Role of Oxidative Stress in *Diabetes Mellitus*

by

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in the

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Program

YOUNGSTOWN STATE UNIVERSITY

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ABSTRACT

Background: The purpose of this study was to investigate the role of oxidative stress in diabetes mellitus. Previous research indicates that oxidative stress and free radical activity has been known to cause damage to cells and organs, especially as we age. In this study, we used urine levels of nitric oxide metabolites such as nitrate and nitrite to measure oxidative stress.

Method: Nitrate/Nitrite Colorimetric Assay was used to measure the total nitrate and nitrite concentration in urine samples among older adults (> 65 years old). Six urine samples were obtained from Discovery Life Sciences (Discovery Life Sciences Inc., CA, USA). Three of the six urine samples were collected from diabetic patients. A nitrate standard curve was prepared in order to quantitate sample nitrate and nitrite concentrations. The total nitrate and nitrite concentration was measured using absorbance values from a plate reader at 550 nm.

Results: Our findings appear to show higher levels of oxidative stress among older diabetic patients. The average total nitrate and nitrite concentration of 716 μM was found in patients with diabetes while those without diabetes had an average total nitrate and nitrite concentration of 246 μM.

Conclusion: This study highlights the role of free radical activity and oxidative stress in the development of diabetes in older adults. Higher nitric oxide levels indicate higher oxidative stress activity. Future research that can focus on slowing down oxidative stress can prove to be quite helpful in aging studies.

Keyword(s): *Diabetes Mellitus*, Free Radicals, Greiss Reaction, Oxidative Stress

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INTRODUCTION

Diabetes Mellitus (DM) is another term used for diabetes. DM is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.[1] Type I diabetes is an autoimmune condition where the immune system wrongly identifies and subsequently attacks the pancreatic cells that produce insulin, leading to little or no insulin production. This is a more serious type of diabetes. Type II diabetes is associated with impaired insulin secretion. Insulin levels affect glucose levels and thus lead to type II diabetes. In type II diabetes, the body usually still produces some insulin, but this is not enough to meet demand and the body's cells do not properly respond to the insulin. Most commonly, this particular type is associated with being overweight or obese. Type II diabetes, which was previously called adult onset, is a more common than type I diabetes. Another name that was previously referred to type I diabetes was juvenile onset. This clearly highlights the differences between both types of DM. This particular study focuses on type II diabetes.

The prevalence of diabetes and hypertension increases with age.[2] Among those who are 65 years or older, more than 25% are known to have diabetes and are at an increased risk for both acute and chronic microvascular and cardiovascular complications of the disease.[3] There are a number of major risk factors for DM, especially type II diabetes. Although some diabetic risks come from genetics, some may be preventable. Diet and lack of exercise are the two common reasons that lead to diabetes. Among older adults, free radical damage and oxidative stress should be

given careful consideration. Free radicals may damage lipids, proteins and DNA, which may lead to critical diseases in the aging.

Oxidative stress and free radical damage are known to be harmful to many cells and enzymes. By definition, oxidative stress is referred to as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses.[4] Oxidative stress and free radical damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, and rheumatoid arthritis.[5] Preliminary review of the literature shows some evidence in both experimental and clinical studies that oxidative stress plays a major role in the pathogenesis of both types of DM.[6]

As one ages, free radicals play a significant role in the aging process. Free radical damage has been proven to be harmful to many cells and enzymes. Dr. Harman first proposed the free radical theory of aging in 1960s. Over the years, this theory has explained many aging related diseases. The free radical theory of aging states that organisms age because cells accumulate free radical damage over time. A free radical is any atom or molecule that has a single unpaired electron in an outer shell. The free radical theory of aging postulates that aging changes are caused by free radical reactions. The data supporting this theory indicate that average life expectancy at birth may be increased by five or more years, by nutritious low caloric diets supplemented with one or more free radical reaction inhibitors.[7]

Two key phrases central to the discussion of oxidative stress and free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•),

consist of radical and non-radical oxygen species formed by the partial reduction of oxygen.[8] Cellular ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation, or they may arise from interactions with exogenous sources such as xenobiotic compounds. In this study, we will primarily focus on RNS. In biological systems, the main source of all RNS is nitric oxide (NO).[9] RNS act together with ROS to damage cells, causing biological stress. Therefore, these two species are often collectively referred to as ROS/RNS.

Another aspect that is important biochemically is the role of antioxidants in defense of free radical damage. Much of the literature also discusses antioxidants and how important they are to fight free radical damage in cells. By definition, antioxidants are substances, which inhibit or delay oxidation of a substrate while present in minute amounts. The main source of antioxidants is nutrition. As we age, antioxidant levels are not as abundant and thus we are at a greater risk for free radical damage. This in turn puts us at a greater risk for type II diabetes. In another review article, it is pointed out that low levels of antioxidants cause damage in pancreatic islet cells for production of insulin.[10] This suggests that not only does free radical activity play a role in diabetes but also levels of antioxidants are also considered very important in determining diabetic complications in older adults as higher levels of antioxidants help fight against free radicals.

Measuring total nitrate/nitrite concentrations can be a way of detecting oxidative stress. Cardiovascular risk factors such as hypercholesterolemia, hypertension, and diabetes mellitus enhance ROS generation, which lead to oxidative stress.[11] Therefore, measurement of nitrate and nitrite production can be used as

detection for oxidative stress. There have been a few studies that have measured nitrate and nitrite levels in biological fluids such as plasma and urine.[12, 13]

In this study, the Griess reaction was the method used for determination of total nitrate and nitrite levels in patient samples. The Nitrate/Nitrite Assay is a simple and sensitive assay for nitrate and nitrite determination based on the commonly used two-step assay method which involves the addition of two cofactors for the nitrate reductase reaction. With the help of additional cofactors, the reductase reaction to convert nitrate to nitrite is accelerated and simultaneously the excess NADPH is degraded to NADP. Thus, the reductase reaction can be completed within thirty minutes and colorimetric determination can be directly measured by the addition of Griess Reagent. This kit is fast, simple and can be used to assay nitrite and nitrate in urine, plasma, serum, saliva, cell lysate and tissue culture medium. Absorbance and colorimetric assays are designed to detect or quantitate the amount of a particular reagent in an assay by measuring the amount of light absorbed by the reagent or chromogenic reaction product at a wavelength of 550 nm. A total of six samples were analyzed using nitrate/nitrite colorimetric assay which included three samples of diabetic and three samples of non-diabetic patients. If oxidative stress and free radical activity is related to diabetes, then diabetic patients will show higher levels of total nitrate/nitrite concentrations than non-diabetic patients.

MATERIALS AND METHODS

Many different methods have been applied for nitrate/nitrite detection, with the most commonly used being the spectrophotometric assay based on the Griess reagent.[14] The Greiss reagent test provides a simple colorimetric assay for nitrites, and nitrates that have been reduced to nitrites, with a detection limit of about 100 nM. For this experiment, Nitrate/Nitrite Colorimetric Assay kit was used from Cayman Chemical (Cayman Chemical Inc., MI, USA). This experiment provides a quantitative colorimetric determination of total nitrate/nitrite in urine, plasma, serum, saliva, cell lysate and tissue culture medium. In this study, urine samples were used for measurement of nitrate/nitrite metabolites using Cayman's kit as it works well for the analysis of fluids. It also provides a fast, simple, and convenient method for the detection of nitrate/nitrite. A total of six urine samples were obtained from Discovery Life Sciences (Discovery Life Sciences Inc., CA, USA). The urine samples were used directly after dilution to the proper concentration in Assay Buffer. Urine contains high levels of nitrate (200-2,000 uM), so dilutions of 1:25 were used. Three samples (1, 2 and 3) were non-diabetic while the other three samples (4, 5 and 6) were diabetic. Table 1 summarizes age and gender information for all samples.

Sample	Diabetes?	Age	Gender
1	No	89	Male
2	No	78	Male
3	No	73	Male
4	Yes	66	Male
5	Yes	81	Male
6	Yes	73	Male

Table 1. Urine Samples

Reagent Preparation

Another part of the pre-assay process was the reagent preparation. Reagent preparation was done prior to performing the assay. The following reagents were prepared:

a) Nitrate/Nitrite Assay Buffer: The contents of the Assay Buffer vial were diluted to 100 ml with Milli-Q water. This Assay Buffer was used for dilution of samples as needed prior to assay.

b) Nitrate Reductase Enzyme: The contents of the vial were reconstituted with 1.2 ml of Assay Buffer and kept on ice during use.

c) Nitrate Reductase Cofactors: The contents of the vial were reconstituted with 1.2 ml of Assay Buffer and kept on ice during use.

d) Nitrate Standard: The vial stopper was removed slowly to minimize disturbance of the lysophilized powder. The contents of the vial were reconstituted with 1.0 ml of Assay Buffer. Once the Assay Buffer was added, the vial was vortexed to ensure all of the powder, including any on the stopper, was in solution.

e) Griess Reagents: There was no water or Assay Buffer added to these reagents, as they were ready to use.

Nitrate Standard Curve

The first step for measurement of total nitrate and nitrite concentration was preparation of nitrate standard curve. A nitrate standard curve was performed in order to quantitate sample nitrate and nitrite concentrations. A volume of 0.9 ml of Assay Buffer was placed in a clean test tube. Then, 0.1 ml of reconstituted Nitrate Standard was added. The concentration of this stock standard was 200 μM. This standard (200 μM) was used for the preparation of the nitrate standard curve.

The nitrate standard curve was used to determine total nitrate and nitrite concentration in the sample. Although the amount of nitrite present in the samples was measured as total NO products (nitrate + nitrite), nitrite standard curve was not needed. The nitrate standard curve was sufficient in determining the total NO products (nitrate + nitrite) in the samples. The nitrate and nitrite standard curves are nearly identical; in practice, however, a small discrepancy often occurs. Table 2 shows the set up for nitrate standard curve.

Well	Nitrate Standard (μL)	Assay Buffer (μL)	Final Nitrate Concentration (μM)
A1	0	80	0
B1	5	75	5
C1	10	70	10
D1	15	65	15
E1	20	60	20
F1	25	55	25
G1	30	50	30
H1	35	45	35

Table 2. Nitrate Standard Dilutions

Nitrate/Nitrite Colorimetric Assay

Three wells were set up as blank wells. These blank wells contained 200 µL of Assay Buffer. The absorbance for these wells was averaged and subsequently subtracted from the absorbance measured in other wells. Once the blank wells were set up with 200 µL of Assay Buffer, proper dilutions were set up in other wells in triplicates. Then, up to 80 µL of sample dilutions were added to the wells. Then, 10 µL of the Enzyme Cofactor Mixture was added to each of the wells (standards and unknowns). Then, 10 µL of the Nitrate Reductase Mixture was added to each of the wells (standards and unknowns). The plate was covered with the plate cover and incubated at room temperature for one hour. After the required incubation time, 50 µL of Griess Reagent R1 was added to each of the wells (standards and unknowns). Immediately, 50 µL of Griess Reagent R2 was added to each of the wells (standards and unknowns). Color was allowed to develop for 10 minutes at room temperature. Finally, absorbance at was read at 550 nm using a plate reader.

Data Analysis

The absorbance values of the blank wells were averaged and subtracted from absorbance values from all of the other wells. The standard curve was then plotted using the absorbance values of the nitrate standard dilutions. Figure 1 shows nitrate standard curve. This standard curve was used to calculate total nitrate and nitrite concentrations for the unknown urine samples. The following equation was used to calculate total nitrate and nitrite concentrations:

$$[\text{Nitrate + Nitrite}]\ (\mu M) = \left(\frac{A_{540} - \text{y-intercept}}{\text{slope}}\right)\left(\frac{200\ \mu l}{\text{volume of sample used } (\mu l)}\right) \text{ x dilutio}$$

RESULTS

The average absorbance reading for three blank wells was 0.027. This value was subtracted from all other wells (standard and unknowns). Using these new absorbance values, a standard curve for nitrate was constructed (shown in Figure 1). The equation for y-intercept was determined to be $y = 0.03x - 0.0094$. R^2 was 0.9049.

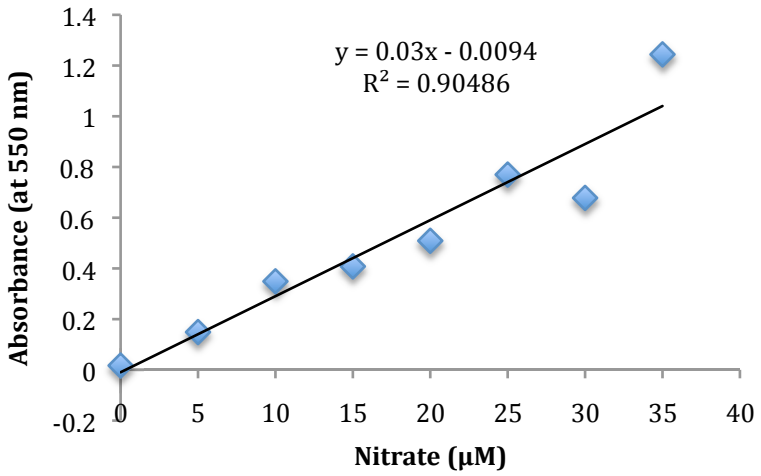

Figure 1: Nitrate Standard Curve

Using the standard curve, total nitrate and nitrite concentration was calculated for the unknown samples (Table 2). Our findings appear to show higher levels of oxidative stress among older diabetic patients. Elevated levels of total nitrate and nitrite concentrations indicate higher oxidative stress levels in urine samples. The average total nitrate and nitrite concentration of 716 µM was found in patients with diabetes while those without diabetes had an average total nitrate and nitrite concentration of 246 µM. All diabetic samples except sample 5 showed higher levels of total nitrate and nitrite concentrations

Sample	Total Nitrate and Nitrite Concentration (μM)
1	91 +/- 5
2	293 +/- 10
3	354 +/- 10
4	1003 +/- 14
5	230 +/- 48
6	914 +/- 45

Table 2. Average Total Nitrate and Nitrite Concentration (μM) of triplicates

DISCUSSION

The Greiss reaction is a popular method for determining total nitrate and nitrite concentrations.[15] The quantification of nitric oxide (NO) metabolites in biological samples provides valuable information with regard to in vivo NO production, bioavailability, and metabolism.[16] Nitrate and nitrite are often used as measurement of NO activity as they are stable metabolites of NO.[17] The results appear to show that patients with diabetes indicate higher levels of oxidative stress in their bodies. This is a novel spectrophotometric method for determining oxidative stress in humans as previous studies have not used this assay. By measuring the levels of total NO products (nitrate + nitrite), it was expected that diabetic patients will show high levels of total NO products in their urine samples than non-diabetic patients. Two out of three patients did show significantly higher levels of NO products in their urine samples. Perhaps, an undisclosed condition like chronic renal failure might be the reason for low levels of nitrate and nitrite in the third patient.[18,19]

One possible limitation with this particular method is that nitrate occurs naturally in urine and therefore more information regarding patients' dietary intake is needed to determine whether their diets affect total NO production levels in urine. It is well known that diet can affect nitrate levels in the urine. Three studies measured urinary nitrate concentrations after fasting that showed significantly decreased nitrate levels.[20] It is widely recognized that dietary nitrate intake can affect nitrate levels in urine.[21]

As other studies have mentioned, there seems to be a possible link between oxidative stress and diabetes. Oxidative stress has been demonstrated in many studies

to participate in the progression of diabetes, which plays important role during diabetes including impairment of insulin action and elevation of the complication incidence. DM is related to endothelial dysfunction, which is caused by free radicals.[22] Antioxidants have already shown to be prospective in the treatment of diabetes both type I and type II. Increase in the levels of oxygen and nitrogen free radicals (ROS/RNS) has been linked with lipid peroxidation, non-enzymatic glycation of proteins and oxidation of glucose which contributes toward diabetes mellitus and its complications. Most of the studies have shown relationship between oxidative stress and diabetes along with their complications related to heart, liver kidney and eye. Thus, oxidative stress seems to be more worrying in metabolic disorders specially type II diabetes. There are some benefits of free radicals ROS/RNS when the body maintains homeostasis but it is the imbalance that leads to oxidative stress.[23]

Other laboratory tests for measuring oxidative stress have been known. These include flow cytometry, nitroblue tetrazolium (NBT) assay and other ways to that detect lipid peroxidation markers.[24] These methods can be expensive and not readily available. Therefore, using Greiss method can be simple, convenient and cost effective. Spectrophotometric assay methods can be used readily with

There were few other possible limitations for this study. One limitation may include that this is a scientific study and therefore it may not account for other social factors that influence oxidative stress in individuals with diabetes. Another limitation for this study is a small sample size of those tested. Future research in this area can help shed light into more possible explanations for diabetes. Perhaps, future studies with aldose reductase, an enzyme involved in diabetes, can prove to be beneficial.

This study highlights the role of free radical activity and oxidative stress in the development of diabetes in older adults. Higher NO levels indicate higher oxidative stress activity. Understanding free radical biology is key to developing and designing nutritional countermeasures against oxidative stress.[25] There are a number of epidemiological studies that have shown inverse correlation between the levels of established antioxidants and phytonutrients present in tissue/blood samples and occurrence of cardiovascular disease, cancer or mortality due to these diseases.[26] Studies that can focus on slowing down oxidative stress can prove to be quite helpful in aging research for years to come.

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APPENDIX A

Youngstown
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May 8, 2018

Dr. Daniel Van Dussen, Principal Investigator
Mr. Anubhav Vinayak, Co-investigator
Department of Sociology, Anthropology & Gerontology
UNIVERSITY

RE: HSRC Protocol Number: 175-2018
Title: Role of Oxidative Stress in *Diabetes Mellitus*

Dear Dr. Van Dussen and Mr. Vinayak:

The Human Subjects Research Committee has reviewed the abovementioned protocol and has determined that it does not require further IRB oversight because it does not meet the definition of human research as stated in the Code of Federal Regulations, 45 CFR 46.

Any changes in your research activity should be promptly reported to the Human Subjects Research Committee and may not be initiated without HSR approval except where necessary to eliminate hazard to human subjects. Any unanticipated problems involving risks to subjects should also be promptly reported to the Human Subjects Research Committee.

The HSRC would like to extend its best wishes to you in the conduct of this study.

Sincerely,

Mr. Michael A. Bripko
Associate Vice President for Research
Authorized Institutional Official

MAH/cc

c: Dr. Matt O'Mansky, Chair
Department of Sociology, Anthropology & Gerontology

APPENDIX B

SchulmanIRB
Offices: Cincinnati, OH | Research Triangle Park, NC
www.sairb.com
CanadianREB Minimal Risk REVIEW Phase 1 REVIEW Central Oncology Review

REAPPROVED: 09/28/2017
EXPIRATION DATE: 09/27/2018

September 29, 2017

FROM: Schulman IRB
TO: Ann Dover, Discovery Life Sciences, Inc.
SUBJECT: Reapproval
SPONSOR: Discovery Life Sciences, Inc.
PROTOCOL NO: DLS13-Collection
PROTOCOL TITLE: Collection Protocol to Support Validation of Novel Biomarkers and Research and Development Programs

This letter is to inform you that the Board reapproved the referenced protocol for another 12 months.

If the study is expected to last beyond the approval period, you must request and receive re-approval prior to the expiration date noted above. A report to the Board on the status of this study is due prior to the expiration date or at the time the study closes, whichever is earlier. It is recommended that you submit status reports at least 4 weeks prior to your expiration date to avoid any additional fees or lapses in approval. You can find the Study Status Report Form at www.sairb.com. Continue to use the latest Schulman approved informed consent(s).

Approved investigators and sites are required to submit to Schulman for review, and await a response prior to implementing, any amendments or changes in: the protocol; advertisements or recruitment materials ("study-related materials"); investigators (PI and Sub-Is); or sites (primary and additional). Refer to www.sairb.com for comprehensive submission requirements.

Approved investigators and sites are required to notify Schulman of the following reportable events, including, but not limited to: unanticipated problems involving risks to subjects or others; unanticipated adverse device effects; protocol violations that may affect the subjects' rights, safety, or well-being and/or the completeness, accuracy and reliability of the study data; subject death; suspension of enrollment; or termination of the study. Refer to the "Event(s) That Investigators Have to Report to Schulman" guidance document available on the Schulman WebPortal/SiteAccess and at www.sairb.com.

Schulman IRB is in compliance with Part C Division 5 of the Canadian Food and Drug Regulations, the Tri-Council Policy Statement (TCPS), the International Conference on Harmonization Good Clinical Practice Guidelines, the regulations of the United States Food and Drug Administration as described in 21 CFR parts 50 and 56, and the United States Department of Health and Human Services regulations 45 CFR part 46, and the Environmental Protection Agency 40 CFR 26.

PLEASE REFERENCE IRB #[redacted] ON ALL CORRESPONDENCE FOR THIS STUDY
WebPortal/Paperless

All dates are in mm/dd/yyyy format.