some new protein that is not yet entered in the 2-DE heart proteome databases. Among the 8 identified proteins showing differential expression, five proteins were known to be involved in the cardiac conduction, which includes potassium voltage-gated channel subfamily G, inward rectifier potassium channel 4, Na(+)/H (+) exchange regulatory cofactor, Tropomyosin beta chain and Protein Lin 7 homolog B [Table 3].

Voltage-gated channel subfamily G is a member subunit of the voltage-gated potassium channel, especially the delayed rectifier type channels, which play role in regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability and epithelial electrolyte depolarization.[25] It is also known to modulate the channel activity by shifting the threshold and half maximal activation to more negative values. This protein was found to be downregulated in both experimental groups suggesting a conduction abnormality. Surprisingly, we found that inward rectifier potassium channel 4 proteins was only expressed in furosemide-treated rats which might be due to the cellular potassium homeostasis, and are characterized by a greater tendency to allow potassium to flow into the cell rather than out of it. This occurs during an increased external potassium level and the voltage range of the channel opening shifts to more positive voltages. The inward rectification is mainly due to the blockage outward current by internal magnesium.[26]

Na(+)/H(+) exchange regulatory cofactor (NHRF2) was found to be upregulated in furosemide and downregulated in KCl-treated rats. It functions as a scaffold protein that connects plasma membranes of the ezrin/moesin/radixin (ERM) family and thereby helps to link them to the actin cytoskeleton and regulate their surface expression. This ERM family of actin-binding proteins acts as signal transducers in responses involving cytoskeletal remodeling.[27] Tropomyosin plays a role in Ca^{++}-dependent muscle contraction and β-Tropomyosin (TMβ) binds to actin filaments in muscle and non-muscle cells, is implicated in stabilizing cytoskeletal actin filaments. Absence of TMβ expression in KCl-treated rats was found in the present study. A similar result in breast cancer cell lines, found its function in tumor suppression and agonist-mediated receptor internalization.[28,29] An overexpression of TMβ was observed in furosemide-treated rats. Prabakar *et al*.,[30] carried out a study on transgenic mice to overexpress TMβ which revealed that high percentage of TMβ replaces alpha-tropomyosin in the heart and resulted in death due to severe cardiac abnormality. Thus, the overexpression of this protein suggests that furosemide treatment might lead to severe cardiac abnormality. Protein lin-7 homolog B plays an important role in establishing and maintaining the asymmetric distribution of channels and receptors at the plasma membrane of polarized cells. The differential expression in the experimental groups implies that the treatment might have altering effect on the channels and the receptors of polarized cells. In addition, this protein forms associated multiprotein complexes that may regulate the delivery and recycling of proteins to the correct membrane domains.

We have shown the differential expression of eight proteins of which five proteins plays an important role in cardiac conductivity in the furosemide and KCl-administered rats. The results suggested that furosemide and KCl alters

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Table 3: Differentially expressed LV proteins in furosemide and potassium chloride treated rats and its function

Spot ID	Identified protein	Database/accession no.	Mowse score I	Mr/pI2-D	Mr/pI database	Peptide sequence	Sequence Coverage %	Function
1	Potassium voltage-gated channel subfamily G member 2 (KCNG2)	NCBInr/gi|157817231	24	114354/6.30	52503/9.58	R. LPGHPEVP GAEPGSAVR. G R. SPCAFRA IVALLR. A R. AEEERG ECSTK. C R.AGLVLRL LRALRVLYVMR. L R. HSLGLRS LGLTVR. R R. SYSELK EQQQR. A	27	Modulates channel threshold and activation
2	Glyceraldehyde-3-phospate dehydrogenase (G3P)	SwissProt/G3P_RAT	49	130249/5.47	35805/8.14	K. FNGTVK. A R. DPANIK. W K. AGAHLK. G K. YDNSLK. I K. LTGMAFR. V R.LEKPAKYDDIK K.V	12	Key role in glycolysis and nuclear function. Participates in apoptosis and assembly of cytoskeleton
3	Phospho-lipid hydroperoxide glutathione peroxidase, mitochondrial (GPX41)	SwissProt/GPX41_RAT	48	521498/4.35	22838/8.74	R. DDWR. C K. VQPKGR. G K. FLIDK. N	13	Protects from toxicity of ingested peroxidase and oxidative damage
4	Inward rectifier potassium channel 4 (IRK4)	SwissProt/IRK4_RAT	24	83647/6.32	50285/5.82	R. NRFVKK. N K. MPRPKK. R R. VGNLRK. S	5	Cellular influx of potassium
7	Na (+)/H (+) exchange regulatory cofactor (NHRF2)	SwissProt/NHRF2_RAT	18	65521/5.37	37688/7.25	K. DVNGPPRE LRPRLCHLRR. G R. HAEVVAR. I R. SDLPGSEK. D R. ATRVNK. R	16	Surface expression and cAMP mediated phosporylation
7b	Alpha-2-macroglobulin receptor-associated protein (AMRP)	SwissProt/AMRP_RAT	18	62358/5.28	42006/6.85	K. YSREK. N R. LHLSPVR. L R. YGLDGRK. D K. LWHKAK. T R. EFLHYKEK. I K. SDTLASK. H K. ELESFR. E K. ELGYK. V	17	Interacts with LRP1/ alpha-2-macroglobulin receptor and glycoprotein 330
8	Tropo-myosin beta chain (TPM2)	SwissProt/TPM2_RAT	7	43654/6.45	32817/4.66	K. ENAIDRAE QAEADK. K R. CKQLEEEQ QALQK. K K. LKGTEDEV EK. Y	92	Striated muscle and smooth muscle contraction
12	Protein lin7 homolog B (LIN7B)	SwissProt/LIN7B_RAT	31	38462/5.15	22943/8.71	R. AVELLER. L R. SGELPPQK LQALQRVLQSRFCSAIR. E R. AHATAK.A R. VVELPK. T R. VIPGGVAD RHGGLKR. G K. AVELLKAA QGSVK. L R. FEKMRSAR. R	43	Establishment and maintenance of asymmetric distribution of receptor of polarized cells

LV= Left ventricular, NCBINR= National center for biotechnology information non-redundant database

the protein expression of the LV cardiocytes resulting in the disruption of polarity in the cells thereby, may lead to abnormal conduction of impulses within the heart, which can lead to arrhythmias. Our finding also presents solution for the furosemide and KCl-induced SCD. We yet regret that we could not confirm 2-DE and MS results by Western blot or immunohistochemical methods as this study is limited to only the proteome analysis of LV. Further, studies are required to confirm the significance of these identified proteins in patient samples. In conclusion, the commonly used drugs, furosemide and KCl in the treatment of various diseases render the differential expression of proteins in the LV tissue, which is involved in the cardiac conductivity. This study helps in the better understanding of furosemide and KCl-induced cardiovascular disease and SCD.

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