Original Article

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Biochemistry Section

Nitric Oxide and Iron- Friends or Foes to Heart?

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ABSTRACT

Introduction: Ischemic Heart Disease (IHD) is the leading cause of cardiovascular death worldwide and will be one of the four major causes of death by 2030. Nitric Oxide (NO) is a locally acting vasodilator that regulates smooth muscle tone and produces relaxation of coronary arteries. Iron (Fe) is an essential nutrient that contributes to many important physiological functions in the body. Abnormalities in the serum levels of iron and NO play a crucial role in the development of the cardiovascular disorder, especially IHD, by promoting atherogenesis.

Aim: To estimate and compare serum iron and NO levels in new and old cases of IHD and also to find the correlation between iron and NO levels in IHD.

Materials and Methods: The present case control study was done in the Department of Biochemistry, KS Hegde Charitable Hospital, from October 2015 to March 2016. A total of 107 subjects were included in the study. Out of 107 subjects, 35 were new IHD cases diagnosed recently, 36 were old IHD cases currently on treatment, and 36 were age and sex matched controls. After obtaining Ethical clearance and taking informed consent, iron and NO levels were analysed. Iron was determined by the Ferrozine method on the autoanalyser. Serum NO was estimated by the Griess reagent method using a Double UV beam spectrophotometer. Results were expressed as mean±SD for normally distributed data and as median (interquartile) for skewed data. The groups were compared using ANOVA for normal data and the Kruskal Wallis test for skewed data. Correlation between different parameters was determined using Karl Pearson's correlation coefficient. A p-value of <0.05 was considered statistically significant.

Results: Both new and old IHD cases had significantly low ($p<0.001$) serum NO levels when compared to controls. The levels were moderately higher ($p<0.05$) in old IHD cases, than those in new IHD cases. Significantly low mean serum iron levels were observed in both new and old IHD cases when compared to controls. However, all the serum iron values were within the normal reference range. There was no statistically significant correlation between serum NO and iron in new and old cases of IHD.

Conclusion: High NO and iron levels reduce the risk of IHD. Serial estimation of these parameters in high risk population may help in early diagnosis and prevention of IHD.

Keywords: Correlation, Free radicals, Ischemic heart disease, Inflammation

INTRODUCTION

The IHD contributes to 12.2% of total deaths worldwide and is expected to be one of the four major causes of death by 2030 [1]. The prevalence of IHD is estimated to be as 23.8 million cases in India [2]. The most common cause of IHD is atherosclerotic disease of the coronary artery, which reduces myocardial blood flow and causes inadequate perfusion of the myocardium supplied by the involved coronary artery [3].

Nitric oxide protects against cardiovascular diseases by regulating blood pressure, inhibiting platelet aggregation, and preventing smooth muscle cell proliferation by activating guanylatecyclase, which causes an increase in the concentration of cyclic Guanosine Monophosphate (GMP) in target cells. Reduced bioavailability of NO causes endothelial dysfunction and leads to loss of cardioprotective effect [4]. Another theory states that at higher levels, NO reacts with superoxide to form peroxynitrite, which causes tissue and cellular injury [5].

Iron (Fe) is an essential nutrient that contributes to many important physiological functions in the body. Increased levels of iron promote the free hydroxy radical generation, which promotes oxidative damage and atherosclerosis [6]. Few studies report low or no difference in iron levels in acute coronary syndrome [7,8].

There exists a delicate homeostatic balance between NO and Fe in biological systems. There is a possibility of increased production of NO, leading to high iron stores by increasing the affinity of Iron Regulatory Protein (IRP) or by increasing the expression of hemeoxygenase. But this increase in cellular iron down regulates the expression of NO synthase and thus NO. Decreased NO levels impair vasodilation, blood pressure regulation, and promotes atherosclerosis [6].

Many studies have been done estimating these parameters in acute cases of IHD compared to controls. In this study, a third group comprising of old IHD cases were also considered so as to understand the effectiveness of treatment in them. Existing data is not clear whether increased or decreased levels of NO and Iron are causing IHD. So, whether the increase in these parameters is preventing (beneficial -friends to the heart) or causing (damage-foes/enemies to the heart), IHD requires research. So this study was taken up to understand the change in levels of these parameters in IHD. Hence, the study aimed to estimate and compare serum iron and NO levels in acute as well as old cases of IHD and also to find the correlation between iron and NO levels in IHD.

MATERIALS AND METHODS

The present case control study was done in the Department of Biochemistry, KS Hegde Charitable Hospital, from October 2015 to March 2016. As per the statistician's suggestion, to test the rejection of the null hypothesis between multiple groups and also to decrease type I error, a minimum sample size of 98 in total was determined by using the statistical formula applicable for ANOVA with post hoc Bonferroni Correction. Ethical clearance was obtained from the Institutional Ethical Committee before the start of the study (Ref. No. KSHEMA/E.C/001/2014-15). A total of 107 subjects were included in the study. Informed consent was obtained from these subjects before the start of the study.

The subjects for the study were recruited among those admitted to the Cardiology ward of KS Hegde Charitable Hospital. The subjects were selected based on the clinical history and clinical examination. The criterion for the diagnosis of IHD was based on the findings in ECG or echocardiogram (presence of regional motional valve abnormality) or elevation of cardiac markers. The most common clinical presentations of IHD are ST-Segment Elevated Myocardial Infarction (STEMI), Non ST-Segment Elevated Myocardial Infarction (NSTEMI) and Unstable Angina (UA). When the ECG is not diagnostic of STEMI, early detection of the presence or absence of wall motion abnormalities by echocardiography can aid in management decisions [9]. Age and sex matched control subjects were recruited from the general population.

Inclusion Criteria

Subjects with haemoglobin >12 gm/dL, between the age group of 30-60 years who were admitted to the Cardiology ward and were diagnosed to have IHD by Cardiologists,were included in the study. Patients with a history of Diabetes and Hypertension (who were on regular treatment) were also included in the study.

Exclusion Criteria

Patients with a history of chronic kidney disease,chronic liver disease, malignancy, or subjects who donated blood in the past three months or on iron therapy or antioxidants, or having Infections, Anaemia, and Polycythemia were excluded from the study.

The study populations of 107 were further divided into 35 new IHD cases with recently diagnosed IHD (before starting treatment), 36 old IHD cases with a history of IHD (currently on therapy), and 36 age and sex matched controls. Subjects with a history of IHD (old IHD subjects) were on treatment with statins, aspirin, and clopidogrel for a minimum period of six months and attended OPD for regular follow-up. Subjects with a history of hypertension and diabetes were on appropriate antihypertensive and antidiabetic medication.

Laboratory Investigations

With aseptic precautions, 5 mL of blood samples were collected from the antecubital veins of the selected subjects in plain red-topped vaccutainer tubes containing clot activator. Blood samples were left undisturbed for 30 minutes following which they were centrifuged for 5 minutes at 3000 rpm, and sera were separated. All the analyses were carried out on serum samples only. Sera were stored at -20°c till analysis for the estimation of NO. The precision of the instrument was checked on many occasions. All the analytical procedures were standardised. Iron was determined by the Ferrozine method [10] on the autoanalyser. Serum NO was estimated by the Griess reagent method [11] using a Double UV beam spectrophotometer. Optical density was measured at 550 nm against blank using a Double UV beam spectrophotometer. The concentration in the sample was calculated from a calibration plot prepared by running a series of standard nitrite solutions.

STATISTICAL ANALYSIS

Data were analysed using Statistical Package for Social Sciences (SPSS) 16. Results were expressed as mean±SD for normally distributed data and as median (interquartile) for skewed data. The groups were compared using ANOVA for normal data and the Kruskal Wallis test for skewed data. Pairwise comparison was made using the Mann-Whitney U test adjusted for p-values using Bonferroni correction. Correlation between different parameters was determined using Karl Pearson's correlation coefficient (r). A p-value of <0.05 was considered statistically significant.

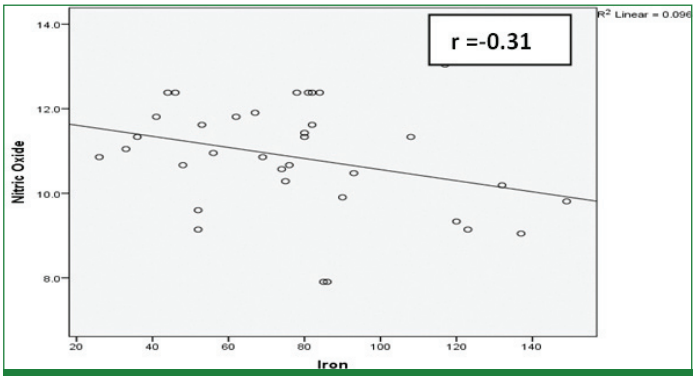
RESULTS

In each study group of IHD, six subjects were females, and the rest were males. The minimum age in new IHD cases, old IHD cases, and controls were 35, 38, and 39 years, respectively, and the maximum age in all the three groups was 60 years. Comparison of Serum NO and Iron levels in IHD cases and controls is shown in [Table/Fig-1]. Both new and old IHD cases had significantly low (p<0.001) serum NO levels when compared to controls. The levels were moderately higher (p<0.05) in old IHD cases, than those in new IHD cases. Significantly low mean serum iron levels were observed in both new and old IHD cases when compared to controls. However, all the serum iron values were within the normal reference range. The decrease was more in old IHD cases (p<0.001) than in new IHD cases (p<0.05). The difference in serum iron levels between new and old IHD cases was not statistically significant. There was no difference observed in mean levels of serum NO and iron in subjects with and without risk factors.

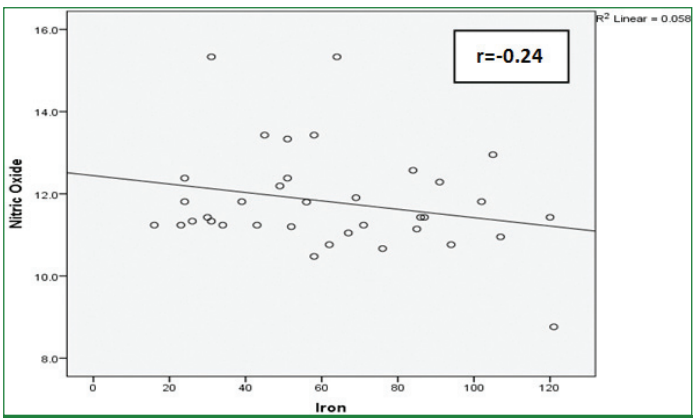
Study groups	Serum nitric oxide levels in µmoles/L, median (interquartile)	Serum iron levels in µg/dL (Mean±SD)
New IHD cases	10.9a*,b** (9.9-11.8)	77.6±30.7^{x*}
Old IHD cases	11.4c** (11.2-12.3)	62.0±29.3^{y**}
Controls	18.1 (15.9-20.7)	94.2±17.8

[Table/Fig-1]: Comparison of Serum NO and Iron levels in IHD cases and controls. a: comparison of new IHD cases with old IHD cases; b: comparison of new IHD cases with controls; c: comparison of old IHD cases with controls; x: comparison of new IHD cases with controls; y: comparison of old IHD cases with controls; *p-value of <0.05; **p-value of <0.001

[Table/Fig-2,3] shows the correlation between serum NO and Iron in new and old IHD cases. There was no significant correlation between serum NO and iron in new (r=-0.31) and old (r=-0.24) cases of IHD.



[Table/Fig-2]: Correlation between serum NO and Iron in new IHD cases.



[Table/Fig-3]: Correlation between Serum NO and iron in old IHD cases.

DISCUSSION

The IHD mortality rates increases with age and are generally higher in men than women [12]. In the present study also, the majority of IHD cases were males, and most of the cases were

seen in subjects with age in the upper half of 30 to 60 years range.

In the present study, IHD cases had significantly low serum NO levels when compared to controls. These results are following the fact that diminished NO levels facilitate vascular inflammation, oxidation of lipoproteins, and formation of atherosclerotic plaque [13]. Rizk A et al., and Bhardwaj S et al., have also observed low NO levels in IHD cases when compared to controls [14,15].

This study also observed an increase in serum NO levels in old IHD cases when compared to new IHD cases. This increase may be because of treatment with statins. Statin treatment causes a decrease in Ras homologous protein (Rho) GTPase activity and an increase in levels of NO [16]. The intermediate Geranyl Pyrophosphate formed during cholesterol synthesis attaches to Rho and activates Rho kinase. Statins inhibit Rho kinase activity, which leads to increased endothelial nitric oxide synthase (eNOS) expression and, finally, NO production [17]. Another mechanism by which statins increase NO levels is the activation of protein kinase B (Akt). Akt modulates caspase-9 and eNOS by phosphorylation and increases NO levels [18]. Laufs U et al., found that there was an increase in endothelial synthase activity and NO levels on treating mice with atorvastatin, and subsequent withdrawal of statins caused a decrease in NO levels [19].

Results of the present study are contradictory to the existing theory that increased levels of free iron promotes the free radical formation and, finally, atherosclerosis and ischemia [6]. The serum iron levels in all the three groups were within the normal range, but mean serum iron levels in the IHD were low in comparison to controls. Kervinen H et al., found an association between low serum iron levels and coronary heart disease [7]. Liao Y et al., also observed that there was an inverse relationship between serum iron and Myocardial Infarction (MI) risk in women [20]. Ekblom K et al., found that there exists a low risk for MI with iron levels in the normal upper range [21]. Contrary to the above studies, Marniemi J et al., found that there was no association between iron levels and coronary heart disease [8].

Serum iron levels in old IHD cases were moderately decreased when compared to new IHD cases in the present study. These results are partially in accordance with studies done by Regnstrom J et al., who found that serum iron levels were low in MI patients below the age of 45 years after a follow-up of 4-6 months post infarction. Still, drug therapy had not been stated [22]. The role of statins in decreasing iron levels is not clear.

In the present study, there were no significant correlations between NO and iron in new and old cases of IHD. These results are contradictory to a study done by Adeyemi OS and Akanji MA, which suggested that increased NO levels increase iron stores, which inturn generates free hydroxyl radicals level and causes atherosclerosis [6].

A study done by Tojo A et al., on rats indicated that NO levels decreased with hypertension, and activity of endothelial NO synthase improved on treatment with antihypertensive drugs [23]. Many subjects in the study group had multiple risk factors of IHD like hypertension, diabetes, smoking, and alcoholism, and the number of subjects having one particular set of risk factors was limited. Hence statistical analysis could not be done. There was no statistically significant difference observed in mean levels of serum NO and iron in subjects with and without risk factors.

Limitation(s)

This study consists of a small sample size. Correlations between serum iron and NO would probably have been better understood if the study was performed on a larger population. Serial estimation of serum iron and NO levels during the acute phase would have given a better understanding of the role of these parameters in acute IHD cases. Ferritin analysis, along with serum iron, could have been a better parameter for understanding the correlations. Follow-up studies were not done. Variations in serum iron and NO in old IHD subjects would have been better understood if follow-up studies were done. The confounding effect of IHD risk factors can be removed when the study is done on a large population. Other confounding factors for the alteration in levels of serum iron in old subjects of IHD should be studied.

CONCLUSION(S)

In the present study, low NO levels were observed despite the treatment in old IHD cases. These low NO levels are a matter of concern as they have been reported to predict future risk of adverse cardiovascular events. Though serum iron levels were decreased in IHD cases when compared to controls, all the values were within the normal reference range. So other confounding factors such as dietary iron that might alter the levels of serum iron in IHD cases should be studied. Serial estimation of NO and iron levels in high risk population may help in early diagnosis and prevention of IHD.

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