

Hemoglobin Oxidation Studies in Diabetics Blood Comparing the Effect of Sodium Nitrite vs. Amyl Nitrite

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Abstract

Comparative studies between sodium nitrite and isoamyl nitrite in nitrite induced methemoglobin formation of diabetic's blood have been undertaken. First the effect of sodium nitrite on human Type 2 diabetics' blood was undertaken using non diabetics' blood as the control group. These studies revealed that diabetics erythrocytes were oxidized at apparently the same rate as the control blood ($P > 0.05$). The sodium nitrite mean oxidation time $\pm$ SEM of diabetics blood was 3.5 ± 0.18 min (sample size (n) is 22) and the mean oxidation time $\pm$ SEM of the non diabetics blood was 3.5 ± 0.16 min (n is 22). Next it was revealed that diabetics erythrocytes were oxidized by amyl nitrite at a significantly greater rate than control erythrocytes ($P < 0.05$). The mean oxidation time $\pm$ SEM of the diabetics blood was 1.5 ± 0.05 min (n is 20) whereas the mean oxidation time $\pm$ SEM of the non diabetics blood was 3.1 ± 0.12 min (n is 20). Thus, these studies demonstrate that while diabetics blood has an enhanced susceptibility to oxidation to methemoglobin by isoamyl nitrite compared to the control group when sodium nitrite is used both diabetics and control blood are oxidized at the same rate. This difference could be attributed to the fact that sodium nitrite reactions with oxyhemoglobin are sigmoidal in nature and involve an induction or lag phase, whereas the isoamyl nitrite reactions with oxyhemoglobin are an immediate reaction wherein a rectangular hyperbolic curve is generated.

This probably means that isoamyl nitrite has greater accessibility to the heme crevice especially in the diabetic presumably due to glycation induced structural modifications present in Hemoglobin A1C (HbA1C).

Keywords: Diabetes; HbA1C; Isoamyl nitrite; Methemoglobin; Oxidation of Hemoglobin; Sodium nitrite

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Introduction

Both sodium nitrite and amyl nitrite are chemical compounds that cause oxyhemoglobin to undergo oxidation, i.e. the iron (II) in the hemoglobin loses an electron to become iron (III). When this occurs the ruby red oxyhemoglobin changes in color to a chocolate brown which signifies it has become methemoglobin or iron (III) hemoglobin. While iron (II) hemoglobin can carry oxygen to the tissues as oxyhemoglobin, iron (III) hemoglobin or methemoglobin cannot carry oxygen to the tissues and is therefore useless in oxygen transport to the tissues [1]. Nitrites are compounds that have long been known to cause this oxidation reaction [2]. Sodium nitrite is a food additive that is used as a preservative. For example, foods containing sodium nitrite and nitrate preservatives, especially

hot dogs and lunch meats, could pose a potentially greater threat to diabetics than non diabetics were the diabetic's blood to have a relatively enhanced susceptibility to oxidation by inorganic nitrites [3]. With wide usage of amyl nitrite in the treatment of coronary heart disease and its effect to cause methemoglobinization and possible side effects there from, this additional comparative study of diabetics' blood's susceptibility to enhanced methemoglobin formation compared to inorganic nitrites appears warranted [4].

As is well known people with diabetes mellitus have hemoglobin that differs from ordinary adult blood in that it is glycated to a level of 6.5% hemoglobin A1C or greater by the abnormally high level of glucose in the untreated diabetic's blood [5]. Recently Moussa reported that methemoglobinization, that is the oxidation of iron (II) of

oxyhemoglobin into iron (III) to form methemoglobin, is an important indication of oxidative stress in certain diabetic patients [6]. Specifically, those afflicted with type 1 diabetes mellitus have higher hemoglobin auto-oxidation rate than those with type 2 diabetes mellitus or non diabetics [7]. Increased susceptibility to the oxidation of diabetics' blood by sodium nitrite would be worthwhile information to establish due to supporting evidence that dietary inorganic nitrites are associated with the development of type 1 diabetes [8]. This would then become a good reason to remove inorganic nitrites and nitrates from foods. In addition these studies may help confirm that certain diabetics blood (e.g., those with type 2 diabetes mellitus) is less stable than those of a normal adult which could put them at even greater risk of all heart disease (both angina pectoris and myocardial infarction) due to hemoglobin with a higher auto-oxidation rate.

Table 1: Characteristics of Typical Patients Used in the Nitrite Oxidation Studies

Sample ID	Sample Type	HbA1C (%)	Age	Gender	Weight	Smoker status
B000046	Diabetic ACD Blood	9.3	49	Male	276	Smoker
W000853	Diabetic ACD Blood	10.2	62	Male	342	Non-Smoker
W000854	Diabetic ACD Blood	11	39	Female	169	Non-Smoker
W000855	Diabetic ACD Blood	9.7	27	Female	204	Smoker
W000861	Diabetic ACD Blood	12.4	54	Female	218	Non-Smoker
W000862	Diabetic ACD Blood	10.9	42	Female	286	Smoker
W000849	Normal ACD Blood	5.5	33	Female	141	Smoker
W000850	Normal ACD Blood	5.3	37	Male	205	Smoker
W000851	Normal ACD Blood	5.7	32	Male	230	Smoker
W000856	Normal ACD Blood	5.1	31	Male	165	Smoker
W000857	Normal ACD Blood	5.7	30	Male	179	Smoker
W000858	Normal ACD Blood	5.8	32	Male	234	Non-Smoker

Materials

Sodium nitrite was purchased from Fisher Scientific. Isoamyl nitrite was purchased from Across Organics. Other required chemicals were obtained from the Sigma and Aldrich Chemical Company. Blood products such as normal adult blood and diabetics' blood were purchased from Physicians Plasma Alliance (PPA). All blood was tested and certified to be non-viral by PPA.

For the sodium nitrite studies: the data was obtained from 44 donors 22 of whom had type 2 diabetes mellitus and 22 of whom were non diabetics.

For the isoamyl nitrite studies: the data was obtained from 40 donors 20 of whom had type 2 diabetes mellitus and 20 of whom were non diabetics. The samples were provided as matched sets of diabetics and non diabetics blood wherein the two groups were evenly matched with respect to age, gender, number of obese and number of cigarette smokers. Also these donors took similar vitamins and medications according to their medical histories. In Table 1 the characteristics of typical patients used in these studies are presented, i.e., age, gender, weight and smoker status are noted. All blood was drawn into Acid-Citrate-Dextrose (ACD) tubes and stored at 2-4 C prior to use.

Methods

The Hemoglobin A1C (HbA1C) percentages were determined using a Bayer DCA-2000 test kit. Diabetes was assessed as a HbA1C percentage greater than 6.5% [5]. A laboratory spectrophotometer equipped with a strip chart recorder was employed to monitor the formation of methemoglobin at 436nm. A small table top centrifuge was used to separate plasma from the red blood cells. To determine the oxidation time's blood samples were centrifuged for 2000g for 20 min to remove any remaining plasma. The remaining packed Red Blood Cells (RBCs) were aerated and washed in 20mM Phosphate Buffer Saline (PBS) at pH 7.2 followed by another centrifugation to remove the saline. This procedure of centrifugation, aeration and washing was repeated. The RBCs were then resuspended in 20mM PBS (pH 7.2) for a maximum of 60 min prior to testing.

For the sodium nitrite studies: A 0.01 mL portion of resuspended RBCs was hemolyzed by the addition of 1 mL of distilled water and adjusted to a final volume of 2.6mL by the addition of 20mM PBS (pH 7.2). The hemoglobin solutions were then adjusted to a standard absorbance (A) (e.g., $A = 1.0 \pm 0.2$) at a wavelength of 436nm with more 20mM PBS (pH 7.2). The 2.6mL aliquot of this hemoglobin solution was then added to a 0.1 mL aliquot of a 0.2% aqueous sodium nitrite solution. A final concentration of 10.7 mill moles per liter of sodium nitrite was obtained after its addition to the hemoglobin solution. The above gave a final hemoglobin concentration between 6 and 9 micromoles per liter [9].

For the isoamyl nitrite studies: A 0.01 mL portion of resuspended RBCs was hemolyzed by the addition of 1 mL of distilled water and adjusted to a final volume of 2.6mL by the addition of 20mM PBS (pH 7.2). The hemoglobin solutions were then adjusted to a standard absorbance (e.g., $A = 1.0 \pm 0.2$) at a wavelength of 436nm with more 20mM PBS (pH 7.2). The 2.6mL aliquot of this hemoglobin solution was then added to this 0.05mL aliquot of a 0.1% isoamyl nitrite in ethanol solution. A final concentration of 140 micromoles per liter of amyl nitrite was obtained after its addition to the hemoglobin solution. The above gave a final hemoglobin concentration between 6 and 9 micromoles per liter [9].

All of the above solutions were then placed in cuvettes and the reaction measured in a spectrophotometer equipped with a chart recorder set at a wavelength of 436nm. This is a suitable wavelength for measuring and distinguishing oxyhemoglobin and methemoglobin. The spectrophotometer chart recorder then generated graphic representations of the conversion of oxyhemoglobin into methemoglobin as a function of time. The terminal period or asymptotic phase corresponds to essentially 100% methemoglobin formation. The final absorbance was found to be approximately $A = 0.5 \pm 0.1$.

According to Colton [10] the appropriate test to use for these data is the Student's t-test for independent samples. The data was analyzed using a statistical packet on a Microsoft computer.

Significance level has been considered to be a Probability (P) of less than (<) 0.05.

Results and Discussion

For the sodium nitrite studies the findings of the HbA1C percentages revealed that the diabetics blood mean $\pm$ SEM was $11.3 \pm 0.28\%$, while that of the non diabetics blood had a mean $\pm$ SEM of $5.4 \pm 0.05\%$. Thus, the percentage differences between the two populations was statistically significant ($P < 0.05$) and this means that these two populations (sample size(n) = 22) are good groups on which to undertake the sodium nitrite oxidation studies as is shown in the column comparison of the means $\pm$ SEM in Figure 1 [10]. The mean oxidation time of the diabetics blood $\pm$ SEM was 3.5 ± 0.18 min (sample size (n) = 22) whereas the mean oxidation times of the non diabetics blood $\pm$ SEM was 3.5 ± 0.16 min (n = 22). Thus, the comparative study of human adult diabetics' blood revealed that the diabetics' oxyhemoglobin was oxidized by sodium nitrite at about the same rate as non diabetics blood as shown in the column comparison of the means $\pm$ SEM in Figure 2. Based on an independent Student's t-test, the time taken for diabetics erythrocytes to undergo oxidation was not significantly shorter ($P > 0.05$) than the non diabetic controls.

Figure: 1

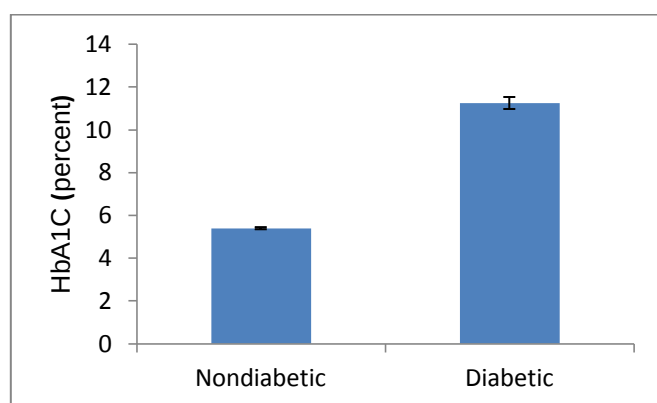


Figure 1: COLUMN COMPARISON OF MEANS FOR THE PERCENT HbA1C OF THE HEMOGLOBIN OF DIABETICS AND NONDIABETICS BLOOD USED IN SODIUM NITRITE STUDIES*

* The mean $\pm$ SEM for diabetics HbA1C was $11.3 \pm 0.28\%$ and the mean $\pm$ SEM for non diabetics HbA1C was $5.4 \pm 0.05\%$. Based on Student's t-test a T value of 21.1 with 42 degrees of freedom was obtained with a difference between the two means that is statistically significant ($P < 0.05$).

Figure: 2

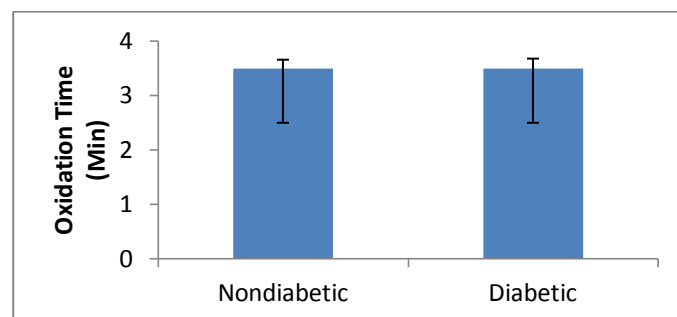


Figure 2: COLUMN COMPARISON OF MEANS FOR THE OXIDATION TIMES OF THE HEMOGLOBIN OF DIABETICS AND NONDIABETICS BLOOD BY SODIUM NITRITE*

* The mean $\pm$ SEM for diabetics oxidation time was 3.5 ± 0.18 min and the mean $\pm$ SEM for non diabetics oxidation time was 3.5 ± 0.16 min. Based on Student's t-test, a T value of 0.0 with 42 degrees of freedom was obtained with a difference between the two means that is not statistically significant ($P > 0.05$).

For the isoamyl nitrite studies the findings of the HbA1C percentages revealed that the diabetics blood mean $\pm$ SEM was $11.4 \pm 0.26\%$ (n = 20), while that of the non diabetics blood mean $\pm$ SEM was $5.5 \pm 0.04\%$ (n = 20). Thus, the percentage differences between the two populations was statistically significant ($P < 0.05$) and this means that these two populations (n = 20) are good groups on which to undertake the isoamyl nitrite oxidation studies as is shown in the column comparison of the means $\pm$ SEM in Figure 3 [10]. The mean oxidation time $\pm$ SEM of the diabetics' blood was 1.5 ± 0.05 min whereas the mean oxidation time's $\pm$ SEM of the non diabetics' blood was 3.1 ± 0.12 min. Thus, the comparative study of human adult diabetics' blood revealed that the diabetics' oxyhemoglobin was oxidized by isoamyl nitrite at about twice the rate of non

diabetics' blood as shown in the column comparison of the mean $\pm$ SEM in Figure 4. Based on an independent Student's t-test, the time taken for diabetics erythrocytes to undergo oxidation was significantly shorter ($P < 0.05$) than the non diabetic controls.

Figure: 3

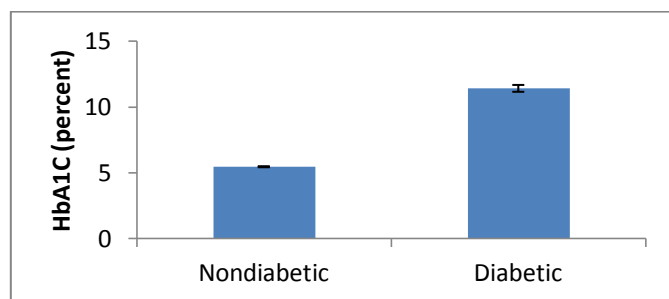


Figure 3: COLUMN COMPARISON OF MEANS FOR THE PERCENT HbA1C OF THE HEMOGLOBIN OF DIABETICS AND NONDIABETICS BLOOD USED IN ISOAMYL NITRITE STUDIES *

* The mean $\pm$ SEM for diabetics HbA1C was $11.4 \pm 0.26\%$ and the mean $\pm$ SEM for non diabetics HbA1C was $5.5 \pm 0.04\%$. Based on Student's t-test a T value of 12.0 with 38 degrees of freedom was obtained with a difference between the two means that is statistically significant ($P < 0.05$).

Figure: 4

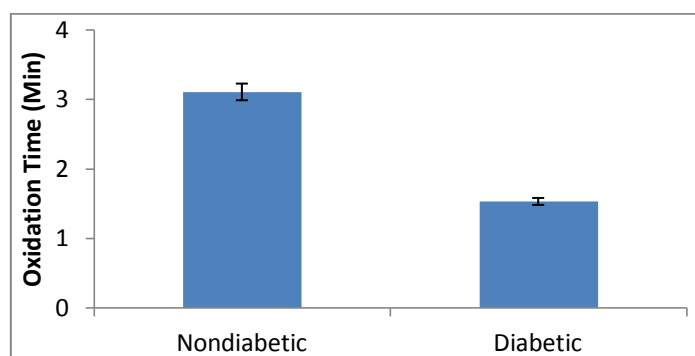


Figure 4: COLUMN COMPARISON OF MEANS FOR THE OXIDATION TIMES OF THE HEMOGLOBIN OF DIABETICS AND NONDIABETICS BLOOD BY ISOAMYL NITRITE*

* The mean $\pm$ SEM for diabetics oxidation time was 1.5 ± 0.05 min and the mean $\pm$ SEM nondiabetics oxidation time was 3.1 ± 0.12 min. Based on Student's t-test, a T value of 23.3 with 38 degrees of freedom was obtained with a difference between the two means that is statistically significant ($P < 0.05$).

Interestingly the enhanced susceptibility to isoamyl nitrite induced oxidation occurred in Type 2 diabetics blood which implies that HbA1C oxidation to methemoglobin is a direct function of the amount of HbA1C present as opposed to metabolic differences in the type 1 and type 2 diabetes [7]. This finding appears to be well supported by the fact that glycation of hemoglobin is an irreversible chemical reaction that is non-enzymatic in nature irrespective of the type of diabetes. Essentially, any untreated diabetic simply has a greater percentage of HbA1C than a non diabetic, e.g. 11.4% vs. 5.5% in this study as shown in Figure 3. Thus, these preliminary findings indicate that diabetics have hemoglobin that exhibit greater oxidative stress to isoamyl nitrite owing to a higher percentage of HbA1C. In fact HbA1C has been reported to be more thermolabile than non glycosylated Hemoglobin (HbA0). This supports the view that structural modification of hemoglobin due to glycation causes the HbA1C to become less stable and more prone to oxidation by glycation-induced structural modification of hemoglobin which leads to a functional modification resulting in oxidative stress in diabetic patients [11].

In addition, these preliminary findings demonstrate that the diabetics and non diabetics blood have the same susceptibility to oxidation when sodium nitrite is used to oxidize their blood ($P > 0.05$). This difference could be attributed to the fact that the sodium nitrite reactions involve an induction or lag phase, whereas the isoamyl nitrite reactions with hemoglobin are immediate wherein a rectangular hyperbolic curve would be generated from a strip chart recording of this reaction [12]. This implies that isoamyl nitrite has greater accessibility to the heme crevice especially due to the glycation induced structural modifications present in the HbA1C. This is supported by the fact that this crevice is hydrophobic which means that an

organic nitrite such as isoamyl nitrite could more rapidly gain access to it as opposed to an inorganic nitrite such as sodium nitrite. Moreover steric changes in the HbA1C from its glycation reaction with glucose could also allow for even more rapid access of isoamyl nitrite as compared to normal human hemoglobin. This would then lead impetus to look at Diabetes Mellitus as a hemoglobinopathy in addition to a metabolic disorder.

References

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