Salivary Nitrite in Patients with Type 2 Diabetes Mellitus: Role of Diabetic Pharmacotherapy

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Submission: January 6, 2017; Published: February 06, 2017

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Abstract
Salivary nitrite is derived from salivary nitrate that obtained from ingested nitrate since 25% of nitrate is secreted through saliva thus; salivary nitrate is 10-20 times higher than plasma nitrate. Nitrate-nitric-NO pathway plays a role in prevention of insulin resistance and progression of diabetes mellitus. Because of salivary nitrite is also generated from NO metabolism therefore, salivary nitrite may reflect the endogenous NO production and endothelial function in various diseases thus; the aim of present study was evaluation of salivary nitrite in controlled and complicated T2DM regarding the current diabetic pharmacotherapy. In this study a total number of 50 patients with T2DM were selected randomly compared with 27 healthy subjects. 10ml of venous blood from all patients and healthy subjects after an overnight fasting was drawn, lipid profile, fasting blood glucose, plasma nitrate, plasma nitrite, nitric oxide NO and salivary nitrite were determined in patients with T2DM regarding specific diabetic pharmacotherapy and complications compared to healthy control. Salivary nitrite was high in patient with complications p=0.04 compared with control and near normal in diabetic patient without complications. Metformin increases salivary nitrite more than glimepiride but combination of metformin plus glimepiride produced significant amelioration in salivary nitrite levels.

Conclusion: Salivary nitrite levels were high in complicated T2DM and low in controlled T2DM compared to the control. Metformin increases salivary nitrite levels whereas glimepiride alone or in combination with metformin reduce salivary nitrite levels in T2DM patients.

Keywords: Salivary nitrite; Metformin; glimepiride; T2DM

Abbreviations: T2DM: Type 2 Diabetes Mellitus; MIP: Macrophage Inflammatory Protein; GLUT4: Glucose Transporter 4; VEGF: Vascular Endothelial Growth Factor

Introduction
Type 2 diabetes mellitus (T2DM) is an endocrine and metabolic disorder characterized by hyperglycemia and inflammatory changes that cause systemic complications due to nitric oxide disorders that cause endothelial dysfunction [1]. Inorganic nitrite and nitrate are found in vegetable food and drinking water; this nitrate/nitrite compounds are responsible for restoring of nitric oxide NO which play an important role in prevention of insulin resistance and development of T2DM [2]. Previously, nitrate/nitrite compounds were reported to be a risk factor in development of T2DM [3]. Salivary nitrite is derived from salivary nitrate that obtained from ingested nitrate since 25% of nitrate is secreted through saliva thus; salivary nitrate is 10-20 times higher than plasma nitrate [4]. Moreover, nitrite is responsible for synthesis of NO via independent-nitric oxide synthase pathway that called nitrate-nitric-NO pathway, so diet deficient nitrate lead to cardiac and hepatic damage due to ischemic-reperfusion injury [5].

Nitrate-nitric-NO pathway plays a role in prevention of insulin resistance and progression of diabetes mellitus since; NO increase insulin sensitivity and secretion at post-receptor signaling also, NO improves insulin action through reduction of proinflammatory cytokines that down-regulate glucose-transporter 4(GLUT4) thus; NO inhibits mitochondrial reactive oxygen species production which are implicated in the induction
of insulin resistance [6]. Indeed, the impact of T2DM on NO production is revealed through different mechanisms which are:

i. Longstanding hyperglycemia leads to induction of oxidative stress and buildup of advance glycation end products that inhibit nitrate-nitric-NO pathway [7].

ii. Chronic hyperglycemia induces production of dimethylarginine which inhibit nitrate-nitric-NO pathway [8].

iii. Stimulation of arginase activity that decrease the availability of arginine for NO synthesis [9].

iv. Inhibition of NO synthesis due to insulin impairment [10].

Regarding diabetic pharmacotherapy, metformin is a biguanide approved for management of T2DM, it possess significant anti-inflammatory and anti-oxidant effects that reducing the cardio-metabolic complications [11], additionally, metformin improves endothelial function through regulation of endothelial NO and attenuating the inflammatory-induced vascular endothelial function [12]. Alternatively, glimepiride which is a long acting secretagogus sulfonylurea acts through augmentation of peripheral insulin sensitivity and stimulation of insulin secretion from pancreatic β-cells [13]. Generally, sulfonylurea reduced NO production [14] but it is little known about glimepiride effect on nitrate-nitric-NO pathway. Because of salivary nitrite is also generated from NO metabolism therefore, salivary nitrite may reflect the endogenous NO production and endothelial function in various diseases thus; the aim of present study was evaluation of salivary nitrite in controlled and complicated T2DM regarding the current diabetic pharmacotherapy.

Materials and Methods

In this observational study a total number of 50 patients with T2DM were selected randomly from Iraqi endocrinology center, compared with 27 healthy subjects. Moreover, T2DM patients were subdivided into two groups: group 1 involved 22(44%) patients with diabetic complications; group 2 involved 28 (56) % patients without complications. All patients and control subjects gave a verbal consent for their participation in this study. This study was approved by scientific jury and ethical committee. A full history was been undertaken for all patients regarding disease duration, current pharmacotherapy, dietary schedule (avoiding high nitrate food for 12 prior to the test) and associated diseases, and any patient with end stage or severe complications were excluded.

Biochemical measurements

10ml of venous blood from all patients and healthy subjects after an overnight fasting was drawn, after centrifugation the sera were stored at -20°C for analysis. Lipid profile was estimated by specific kit method according to the kit instructions; VLDL and LDL were estimated according to specific formula [15]. Fasting blood glucose was determined by fasting capillary method [16]. Plasma nitrate, plasma nitrite in μM and nitric oxide NO μmol/L was determined according to the specific method [17]. Direct strip method was used for estimation of salivary nitrite (μmol/L) according to the specific method [18]. the samples are taken at 8.00 am and immediately used to avoid the diurnal variations.

Statistical analysis

Data of the present study were expressed as mean± SD, percentages and numbers. Unpaired t test was used for determination the significance of differences compared with control, whereas ANOVA test was used to detects the significance of differences among the diabetic patients and control subjects, regarding p is significant when it <0.05. SPSS version 21 was used for analysis the data of the present study.

Results

Table 1: Characteristics of the present study.

| Variables | The Characteristics |
|-----------|---------------------|
| Number    | 77                  |
| Diabetic patients | 50 (64.93) |
| Control   | 27 (35.06)          |
| Gender M:F ratio | 28 male (71.42), 22 female (28.57) |
| Age (years) | 49.66±11.31        |
| Duration of type 2DM (years) | 6.33±2.59         |
| Patients with complicated type 2DM | 22 (44)         |
| Patients with non-complicated type 2DM | 28 (56)         |
| Associated Diseases |
| Hypertension | 16 (32)           |
| Ischemic heart diseases | 14 (28)       |
| Dyslipidemia | 20 (40)           |
| Asthma     | 2 (4)              |
| Diabetic Pharmacotherapy |
| Metformin  | 12 (24)            |
| Sulfonylurea | 11 (22)      |
| Sitagliptin | 3 (6)            |
| Metformin + Sulfonylurea | 14 (28)       |
| Metformin + Sitagliptin | 1 (2)             |
| Insulin    | 10 (20)            |
| Others     |
| Statins    | 20 (40)            |
| CCB        | 11 (22)            |
| ACEI       | 5 (10)             |
| Anti platelets | 39 (78)     |
| Theophylline | 2 (4)            |
| Smoking    | 41 (82)            |

Data are expressed as Mean±SD, n (%); CCB: calcium channel blockers; ACEI: angiotensin converting enzyme inhibitors.
A total number of 50 patients compared with 27 normal healthy subjects were included in this study. The duration of T2DM was 6.33±2.59 years, 44% of diabetic patients (n=22) presented with complications whereas; 56% of diabetic patients (n=28) not associated with diabetic complications. The associated diseases with T2DM in this study were hypertension 16 (32%), ischemic heart diseases 14 (28%), dyslipidemia 20 (40%) and asthma 2 (4%). Most of diabetic patients were on current therapy of metformin 24% and metformin plus sulfonylurea 28% also, 82% of diabetic patients (n=41) were smokers. Other treatments that are received by the diabetic patients were calcium channel blockers, angiotensin converting enzyme inhibitors, statins, antiplatelets and theophylline, Table 1 showed the characteristics of enrolled patients. In T2DM patients without complications there was well-controlled fasting blood glucose, HbA1c p>0.05 as compared with control but in those patients there is dyslipidemic state p<0.05 compared with control without significance of differences regarding plasma nitrate, plasma nitrite, salivary nitrite and endogenous NO levels p>0.05 compared with control.

Table 2: Differences in the salivary nitrite levels and plasma nitrate-nitrite-NO pathway in T2DM patients with and without complications compared with control.

| Variables          | Control (N=27) | Diabetic without Complications (N=28) | Diabetic with Complications (N=22) | ANOVA |
|--------------------|----------------|--------------------------------------|------------------------------------|-------|
| FBG (mg/Dl)        | 91.22±13.23    | 125.68±12.63                        | 166.89±18.48*                     | <0.01 |
| HbA1c (%)          | 4.6±1.33       | 6.55±2.58*                          | 9.82±3.52*                       | <0.01 |
| TC (mg/Dl)         | 110.83±11.62   | 144.64±14.82*                       | 215.68±19.22*                    | <0.01 |
| TG (mg/Dl)         | 133.93±16.69   | 158.61±17.22*                       | 299.61±18.29*                    | <0.01 |
| HDL (mg/Dl)        | 54.73±9.41     | 46.97±10.61*                        | 36.91±6.11*                      | <0.01 |
| VLDL (mg/Dl)       | 29.79±8.41     | 31.72±8.69                          | 59.92±8.52*                      | <0.01 |
| LDL (mg/Dl)        | 29.31±6.39     | 65.94±9.55*                         | 118.84±14.69*                    | <0.01 |
| Plasma nitrate (Mm)| 25.13±6.56     | 23.22±6.33                          | 21.82±6.82                       | 0.212 |
| Plasma nitrite (Mm)| 3.29±1.44      | 2.77±1.39                           | 4.27±1.33**                      | 0.001 |
| Salivary nitrite (μmol/L)| 55.63±11.85 | 56.81±10.49                          | 61.99±9.31**                     | 0.37  |
| NO (μmol/L)        | 88.51±18.69    | 79.53±18.35                         | 99.99±21.71**                    | 0.015 |

Unpaired t test: *p<0.01, **p<0.05 as compared with control. FBG: fasting blood glucose; HbA1c: glycated hemoglobin; TC: total cholesterol; TG: triglyceride; HDL: high density lipoprotein; VLDL: very low density lipoprotein; LDL: low density lipoprotein; NO: nitric oxide.

In T2DM patients with complications there were significant hyperglycemia, dyslipidemia, elevated plasma nitrite, reduced plasma nitrate, elevated salivary nitrite and elevated endogenous NO levels therefore; salivary nitrite was high in patient with complications p=0.04 compared with control and near normal in diabetic patient without complications (Table 2). Regarding the diabetic pharmacotherapy, metformin increases salivary nitrite more than glimepiride but combination of metformin plus glimepiride produced significant amelioration in salivary nitrite levels. In the present study most of diabetic patients without complications were on combination therapy whereas most of diabetic patients with complications were on monotherapy of either metformin or glimepiride (Figure 1). Regarding the significance value of salivary nitrite test in this study it was highly sensitive with moderate specificity with comparable positive and negative predictive value (Table 3).

Table 3: Sensitivity and specificity of salivary nitrite test in patients with type 2 DM.

| Salivary Nitrite Value + 95% CI (Upper-Lower Limits) | Sensitivity | Specificity | Positive likelihood ratio | Negative likelihood ratio | Positive predictive value | Negative predictive value |
|-----------------------------------------------------|-------------|-------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Sensitivity                                         | 95.24%±(83.84-99.42) | 62.50%±(24.49-91.48) | 2.54±(1.04-6.23)       | 0.08±(0.02-0.33)     | 93.02%±(89.94-98.54) | 71.43%±(29.04-96.33) |

Discussion

The present study illustrated that salivary nitrite was low in patients with controlled T2DM and high in T2DM patients with complications compared with normal healthy control subjects, these findings were corresponded with Francesconi, et al. [19] study that showed changes in nitrate and nitrite levels may reflect the endogenous NO production and metabolic alterations induced by hyperglycemia [19] Moreover, elevation and reduction in nitrate and nitrite levels were revealed in...
T2DM compared with control due to metabolic disturbances and diabetic-induced complications such as nephropathy that causing significant elevation in nitrate/nitrite levels due to activation of inducible NO synthase enzyme, inflammatory changes and insulin resistance [20].

Indeed, changes in salivary nitrite in the patients with T2DM may be due to diabetic-induced alterations in the salivary flow and compositions [21]. Results of present study as well showed significant changes in NO serum levels, high in complicated and low in non-complicated T2DM since; increased nitrate/nitrite plasma levels with increased NO production could be a compensatory mechanism against insulin resistance and diabetic-induced oxidative stress [22] thus; augmented nitrate-nitrite-NO pathway was reflected via increasing in the salivary nitrite levels whereas; well controlled diabetic patients exhibited low nitrate/nitrite levels [23].

Moreover, the present study showed that diabetic-induced dyslipidemia was more observed in patients with complicated T2DM, this dyslipidemia may affect the endothelial function and NO production [24]. Therefore, augmentation in the nitrite/nitrate plasma levels in complicated T2DM could have a beneficial effect in prevention the progression of diabetic complications since; plasma nitrite/nitrate has interesting antioxidant and anti-inflammatory actions that ameliorate endogenous catalase and glutathione activity as well as improves the level of pro-inflammatory cytokines [25].

On the other hand, 81% of recruited patients were smokers that may affect the nitrite/nitrate/NO pathway as illustrated by Retterstol, et al. [26] study that showed low level of nitrate and nitrite levels in smokers healthy subjects [26] but in our study high level of nitrite/nitrate/NO pathway was showed in complicated T2DM smoker patients since; chronic nicotine smoking leads to significant endothelial dysfunction [27]. Regarding T2DM pharmacotherapy in the present study, most of patients were treated by either metformin and/or glimepiride these agents may also affect nitrite/nitrate/NO pathway seeing as; metformin improves endothelial function in T2DM patients that reflected by increase in the NO levels with subsequent augmentation in nitrite/nitrate plasma levels that excreted though salivary rout as salivary nitrite [28,29] thus; metformin improves NO through AMP-activated protein kinase activation that responsible for up regulation of eNOS and then NO production and inhibition of Rho kinases which implicated in the development of endothelial dysfunction and reduction of endothelial NO production [30]. Indeed, glimepiride have potent anti-inflammatory and anti-oxidant activities through reduction of vascular endothelial growth factor (VEGF) and macrophage-inflammatory protein (MIP) that contributes into prevention of diabetic-induced endothelial dysfunction and the alteration in the NO production [31] Furthermore, salivary nitrite in the present study was highly sensitive but less specific with high positive predicative value since; all samples were taken at morning and immediately used for analysis in view of the fact that salivary nitrite levels are highs affected by sample time, storage and analysis time [32,33].

Conclusion
Salivary nitrite levels were high in complicated T2DM and low in controlled T2DM compared to the control. Metformin increases salivary nitrite levels whereas glimepiride alone or in combination with metformin reduce salivary nitrite levels in T2DM patients.

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How to cite this article: Hayder M A, Ali I Al-G, Marta C M, Salah A Al-w, Huda J. Salivary Nitrite in Patients with Type 2 Diabetes Mellitus: Role of Diabetic Pharmacotherapy. Glob J Pharmaceu Sci. 2017; 1(2) : 555558. DOI: 10.19080/GJPPS.2017.01.555558

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DOI: 10.19080/GJPPS.2017.01.555558