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Research Article

Status of Serum Nitrite/Nitrate in Patients with Medullary Thyroid Carcinoma

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Abstract

Objective: Recently, Nitric Oxide (NO) has been suggested as a ubiquitous critical molecule in physiological and pathological processes. Changes in serum levels of NO were reported in some cancers. NO is a pleiotropic mediator and its role in different human malignancies has been considered. The aim of this study was the assessment of NO metabolites, Nitrite/Nitrate, levels in serum of medullary thyroid cancer patients.

Methods: In this study 90 participants, including 45 MTC patients and 45 controls were selected. Serum Nitrite/Nitrate was determined using Nitrite/Nitrate assay kit.

Results: The normal distribution of serum Nitrite/Nitrate levels was assessed using the Shapiro-Wilk test. Median of Nitrite+Nitrate in patient and control groups were 17.2 and 17.8 μM respectively (95% CI for was 19.5-30.3 for MTC group and 16.9-24.5 for control group) and no significant difference was found using Mann-Whitney test (P=0.301). There was a significant difference for Nitrate (P=0.049) and Nitrite levels (P=0.03) separately between control and MTC groups. No correlation was found between age/BMI and serum levels of Nitrite/Nitrate.

Conclusion: Total nitrite and nitrate are not a good indicator for discriminate between the control and MTC groups. However, each alone can be used to diagnose. Therefore, unlike most studies, it is recommended that instead of measuring nitric oxide, we do not neglect measuring its components (nitrite and nitrate).

Keywords: Medullary thyroid cancer; Nitric oxide; Biological marker; Nitrite; Nitrate

Highlight: In this study, it is showed that instead of Nitric oxide, its components may be good markers for a clinical situation such as MTC.

Introduction

Recently, incidence of the most common endocrine system malignancy, thyroid gland carcinoma, has been increased and it is attributed to different kinds of factors such as lifestyle and environmental changes [1]. Thyroid gland cancers comprise two main groups: 1) follicular thyroid cells derived cancers and 2) para-follicular thyroid cell derived carcinoma. The first one contains Follicular (FTC), Papillary (PTC) and Anaplastic (ATC) thyroid cancers and second one includes Medullary Thyroid Cancer (MTC) [2]. MTC accounts roughly 5% of all human thyroid cancers and it may take place in sporadic (s-MTC, 75% of cases) and hereditary (25% in of cases) forms [3]. In addition to thyroid, parathyroid and adrenal glands are involved in hereditary MTC [4,5] and it manifests in three clinical forms of Multiple Endocrine Neoplasia (MEN) type 2A, MEN 2B and Familial MEN (FMEN) [3]. MTC is the main feature of MEN2 and its hereditary pattern is autosomal dominant [6-8]. The s-MTC is identified by the lack of a familial history for MTC and absence of RET germline mutations and other MEN2A-related tumors. The s-MTC patients are usually diagnosed by physical examination, high serum levels of calcitonin and FNA cytology. The slow growth and quick spread to the regional cervical lymph nodes are the main features of s-MTC. Local cervical metastasis may be observed in more than 70% of cases at the time of diagnosis and a distant metastasis may occur in the liver, lung, bone and brain [9]. In order to confirm of MTC, cytological and pathological evidences and for determining of tumor size Ultra Sound (US), Computerized Tomography Scan (CTS) and Mmagnetic Resonance Imaging (MRI) are required [10]. However, owing to the some ambiguous problems around assay methodology, sensitivity, specificity and cost effectiveness, the routine assessment of serum calcitonin remains controversial yet [11]. During two past decades, Nitric Oxide (NO) has been suggested as a ubiquitous critical molecule in physiological processes [12-14] and also in pathological states including diabetes, metabolic syndrome, hypertension and heart failure [15]. NO also plays substantial roles in roughly every biological system. Recently it has been suggested as an endocrine molecule [16]. NO is a pleiotropic mediator [12] and its various roles in different human cancers such as breast [17], cervical [18,19], prostate [20], head and neck [21] and gastric [22] cancers have been studied. Besides, despite the controversial role of NO in tumorigenesis, some researchers believe that the unwinding of the complexities of different actions of NO at the molecular level in these

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cancers will be helpful for development of diagnostic or prognostic markers based on NO [23]. In this paper, NO metabolites levels in MTC patients and its correlation with age, sex and BMI have been studied and it is endeavored to answer whether Nitrate or Nitrite or Nitrate+Nitrite can be used as a marker or co-marker in the diagnosis and prognosis of MTC.

Materials and Methods

Patients and sample preparation

MTC patients (all of the patients had previously undergone to the thyroidectomy) were referred from different health centers to the Cellular and Molecular Endocrine Research Center, Research Institute for Endocrine Sciences. In the present study, 90 subjects were selected. Forty five of the 90 participants were MTC patients (19 male 30.31±7.78 years, 26 females 35.57±12.56 years) and 45 individuals (19 male 31.05±9.58years, 26 female 33.03±10.46 years) were volunteer controls without any history of cancer and systemic disease. The criteria for entering the study include having sporadic MTC (according to the pathologist confirmation and RET mutation analysis) and having no other disease or cancer other than MTC. After obtaining written informed consent, under aseptic condition 2ml venous blood was collected from participants and then, in order to clot formation the samples were incubated 5min at room temperature. Serum was separated by centrifuge (Centrifuge 5702R, Eppendorf AG, Hamburg, Germany) at 3000 rpm for 10 min and stored at 80°C before analysis. After weight and height measurement, the subjects' Body Mass Index (BMI) was calculated.

NO metabolites assessment

Direct determination of NO is difficult owing to its short biological half-life, therefore the assessment of nitrite/nitrate in biological fluids is the most appropriate and common method to determine the NO levels. In this study serum nitrite and nitrate was determined in both MTC and control groups using total nitrite/nitrate assay kit (ZellBio GmbH, Ulm, Germany), the assay sensitivity was 1μM and the intra assay coefficient of variation was 3.5% with eight replications. The NO metabolites level is quantified based on the conversion of nitrate to nitrite by reducing agent and colorimetric detection of nitrite in Griess reaction.

Statistical analysis

The present research is a descriptive and case-control study. In order to study design, the sample size was calculated by MedCalc software. Continuous quantitative variable analysis and comparison of two means (two groups' means) were done by using Shapiro-Wilk and Mann-Whitney respectively. The coefficients were determined between serum levels of nitrite/nitrate and BMI and age in the both MTC and control groups.

Results

The sample size was calculated 45 subjects for each group (overall 90 participants). The power of the study (with type II error, β=0.2), confidence interval (type I error, α=0.05), mean variation and standard deviation were evaluated 80%, 95%, 1.2 and 2 respectively. According to the Shapiro-Wilk test, the distribution of age and BMI as continuous quantitative variables was normal and the independent "t" test did not show a significant difference between means of MTC and control groups. Normal distribution of nitrite/nitrate serum levels was observed for female and male case and control groups separately, but serum levels of NO metabolites (Nitrate+Nitrite) had not a normal distribution in case and control groups. The subjects' demographic/anthropometric and biochemical data were demonstrated in (Table1 and Table2) respectively. Independent "t" test was used for the comparison of mean between MTC and control groups and statistical significance of the test considered $p<0.05$. The statistical significant difference was not observed between BMI and age means in the MTC and control groups. Distribution of Nitrate, nitrite and total Nitrate+Nitrite for the Case and Control groups showed ad a Pie or Doughnut chart (Figure 1). Median level of Nitrate+Nitrite in-patient and control groups were 17.2 and 17.8 μM respectively (P=0.301), showing that there was not statistically significant difference between two groups (Figure 2). Also, there was no significant difference mean of serum levels of NO metabolites between females of control and MTC groups (P=0.08) and between males of control and MTC groups (P=0.22). Logarithmic tests showed no correlation between Age and BMI with serum levels of nitrite/nitrate (Figure 3).

Discussion

The aim of the present study was to determine the levels of nitric oxide metabolites (Nitrate/Nitrite), separately and sum of them in medullary thyroid cancer patients and compare them with normal subjects and correlate these levels with the disease. By assessment of serum levels of nitrite/nitrate in MTC patients and healthy control cases, we found that there is no statistically significant difference between the serum NO metabolites levels in MTC patient and control groups and any correlation was not observed between nitrite/nitrate serum levels and MTC. But fortunately, both Nitrate and Nitrite

Table 1: Demographic, anthropometric and biochemical data of MTC patients and controls.

Variables	Unit	Subjects	Minimum	Maximum	Mean	SD	Female	Male
Age	year	control	20	60	32.2	10.03	33.03±10.46	31.05±9.58
		case	20	59	33.35	11.01	35.57±12.56	30.31±7.78
BMI	kg/m^2	control	24	27.9	25.84	1.09	25.72±0.86	25.99±1.36
		case	24.1	28.9	26.25	1.26	26.38±1.15	26.06±1.41

*SD: Standard Deviation, BMI: Body Mass Index.

Table 2: Serum NO metabolites levels in MTC patients and controls.

Variables	Unit	Subjects	Minimum	Maximum	Median	Female	Male
Nitrite/Nitrate	*μM*	control	5.5	56.9	17.2	17.02±9.9	25.4±15.1
		case	8.4	91.0	17.8	22.89±13.5	19.7±11.7

*SD: Standard Deviation

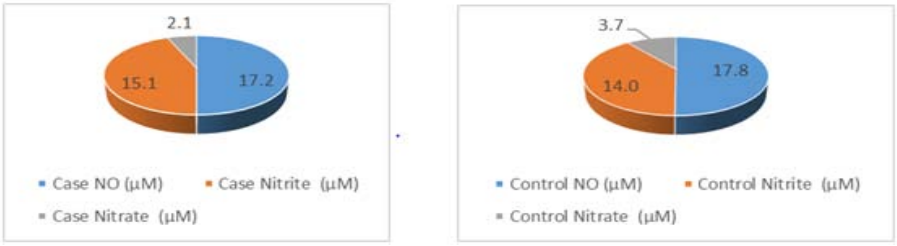

Figure 1: Distribution of Nitrate, nitrite and total Nitrate+Nitrite in the Case and Control groups.

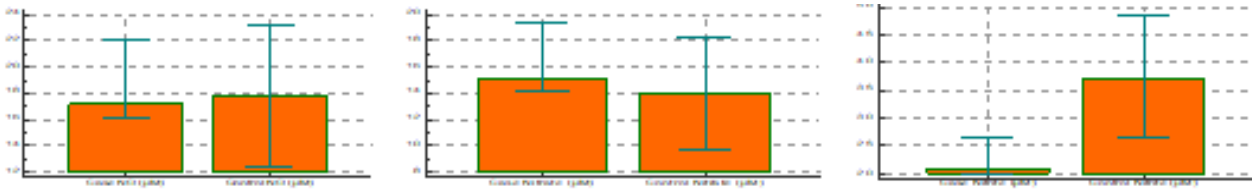

Figure 2: Mann-Whitney t-test showed no significant difference between serum level of nitric oxide in MTC and control groups. But a significant difference revealed between Nitrite and also Nitrate between the control and MTC groups.

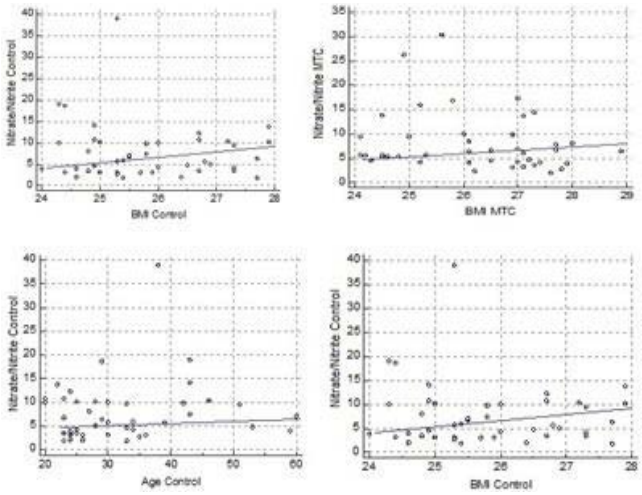

Figure 3: Spearman test showed no correlation between age/NO metabolites and BMI/NO metabolites levels in both control and MTC groups.

levels separately showed a significant difference between MTC and the control group. This suggests measuring the components of Nitric Oxide as a marker for MTC.

In mammals, the enzyme Nitric Oxide Synthases (NOS) synthesizes NO. This enzyme has three isoforms including: Neuronal (nNOS or NOS1), Inducible (iNOS or NOS2) and Endothelial (eNOS or NOS3). nNOS and eNOS regulate neural and vascular function respectively and there function depends on calcium. iNOS, the second isoform, is calcium-independent and inducible [23]. Recently, dual effects of NO in different types of diseases have been reported. For instance, Gasemi [24] and Gheibi [25] demonstrated its therapeutic effects on diabetes and obesity. On the other hand, increased iNOS expression has been reported in many tumors, however, the role of NO during tumor development is also very controversial and both promoting and inhibiting actions have been described [23]. In 1995, Thomsen et al assessed the activity and distribution of NO synthase in human breast tumors and in normal breast tissue. They found that NOS activity and NO biosynthesis in invasive tumors were in high levels compared with normal tissue. According to their findings, NOS and subsequently NO biosynthesis correlated with tumor grade [17]. The results of a performed study by Hiraku et al in 2007, suggested that increased levels of NO owing to HPV infection has an important role and intrinsic mutagenic activity in cervix carcinogenesis [18]. In the other study, Wei et al demonstrated that increased release of NO in women's cervixes attributed to the presence of HR-HPV and could hinder the tumor growth by enhancing the apoptosis in cervical cancer cells [26]. This study was in contrast to the results of Hiraku's study. In 1998, a research on prostate cancer tissue showed a high NO generation due to iNOS expression. According to this finding Klotz et al., suggested that iNOS could be a specific immunohistochemical marker for prostate cancer [20].

In 2006, nitrotyrosine (as a biomarker for peroxynitrate formation from NO), was detected in all Papillary Thyroid Cancer (PTC) patients using of immunohistochemistry. In this study, Nakamura et al studied NO effects on Vascular Endothelial Growth Factor-D (VEGF-D) in PTC cell line. VEGF-D is one of the crucial growth factors playing an important role in lymph node metastasis by lymphangiogenesis in PTC. Their data showed that NO stimulated expression of VEGF-D *in vitro* and they suggested that this process could prompt lymph node metastasis in PTC [27]. In the other similar study, Yasouka et al., examined NO effects on CXC Chemokine Receptor 4 (CXCR4) in PTC cell line. They indicated that NO could stimulate CXCR4 and this process showed a correlation with lymph node metastasis induction by CXCR4 in PTC [28]. According to these findings, it seems that NO may play a significant role in angiogenesis and promotion of PTC. In a study carried out in 2000, NOS gene was transferred to the MTC rats. According to this fact that produced NO by iNOS is the most important mediator of the tumoricidal macrophages, Soler et al., demonstrated that iNOS gene seems to be a promising gene therapy of MTC [29]. Although, several studies have investigated the role of produced NO by innate immune cells in different cancers promotion, but its effect on MTC is ambiguous

in this research and before intervention (gene therapy), the levels of produced NO by innate immune cells were not determined.

In a research on the breast cancer, Mehdi et al. determined a high value of NO in breast cancer patients and they demonstrated that serum assessment of NO may be applicable in diagnosis and prognosis of the disease [30]. Despite this finding, Kilic et al., evaluated serum levels NO in patients with bladder cancer looking for a marker. Although they found an increase in NO production locally and peripherally in bladder cancer patients, but according to the results, they suggested that owing to the inadequate post therapeutic monitoring, NO serum levels could not consider as bladder cancer marker [31]. In the most carried out studies on the assessment of NO levels, we can find the subsequent increases of NO levels following the cancers initiations, which can be attributed to the activity of the innate immune cells (macrophages). Some of the investigations (which were performed to find a correlation between NO and a distinct cancer), had been carried out on tumor tissue or cancerous cell lines. In order to find an appropriate diagnostic marker we decided to assess NO levels in serum because one of the important criteria to confirm a molecule as a diagnosis or prognosis is its noninvasive availability and applicability. In the present research, we could not find a statistically significant relationship between serum levels of NO in MTC patients and healthy controls. According to this finding, two hypotheses can be proposed: 1) the innate immune cells are not activated in MTC tissue and they do not secrete NO and 2) the innate immune tumorcidal cells are activated in MTC tissue but the produced NO by these cells is not secreted into the blood. A highlight of this study was determining and considering the components of Nitric oxide separately and it is showed that Nitrate or Nitrite levels each alone may be a good markers for a clinical situation such as MTC.

In overall, the small sample size and lack of patient staging stratification owing to the disease low incidence are the study limitations, therefore the findings in this study cannot be generalized to the general population. Complimentary study on a wide sample with the exact pathological staging is suggested for the future studies.

Conclusion

In conclusion, NO and its role in promotion or inhibition of different diseases is challenging and controversial. Although NO may play a role in MTC promotion, but it couldn't be presumably considered as an appropriate diagnostic marker for the present time and more studies with a wide sample size are required to confirm or to reject this hypothesis and also to generalize the results to the population. In contrast, of many published papers, it is recommended in addition of total nitrate/Nitrite as nitric oxide metabolite, each of them, nitrate and nitrite separately assess in various clinical situations.

Compliance with Ethical Standards

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Registering No: This study was registered by Cellular and Molecular Endocrine Research Center, Research Institute of Endocrine Sciences, Shahid Beheshti University of Medical Sciences and Tehran, Iran. With Registered Number 852.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of Institutional Review Board and Ethics Committee of Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences and Tehran, Iran and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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