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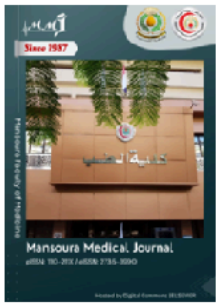

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EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN ISCHEMIC HEART DISEASES AND MYOCARDIAL INFARCTION

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EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN ISCHEMIC HEART DISEASES AND MYOCARDIAL INFARCTION

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ABSTRACT

There is increasing evidence that alterations in nitric oxide synthesis are of pathophysiological importance in heart diseases. So, the present study was conducted to evaluate the role of inducible nitric oxide synthase (iNOS) in coronary heart diseases either ischemic heart disease (IHD) or myocardial infarction (MI) and to detect the expression of iNOS gene in these diseases. The current study was conducted on 50 patients (25 patients with ischemic heart disease and 25 patients with myocardial infarction) with mean age 55.53 ± 4.18 years and 12 healthy volunteers of matched age and sex were taken as control.

The serum levels of nitrite and cGMP were measured by spectrophotometric method and ELISA assay, respectively and also detection of the iNOS gene expression in the total RNA extracted from different studied groups were done. Results of the present study have demonestrated that: a significant increase in nitrite and cGMP levels in myocardial infarction group compared to control group. Anon-significant increase in nitrite and cGMP levels in myocardial infarction group compared to control group. Anon-significant increase in nitrite and cGMP levels in ischemic heart disease group compared to control group. Also, iNOS gene expression was found to be positive in 76% of myocardial infarction group and negative in ischemic heart disease group. Positive correlation between serum nitrite and serum cGMP in all patients groups. Also, positive correlation between serum nitrite, serum cGMP and iNOS gene expression. So, in conclusion, iNOS may have a role in the pa-

tho-genesis of coronary heart diseases and the use of iNOS gene transfer or the use of antisense technology aiming at inhibiting the expression of iNOS may be of beneficial therapeutic values in these conditions.

INTRODUCTION

The historical discovery of the enzyme family of nitric oxide synthase (NOS) in 1989 is relatively recent and we know that there are 3 distinct isoforms of 3 distinct forms of: neuronal NOS (nNOS), inducible NOS (iNOS) and constitutive NOS (cNOS). These 3 isoforms were discovered in the above order from 1991-1994 (Qianhong et al., 2003). Each NOS isoform is transcribed from a separate gene. The genes encoding eNOS, nNOS and iNOS are located on chromosome 7,12 and 17 respectively. (Qingping et al., 2001).

All three NOS isoforms play distinct roles in the regulation of vascular tone. Whereas eNOS and nNOS are calcium-dependent enzymes and produce small amounts of NO on stimulation, iNOS is a calcium-independent enzyme often induced by cytokines and produces high levels of NO (Massion et al., 2003). Basal generation of NO by eNOS plays an important role in the regulation of basal vascular tone, blood pressure, and tissue perfusion. High levels of NO produced by activated macrophages not only may be toxic to undesired microbes, parasites, or tumor cells but also may harm healthy cells. (Qingping et al., 2001 and Vejlstrup et al., 1998).

Atherosclerosis accounts for half of the morbidity and mortality in western countries (Ross, 1993). Also, cardio-vascular disease accounts for at least 85 percent of deaths. Additionally, 75 percent of these deaths are due to ischemic heart disease (Hayden and Tyagisc, 2003). NO inhibits many key steps in the atherogenic process for example platelet adhesion and aggregation, adhesion molecules, chemokine expression, inflammatory cell infiltration, smooth muscle cell migration and proliferation. (Sar Kar et al., 1996).

The early NO deficit may facilitate atherosclerosis progression. Although the eNOS pathway appears to be involved in atherosclerosis, little is known about the role of iNOS in human atherosclerosis, and coronary heart diseases as ischemic heart disease and myocardial infarction.

(Luscher and Noll, 1995).

AIM OF THE WORK

So, this study was designed to detect the expression of iNOS gene in patients with coronary heart diseases either ischemic heart disease and/or myocardial infarction. Also, to evaluate the role of iNOS in the pathogenesis of these diseases.

SUBJECTS AND METHODS

** Subjects :*

The present study was conducted on 25 patients with ischemic heart disease (IHD) and 25 patients with myocardial infarction attending Mansoura University Hospitals. Their age ranged from 48-62 with mean ± SD 55.53 ± 4.18 years.

The study also included 12-subjects of matched age as a control group. Their age ranged from 48-60 years with meant ± SD 53.56 ± 4.09 years.

** Sampling :*

5ml Fasting venous blood samples were taken after consent of the patients and separated into 2 parts:

1- I ml whole blood sample was taken into EDTA containing tube used for RNA extraction.

2- The rest of the sample was taken into dry tube and allowed to clot at room temperature for 30 minutes. Serum was separated by centrifugation at 3000 rpm for 15 min then frozen at $-20^{o}c$ until the time of assay of nitrite and cGMP.

** Assay:*

- Nitrite determination :

The concentration of nitrite was measured by a spectrophotometric method based on the Greiss reaction as described by Green et al., (1982). Nitrite measurement reflects the amount of NO production. The data were expressed as umol/L.

- cGMP determination:

The concentration of cGMP was measured by using ELISA assay Kit according to Gettys, (1991). the data were expressed as pmol/m1.

- Determination of iNOS gene expression:

** RNA extraction:*

total RNA was extracted from the whole blood samples using promega (USA) SV total RNA isolation kit, according to the method of Otto (1998).

In brief, centrifugation at 10.000 rpm for 10 min. the supernatant was

transferred to the spin column and 50ul of the prepared DNAase was added for 5min and centrifuged. The column was washed twice with wash solution, using 600ul in the first wash and 250ul in the second. The RNA was eluted in 100ul of nuclease free water.

RNA was quantified spectrophotometrically at 260nm and stored at –20°c. 2ul of the extracted RNA was subjected to RT-PCR.

- RT-PCR and analysis of the products:-

• Access RT-PCR Kit supplied by promega USA, was used according to method of Miller and Storts (1995).

• RT-PCR was carried out on total RNA isolated from the different studied groups.

• All primers were synthesized at the Biosource Europe laboratories. The sequences of the two primers used, as designed by Bonmann et al., (1997) are shown as follow;

a- Sense primer:
5-TGATGTGCTGCCTCTGGTCT-3

b- Anti sense primer:
5-ACTTCCTCCAGGATGTTGTA-3

• In a total reaction volume 50ul, containing 1um, primer a and b each, AMV/TFI reaction buffer, 2mM MgSo4, 0.2mM dNTPs, 0.1unit AMV reverse transcriptase and 0.1unit TFI DNA polymerase. The mixture was overlayed with 50 ul mineral oil and RT-PCR was performed in a programmable thermal minicycler.

• The cycle conditions were as follow: 45min at 48°c and 2min at 95°c for RT-reaction followed by 40 cycles of 30sec at 94°c (denaturation), 1min at 60°c (annealing) and lastly 2min at 68°c (extension). Then one cycle 7min at 68°c for (final extension). Then the samples were then rapidly cooled to 40°c.

• The solution containing the PCR product (9ul) was mixed with 1ul of loading dye (0.1 Bromophe nol blue and 30% glycerol in water) poured into agarose gel containing 0.5ug/ml ethidium bromide in 1XTBE buffer. The samples were run in 1XTBE buffer for 30min at 140V in a mini-gel apparatus. A DNA marker VIII (Boehringer manheim) was run in parallel as size marker. The specificity of the amplified bands was validated by their predicted size. The resulting bands were visualized by UV-Light and photographed.

STATISTICAL ANALYSIS

Results were expressed as mean ± SD for (n) experiments. Statistical significance between groups were determined using student-t-test. Correlation coefficients (r) between the different variables were calculated with spearmann rank test. P valuses less than 0.05 were considered significant. These tests were done on an IMB compatible personal computer using the statistical pachage for social scientists (SPSS version 10).

RESULTS

- A non significant increase in levels of nitrite and cGMP in serum of IHD cases as Shown in table (1).

- A highly significant increase in levels of nitrite and cGMP in serum of M.I cases as Shown in table (2)

- More expression of iNOS gene in cases of M.I in comparison with cases of IHD as Shown in table (3).

- Positive correlation between serum nitrite level and both cGMP level in serum and expression of iNOS gene. Also there is positive correlation between serum levels of cGMP and iNOS gene expression as Shown in table (4 & 5).

PCR for induced nitric oxide synthase (iNOS)-cDNA

The product of PCR amplification of extracted total RNA with formation of cDNA and using the iNOS-specific sense and antisense primers were electrophorised through 1.8% agarose gel containing ethidium bromide and visualized by an UV transilluminator.

No cDNA was amplified from control group (lane2). iNOS specific amplification of cDNA yielded a PCR product of 279 bp in lane (3,5,7) in myocardial infarction cases (M.I). But in ischemic heart disease cases (IHD) no cDNA was amplified as shown in lane (4,6,8). The marker was put in lane (1).

Table (1): nitrite and cGMP levels in ischemic heart diseases (IHD) cases.

	IHD	Control	P
1- nitrite (umol/L)	11.87±3.7	7.6±0.9	0.102
2- cGMP (pmol/ml)	13.1±3.4	9.9±1.6	0.104

Table (2) nitrite and cGMP levels in mycocardial infarction (M.I) cases.

	M.I	Control	P
nitrite (umol/L)	25.6±3.3	7.6±0.9	<0.001
cGMP (pmol/ml)	37.03±2.9	9.9±1.6	<0.001

It is shown that there is a highly significant increase in levels of nitrite and cGMP in serum of M.I cases.

Table (3) inducible NOS gene expression in the patients groups.

Gene expression	IHD	M.I
Positive	28% (n=7)	76%(n=19)
Negative	72%(n=18)	24%(n=6)

It is shown that there is more expression of iNOS gene in cases of M.I in comparison with cases of IHD.

Table (4): comparisom between cGMP and nitrite levels in M.I and IHD patients groups.

	t	P	Significance
CGMP	23.55	<0.0001	high sig
Nitrite	12.401	<0.0001	high sig

Table (5) correlation between serum levels of nitrite, cGMP and iNOS gene expression in all studied cases.

	CGMP		iNOS gene expression	
Nitrite (umol/l)	r	p	r	p
	0.69	<0.001	0.58	<0.001
CGMP (pmol/ml)	-	-	0.71	<0.001

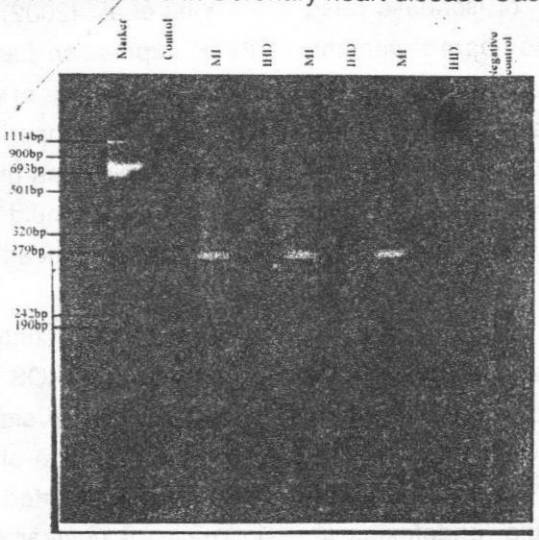

Figure (1)

MI= Myocardial infarction
IHD = Ischemic heart disease

DISCUSSION

Coronary heart diseases remain the leading cause of death in most developed countries as seen over the past quarter-century. Reducing the mortality rate and prevention of myocardial infarction (M.I) are of utmost importance. (Sun et al., 2002).

There is increasing evidence that alterations in nitric oxide synthesis are of pathophysiological importance in heart failure. A number of studies have shown altered nitric oxide production by the endothelial constitutive isoform of nitric oxide synthase (cNOS), but there is very little information on the role of the inducible isoform (iNOS) (Guy et al., 1996).

So, this study aimed at evaluation of the role of iNOS in patients with coronary heart disease and detection of the expression of its gene in both ischemic heart disease and myocardial infarction.

In the present study, levels of nitrite, cGMP were found to be significantly higher in patients group of myocardial infarction when compared with control group as shown in table (2). But there is a non significant increase of both nitrite, cGMP serum

levels in cases of ischemic heart diseases when compared with the control group as shown in table (1). Also, iNOS gene expression was found to be positive in about 76% of cases of myocardial infarction versus 28% of cases of ischemic heart diseases.

It is reported that iNOS expression is seen mainly in conditions of infection or inflammation and that this inducible isoform of NOS can be rapidly induced by microbial endotoxins or cytokine stimulation (Qianhong et al., 2003).

Also expression of iNOS in active atherosclersis plaques has also been detected. It is possible that this iNOS contributed to tissue damage or other features of plaque development or stability. (Thoenes et al., 1996).

NO produced by iNOS reacts with superoxide anions in the plaque to yield reactive oxidant species such as peroxynitrite contributing to cytotoxicity and tissue injury. Another possible outcome for NO coming from iNOS could be the activation of matrix metalloproteinase and induction of apoptosis in smooth muscle cells and macrophages. (Drexler et al., 1998).

Ying et al., (2002) suggested that, iNOS expression has been found in cardiac myocytes of patients with dilated cardiomyopathy, myocarditis and ischemic heart disease, suggesting that iNOS could be a source of NO in the failing heart.

The present study has revealed that the level of iNOS and its gene expression increase significantly within the patients group of myocardial infarction. It is reported that it is due to increase of nuclear factor kappa B (NF-B) which occur by increase of cytokines that act directly or by phosphorylation of NF-B, inflammation, injury or immune response and M.I. is characterized by increase in susceptibility to injury and inflammation. (Guy et al., 1996).

Also, a statistically significant positive correlation has been detected between serum nitrite and serum cGMP levels as shown in table(5).

cGMP has been reported to be associated with endothelial dysfunction and enhanced expression of iNOS with enhanced production of superoxide anion that shares in the formation of powerful oxidant, peroxy-nitrite that initiates lipid peroxidation with produc-

tion of lipoperoxides, a marker for oxidative stress. (Hitoshi et al., 2000).

It is revealed that impaired endothelial dependent vasodilatation associated with abnormal iNOS may be an important factor with development and progression of atherosclerosis. In addition endothelial dysfunction in myocardial infarction patients may initiate vascular inflammation that leads to cytokine-induced activation of iNOS which favours the formation of peroxynitrite contributing to cytotoxicity and tissue injury. (Stephen et al., 1997).

In conclusion, iNOS, the important signaling molecule in conditions of inflammation, atherosclerosis and tissue injury, was found to be higher with more gene expression in patients with myocardial infarction more than in patients with ischemic heart disease. Also it is associated with presence of increasing cGMP serum level.

Now, what is the value of detection of iNOS gene expression and determination of serum level of nitrite and cGMP ?.

Recently with the availability of efficient transduction systems for invivo gene transfer, as well as other methods of gene manipulation for NOS gene therapy. Firstly, restoring the beneficial effects of NO activity (gain of function) may provide potential therapeutic benefit for the treatment of several forms of cardiovascular diseases. (Walter et al., 2001).

Transfering the human iNOS gene using a retroviral vector resulted in, prevention of neointimal lesion formation and inhibition of stent induced lesion formation by 50%, autoregulatory feedback inhibition of vascular inflammation and inhibition of the development of vein graft disease and trans-plant vasculopathy. (Sun et al., 2002).

Also, targeting NOS by a "loss of function" approach is aimed at inhibiting the expression of specific NOS isoform gene using antisense technology. Thus inhibition of iNOS using antisense oligonucleotides has been shown to improve nitric oxide mediated hemodynamic changes in experimental models of septic shock. (Qingping et al., 2001).

NOS gene transfer enables the achievement of therapeutic concentrations of NO locally in the target tis-

sue, without the potential adverse effects of the excessively high blood levels that occur when using systemic therapy. (Kohji A et al., 2001).

Data suggested that both eNOS and iNOS are effective and safe. It is surprising that iNOS gene transfer is not associated with the cytotoxicity observed with the activation of the endogenous gene. (Birgitt et al., 1998).

Furthermore, it has been shown that the efficacy of iNOS gene therapy in experimental vascular disease may be related to a lower level of NO achieved with exogenous gene transfer compared with endogenous gene activation, to the intracellular distribution of NO as the result of the expression of exogenous gene construct versus endogenous native gene and/or to the microenvironment of the iNOS gene product accmulation. The higher turnover rate of iNOS may be preferable for clinical use because low concentrations could be used, thereby minimizing the risk of systemic distribution of relevant amounts of DNA vector. (Vonder Leyden and Dzau, 2001).

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تمثيل الإنزيم المحفز لأكسيد النيتريك فى أمراض قصور الشريان التاجى أو الجلطة التى تصيب القلب

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*أقسام الكيمياء الحيوية الطبية والباطنة**

كلية الطب - جامعة المنصورة

تم فى هذا البحث دراسة الدور الذى يقوم به الإنزيم المحفز لتكوين أكسيد النيتريك فى مرضى الشريان التاجى سواء فى حالات قصور الشريان التاجى (الذبحة الصدرية) أو فى حالات الجلطة التى تصيب القلب نتيجة انسداد كلى فى الشريان التاجى. وأيضاً تم الكشف عن مدى تمثيل الجين الخاص بهذا الإنزيم فى هؤلاء المرضى.

الخطوات المتبعة فى إجراء البحث :-

لقد أجرى هذا البحث على ٢٥ مريض من حالات الذبحة الصدرية وأيضاً ٢٥ مريض من حالات جلطة القلب وقد كان متوسط أعمار المرضى ٥٣ر٥٥ + ١٨ر٤ سنه. كما شملت هذه الدراسة أيضاً مجموعة من الأشخاص الأصحاء متوسط أعمارهم ٥٦ر٥٣ + ٩ .ر٤ سنة ويمثلوا المجموعة الضابطة.

وقد تم قياس مايلي فى عينات دم المرضى :

- قياس النيتريت .
- قياس الجو انزين أحادى الفوسفات الحلقى.
- الكشف عن الجين الممثل للإنزيم المحفز لأكسيد اليتريك.

نتائج البحث :

١- زيادة مستوى النيتريت ومستوى الجو انزين أحادى الفوسفات الحلقى زيادة ذات دلالة إحصائية فى عينات دم مرضى الجلطة القلبية مقارنة بالمجموعة الضابطة. كما أن الجين الممثل للإنزيم المحفز لأكسيد النيتريك قد تم ظهوره فى ٧٦٪ من الحالات .

٢- زيادة مستوى النيتريت ومستوى الجو انزين أحادى الفوسفات الحلقى فى عينات دم مرضى الذبحة الصدرية مقارنة بالمجموعة الضابطة ولك ن ليست ذات دلالة إحصائية مع عدم ظهور الجين الخاص بالإنزيم المحفز لأكسيد النيتريك فى معظم هؤلاء المرضى.

٣- وجود علاقة طردية ذات دلالة إحصائية بين مستوى كل من النيتريت والجو انزين أحادى الفوسفات الحلقى فى الدم. وأيضاً يوجد علاقة طردية ذات دلالة إحصائية بين كل من النيرتيت والجو انزين إحادى الفوسفات الحلقى وبين الجين المكون للإنزيم المحفز لأكسيد النيتريك.

الخلاصة :

نظرا أن هذا البحث قد كشف عن وجود إرتفاع فى مستوى الإنزيم المحفز لأكسيد النيتريك بالدم فى مرضى الجلطة القلبية وأيضاً ظهور الجين الخاص بهذا الإنزيم فى نفس الحالات فإن إستخدام العلاج بالچينات مثل نقل الجين الخاص بالانزيم المحفز لأكسيد النيتريك قد يمنع تكوين هذا الإنزيم ويكون ذو فائدة علاجية فى حالات الجلطة القلبية .