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Research Article

Detection of Endothelin (ET-1 and ET-3) as a novel biomarker of cardiovascular stress in patients of liver cirrhosis with ascites

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Abstract
Endothelin (ET) is an endothelial cell-derived peptide with greater vasoconstrictive potency involved in the control of systemic blood pressure (BP) and vascular tone. Dysfunctioning of ETs (endothelin-1 and endothelin-3) is considered a key risk factors for cardiovascular disease. The present study was aimed to find the correlation of endothelin and oxidative stress with cardiovascular stress. 50 Blood and ascitic fluid samples were collected from the patients of liver cirrhosis with ascites and 25 blood samples from normal individuals from General Hospital Lahore Pakistan. Endothelin-1 and endothelin-3 were quantified in serum and ascetic fluid by Real time PCR. Nitric oxide (NO), creatine kinase (CK), creatine kinase-MB (CK-MB), cholesterol, and C-reactive protein (CRP) were estimated as cardio vascular stress parameters and glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MAD) were estimated as biomarker of oxidative stress. ET-3 was quantified while ET-1 was not quantified due to very low concentration both in serum and ascitic fluid. Overall positive and highly significant ($P<0.05$) correlation of oxidative stress and cardiovascular stress with ET-3 was found. It was inferred that increase production of endothelin (ET-3) under oxidative stress generates cardiovascular stress in patients of liver cirrhosis with ascites.

Keywords: Endothelin, superoxide dismutase (SOD), malondialdehyde (MAD), catalase (CAT), glutathione (GSH), antioxidative enzymes, cardiovascular disease.

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Introduction

Endothelins are from the family of peptides, which comprises endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), each comprises 21 amino-acids [1]. The overall function of endothelin is to increase blood pressure and vascular tone. Endothelins are proteins that constrict blood vessels. They are normally kept in balance by other mechanisms; however, when they are over-expressed, they contribute to high blood pressure and heart disease. ET production is stimulated in a variety of different cell types under the influence of risk factors for cardio vascular stress and during development of CVD [2]. Endothelial dysfunction and the bioavailability of nitric oxide (NO) have been documented as one of many risk factors for cardiovascular disease [10]. Mechanisms that participate in the reduced vasodilatory responses in endothelial dysfunction include nitric oxide generation, oxidative stress, and reduced formation of hyperpolarizing factor [13].

Endothelin-1 and endothelin-3 has been implicated as an important factor in the development of vascular dysfunction and cardiovascular disease. In patients of liver cirrhosis with ascites there is induced production of oxidative stress with the production of endothelins. Portal hypertension is produced due to vasoconstriction. Splanchnic vascular and peripheral arterial vasodilation takes place which generates NO overproduction. In cirrhosis arteriolar vasodilation causes under filling systemic arterial vascular space. This event, through a decrease in effective blood volume leads to a drop in arterial pressure. Consequently baro-receptor activated renin-angiotensin aldosterone system (RAAS) occurs to restore normal blood homeostasis [5].

Splanchnic vasodilation causes increase lymph production that exceeds the lymph transportation system capacity and lead to lymph leakage in to the peritoneal cavity. RAAS produces vasoconstriction as a result cardiovascular stress is generated in patients of liver cirrhosis with ascites [12]. Keeping in view the role of endothelins in generating cardiovascular complications in liver cirrhosis, the present study was conducted to

estimate Endothelin (ET-1 and ET-3) as a novel biomarker of cardiovascular stress in patients with liver cirrhosis.

Materials and Methods

Human sera and ascitic fluid were obtained from patients of liver cirrhosis with ascites from General Hospital Lahore Pakistan, after approval of the protocol by the Ethics Review Committee of hospital, along with blood samples of normal individuals and were stored at -20 ^{o}C until further use. 25 samples of blood and 25 ascetic fluids were taken from the patients of liver cirrhosis with ascites. 25 blood samples were collected from normal individuals. The samples were processed and analyzed for detection of ET-1, ET-3, SOD, GSH, CAT, MDA, NO, cholesterol CRP, CK and CK-MB. Patients with hypertension, renal failure, diabetes, CVD, and pregnant women were excluded from the study.

Detection of ET-1 and ET-3

A multiplex PCR was employed to genotype the serum and ascitic fluid specimen. The remaining samples were separated and stored at -20 ^{o}C until further use. RNA was extracted from serum and ascitic fluid samples by using RNA isolation Kit Cat No: FAVK001-2 provided by Favorgen Biotech Corp, according to the kit protocol with little modification. 570 µl of VNE (carrier RNA added) was added to the serum and ascitic fluid samples, after vortexing incubated for 10 minutes at room temperature 570 µl of ethanol (96-100%) was transferred to samples. 500 µl of wash buffer 1 (ethanol) was added to VNE column, and was centrifuged at 8000rpm for 1 minute. Then 50 µl of wash buffer 2 (ethanol added) was added to VNE column and was centrifuged at 8000rpm for 1 min before discarded the flow-through. Whole sample mixture was centrifuged at full speed 13,000 rmp for 1 minute. 50 µl of RNase-free water was transferred to membrane center of VNE column to elute nucleic acid from collection tubes after centrifugation at 10,000 rpm for 2 minutes and nucleic acid was stored at -70°C.

Amplification conditions

For ET-1 and ET-3 primers were designed by primer3 software (**Table 01**). After reconstitution of primers PCR reaction was carried out in thermal cycler with Taq DNA polymerase (5U). The amplification was performed with 2 µl of cDNA by using 7.8 µl first round PCR mix. 8 µl reaction mixture was added to form 10 µl total reaction. Cycling condition for amplification of ET-1 and ET-2 was kept same. With 35 cycles at 50 ^{o}C 7 µl Mix 2 of ET-1, 1µl taq (2U) and 2 µl of template was carried out. In 2nd Round of Amplification of ET-3 7.8 µl Mix 2 of ET-3, 0.2 µl Taq (5U) and 2 µl of template was added and reaction was carried at 54 with 35 cycles. Detection of PCR amplification products (ET-1 and ET-3) was detected on 2% agarose gel.

Quantification of ET-1 and ET-3

Reaction mixture was prepared by adding 12.5 µl SYBET Green, 2.5 µl, Forward Primer 10 (pm/µl), 2.5 µl Reverse Primer, 2.5 µl water (nuclease-free) and 5µl cDNA. Master mix was mixed thoroughly. For ET-1 and ET-3, in Stage 1 at 95°C for 600 seconds and in stage 2:3 95.0^{o}C for 15 seconds, 50.0^{o}C for 30 seconds and 72.0^{o}C for 30 seconds, thermal conditions were applied.

Estimation of MDA through standard curve by using TBARS

Thiobarbituric acid reactive substance was used for estimation of MDA level in serum and ascitic fluid [11]. 1 ml of both samples was taken and a 10% (w/v) homogenate was prepared in 10 mM buffer (pH 7.4). The supernatant was used for immediate thiobarbituric acid reactive substances. In this test 200 µl of serum sample, 200 µl of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 0.8% TBA, 1.5 ml of 20% acetic acid solution (pH 3.5) and 4.0 ml distilled water and 5.0 ml of n-butanol were used. Its absorbance at 532 nm was recorded.

Estimation of SOD, catalase and glutathione activity through standard curve

Serum and ascitic fluid were taken and homogenate was prepared in 50% TCA and centrifuged at 13000 rpm. The supernatant was used for immediate SOD and other enzyme activity evaluation. In this test 100 µl of serum sample,1.2 ml of sodium phosphate buffer (pH 8.3, 0.052M),100 µl of Phenazinemethosulphate (186µm), 300 µl of nitro blue tetrazolium (300 µm), 200 µl of NADH (750 µm) and 4.0 ml of n-butanol were used. Reaction was started by

addition of NADH. .Its absorbance at 560 nm was recorded against butanol. All procedure for estimation of antioxidant enzyme superoxide dismutase was performed in ice bath.

Catalase activity was measured by the method earlier described by Aebi et al [14]. 1 ml of serum and ascitic fluid was taken and a 10% (w/v) homogenate was prepared in 10 mM buffer (pH 7.4) and was centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was used for the estimation of CAT. 100 µl of supernatant and 1.9 ml of 50mM phosphate buffer and freshly prepared 30 mM H2O2 were used. The rate of decomposition of H2O2 was measured spectrophotometer at 240 nm. Activity of catalase was expressed as unit/ml of protein.

0.1 ml of both samples (serum and ascitic fluid) was added in test tubes. 2.4 ml of 0.02M EDTA was added in each test tube and was kept in ice bath for 10 minutes. Then 2.0 ml of dH_2O (distilled water) was added in each test tube. After addition of dH_2O 0.5 ml 50% TCA was added in each test tube and was again kept in ice bath for 10-15 minutes. The mixture was centrifuged at 3000-3500 rpm for 10 minutes. 1.0ml supernatant was taken in separate tubes and 2.0 ml 5 M Tris HCl and 0.05 ml DTNB was added. The mixture was vortexed and absorbance was taken after 2-3 minutes at 412 nm. The absorbance was compared with standard curve generated by the known GSH level. The GSH was measured in µg/ml.

Measurement of cardio vascular stress: Estimation of nitric oxide, cholesterol, CK, CK-MB and CRP

100 µl of Gries Reagent, 300 µl of the nitrite-containing samples (serum and ascitic fluid) and 2.6ml of deionized water was added in each test tube. Absorbance was measured at 548 nm. Absorbance was compared with standard curve generated by the known NO level.

20 µl of serum samples, 1000 µl of reagents and 200 mg/dl cholesterol aqueous primary standard was and absorbance was noted at 340 nm by Techno plus Chemistry Analyzer.

CK level was determined by Randox (Creatine Kinase Manual RX MONZA) kit from serum samples of patients of liver cirrhosis with ascites. 0.8 ml enzyme reagent (L1), 0.05 ml sample were mixed in test tube after adding 0.2 ml of starter reagent (L2) . Absorbance was noted at 340 nm by Techno plus Chemistry Analyzer.CK-MB level was determined by Global,s Creatine Kinase (Immunoinhibition / Modified IFCC) kit in serum samples of patients of liver cirrhosis with ascites. 0.8 ml enzyme reagent (L1), 0.05 ml sample and 0.2m of starter reagent (L2) was added in test tubes and absorbance was noted at 340nm by Techno plus Chemistry Analyzer.

CRP was determined by Latex test (CRP/010) kit. Agglutination was noted from each sample of serum.

Statistically designing and data analysis

The obtained data was evaluated statistically by using t-test and Pearson correlation.

Results

Both proteins ET-1 and ET-3 were detected in blood and ascetic fluid. ET-1 was detected 30% and detection of ET-3 was 35%.

ET-1 was not quantified in any of sample while ET-3 was quantified in serum and ascitic fluid samples. ET-3 was quantified at Ct= 295.5, 33.30, 34.63. Amount of ET-3 was calculated by WHO standards and amount of ET-3 was 59726.7 cps/µl, 4311.9 cps/µl and 315.9 cps/µl for three endothelin gene polymorphisms.

The mean value of serum catalase (U/dl) level in normal individuals was recorded as 164±10.86 and 142±10.59 in patients of liver cirrhosis with ascites. Mean SOD (U/dl) levels in normal individuals were measured as 9.55±2.21 while in patients of liver cirrhosis with ascites it was assessed as 7.91±2.03. GSH levels in patients were observed 3.96±1.39 as compared to control group 9.96±2.48. MDA (U/dl) levels in patients of liver cirrhosis with ascites were observed as 5.41±1.16 as compared to control group 3.68±1.22. The MDA levels were significantly increased ($p < 0.05$) in patients as compared to control group. While significant ($p < 0.05$) decreased level of serum CAT, GSH and SOD was observed in patients of liver cirrhosis as compare to control group.

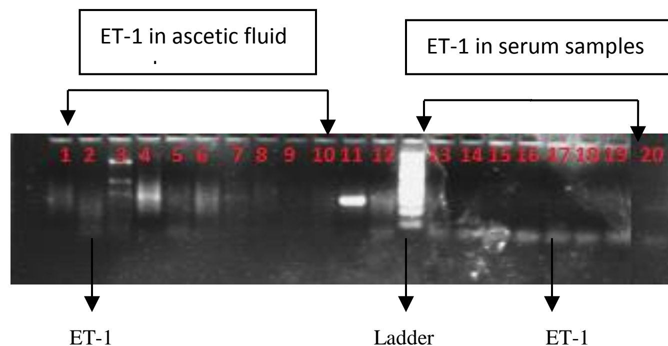
The mean values of catalase (U/dl) in serum and ascetic fluid of patients were evaluated as 143±10.59 and 119±13.69 respectively. The catalase level was significantly increased ($P<0.05$) was observed in serum as compared to ascetic fluid of patients of liver cirrhosis with ascites. The non-significant evaluated mean values ($P>0.05$) of

SOD (U/dl) were evaluated as 7.91±2.03 in serum and ascetic fluid 7.03±2.349. The mean values of GSH (U/dl) in patient's serum were estimated as 3.95±1.39, while in ascetic fluid it was 3.39±1.32. There was no significant difference was observed in GSH levels in serum as compare to ascetic fluid of patients of liver cirrhosis with ascites. The MDA levels in serum patients were observed as 5.41±4.05 as compared to ascetic fluid of patients 4.05 ± 1.22 U/dl. The MDA levels were significantly ($P<0.05$) elevate in serum as compared to ascetic fluid.

Table1: Primer sequences for amplification of ET-1, ET-2 and ET-3

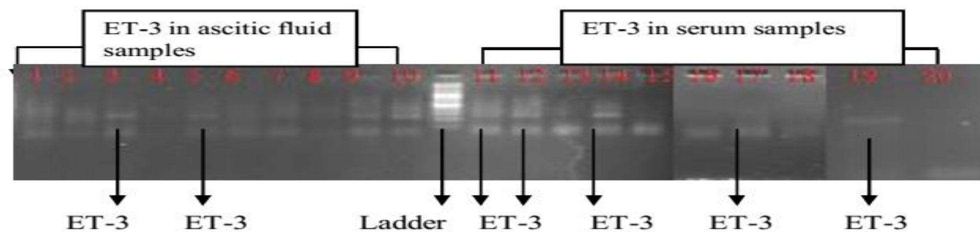
Sr #	Primer Names	Sequence 5' to 3'	Length (bps)
1	OS-E1UL	GCTCGTCCCTGATGGATAAA	20
2	OAS-E1UL	AGTCAGGAACCAGCAGAGGA	20
3	IS-E1UL	TTCCGTATGGACTTGGAAGC	20
4	IAS-EUL	TTTGACGCTGTTTCTCATGG	20
5	OS-E3UL	GATCTGAGGGGGACTGTGAA	20
6	OAS-E3UL	TTTTTCTGCCGTCCTTGAAT	20
7	IS-E3UL	AGCACTGCAGGGTCCAAG	18
8	IAS-E3UL	GCAGGCCTTGTCATATCTCC	20

Figure 1: Gel photograph of PCR product showing endothelin-1(ET-1) in serum and ascetic fluid



Note: 2.0% agarose gel showing cDNA amplified with ET-1 in ascetic fluid and serum with specific primers. The amplified fragments resulted to be of the expected size, which was 320 bp for ET-1.

Figure 2: Gel photograph of PCR product showing endothelin-3(ET-3) in serum and ascetic fluid



Note: 2.0 % agarose gel showing cDNA amplified with ET-3 in ascetic fluid and serum with specific primers.

The serum mean values of NO in patients of liver cirrhosis with ascites were calculated as 48.54±14.82 was increased as compared to normal

individuals (28.72±3.92). The overall significantly high values of NO were observed obtained in serum and ascetic fluid of patients liver cirrhosis with ascites 48.54±14.80 and 21.35±8.21 respectively. CK were assessed as 120±146 in patients as compared to normal individuals i.e. 28.8±3.96. The mean values of serum CK-MB in control group were calculated as 15.8±2.38 and in patients of liver cirrhosis with ascites as 20.83±10.77. The estimated significant ($P<0.05$) difference in mean values of CK and CK-MB were observed in serum of patients as compared to control group. The estimated cholesterol levels in serum of patients were 154.80±43.95 as compare to normal individuals 178.40±28.39. The overall non-significant ($P>0.05$) results of cholesterol were observed in serum of patients as compared to normal individuals.

Pearson correlation was performed to find out the correlation among all the parameters of oxidative stress with each other and with ET-3. The Pearson correlation showed highly significant ($P<0.05$) and positive correlation between oxidative stress parameters (CAT, GSH, MDA and SOD) and cardio vascular stress parameters (NO, CK, CK-MB, and Cholesterol) with ET3.

CRP test was performed on 20 samples of serum and ascetic fluid of patients of liver cirrhosis with ascites and 10 samples of control. The positive results were obtained from serum and acetic fluid of patients as compared to normal individuals

Discussion

In the present study oxidative stress and cardio vascular stress was determined in relation to the level of endothelin particularly ET-3 as ET-1 in blood and ascetic fluid. Oxidative stress was measured in serum and ascetic fluid of patients of liver cirrhosis with ascites. The outcomes of CAT, GSH, SOD and MDA showed significant ($P<0.05$) differences in CAT, GSH, SOD and MDA levels in serum of patients of liver cirrhosis with ascites as compared to control group which was correlated with the work of Lee et al [15] in which significant ($P<0.05$) difference of CAT, GSH, SOD and MDA level were observed in cirrhotic patients. Due to excessive ROS generation antioxidative defense mechanism gets activated to neutralize the ROS. As a result level of GSH, CAT and GSH gets decreased while increased levels of MDA indicate lipid per oxidation (LEE et al 2010 [15].

Analysis of oxidative stress in serum and ascetic fluid of patients of liver cirrhosis showed significantly ($P<0.05$) elevated levels of catalase in serum of patients as compared to ascetic fluid of patients of liver cirrhosis with ascites. Insignificant ($P>0.05$) difference in SOD and GSH was observed in serum as compared to fluid of patients. Elevated levels of catalase and MDA in serum as compare to the ascetic fluid of patients was may be due to less conversion of hydrogen peroxide into water and oxygen while insignificant MDA levels could be due to less lipid per oxidation and tissue is more susceptible to oxidative damage.

For the estimation of cardio vascular stress, NO in serum and ascetic fluid while CK, CK-MB and cholesterol in serum were assessed. The results showed significant ($P<0.05$) difference in NO, CK, CK-MB levels in patients as compare to normal individuals. nt ($P>0.05$) difference was assessed in serum cholesterol level of patients as compared to normal individuals because biosynthesis of cholesterol has been reduced in cirrhotic patients. The obtained result was in line with work of MuhammadReza et al.[16] in which insignificant cholesterol level was observed in cirrhotic patients. Due to activation of vasodilatory mechanisms splanchnic and systemic circulation takes place due to overproduction of NO in patients of liver cirrhosis with ascites.

Correlation of ET-3 with oxidative stress and with development of cardio vascular stress was also measured by Pearson correlation. Correlation was determined between oxidative stress parameters and ET-3 and between the individual parameters of oxidative stress which showed positive and highly significant ($P<0.05$) correlation of CAT, SOD, MDA, and GSH levels with ET-3 and with each other. Endothelin is produced as a result of oxidative stress. NADPH oxidase form ROS by the activation of endothelin. The findings were in line with work of Geetha et al.[6] in which significant difference in antioxidative enzymes and MDA were obtained.

Pearson correlation was also found between ET-3 level and the development of cardio vascular stress Viz CK, CK-MB and cholesterol levels estimation and it was found that highly significant ($P<0.05$) and positive correlation of ET-3 was found with NO, CK, CK-MB except cholesterol. The outcomes were correlated with work of Salimeh et

al. [8] in which significant results of cardiac biomarkers (CK and CK-MB) were observed.

Considering the potentially important role of ET-3 in the development of cardio vascular stress in patients of liver cirrhosis with ascites further study can be conducted to develop endothelin receptor antagonists.

Conclusion

Our results showed that the increased production of ET-3 in oxidative stress generated during liver cirrhosis leads to the generation of cardio vascular stress. Therefore T-3 can be considered as an important novel biomarker of cardio vascular stress in patients of liver cirrhosis with ascites as compared to ET-1.

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