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(54) Title: METHOD OF INCREASING THE CONCENTRATION OF NITRIC OXIDE IN BLOOD

(57) Abstract

A method of increasing the nitric oxide concentration in the blood which comprises contacting blood with a nitric oxide concentration-increasing effective amount of ozone gas and ultraviolet radiation. Blood prepared by the method of the invention is useful for treating a variety of conditions benefitted by increased blood levels of nitric oxide.

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"METHOD OF INCREASING THE CONCENTRATION OF NITRIC OXIDE IN BLOOD"

THE PRESENT INVENTION relates to method of increasing the concentration of nitric oxide in blood.

Platelets are the smallest of the formed elements of the blood. Every cubic millimetre of blood contains about 250 million platelets, as compared with only a few thousand white cells. There are about a trillion platelets in the blood of an average human adult. Platelets are not cells, but are fragments of the giant bone-marrow cells called megakaryocytes. When a megakaryocyte matures, its cytoplasm breaks up, forming several thousand platelets. Platelets lack DNA and have little ability to synthesize proteins. When released into the blood, they circulate and die in about ten days. However, platelets do possess an active metabolism to supply their energy needs.

Because platelets contain a generous amount of contractile protein (actomyosin), they are prone to contract much as muscles do. This phenomenon explains the shrinkage of a fresh blood clot after it stands for only a few minutes. The shrinkage plays a role in forming a hemostatic plug when a blood vessel is cut. The primary function of platelets is that of forming blood clots. When a wound occurs, platelets are attracted to the site where they activate a substance (thrombin) which starts the clotting process. Thrombin, in addition to converting fibrinogen into fibrin, also makes the platelets sticky. Thus, when exposed to collagen and thrombin, the platelets aggregate to form a plug in the hole of an injured blood vessel.

Platelets not only tend to stick to one another, but to the walls of blood vessels as well. Because they promote clotting, platelets have a key role in the formation of thrombi. The dangerous consequences of thrombi are evident in many cardiovascular and cerebrovascular disorders.

The precise function of blood platelets in various human disease states has recently become increasingly understood as advances in biochemistry permit the etiologies of diseases to be better understood.

For example, many attempts have been made to explain the process of atherogenesis, that is, the creation of plaque which narrows arteries and, of particular concern, the coronary arteries. Recently, there has been

increasing interest in the possible role of platelets in atherosclerosis.

In this regard, it has become recognized that nitric oxide (NO) in the blood inhibits blood clotting by preventing the aggregation of blood platelets. See, e.g., Snyder et al., "Biological Roles of Nitric Oxide," Scientific American, May 1992, pages 68-77, the disclosure of which is incorporated herein by reference.

As the precise biological role of nitric oxide has been explored, it has become known that nitric oxide serves as an important messenger molecule in the brain and other parts of the body, governing diverse biological functions. In blood vessels, the principal endothelium-derived relaxing factor (EDRF) is nitric oxide, which stimulates vasodilation. Nitric oxide also inhibits platelet aggregation and is partially responsible for the cytotoxic actions of macrophages.

In the brain, nitric oxide mediates the actions of the excitatory neurotransmitter glutamate in stimulating cyclic GMP concentrations. Immunohistochemical studies have localized nitric oxide synthase (NOS) to particular neuronal populations in the brain and periphery. Inhibitors of nitric oxide synthase block physiological relaxation of the intestine induced by neuronal stimulation, indicating that nitric oxide has the properties of a neurotransmitter. In this regard, nitric oxide appears to be a novel type of neuronal messenger, in that, unlike conventional neurotransmitters, nitric oxide

is not stored in synaptic vesicles and does not act on typical receptor proteins of synaptic membranes. One function of nitric oxide may be to protect neurons from ischemic and neurotoxic insults. See, Bredt et al., "Cloned and Expressed Nitric Oxide Synthase Structurally Resembles Cytochrome P-450 Reductase", Nature, Vol. 351, June 1991, pages 714-718.

Thus, in addition to platelet aggregation associated diseases, a number of other disease states in humans are presently believed to be associated with inadequate nitric oxide levels in the blood. These nitric oxide associated conditions include: high blood pressure, neurological conditions such as depression, tumours, bacterial and fungal infections, and impotence.

According to this invention there is provided a method of increasing the nitric oxide concentration in blood, which comprises contacting blood with a nitric oxide concentration-increasing effective amount of ozone gas and ultraviolet radiation.

Preferably the ozone gas has a concentration of from about 0.5 μg/ml to about 100 μg/ml in the blood.

Advantageously the ultraviolet radiation has a wavelength of about 253.7 nm.

Preferably the blood is maintained at a temperature of from about 0^0C to about 56 ^{0}C while being contacted with the ozone gas and ultraviolet radiation. The preferred temperature range is 37^0C to 43^0C, the most preferred temperature being 42.5^0C.

The quantity of blood treated may be from 0.01 ml to 400 ml and is preferably about 10 ml, and the blood is contacted with the ozone gas and ultraviolet radiation preferably for a period of about 3 minutes.

The blood may be human blood.

The invention relates to blood with an increased nitric oxide concentration prepared by a method as described above and also relates to the use of blood as described above and the use of a method as described above in the preparation of a medicament. The medicament may be for the treatment of high blood pressure, a neurological condition, depression, a tumour, a bacterial infection, a fungal infection, impotence, a viral infection or a protozoal infection.

The invention will now be described in greater detail.

Examples 1 and 2 below show that an inhibition of blood platelet aggregation can only be achieved when the blood is treated with a combination of ozone gas and ultraviolet radiation (UV). Treatment of blood solely with ozone gas produces minimal inhibition of blood platelet aggregation. Treatment of blood solely with

ultraviolet light produces no inhibition of platelet aggregation whatsoever. Moreover, Examples 3 and 4 show that the inhibition of blood platelet aggregation proceeds via a nitric oxide mediated mechanism, and that treatment of blood with ultraviolet light and ozone according to the invention increases nitric oxide concentrations in the blood.

The combined treatment with ozone gas and ultraviolet light has therefore been unexpectedly found to produce a notable increase in the blood concentration of nitric oxide, which may be useful in treating a variety of disorders that are benefitted by increased blood levels of nitric oxide.

The ozone gas may be provided by any conventional source known in the art, such as an ozonizer. The ozone gas used in connection with the inventive method has a concentration of ozone of from about 0.5 to about 100 µg/ml. Preferably, the ozone gas has a concentration of from about 5 to about 50 µg/ml. The ozone gas is preferably delivered to the blood by means of a medical oxygen carrier, and is preferably contacted with the blood by any means known in the art, preferably by bubbling the ozone/oxygen mixture through the blood sample.

The ultraviolet radiation may be provided by any conventional source known in the art, for example by a plurality of low-pressure ultraviolet lamps. The invention preferably utilizes a standard UV-C source of ultraviolet radiation. Preferably employed are low-

pressure ultraviolet lamps that generate a line spectrum wherein at least about 90% of the radiation has a wavelength of about 253.7 nm. It is believed that ultraviolet radiation having emission wavelengths corresponding to standard UV-A and UV-B sources would also provide acceptable results.

The blood to be treated with UV/ozone is preferably heated to a temperature of from about 0 to about 56 °C while being contacted with the ozone gas and ultraviolet radiation. Any suitable source of heat known in the art may be employed to heat the blood, preferably one or more infrared lamps. The blood may be heated to about 37-43 °C, most preferably about 42.5 °C, prior to being contacted with the ozone gas and ultraviolet radiation. Preferably, the temperature of the blood is then maintained at about 42.5 °C during the treatment with UV/ozone.

Alternatively, the blood sample is heated while being subjected to UV radiation, until the blood reaches a predetermined temperature (preferably about 42.5 °C), at which point bubbling of ozone gas through the blood is commenced. The concurrent UV/ozone treatment is then maintained for a predetermined period of time, preferably about 3 minutes.

Another alternative method involves subjecting the blood to UV/ozone while heating the blood to a predetermined temperature (preferably about 42.5 °C), then either ending the treatment once the predetermined

temperature is reached, or continuing UV/ozone treatment for a further period of time, most preferably about 3 minutes.

Heating the blood to about 42.5 °C with the infrared lamps preferably employed according to the invention has been found to take from about one minute and fifty seconds to about two minutes and ten seconds.

It will be understood that the source of blood treated according to the invention may be blood from an outside source, such as a blood donor of compatible blood type, which is treated with UV/ozone and then administered to a patient. Alternately, and preferably, the blood to be treated may be withdrawn from the human patient as an aliquot, treated with UV/ozone, then readministered to the patient from whom the aliquot of blood was taken. All or a portion of the blood removed from the patient may be treated and then readministered to the patient.

In general, from about 0.01 to about 400 ml of blood may be treated according to the invention. Preferred amounts are in the range of about 0.1 to 200 ml, and more preferably from about 1 to 50 ml of blood. The method most preferably involves treating about 10 ml of blood with ozone gas and ultraviolet radiation, then administering (or readministering) the treated blood to the patient by intramuscular injection.

Other conventional techniques known in the art for administering blood may be employed, such as inter-arterial injection, intravenous injection, subcutaneous

injection, and intraperitoneal injection. The administration of small volumes of host blood in this fashion is termed micro-auto-hemotherapy.

The invention also contemplates an embodiment wherein blood is continuously removed from a patient's body and circulated through an apparatus which treats the blood with ozone gas and ultraviolet light as described above, before returning the blood to the patient. This procedure would have particular utility, for example, during the performance of operative procedures, such as coronary bypass surgery.

The blood is contacted with the ozone gas and ultraviolet radiation for a period of time sufficient to effectively raise the nitric oxide blood concentration in the patient. A treatment period of from about a few seconds to about 60 minutes, preferably from about 0.5 minutes to about 10 minutes, and most preferably about 3 minutes, has been found to provide satisfactory increase in nitric oxide blood levels. The blood is preferably maintained at a temperature of about 42.5 °C during the three minute treatment period.

The method should be carried out under sterile conditions known to those of ordinary skill in the art. The method of the invention may be carried out using conventional apparatus for ozonating blood and irradiating blood with ultraviolet radiation known to those skilled in the medical art. Preferably, an apparatus similar to that disclosed in U.S. Patent No. 4,968,483 is employed to

carry out the method of the invention. The disclosure of U.S. Patent No. 4,968,483 is incorporated herein in its entirety by reference.

Those skilled in the art will appreciate that the method of increasing nitric oxide blood concentration provided by the invention will have therapeutic utility for treating a wide range of disease states which may be benefitted by increasing the levels of nitric oxide in the blood.

The term "treating" as used herein refers to the alleviation of prevention of a particular disorder. In the case of traumatic conditions such as stroke, preventative treatment is obviously preferred. Also, although the term "human" is used to describe the preferred host, those skilled in the art will appreciate that the methods of the invention would have similar utility with other mammals.

The following diseases are illustrative of known conditions which are potentially treatable according to the inventive method: high blood pressure; neurological conditions such as depression; tumours; bacterial, viral, protozoal and fungal infections; and impotence. This list is merely illustrative; those of ordinary skill in the art will appreciate that other disease states benefitted by increasing the concentration of nitric oxide in the blood may be treated with the inventive technique.

Peripheral vascular disease is thought to be associated with a reduction of endothelial-derived relaxing factor (EDRF), low levels of which lead to a contraction of the smooth muscle of blood vessels, and hence a reduction in the diameter of the lumen of the vessel and a reduction in blood flow. the major naturally occurring EDRF is nitric oxide. In addition, nitric oxide stabilises blood

platelets, reducing their aggregation. An increase in EDRF (nitric oxide) levels, therefore, has a double beneficial effect on the circulatory system: it inhibits aggregation of platelets, making the blood more fluid, and it enlarges the diameter of the vessels, improving the flow. The reverse, a reduction in nitric oxide levels, may be present in peripheral vascular disease, and the other conditions described above which may be benefitted by increasing the blood concentration of nitric oxide.

As illustrated in the examples below, the method of the invention is believed to increase nitric oxide levels in the blood, which may explain the mode of action in the inventive treatment of peripheral vascular disease and other conditions associated with blood platelet aggregation and nitric oxide deficiency.

The following examples are given to illustrate the invention but are not deemed to be limiting thereof.

EXAMPLE 1

Inhibition of Blood Platelet Aggregation

The following experiment was conducted to study the effects of ozone/ultraviolet light treatment on blood platelet activity.

Experimental Procedure

Samples (20 ml) of peripheral blood were taken from 10 individuals for 13 separate experiments. Each sample was divided into two aliquots. The first aliquot was treated according to the inventive technique, as follows:

The 10 ml aliquot was treated in vitro for three minutes with ozone gas (variable ozone concentration of 5-50 μg/ml) and ultraviolet light (253.7 nm), at a temperature of 42.5°C. An apparatus as disclosed in U.S. Patent No. 4,968,483 was utilized to carry out the treatment of the blood sample.

The second 10 ml aliquot from each sample served as an untreated control.

Platelets were isolated from the control or treated samples by centrifugation, and their ability to aggregate in response to different concentrations of ADP (a natural platelet stimulator) was measured in an aggregometer. A sample of both ozone-treated and untreated blood was used for quantitation of platelet numbers, using a Coulter counter. In some of the experiments described below, aliquots of the blood were treated with different concentrations of ozone. In other experiments performed,

the blood was treated in the presence and absence of UV-light irradiation.

Platelet aggregation in the ozone-treated blood was expressed as a percentage of aggregation in the same-person untreated control blood.

Results

As shown in Table 1, the results of the experiments indicate that treatment of blood with ozone and ultraviolet light according to the invention inhibits the aggregation of blood platelets. Furthermore, there is an indication that this inhibition is dose related to the ozone concentration (see Table 2).

The effect of high levels of ozone on ADP-stimulated blood platelets

High levels of ozone (between 35 and 50 μg/ml) caused a measurable inhibition of ADP-induced platelet aggregation (arbitrarily taken as 33.3% inhibition) in 11 of the 13 experiments (8 of the 10 individuals). Taking all the data on all 10 individuals, the mean inhibition of platelet aggregation was 49.2 +/- 27.8% (mean +/- sd). There was no significant difference between the inhibitory effects on blood taken from males and females (mean inhibition 48.1% and 50.7%, respectively).

This inhibition appears to relate to the concentration of ADP (aggregation stimulator) over the concentration range of 0.01-0.1mM ADP, with lower inhibition at higher concentrations of platelet agonist. However, this relationship did not hold at higher ADP

concentrations (Table 1) and could be spurious, although the level of inhibition at 0.01mM ADP is significantly greater than at 0.1mM ADP (71% vs. 95%, $p < 0.02$).

TABLE 1

The effect of high levels of ozone on the aggregation of human blood platelets in the presence of varying concentrations of ADP.

Date (Individual)	Concentration of ozone (µg/ml)	Concentration of ADP (mM)	Percent Inhibition of Aggregation	Platelet Count Before Ozone	Platelet Count After Ozone
21.11.91 (F1)	50	10	100		
27.11.91 (M1)	50	5	83.3		
		10	71.4		
		30	75.0		
2.12.91 (F2)	50	10	0		
		30	10.0		
		100	27.3		
3.12.91 (M2)	50	0.5	67.1		
		1	57.1		
		5	50.0		
		30	88.1		
6.12.91 (M3)	50	0.1	0	34	49
		0.1	6.2		
		0.5	4.0		
		0.5	0		
11.12.91 (M4)	50	0.05	67.0	46	93
		0.1	62.4		
		1.0	74.3		
		10.0	50.0		
12.12.91 (M5)	50	0.01	67.0	51	121
		0.1	7.1		
		1.0	35.7		
13.12.91 (F1)	50	0.01	63.4	33	87
		0.05	22.7		
		0.1	30.4		
		0.5	15.4		
		1.0	20.8		
		5.0	20.0		
		10.0	27.6		
9.01.92 (M6)	50	0.01	34.2	34	40
		0.05	31.0		
		0.1	9.8		

			0.5	15.4		
			1.0	26.2		
			5.0	31.3		
	10.01.92 (F3)	50	0.001	71.4	49	64
			0.005	37.5		
			0.01	69.8		
			0.05	33.8		
			0.1	31.2		
			0.5	10.1		
			1.0	21.8		
	13.01.92 (F4)	50	0.005	100	49	52
			0.01	100		
			0.05	95.2		
			0.1	92.9		
			0.5	95.8		
			1.0	91.6		
			5.0	95.8		
			10.0	80.0		
	15.01.92 (F1)	40	0.01	90.0	81	66
			0.05	71.4		
			0.1	40.7		
			0.5	87.0		
			1.0	81.8		
			5.0	95.5		
			10.0	85.2		
			50.0	84.0		
			100.0	79.1		
	21.01.92 (M2)	35	0.01	67.1	68	79

The following is a summary of the data set forth in Table 1:

ADP mM	0.01	0.05	0.10	0.50	1.00	5.00	10.0
% inhibition of aggregation	70.8 +/-20.9	53.5 +/-26.1	34.7 +/-28.4	37.6 +/-38.4	50.3 +/-28.7	60.7 +/-35.2	60.7 +/-30.4
N=	6	6	8	7	7	4	4

<u>The effect of high levels of ozone on total whole blood platelet counts</u>:

As any apparent reduction in platelet aggregation following ozone treatment of whole blood could be caused by a loss of platelets from the blood during treatment,

total whole platelet counts were performed on the treated and untreated whole blood samples in 9 experiments on blood from 8 individuals. Overall, the platelet count was 115.5 +\- 59.8% of the untreated level following ozonization (range 82-264%).

Thus, the total platelet counts before and after ozone/UV treatment do not indicate a major loss of platelets from the blood as a result of ozonization.

The effect of different concentrations of ozone on the inhibition of aggregation of human blood platelets stimulated with ADP:

Three different concentrations of ozone (5, 25, and 50 µg/ml) were used at a range of ADP concentrations in 4 experiments on 4 different individuals. Bulking the data for different ozone concentrations from each individual and calculating the mean for the data from the 4 experiments indicated that there was some dose response relationship between the concentration of ozone used and the inhibition of platelet aggregation (see Table 2).

Although overall these differences were not significant, in two of the four individuals there was a significantly greater inhibitory effect of ozone at 50 µg/ml then at 5 µg/ml (see Table 3).

TABLE 2

The effect of different concentrations of ozone on inhibition of platelet aggregation in the presence of ADP.

Date (Individual)	Concentration of ozone (µg/ml)	Concentration of ADP (mM)	Percent Inhibition of Aggregation	Platelet Count Before Ozone - After Ozone
3.12.9 (M2) .	15	0.1	27.3	
	25	0.1	100	
	5	0.5	0	
	25	0.5		
	50	0.5	67.1	
	5	1.0	0	
	25	1.0	28.6	
	50	1.0	57.1	
	5	5.0	0	
	25	5.0	25.0	
	50	5.0	50.0	
	5	30.0	50.0	
	25	30.0	62.0	
	50	30.0	88.1	
9.01.92 (M6)	5	0.01	20.1	34 43
	25	0.01	28.9	45
	50	0.01	34.2	40
	5	0.05	0	
	25	0.05	5.2	
	50	0.05	31.0	
	5	0.1	9.8	
	25	0.1	1.4	
	50	0.1	9.8	
	5	0.5	0	
	25	0.5	0	
	50	0.5	15.4	
	5	1.0	22.5	
	25	1.0	13.7	
	50	1.0	26.2	
	5	5.0	0	
	25	5.0	17.8	
	50	5.0	31.5	
10.01.92 (F3)	5	0.001	57.1	49 73
	25	0.001	85.7	90
	50	0.001	71.4	64
	5	0.005	37.5	
	25	0.005	80.0	

		50	0.005	37.5		
		5	0.01	66.4		
		25	0.01	83.2		
		50	0.01	69.8		
		5	0.05	44.9		
		25	0.05	66.9		
		50	0.05	33.8		
		5	0.1	29.3		
		25	0.1	61.0		
		50	0.1	31.2		
		5	0.5	39.4		
		25	0.5	54.5		
		50	0.5	10.1		
		5	1.0	21.8		
		25	1.0	52.9		
		50	1.0	21.8		
	13.01.92	5	0.005	100	49	60
	(F4)	25	0.005	100		85
		50	0.005	100		52
		5	0.01	100		
		25	0.01	87.5		
		50	0.01	100		
		5	0.05	84.8		
		25	0.05	97.1		
		50	0.05	95.2		
		5	0.1	82.9		
		25	0.1	91.4		
		50	0.1	92.9		
		5	0.5	83.3		
		25	0.5	95.8		
		50	0.5	95.8		
		5	1.0	83.2		
		25	1.0	89.5		
		50	1.0	91.6		
		5	5.0	79.2		
		25	5.0	91.7		
		50	5.0	95.8		
		5	10.0	85.3		
		25	10.0	80.0		
		50	10.0	80.0		

The following is a summary of the data set forth in Table 2:

Concentration of ozone (μg/ml)	5	25	50
Platelet aggregation (%) (mean +/- sd, n=4)	38.5+/-30.9	56.5+/-29.4	55.9+/-26.4

TABLE 3

The effect of different concentrations of ozone on inhibition of platelet aggregation in two individuals

Concentration of ozone (μg/ml)	5	25	50
Platelet aggregation M2 (%)	15.5+/-20.2	53.9+/-30.0	65.6+/-14.4
Difference from 5 μg/ml		ns	p<0.01
Platelet aggregation M6 (%)	8.7+/-9.6	11.2+/-10.2	24.7+/-9.0
Difference from 5 μg/ml		ns	p<0.02

ns=not significant

The effect of UV light on the response of platelets to ozone:

The effect of ozone on the aggregation of human blood platelets was investigated at different concentrations of ADP, in the presence or absence of UV light. The results, shown in Table 4, indicate that, although there may be some platelet aggregation-inhibitory response to ozone alone, this is nearly always greater in the presence of UV light and the effect of UV light was highly significant ($p<0.001$) in this single experiment. This result was also repeated in a second experiment, using a single concentration of ADP (0.01 mM). The results of this second experiment are set forth in Table 5.

TABLE 4

The effect of UV light on the inhibition of ADP-induced platelet aggregation by ozone at a concentration of 40 μg/ml. (Experiment date 15.01.92, individual F1)

Concentration ADP (mM)	Inhibition of platelet aggregation (%)	
	+UV	-UV
0.01	90.0	60.0
0.05	71.4	0
0.1	40.7	40.7
0.5	87.0	0
1.0	81.8	0
5.0	95.5	19.4
10.0	85.2	18.5
50.0	84.0	16.0
100.0	79.1	4.2
Mean +/- sd	79.4+/-15.1	17.6+/-19.6 ($p<0.001$)

TABLE 5

The effect of UV light on platelet aggregation induced by ADP (0.01 mM) in the presence or absence of ozone. (Experiment date 21.01.92, individual M2)

Percent inhibition of platelet aggregation		
Ozone 35 μg/ml + UV	Ozone 35 μg/ml - UV	No ozone, UV alone
83.4%	11.2%	0%

In summary, the results of Example 1 indicate that the *in vitro* treatment of an aliquot of blood with ozone gas and ultraviolet light inhibits the aggregation of blood platelets. This platelet inhibition has been found

to be dose related to the ozone concentration. Further, platelet inhibition was found to critically depend on the combined treatment of ultraviolet light and ozone gas, as evidenced in Tables 4 and 5. Treatment with ozone gas alone resulted in minimal inhibition of platelet aggregation, while treatment with ultraviolet light alone produced no inhibition of platelet aggregation.

EXAMPLE 2

Measurement of Nitric Oxide

In order to elucidate the mechanism whereby ozonization/UV light affects the aggregation of platelets in treated blood, the concentration of certain oxidized forms of nitrogen were measured.

The direct measurement of nitric oxide is difficult to achieve. However, nitric oxide is an intermediate in a metabolic pathway in which arginine is converted to citrulline. Other stable end-products are nitrates and nitrites.

Accordingly, the nitric oxide content for several samples of blood treated with ultraviolet light and ozone gas according to Example 1 were indirectly determined by measuring the combined nitrate plus nitrite concentrations in the samples before and after treatment with ozone/UV light, after converting nitrate to nitrite.

The results show that there is a small increase in nitrate plus nitrite concentrations after treatment according to the invention. This increase was consistently found in samples treated with ozone gas/UV

light. Thus, nitric oxide levels may be enhanced by the treatment with ozone gas/UV light, and this may be part of the mode of action by which an inhibition of blood platelet aggregation is achieved by the invention. This therapeutic effect would be consistent with the etiology of peripheral vascular disease described above.

Conclusions

The data of Examples 1 and 2 suggest that the treatment of blood with ozone gas and ultraviolet light according to the invention is actually inducing an inhibition of platelet aggregation for the following reasons:

1. The inhibitory effect is at least partially dependent on the concentration of ADP, ozone being more inhibitory at lower ADP concentrations. This may be interpreted as the higher agonist concentrations partially overcoming the inhibitory effect of ozone by "hyperstimulating" the platelets. This suggests that the inhibition is at least partially reversible, and is probably not acting by destroying the platelet's ability to aggregate.

2. The inhibitory effect appears to be dose related to ozone concentration, with higher concentrations of ozone resulting in a greater inhibition of platelet aggregation.

3. The inhibitory effect is UV-dependent, suggesting that this is not a non-specific toxic effect caused by the oxidative capacity of the ozone gas.

EXAMPLE 3

Venous blood (20 ml), taken from 13 healthy non-smoking volunteers, 6 females and 7 males, age 20-50 years, was collected into sodium citrate anticoagulant. None of the volunteers had taken any medication for at least one week prior to the investigation. The blood was divided into two 10 ml aliquots. One aliquot was treated with ozone/UV as described below, the other was an untreated control sample.

Ozone treatment of blood samples

Blood was treated according to the invention with different concentrations of ozone using a device similar to that described in U.S. Patent No. 4,968,483. Ozone in medical oxygen was bubbled through the blood sample at a rate of 0.3 l/min for a fixed period of about 3 minutes. The blood was heated to a temperature of 42.5 °C and exposed to ultraviolet light at a wavelength of 253.7 nm. The concentration of ozone in the oxygen carrier was variable between about 5 and 50 μg/ml, and was measured using an ozone monitor (Humares, Karlsruhe, Germany).

Platelet aggregation studies

Platelet aggregation was measured essentially by the end point turbidimetric method of Born.

Platelet rich plasma (PRP) was prepared by centrifuging 10 ml of blood (either ozone-treated or untreated control blood) at room temperature for 20 minutes at 200 x g. Four ml of PRP was diluted by the addition of 1.0 ml of phosphate-buffered saline.

Diluted PRP (0.225 ml) was placed in an aggregometer cuvette containing a small magnetic stirrer bar (aggregometer model 1002, ADG Instruments Ltd., Codicote, Herts., U.K.) and equilibrated at 37 °C. After stirring was commenced, 0.025 ml of agonist (either ADP - ADP platelet aggregation reaction or collagen - collagen platelet aggregation reagent; both from Sigma Chemical Co., Poole, Dorset, U.K.) was added to the final concentrations indicated in the results section below. The maximum change in light transmission was measured. Platelet aggregation in the ozone-treated samples was expressed as a percent of the aggregation in the control samples for each individual experimental condition.

Blood platelet counts were performed on whole blood, using a Coulter counter, provided by the Department of Hematology, Northern General Hospital, Sheffield, U.K.

Results

Following treatment of whole blood with 35-50 μg/ml of ozone in oxygen at a flow rate of 0.3 l/min for 3 min (total mass of ozone reacted: 31.5-45 mg) with exposure to UV, there was an apparent overall increase in the platelet count, to 1461/57% (mean +/- standard deviation, range 81-202%) of the control value in the 12 individuals investigated. This suggests that, under the conditions used in these experiments, the treatment of whole blood with ozone does not destroy the blood platelets. Furthermore, following visual assessment, no marked hemolysis was observed in the treated blood compared with

the control blood samples, indicating that the treatment regime had little effect on erythrocyte integrity.

Platelets from blood treated with 35-50 µg/ml ozone as above showed a reduction in their ability to aggregate in response to ADP (0.001-100 mmol/l concentrations). The overall inhibition of aggregation was 53.1 +/- 31.1% (mean +/-. standard deviation, n=13). The inhibition was variable between individuals, ranging from 2.6% to 100%.

This inhibitory effect of ozone/UV treatment was dependant on the concentration of ADP, showing a higher level of inhibition of platelet aggregation at low concentrations of ADP (see Table 6). The inhibitory effect at 0.01 mmol/l ADP was significantly greater ($p<0.02$) than at 0.1 mmol/l of this agonist (see Table 6).

With collagen as an inducer of platelet aggregation, platelets treated with 35-50 µg/ml ozone in oxygen also showed a high level of inhibition of aggregation: 74.2 +/- 43.3% with 1 mg/ml collagen and 76.4 +/- 25.2% with 10 mg/ml collagen (n=5).

A reduction in the concentration of ozone in the oxygen bubbled through the blood sample resulted in a reduction in the effect of treatment on the inhibition of platelet aggregation. This difference was significant in individual responses to treatment, although the overall mean values of the four individuals investigated were not significantly different (see Table 7).

TABLE 6

The effect of different concentrations of the platelet agonist ADP on the inhibition of ADP-induced platelet aggregation by treatment of blood in vitro with ozone at a concentration of 50 µg/ml in oxygen and UV irradiation.

Subject	Conc. ADP(mmol/l)	Percent Inhibition of Platelet Aggregation
Male 1	0.5	67.1
	1.0	57.1
	5.0	50.0
Female 1	0.001	71.4
	0.01	69.8
	0.1	31.2
	1.0	21.8
Female 2	0.01	63.4
	0.1	30.4
	1.0	20.8
Mean	0.01	70.8+/-20.9, n=6
	0.05	53.5+/-26.1, n=6
	0.1	34.7+/-28.4, n=8*

* significantly different from 0.01 mmol/l, $p<0.02$

TABLE 7

The effect of different concentrations of ozone on the inhibition of ADP-induced platelet aggregation by treatment of whole blood in vitro with ozone in oxygen and UV irradiation.

	Percent Inhibition of Platelet Aggregation		
Subject	5 µg/ml Ozone	25 µg/ml Ozone	50 µg/ml Ozone
Male 1	15.5	53.9	65.6**
Male 2	8.7	11.2	24.7*
Mean (n=4)	38.5	56.5	55.9
sd	30.9	29.4	26.4

** significant at $p<0.01$
* significant at $p<0.02$

EXAMPLE 4

The Effect of UV/Ozone Treatment of Blood in Vitro on the Plasma Nitric Oxide Concentration

Experimental Outline

Blood (10ml), anticoagulated with sodium citrate, from 14 normal healthy individuals, was treated with UV/ozone gas as described in Example 3, with oxygen containing ozone at a concentration of 20-50 µg/ml. Control blood from each individual was not treated. After removal of the cellular components of the blood by

centrifugation at 15 000 x g for 30 seconds, the plasma was stored at -20 °C.

Nitric oxide, produced metabolically from L-arginine, is unstable and reacts with oxygen to form nitrate and nitrite. Total nitrate plus nitrite was measured after conversion of nitrate to nitrite using a cadmium catalyst. Nitrite was measured colorimetrically using the Griess reagent by a method based on that published by Green, Wagner, Glogowski, Skipper, Wishnok & Tannenbaum in 1982 (Analytical Biochemistry, Vol. 126, pages 131-138). All treated and control samples were measured in a single assay run.

Results

The actual values of nitrite concentration varied widely between individuals, ranging from 0.6 - 27.6 µmol/l. To enable comparisons between individuals, the concentration of nitrite in the ozone-treated sample was expressed as a percent of the concentration in the corresponding untreated control. A summary of the results is as follows:

Individual No.	Percent of Nitrite in Ozonated Sample Compared to Control
1	28.0
2	36.7
3	48.9
4	76.4
5	110.0
6	133.3
7	157.6
8	162.9
9	175.8
10	350.0
11	845.5
12	985.7
13	2075.0

14	3067.0
Arithmetic mean	589.5%

These values do not form a normal distribution. However, an approximate normal distribution can be attained after logarithmic transformation of the data. Following logarithmic transformation, the level of nitrite in the UV/ozone-treated blood samples is significantly greater than in the untreated samples ($p<001$).

Inhibition Studies

Nitric oxide inhibits platelet aggregation -- this is one of its physiological activities. It is known that the effect of nitric oxide on platelets can be inhibited by free oxyhemoglobin (Salvemini, Radziszewski, Korbut & Vane, Br. J. Pharmacol., Vol. 101, pages 991-995, 1990). We therefore investigated the effect of oxyhemoglobin on the platelet aggregation inhibitory action of treatment of whole blood with UV/ozone gas.

Experimental Outline

Platelet rich plasma was prepared from whole blood, either treated with UV/ozone or untreated (control), by centrifugation at 200 x g for 20 minutes at room temperature. Platelet aggregation in response to ADP, collagen or thrombin as stimulators was measured in an aggregometer. Oxyhemoglobin was added to the platelet rich plasma subsequent to ozonization and before measuring platelet aggregation activity. If treatment of blood with UV/ozone to inhibit platelet aggregation is acting via a nitric oxide-mediated mechanism, then the addition of

oxyhemoglobin should prevent the inhibition of platelet aggregation caused by UV/ozonization. The results are set forth in Table 8 below.

TABLE 8

Subject	Platelet agonist	Percent inhibition of platelet aggregation after ozone/UV treatment: No Hb	10 µmol/l Hb
Female a	Thrombin 100 iu	23	4
Female b	Thrombin 100 iu	57	0
	Thrombin 10 iu	97	57
	ADP 1 mmol/l	36	0
Male a	Thrombin 10 iu	80	79
	Collagen 1 mg/ml	95	80
	ADP 1 mmol/l	16	0
	ADP 0.1 mmol/l	26	9

Although rather variable, two of the three subjects showed consistent reductions of post-UV/ozone therapy platelet aggregation inhibition in the presence of haemoglobin, and the third subject showed some reduction with 3 of the 4 conditions of aggregation used. The overall means of platelet aggregation were 54% without haemoglobin and 29% in the presence of this inhibitor of nitric oxide activity.

Conclusions

The above data show that ozonization of blood raises the level of nitrite (the stable metabolite of nitric oxide), and that the inhibition of platelet aggregation caused by ozonization of blood can be reversed by haemoglobin, an inhibitor of nitric oxide activity. Taken together, these data strongly suggest that the treatment of blood with UV/ozone according to the invention

increases the in vivo blood levels of nitric oxide, and inhibits the aggregation of platelets via a nitric oxide mediated mechanism.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications are intended to be included within the scope of the following claims.

CLAIMS:

1. A method of increasing the nitric oxide concentration in blood, which comprises contacting blood with a nitric oxide concentration-increasing effective amount of ozone gas and ultraviolet radiation.

2. The method of Claim 1, wherein the ozone gas has a concentration of from about 0.5 μg/ml to about 100 μg/ml in the blood.

3. The method of Claim 2 wherein the ozone gas has a concentration of from about 5 μg/ml to about 50 μg/ml.

4. The method of any one of Claims 1 to 3 wherein the ultraviolet radiation has a wavelength of about 253.7 nm.

5. The method of any one of the preceding Claims wherein the blood is maintained at a temperature of from about 0^0C to about 56 ^{0}C while being contacted with the ozone gas and ultraviolet radiation.

6. The method of Claim 5, wherein the blood is maintained at a temperature of from about 37°C to about 43^0C while being contacted with the ozone gas and ultraviolet radiation.

7. The method of Claim 6, wherein the blood is maintained at a temperature of about 42.5 ^{0}C while being contacted with the ozone gas and ultraviolet radiation.

8. The method of any one of the preceding Claims wherein the blood treated comprises from about 0.01 ml to about 400 ml.

9. The method of Claim 8 wherein the blood treated comprises about 10 ml of blood.

10. The method of any one of the preceding Claims wherein the blood is contacted with the ozone gas and ultraviolet radiation for a period of about 3 minutes.

11. The method of any one of the preceding Claims wherein the blood is human blood.

12. Blood with an increased nitric oxide concentration prepared by a method according to any one of the preceding Claims.

13. The use of blood according to Claim 12 in the preparation of a medicament.

14. The use of a method according to any of Claims 1 to 11 in the preparation of a medicament.

15. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of high blood pressure.

16. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of a neurological condition.

17. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of depression.

18. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of a tumour.

19. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of a bacterial infection.

20. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of a fungal infection.

21. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of impotence.

22. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of a viral infection.

23. Use according to Claim 13 or 14 in the preparation of a medicament for the treatment of a protozoal infection.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00259

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61M1/36; A61K35/14; A61K41/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61M ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	PATENT ABSTRACTS OF JAPAN vol. 8, no. 34 (C-210)15 February 1984 & JP,A,58 198 466 (TEIJIN KK) 18 November 1983 see abstract ---	1
A	DE,A,2 926 523 (STADTLAENDER) 22 January 1981 see page 2, line 18 - line 22 see page 5, line 32 - page 6, line 16; claim 1; figures ---	1
A	US,A,4 968 483 (MÜLLER ET AL.) 6 November 1990 cited in the application see abstract; figures see column 4, line 12 - line 27; claims 8,17 --- -/--	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 MAY 1993	19.05.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ZEINSTRA H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A,P	DATABASE WPIL Week 9307, Derwent Publications Ltd., London, GB; AN 93-058408 & US,A,7 764 906 (US DEPT HEALTH & HUMAN SERVICE) 15 December 1992 see abstract	1
A	US,A,3 325 641 (JONES) 13 June 1967 see column 1, line 15 - line 35; figures	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9300259
SA 70191

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/05/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-2926523	22-01-81	None	
US-A-4968483	06-11-90	DE-U- 8704467	26-05-88
		AU-B- 613333	01-08-91
		AU-A- 1000288	28-07-88
		GB-A,B 2242367	02-10-91
US-A-3325641		None	