Original article:

EVALUATION OF ADENOSINE DEAMINASE (ADA) ISOENZYMES ACTIVITY AND TUMOR NECROSIS FACTOR-\(\alpha\) (TNF\(\alpha\)) CONCENTRATION IN CHRONIC HEART FAILURE

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ABSTRACT

Introduction: Chronic heart failure (CHF) has recently been considered as an inflammatory disease. Enhanced production of tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) in CHF patients has been proved. To compensate deleterious effects of TNF\(\alpha\), the concentration of adenosine is increased in CHF. However, concurrent determination of serum TNF\(\alpha\) and enzymatic activities of ADA and its ADA1 and ADA2 isoenzymes, as the main regulators of adenosine concentration, has not yet been carried out.

Materials and Methods: Blood samples were collected from 52 CHF patients and 55 healthy controls. Laboratory routine tests were performed, and after determining the concentration of TNF\(\alpha\), total ADA (tADA) as well as ADA1 and ADA2 isoenzyme activities were measured.

Results: Mean concentration of TNF\(\alpha\) increased over 2-fold in CHF patients (12.54 \(\pm\) 11.69 pg/ml compared with 6.0 \(\pm\) 6.58 pg/ml in controls). The highest level of TNF\(\alpha\) was observed in patients with the final stage of the disease (NHYA-IV subgroup), according to the New York Heart Association classification. tADA activity was significantly lower in CHF patients compared with controls (19.29 \(\pm\) 9.73 and 24.3 \(\pm\) 6.01 U/L, respectively). ADA2 activity markedly decreased in CHF patients and showed a direct correlation with tADA (r = 0.641, P = 0001). In addition, the lowest levels of tADA and ADA2 activities were observed in patients from the 4\(^{th}\) quartile of NYHA classification.

Conclusion: Adenosine deaminase activity is reduced in CHF patients to give rise to the concentration of adenosine, thereby attenuating pathologic consequences of CHF. Therefore, it is concluded that ADA activity is of paramount importance in pathophysiology of heart failure and might be used for diagnostic purposes or treatment targets.

Keywords: Adenosine deaminase, heart failure, isoenzymes, tumor necrosis factor-alpha
**INTRODUCTION**

Chronic heart failure (CHF) is delineated by reduced cardiac output accompanied by molecular changes that may result from any structural or functional cardiac disorders which lead to progressive heart failure and premature death due to cardiomyocytes and impair the ability of heart to support circulation (Katz and Konstam, 2008; NICE-Clinical-Guidelines, 2004). CHF is characterised by symptoms like shortness of breath and fatigue, and signs such as fluid retention. Due to high rate of mortality, CHF is considered as one of the most current problems and epidemic of human societies (Katz and Konstam, 2008; NICE-Clinical-Guidelines, 2004). Its incidence and prevalence has continued to increase with the aging of the population (NICE-Clinical-Guidelines, 2004) and has an enormous impact on modern societies due to its high mortality and morbidity (Katz and Konstam, 2008). About 1 in 35 people aged 65–74 years have experienced heart failure (NICE-Clinical-Guidelines, 2004). The risk of heart failure is higher in men than in women in all age groups, however because of the population demographics; however, more women than men are now suffering from heart failure (NICE-Clinical-Guidelines, 2004). Coronary heart disease has been considered as an inflammatory and immunizing disease during the past decade and immunity and inflammation may play an important role in the onset and progress of this disease (Libby et al., 2002; Tang et al., 2006). Several factors including neurohumoral mediators (Packer, 1992), catecholamines (Cohn et al., 1984; Francis et al., 1990), and particularly pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (Torre-Amione et al., 1996a, b) play pathogenic roles in the progression of CHF. It is believed that activation of immune system after myocardial injury may be responsible for the release of cardio-depressant cytokine, TNFα (Kaur et al., 2009). On the other hand, development of inflammatory responses and cytokine production is partially controlled by the plasma adenosine (Zidek, 1999).

Several lines of evidence support the idea that adenosine is cardio-protective against deleterious sequels in pathophysiological conditions of the heart, such as chronic heart failure (Hori and Kitakaze, 1991; Kitakaze et al., 1993, 1999) via inhibition of TNFα production, attenuation of release of catecholamines, will increase in coronary blood flow; and inhibition of platelet and leukocyte activation (Asakura et al., 2007; Hori and Kitakaze, 1991; Kinugawa et al., 2006; Kitakaze et al., 1993).

Although several enzymes, including 5'-nucleotidase, adenosine deaminase (ADA), and adenosine kinase (AK) are involved in the metabolism of adenosine (Asakura et al., 2007), its concentration is mainly regulated by the hydrolytic activity of adenosine deaminase which converts adenosine and deoxyadenosine nucleosides into inosine and deoxyinosine, respectively (Phillis, 1991). Two major isoforms of ADA are isolated with different characteristics. ADA1 exists in all human tissues and accounts for the main ADA activity in most of the tissues. ADA2 on the other hand, is the main ADA isoenzyme in serum originated mainly from monocyte-macrophage system (Gakis, 1996). Alteration in serum ADA activity has previously been reported in a wide array of diseases such as rheumatoid arthritis, tuberculosis, systemic lupus erythematosus, HIV-HBV coinfection, chronic obstructive pulmonary disease (Asakura et al., 2007; Goodarzi et al., 2010; Khodadadi et al., 2011; Zuckerman et al., 1980). The probably reduced activity of ADA in patients with CHF enhances intracellular adenosine levels and these changes may compensate for the pathophysiology of CHF.

Although the rise of TNFα concentration (Kaur et al., 2009) in failing heart and the inhibitory effect of adenosine on TNFα production have separately been shown (Kaur et al., 2009; Zidek, 1999),
there is a paucity of information on the concurrent determination of ADA activity, in particular ADA1 and ADA2 isoenzyme activities, and the concentration of TNFα in chronic heart failure. Therefore, the present study attempts to assess both the possible contribution of ADA in chronic heart failure by investigating alteration of serum total ADA as well as ADA1 and ADA2 isoenzyme activities and the concentration of TNFα in patients with CHF compared with control subjects.

MATERIAL AND METHODS

Subjects
All 52 consecutive patients (42 males and 10 females) with an acute exacerbation of CHF who admitted to the Sanandaj Tohid Hospital (Kurdistan, Iran) were enrolled in this case-control study. In addition, fifty five age-matched healthy controls were recruited as well. Written informed consent for participation was obtained and the project was approved by the Research Ethics Committee of Kurdistan University of Medical Sciences (Iran) and conformed to the Declaration of Helsinki. Demographic, clinical and laboratory data, and information on the specific therapy as well as data from physical and clinical examinations were recorded. Clinical examinations of patients showed different causes of heart failure including, idiopathic dilated cardiomyopathy, old myocardial infarction, hypertensive heart disease, and coronary artery disease. Similar physical, clinical, and laboratory examinations were carried out on 55 healthy subjects to exclude any exhibits stricken from the record of cardiovascular disease in control group. Drugs were used by patients group, Digoxin (23 patients), Beta-blockers (3 patients), Diuretics (21 patients), ASA (30 patients), Anticoagulant (1 patient), Amiodaron (3 patients) and Statins (18 patients). Patients were categorized into 4 groups according to the NYHA functional classes (NICE-Clinical-Guidelines, 2004) named as class NYHA-I to NYHA-IV (Table 1). Subjects with recent myocardial infarction, postinfarct angina, significant aortic stenosis, peripheral arterial disease, or patients receiving allopurinol and those who had left-ventricular ejection fraction over 40 %, as assessed by echocardiography, were excluded from the study.

Materials
Adenosine and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) were purchased from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA) and ELISA kit for determination of tumor necrosis factor-α (TNFα) was obtained from Boster Technology (Boster Biological Technology Ltd., Wuhan, China) whereas Pars Azmoon Company (Pars Azmoon Ltd., Tehran, Iran) supplied all commercial enzymatic kits for hematological and biochemical tests unless otherwise stated.

| Subjects                  | Number | Age (year) |
|---------------------------|--------|------------|
|                           |        | Range      | Mean ± SD   |
| Controls                  | 55     | 40-80      | 62.9 ± 11.1 |
| Chronic heart failure     | 52     | 40-87      | 67.3 ± 12.5 |
| NYHA-I                    | 9      | 42-83      | 60.2 ± 13.6 |
| NYHA-II                   | 15     | 52-87      | 70.3 ± 11.3 |
| NYHA-III                  | 13     | 45-79      | 64.3 ± 11.8 |
| NYHA-IV                   | 15     | 40-84      | 71.1 ± 12.3 |

NYHA: New York Heart Association
Blood sample collection and analysis

After the collection of blood samples, serums were separated, and stored at -70 °C pending assay. Aliquots of the EDTA-whole blood were used for white blood cell count by cell counter (Sysmex hematology analyzer kx-21, Sysmex Canada, Inc.). Serum samples were used for routine diagnostic tests such as fasting blood glucose (FBG), blood urea nitrogen (BUN), creatinine, electrolytes, lactate dehydrogenase (LDH), creatine phosphokinase (CK) and uric acid using commercial enzymatic kits, according to the manufacturer's instructions.

Determination of tumor necrosis factor-α (TNFα)

Serum TNFα was determined based on standard sandwich enzyme-linked immunosorbent assay technology. Briefly, human TNFα-specific monoclonal antibody was precoated onto plates. Serum TNFα and biotinylated human specific detection polyclonal antibodies were then added to the wells subsequently followed by the addition of Avidin-Biotin-Peroxidase Complex. Finally, horse raddish peroxidase (HRP) substrate was used to visualize HRP enzymatic reaction and the concentration of serum TNFα was expressed as pg/ml. Based on manufacturer's instruction limit of detection for this method was 5 pg/mL.

Determination of adenosine deaminase activity

Adenosine and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) were purchased from Sigma-Aldrich (Sigma, Missouri, USA). Serum total ADA (tADA) activity was determined according to the Giusti method (Giusti, 1984). In brief, based on the Bertholet reaction, the colored indophenol complex formed from ammonia liberated from adenosine was quantified spectrophotometrically. To determine ADA2 isoenzyme activity, serum ADA1 activity was selectively inhibited by the addition of EHNA into the serum samples and estimated ADA1 activity was calculated by subtracting of ADA2 from tADA activities (Giusti, 1984). Finally, enzyme activities were expressed in unit per liter (U/L).

Statistical analysis

Data was analyzed by SPSS 16 (SPSS Inc., Chicago, USA). One-Sample Kolmogorov-Smirnov test was applied to determine normal distribution of data. Results were presented as Mean ± SD and independent samples T-test used to compare mean differences. One-Way ANOVA followed by PostHoc, Tukey, and Dunnett tests were used to analyze differences between groups and the P value less than 0.05 was considered as significant. For variables with abnormal distribution equivalent non-parametric tests, Kruskal-Wallis and Chi-Square were performed and Correlation Coefficient (r) was determined to show the correlation between variables.

RESULTS

Fasting blood glucose, blood urea nitrogen, creatinine, uric acid, and concentration of electrolytes were detected. The enzymatic activities of lactate dehydrogenase and creatine phosphokinase as well as serum lipid profile and white blood cell count were determined as shown in Table 2.

Altering of serum TNFα in chronic heart failure: Mean concentration of TNFα increased more than 2-fold in chronic heart failure and CHF patients showed significantly (P=0.003) higher serum tumor necrosis factor-α level than controls (12.54 ± 11.69 and 6.0 ± 6.58 pg/ml, respectively). Data analysis also confirmed that concentration of TNFα significantly (P=0.006) differs in CHF patients from different subclasses of NYHA classification and a clear increasing trend in TNFα level was observed from 1st to 4th subclasses (Table 3) being the lowest in NYHA-I patients (8.62 ± 5.84 pg/ml) and the highest in 4th quartile by nearly 3-fold increase (17.23 ± 13.15 pg/ml).
### Table 2: Comparison of some serum biological parameters in CHF patients and healthy subjects

| Subjects                        | Control (n=55) | CHF (n=52) | p value |
|--------------------------------|----------------|------------|---------|
| FBG (mg/dL)                     | 99 ± 17        | 141 ± 71   | 0.001   |
| BUN (mg/dL)                     | 20.12 ± 7.98   | 29.80 ± 25.93 | 0.013 |
| Creatinine (mg/dL)              | 1.26 ± 0.39    | 1.87 ± 2.19 | NS      |
| Na⁺ (mmol/L)                    | 139 ± 3        | 137 ± 3    | NS      |
| K⁺ (mmol/L)                     | 4.12 ± 0.28    | 4.01 ± 0.52 | NS      |
| LDH (U/L)                       | 311 ± 108      | 581 ± 649  | 0.003   |
| CK (U/L)                        | 102 ± 42       | 157 ± 175  | 0.028   |
| Uric acid (mg/dL)               | 5.04 ± 1.36    | 8.33 ± 9.79 | NS      |
| TAG (mg/dL)                     | 148 ± 91       | 95 ± 35    | 0.004   |
| HDL-Cholesterol (mg/dL)         | 45.6 ± 12.98   | 40.6 ± 7.22 | NS      |
| WBC (×10⁹ cells/L)              | 6.96 ± 1.73    | 8.47 ± 2.85 | 0.002   |

Data is presented as Mean ± SD and p value less than 0.05 accepted as significant difference between groups. NS: not significant

### Table 3: Serum adenosine deaminase and TNFα concentration in patients and healthy subjects

| Subjects                        | Number | Adenosine deaminase activity (U/L) | TNFα (pg/mL) |
|--------------------------------|--------|------------------------------------|--------------|
|                                |        | tADA | ADA1 | ADA2 |                  |
| Controls                       | 55     | 24.30 ± 6.02<sup>a,b,c</sup> | 6.43 ± 4.07  | 16.19 ± 7.07<sup>a</sup> | 6.01 ± 6.58<sup>a</sup> |
| Chronic heart failure          | 52     | 19.29 ± 9.73<sup>a</sup> | 7.92 ± 5.84  | 11.37 ± 7.61<sup>a</sup> | 12.54 ± 11.69<sup>a</sup> |
| NYHA-I                         | 9      | 23.49 ± 14.53  | 10.92 ± 6.11 | 12.56 ± 10.14 | 8.62 ± 5.84 |
| NYHA-II                        | 15     | 20.74 ± 10.19  | 6.72 ± 5.05  | 14.02 ± 7.26 | 11.09 ± 11.57 |
| NYHA-III                       | 13     | 18.93 ± 7.65<sup>o</sup> | 6.77 ± 5.04  | 12.24 ± 7.92 | 12.96 ± 13.96 |
| NYHA-IV                        | 15     | 15.63 ± 6.52<sup>c,a</sup> | 8.32 ± 6.87  | 7.24 ± 4.28<sup>o</sup> | 17.23 ± 13.15<sup>o</sup> |

Data is presented as mean ± SD and similar a,b,c or d letters in each column represent significant difference (p<0.05) between groups. NYHA: New York Heart Association classification

**Correlation of adenosine deaminase activity with severity of CHD:** Mean serum total adenosine deaminase activity (tADA) decreased 20% in chronic heart failure and was significantly lower in CHF patients compared with healthy subjects (P=0.002). One-Way ANOVA analysis showed a significant difference in tADA activity between NYHA subclasses (P=0.003) with a trend to a reduced tADA activity from 1<sup>st</sup> to 4<sup>th</sup> quartile in CHF patients. In addition, NYHA-IV subclass of CHF patients showed the lowest level of tADA activity with over 35% reduction in enzyme activity (15.63 ± 6.52 U/L) whereas the highest activity (23.49 ± 14.53 U/L) was observed in the 1<sup>st</sup> quartile of NYHA classification (Table 3). A relatively similar pattern of alteration in enzyme activity was also observed for ADA2 isoenzyme with lower enzymatic activity in CHF patients compared with controls (P=0.001). Again, analysis confirmed that ADA2 activity significantly differs in patients from different subclasses of NYHA category being lowest in NYHA-IV subclass (7.25 ± 4.28 U/L). Unlike tADA and ADA2 activities, serum ADA1 isoenzyme activity neither differed between CHF patients and controls nor between patients from different NYHA subclasses (Table 3). A significantly positive correlation (P<0.0001) with Correlation Coefficient of r=0.641 was observed between tADA and ADA2 enzyme activities in CHF.
patients (Figure 1) but no strong correlation was found between enzymatic activities of tADA and ADA1 isoenzyme (data not shown).

**DISCUSSION**

There are several hypotheses with respect to the source of TNFα in heart failure including, the activation of immune system after myocardial injury, production of TNFα within myocardium, and elevation of TNFα due to the decreased cardiac output (Kaur et al., 2009). Therefore, it is believed that the abnormal inflammatory responses such as the over-expression of TNFα may play a role in the progression and clinical deterioration of chronic heart failure (Kleinbongard et al., 2010; Oikonomou et al., 2011). Studies from various laboratories demonstrated that patients with heart failure have increased concentration of serum TNFα which is directly correlated with a worsening prognosis (Flores-Arredondo et al., 2011; Heymans et al., 2009; Seta et al., 1996). Likewise, the results of the present study showed a significant increase in serum TNFα concentration in CHF patients in comparison to the controls confirming the contribution of TNFα in clinical complication of CHF. Moreover, a clear increasing trend in TNFα level was observed from 1st to 4th subclasses of NYHA classification. This observation is in line with the results of SOLVD (study of left ventricular dysfunction) and VEST (Vesnarinone) trials indicating that levels of TNFα is correlated to the severity of heart failure (Deswal et al., 2001; Flores-Arredondo et al., 2011; Kaur et al., 2009; Torre-Amione et al., 1996a).

It has been reported that for compensation of harmful effects of elevated TNFα in patients with heart failure, an enhancement in the concentration of adenosine is occurred (Asakura et al., 2007; Kitakaze et al., 1999). Since adenosine's concentration is mainly regulated by adenosine deaminase (Phillis, 1991) it is mandatory to consider the enzymatic activity of ADA to further pursue the hypothesis of the concurrent elevation of TNFα concentration and ADA activity in heart failure.

**Figure 1:** Correlation of serum enzymatic activities of total adenosine deaminase (ADA) with ADA2 isoenzyme in CHF patients
Adenosine deaminase (adenosine amidohydrolase, EC 3.5.4.4), plays important roles in human physiology and pathology and numerous studies have been undertaken to delineate its mechanisms of action in health and diseases. Therefore, an elevated level of ADA activity has been reported in a wide array of diseases (Goodarzi et al., 2010; Khodadadi et al., 2011; Tang et al., 2006; Zuckerman et al., 1980). In particular, down-regulation of ADA gene expression together with the reduction in ADA enzyme activity has been shown in coronary heart failure compared with healthy subjects (Asakura et al., 2007). Likewise, in the present study we showed a lower serum tADA activity in CHF patients compared with controls. In addition, the lowest level of tADA activity was observed in patients from 4th subclass of NYHA categorization who were associated with a worse prognosis of disease. These findings are explained by involvement of ADA in the pathophysiology of chronic heart failure. The reduction of serum tADA activity, as observed here in CHF patients, lowers degradation of adenosine nucleotides and gives rise to the concentration of adenosine allowing this endogenous nucleoside to exhibit its cardio-protective potency (Goodarzi et al., 2010; Khodadadi et al., 2011; Zuckerman et al., 1980).

Similar to the reduction in serum tADA, we showed a significant decline in ADA2 isoenzyme activity in CHF patients. The observed accompanied reduction of tADA and ADA2 activities can be explained by the fact that the predominant isoenzyme in serum is ADA2 and plasma ADA activity results from ADA2 isoenzyme (Kowalczyk et al., 2008; Ungerer et al., 1992) therefore; reduction of total ADA activity might be due to a decline in the ADA2 isoenzyme activity. The concurrent elevation of serum total ADA and ADA2 isoenzyme activities has also been reported in other studies (Kowalczyk et al., 2008) confirming a strong direct correlation between these enzyme activities, as observed in the present study.

We showed a significant decline of tADA activity in CHF patients by worsening the disease. Patients in NYHA-IV subclass exhibited a greater reduction in tADA activity in comparison to those subjects in NYHA-I, -II, and -III subclasses. Similar to the activity of total adenosine deaminase, ADA2 isoenzyme activity was also lowest in the patients with worst heart failure (NYHA-IV). Therefore, it can be concluded that serum total ADA and ADA2 activities decrease with increasing pathologic signs of disease.

Our results proposed that ADA activity might be a useful biomarker for determination of CHF severity. Measurement of tADA activity as a simple, rapid, and inexpensive diagnostic marker makes it a usefulness tool for monitoring of CHF patients. Furthermore, among the other targets for therapy to ameliorate the cardiac function and improve long-term prognosis in CHF patients, ADA2 might be considered as a new treatment target. However, further studies are needed to substantiate this claim.

In conclusion, serum TNFα concentration increased with worsening prognosis in CHF patients. Significantly lower activities of total ADA and ADA2 isoenzyme were also observed in CHF patients, decreasing with worsening of disease according to the NYHA categorization. It is supposed that decreasing of ADA activity augments concentration of adenosine to compensate cardiodepressant effects of increased TNFα in CHF patients.

Due to time and funding constraints, data from a large-scale population could not be collected to achieve more clear differences in ADA activity and TNFα concentration in patients from different subclasses of NYHA classification. It should also be noted that although CHF patients with different causes of heart failure including, idiopathic dilated cardiomyopathy, old myocardial infarction, hypertensive heart dis-
ease, coronary artery disease, and others were included in the study, determining the deleterious impact of alteration in ADA activity and TNFα concentration in each type of heart failure were beyond the scope of this study.

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Potential conflict of interest:
None declared.

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