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Comparative Studies on the Effects of Ethyl Nitrite and Hexyl Nitrite Induced Hemoglobin Oxidation of Diabetics Blood and Normal Blood

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Abstract: *The effect of ethyl 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 a significantly greater rate than the control blood (P<0.05). The ethyl nitrite mean oxidation time ± SEM of diabetics blood was 1.5 ± 0.05 min (sample size (n) is 20) and the mean oxidation time ± SEM of the non-diabetics blood was 4.5 ± 0.05 min (n=20). Next the effect of hexyl nitrite on human Type 2 diabetics blood was undertaken using non-diabetics blood as the control group. It was revealed that diabetics erythrocytes were oxidized by hexyl nitrite at a significantly greater rate than control erythrocytes (P<0.05). The mean oxidation time ± SEM of the diabetics blood was 1.5 ± 0.04 min (n=20) whereas the mean oxidation time ± SEM of the non-diabetics blood was 3.7 ± 0.07 min (n=20). Thus, these studies demonstrate that diabetics blood has an enhanced susceptibility of oxidation into methemoglobin by both ethyl nitrite and hexyl nitrite compared to their respective control groups. This similar finding could be attributed to the fact that both ethyl nitrite and hexyl nitrite are organic nitrites wherein the hexyl nitrite contains a saturated six hydrocarbon chain and ethyl nitrite contains a saturated two hydrocarbon chain. Thus the difference of four methylene molecules had no statistically significant effect on the rate of oxidation on either human diabetics blood or human non-diabetics blood (P>0.05).*

Keywords: *Ethyl nitrite; Diabetes; HbA1C; Hemoglobin Oxidation; Hexyl nitrite; Methemoglobin*.

1. Introduction

Both ethyl nitrite and hexyl nitrite belong to a class of compounds called alkyl nitrites that cause oxyhemoglobin to undergo oxidation, i.e. the iron (II) in the hemoglobin loses an electron to become iron (III) and 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 induce this oxidation reaction [2].

In addition both ethyl nitrite and hexyl nitrite are used as inhalants which are inexpensive and easy to obtain but can cause the heart to beat quickly and irregularly and then suddenly stop (cardiac arrest). An overdose via ingestion, rather than inhalation, may result in cyanosis and even death from methemoglobinemia [3-6]. With wide usage of alkyl nitrites for both medicinal and recreational uses and possible side effects there from a study of diabetics blood due to their increased susceptibility by amyl nitrite [7,8] appears warranted.

2. Materials and Methods

Hexyl nitrite (≥ 95%) was purchased from Fisher Scientific. Ethyl nitrite (10-20 wt. % in ethanol) was purchased from the Sigma-Aldrich Chemicals Company. Other required chemicals were obtained from Fisher Scientific. Blood products such as normal adult blood and diabetics' blood were purchased from Physicians Plasma Alliance (PPA). The procedures followed by PPA for this sample collection study were in accordance with the ethical standards of the Hummingbird IRB Protocol wherein all subjects used in these studies gave voluntary informed consent. All blood was tested and certified to be non-viral by PPA.

For both alkyl nitrite studies**:** the data were 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 matched with respect to age, gender, number of obese and number of cigarette smokers as evenly as possible. Also these donors took similar vitamins and medications according to their medical histories. In Tables 1-4 the characteristics of the patients used in these studies are presented, i.e., HbA1C, 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.

Table1. *Characteristics of Diabetic Patients in the Ethyl Nitrite Oxidation Studies*

Sample ID	HbA1C	Age	Gender	Weight	Smoker status	Oxidation Time
	(%)	(yrs)		(lbs)		(min)
GWB002687	10.5	24	Female	258	Non-Smoker	1.4
GWB002677	11.2	22	Male	128	Smoker	1.3
GWB002685	11.6	42	Female	145	Non-Smoker	1.5
GWB002682	12	36	Female	174	Smoker	1.5
GWB002676	14.4	52	Male	136	Non-Smoker	1.6
GWB003977	11.3	47	Female	254	Non-Smoker	2.0
GWB003975	13.4	37	Female	175	Smoker	1.5
GWB003985	12.2	28	Female	206	Non-Smoker	1.9
GWB003978	13.9	48	Male	226	Non-Smoker	1.3
GWB003983	12.4	46	Male	288	Non-Smoker	1.8
GWB004097	10.0	43	Female	290	Non-Smoker	1.7
GWB004103	10.0	32	Male	236	Smoker	1.3
GWB004104	11.3	19	Male	282	Non-Smoker	1.7
GWB004116	14.7	29	Male	220	Non-Smoker	1.4
GWB004117	12.4	30	Male	241	Non-Smoker	1.4
GWB004119	12.4	40	Male	338	Non-Smoker	1.2
GWB004120	13.8	47	Male	400	Smoker	1.3
GWB004113	13.8	43	Female	136	Smoker	1.6
GWB004114	11.2	25	Female	258	Non-Smoker	1.4
GWB004123	11.5	31	Male	188	Smoker	1.3
Mean	12.2	36.1		229.0		1.5
SEM	0.31	2.14		15.53		0.05

Table2. *Characteristics of Non-diabetic Patients in the Ethyl Nitrite Studies*

Sample ID	HbA1C	Age	Gender	Weight	Smoker status	Oxidation Time
	(%)	(yrs)		(lbs)		(min)
GWB002712	5.6	24	Male	218	Non-Smoker	4.7
GWB002713	5.1	41	Male	196	Non-Smoker	5
GWB002714	5.6	58	Male	204	Non-Smoker	5
GWB002708	5.6	62	Male	225	Non-Smoker	4.3
GWB002709	6	48	Male	195	Smoker	4.2
ESG000232	5.3	27	Male	168	Non-Smoker	4.3
ESG000239	4.9	35	Male	217	Non-Smoker	4.3
ESG000235	5.6	25	Male	191	Non-Smoker	4.6
ESG000237	5.3	38	Female	192	Smoker	4.6
ESG000234	5.6	20	Male	240	Smoker	4.3
GWB004098	5.5	57	Female	236	Non-Smoker	4.3
GWB004099	5.5	31	Male	131	Smoker	4.3
GWB004100	5.5	35	Male	228	Non-Smoker	5
GWB004102	5.3	20	Female	146	Smoker	4.5
GWB004107	5.4	39	Male	197	Non-Smoker	4.4
GWB004145	5.5	35	Male	178	Non-Smoker	4.6
GWB004110	5.5	53	Female	160	Non-Smoker	4.6
GWB004115	5.5	57	Male	190	Non-Smoker	4.5
GWB004118	5.5	65	Male	174	Non-Smoker	4.6
GWB004121	5.4	25	Female	285	Smoker	4.6

Mean	5.5	39.8		198.6		4.5
SEM	0.05	3.19		7.73		0.05

Table3. *Characteristics of Diabetic Patients in the Hexyl Nitrite Studies*

Sample ID	HbA1C	Age	Gender	Weight	Smoker status	Oxidation Time
	(%)	(yrs)		(lbs)		(min)
GWB002687	10.5	24	Female	258	Non-Smoker	1.3
GWB002677	11.2	22	Male	128	Smoker	1.5
GWB002685	11.6	42	Female	145	Non-Smoker	1.4
GWB002682	12	36	Female	174	Smoker	1.3
GWB002676	14.4	52	Male	136	Non-Smoker	1.3
GWB003977	11.3	47	Female	254	Non-Smoker	1.5
GWB003975	13.4	37	Female	175	Smoker	1.8
GWB003985	12.2	28	Female	206	Non-Smoker	1.8
GWB003978	13.9	48	Male	226	Non-Smoker	1.8
GWB003983	12.4	46	Male	288	Non-Smoker	1.6
GWB004097	10.0	43	Female	290	Non-Smoker	1.5
GWB004103	10.0	32	Male	236	Smoker	1.7
GWB004104	11.3	19	Male	282	Non-Smoker	1.7
GWB004116	14.7	29	Male	220	Non-Smoker	1.4
GWB004117	12.4	30	Male	241	Non-Smoker	1.5
GWB004119	12.4	40	Male	338	Non-Smoker	1.3
GWB004120	13.8	47	Male	400	Smoker	1.4
GWB004113	13.8	43	Female	136	Smoker	1.5
GWB004114	11.2	25	Female	258	Non-Smoker	1.3
GWB004123	11.5	31	Male	188	Smoker	1.3
Mean	12.2	36.1		229.0		1.5
SEM	0.31	2.14		15.53		0.04

Table4. *Characteristics of Non-diabetic Patients in the Hexyl Nitrite Studies*

Sample ID	HbA1C	Age	Gender	Weight	Smoker status	Oxidation Time
	(%)	(yrs)		(lbs)		(min)
GWB002712	5.6	24	Male	218	Non-Smoker	4.5
GWB002713	5.1	41	Male	196	Non-Smoker	4
GWB002714	5.6	58	Male	204	Non-Smoker	3.7
GWB002708	5.6	62	Male	225	Non-Smoker	4
GWB002709	6	48	Male	195	Smoker	4.3
ESG000232	5.3	27	Male	168	Non-Smoker	3.5
ESG000239	4.9	35	Male	217	Non-Smoker	3.5
ESG000235	5.6	25	Male	191	Non-Smoker	3.2
ESG000237	5.3	38	Female	192	Smoker	3.5
ESG000234	5.6	20	Male	240	Smoker	3.8
GWB004098	5.5	57	Female	236	Non-Smoker	3.4
GWB004099	5.5	31	Male	131	Smoker	3.6
GWB004100	5.5	35	Male	228	Non-Smoker	3.7
GWB004102	5.3	20	Female	146	Smoker	3.7
GWB004107	5.4	39	Male	197	Non-Smoker	3.5
GWB004145	5.5	35	Male	178	Non-Smoker	3.8
GWB004110	5.5	53	Female	160	Non-Smoker	3.8
GWB004115	5.5	57	Male	190	Non-Smoker	3.9
GWB004118	5.5	65	Male	174	Non-Smoker	3.7
GWB004121	5.4	25	Female	285	Smoker	3.7
Mean	5.5	39.8		198.6		3.7
SEM	0.05	3.19		7.73		0.07

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% [9]. A laboratory spectrophotometer equipped with a strip chart recorder was employed to monitor the formation

of methemoglobin at 436 nm. A small table top centrifuge was used to separate plasma from the red blood cells. To determine the oxidation times 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 20 mM 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 20 mM PBS (pH 7.2) for a maximum of 60 min prior to testing.

A 0.01 mL portion of resuspended RBCs was hemolyzed by the addition of 1.0 mL of distilled water and adjusted to a final volume of 2.6 mL by the addition of 20 mM PBS (pH 7.2). The hemoglobin solutions were then adjusted to a standard absorbance (e.g., A = 1.0 ± 0.2) at a wavelength of 436nm with more 20 mM PBS (pH 7.2). The 2.6 mL aliquot of this hemoglobin solution was then added to a 0.05 mL aliquot of 0.1% ethyl nitrite (or 0.1% hexyl nitrite) in ethanol solution. A final concentration of 38 µmol/L of ethyl nitrite (or 138 µmol/L for hexyl nitrite) was obtained after its addition to the hemoglobin solution. In both studies the above gave a final hemoglobin concentration between 6 and 9 µmol/L [10].

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 436 nm. 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 ± 0.1. All hemoglobin oxidation times (in min) obtained have been included in Tables 1-4 for these samples.

According to Colton [11] the appropriate test to use for these data is the Student's t-test for independent samples. The data was analyzed using an Excel spreadsheet on a Microsoft computer. The significance level has been considered to be P< 0.05.

3. Results and Discussion

For the alkyl nitrite studies the findings of the HbA1C percentages revealed that the diabetics blood mean ± standard error of the mean (SEM) was12.2 ± 0.31%, while that of the non-diabetics blood had a mean ± SEM of 5.5 ± 0.05%. Thus, the percentage differences between the two populations was statistically significant (P<0.05), and this means that these two populations are good groups on which to undertake the alkyl nitrite oxidation studies as is shown in the column comparison of the means ± SEM in Figure 1 [11]. For ethyl nitrite the mean oxidation time of the diabetics blood ± SEM was 1.5 ± 0.05 min whereas the mean oxidation times of the non-diabetics blood ± SEM was 4.5 ± 0.05 min as shown in the column comparison of the mean ± SEM in Figure 2. 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.

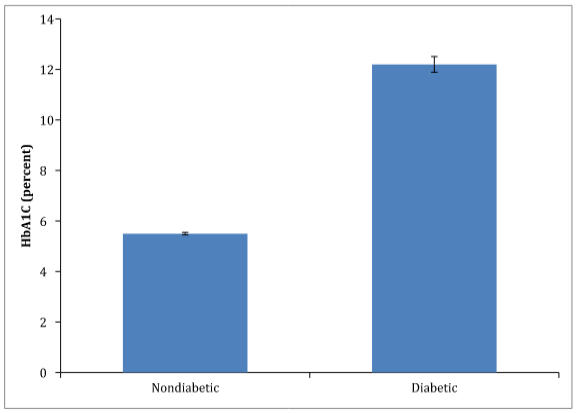

Figure1. *Column comparison of means for the percent HbA1C of the hemoglobin of diabetics and non-diabetics blood used in the alkyl nitrite studies*

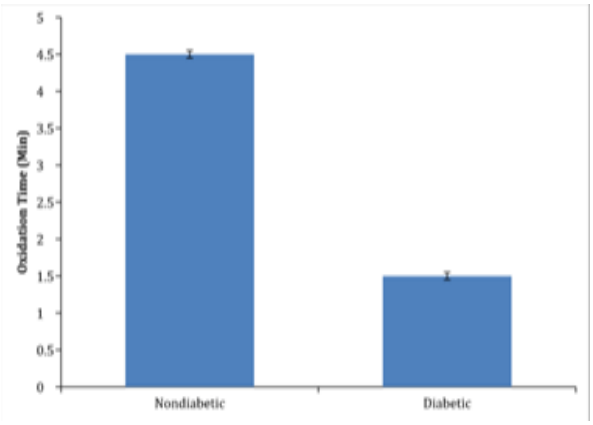

Figure2. *Column comparison of means for the oxidation times of the hemoglobin of diabetics and non-diabetics blood by ethyl nitrite*

For hexyl nitrite the mean oxidation time ± SEM of the diabetics blood was 1.5 ± 0.04 min whereas the mean oxidation times ± SEM of the non-diabetics blood was 3.7 ± 0.05 min as shown in the column comparison of the mean ± SEM in Figure 3. 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. This similar finding could be attributed to the fact that both ethyl nitrite and hexyl nitrite are organic nitrites that contain saturated hydrocarbon chains.

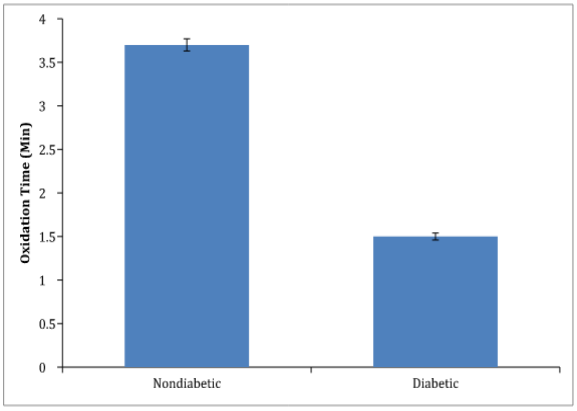

Figure3. *Column comparison of means for the oxidation times of the hemoglobin of diabetics and non-diabetics blood by hexyl nitrite*

Interestingly, the enhanced susceptibility to both alkyl nitrite induced oxidation reactions 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 [12]. Essentially, any untreated diabetic simply has a greater percentage of HbA1C than a non-diabetic, e.g. 12.2% vs. 5.5% in this study as shown in Figure 1. Thus, these preliminary findings indicate that diabetics have hemoglobin that exhibit greater oxidative stress to alkyl nitrite owing to a higher percentage of HbA1C.

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