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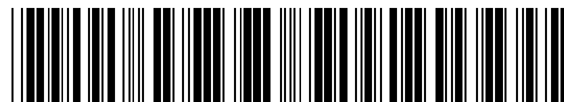
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TITLE:	HAEMATOLOGICA
PUBLISHER/PLACE:	Ferrata Storti Foundation Pavia, Italy :
VOLUME/ISSUE/PAGES:	1990 Nov-Dec;75(6):510-5 510-5
DATE:	1990
AUTHOR OF ARTICLE:	Bocci V; Paulesu L
TITLE OF ARTICLE:	Studies on the biological effects of ozone 1. Indu
ISSN:	0390-6078
OTHER NUMBERS/LETTERS:	
	NLM Unique ID: 0417435
	PubMed ID: 2129118
SOURCE:	PubMed
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HAEMATOLOGICA
1990; 75:510-5.

STUDIES ON THE BIOLOGICAL EFFECTS OF OZONE 1. INDUCTION OF INTERFERON γ ON HUMAN LEUCOCYTES

VELIO BOCCI, LUANA PAULESU

In this study we have investigated the effects of ozone on human blood, as well as on resuspended buffy coats and Ficoll-purified mononuclear cells. Samples were exposed at different ozone concentrations (from 2.2 μg to 108 μg/ml) for 30 sec and then incubated for different times at 37° C in a 95% air-5% CO_2 humidified atmosphere. Supernatants were collected and frozen at-20° C until tested for interferon (IFN) activity. We have determined that the ozone concentration is critical for lymphokine induction. In fact, while low concentrations (2.2 μg/ml) are effective in lymphocytes, they do not induce IFN in either whole or diluted (1:1) human blood, or resuspended buffy coats. In such cases levels as high as 42 μg/ml are required. On the other hand, a very high ozone concentration (108 μg/ml) is not effective and probably toxic. Maximal IFN production occurs 72-96 h after ozone exposure, and the kinetics of IFN release is similar to that after Staphylococcal Enterotoxin B addition. Because ozonization of blood is a medical procedure followed in several countries for treatment of viral diseases, this study can open a new field of investigation that may yield useful results both in biological and practical terms.

KEY WORDS: Ozone, interferon gamma, lymphomonokines.

Ozonization of autologous blood followed by slow reinfusion in the donor has been used for a long time in Western Europe for the treatment of various fungal, viral diseases and tumours [1]. The autohaemotherapy procedure has been standardised on an empirical basis and the therapeutic benefit is unlikely due to the direct virucidal properties of ozone. This diffusible gas is very unstable and, in an aqueous environment, oxygen gives rise to powerful reactive species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1O_2$) [2]. These reactive oxygen species, while having a very brief halflife, can exert an array of deleterious effects on biological substrates and living cells [3-10]; on the other hand, they could oxidize and stimulate cell receptors, leading finally to lymphocyte mitogenesis with possible induction and release of lymphokines. It is known that periodate, hydrogen peroxide and galactose oxidase could trigger the synthesis and release of interferon and other cytokines from monocytes and lymphocytes [11-15].

Therefore the hypothesis can be made that ozone, via free radicals, could lead to the release of the tumor necrosis factor (TNF), interferon (IFN) and interleukins (Ils) from lymphocytes or/and monocytes. IFN is a powerful antiviral protein [16] and, with other cytokines, shares immunomodulatory activities [17] that could produce some beneficial effects in patients with viral diseases. To our knowledge the possibility that ozonization of human blood could act via release of lymphokines has never been entertained, and we thought it worthwhile to begin a series of studies addressing the fundamental questions of whether ozonization of human blood, or of isolated peripheral blood mononuclear cells (PBMC), is capable of inducing the release of IFN.

MATERIALS AND METHODS

Human blood was withdrawn from 26 healthy donors, (age range 25-40 years), hepatitis B surface antigen and HIV negative. In some experiments whole blood was used as such, while in others it was centrifuged at $300 \times g$ at 20° C for 20 min, and the buffy-coat was resuspended in RPMI 1640 medium (GIBCO LTD. Paisley, Scotland) supplemented with 25 mM HEPES, 10 mM $NaHCO_3$ antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 2 mM glutamine at a final leucocyte number of about 1×10^7/ml.

Human PBMC were isolated by the method of Boyum [18] on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). Cell number was determined by light microscope count and viability was assayed by trypan-blue dye exclu-

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Acknowledgments: This work was supported by grants from CNR «P.F. Oncologia» and Italian Ministry of Education (projects 40% and 60%).
Received February 28, 1990; accepted July 9, 1990.
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sion technique. The cells were finally adjusted to 1.5×10^6 viable cells/ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Bocknec, Laboratories INC, Canada).

Treatment of samples

The ozone-oxygen gas mixture at different ozone concentrations was produced by using the Ozonosan PM 80 (Dr. J. Hansler GMBH, Medizintechnik, Germany): 4.5 ml aliquots of either whole blood or buffy coat as PBMC suspensions were insufflated with four different ozone concentrations (0.2, 11.5, 42 and 108 μg/ml) in 25 cm^2 tissue flasks (Falcon, Becton Dickinson, Milano, Italy) for usually 30 sec at room temperature.

Afterwards all samples were aliquoted in 0.2 ml and incubated in 96-well culture dishes (Falcon) at 37° C in a humidified atmosphere of 95% air-5% CO_2 for 24, 48, 72, 96, 114 hr. At each time, samples were centrifuged at $1000 \times g$ and the supernatants were frozen at -20° C until IFN determination.

Controls underwent the same procedure except that sterile air was used instead of ozone. In order to verify cell responsiveness, Staphylococcal Enterotoxin B (SEB, from Sigma, St. Louis, Mo) was added to some of the controls.

IFN titration

Supernatants were titrated for IFN activity with a microplaque reduction assay [19] using human amniotic cells (Wish) and vesicular stomatitis virus (VSV, Indiana strain) as challenge virus. Cell monolayers were infected with 50 plaque-forming units of VSV and all samples were tested at least twice in quadruplicate. Titrations were always performed employing the international reference preparation (IRP) for human IFN-γ (Gg 23-901-530, NIAID, NIH Bethesda, MD). Titres are reported as International Units (IU/ml).

Evaluation of cell viability

PBMC suspended in culture medium previously exposed to 2.2 μg/ml and 11.5 μg/ml of ozone or to air were evaluated for their viability by a colorimetric assay after different periods (0, 24, 48, 72, 96 and 114 hr) of incubation [20]. Briefly, 20 μl of prefiltered [3-(4,5-Dimethylthiazol-2)-2,5-diphenyltetrazolium bromide] (MTT, Sigma) solution (5 mg/ml in PBS) were added to each well and the plate incubated for 4h at 37° C in a humidified atmosphere. Supernatants were then removed and the formazan crystals were dissolved by addition of dimethyl sulfoxide (JT Baker Chemicals BV Deventer, Holland) (DMSO). The supernatants were read after 60 min at 540 nm on a Titertek Multiskan microElisa reader, and values are reported as a percentage of control samples.

Characterisation of the antiviral activity

Neutralisation of IFN was carried out as follows. Samples containing about 50 IU/ml as well as IFN international standards were incubated (60 min at 37° C) in the presence of individual and pooled anti-IFNα, -IFNβ and -IFNγ antisera (G 026-502-568, G 028-501-568 and G 034-501-565) obtained from NIAID, NIH, and the residual antiviral activity was then assayed. For each experiment the amounts of anti-human IFNα, -β and -γ used were sufficient to completely neutralise the corresponding IFNs.

Statistical evaluation

As far as Figures 1-3 are concerned, we have compared the areas (IFN concentration versus time) under the curve (AUC) obtained after various ozone concentrations as opposed to those of controls.

Statistical evaluation of the AUC values was performed using the Student's T test with $P < 0.001$ (two-tailed) as the minimum level of significance.

Results

Although blood is a very heterogenous cell suspension and contains one PBMC for about 2800 erythrocytes, we began our study by carrying out ozonisation of blood in conditions closely resembling the procedure followed in clinical practice.

Five samples of human blood from different donors were ozonised with four ozone concentrations for 30 sec.: 42 μg/ml is the one most frequently used clinically but, in order to explore a possible dose-response effect, we also tested 2.2, 11.5 and 108 μg/ml. Fig. 1 shows that the lowest ozone concentration (2.2 μg/ml) was uneffective and, like the control sample, no IFN was detectable. The highest amount of IFN (71.0 $\pm$ 13.0 IU/ml) was released after 96h incubation of blood samples ozonised with 42 μg/ml; as could be expected, the addition of SEB, a typical inducer of IFN, caused the highest release of IFN between 72 and 96h. On the other hand, ozone concentrations of 11.5 and 108 μg/ml were scarcely effective, probably because they were either too low or too high, respectively.

If whole blood after ozonisation was diluted (1:1) with RPMI 1640, similar results (not shown) to those reported in Fig. 1 were seen except that IFN levels obtained after SEB addition were about onefold higher than on whole blood.

As an intermediate step, we also evaluated IFN production in buffy-coat suspension. In this case the erythrocyte/PBMC ratio decreased from about 2800 to about 500/1 and, owing to the reduced number of erythrocytes, more reactive oxygen species may oxidise PBMC. Although the general pattern remains similar, Fig. 2 shows a few interesting differences: even the lowest ozone concentration (2.2 μg/ml) becomes slightly stimulatory, and 11.5 μg/ml is almost as effective as 42 μg/ml. Once again, the highest

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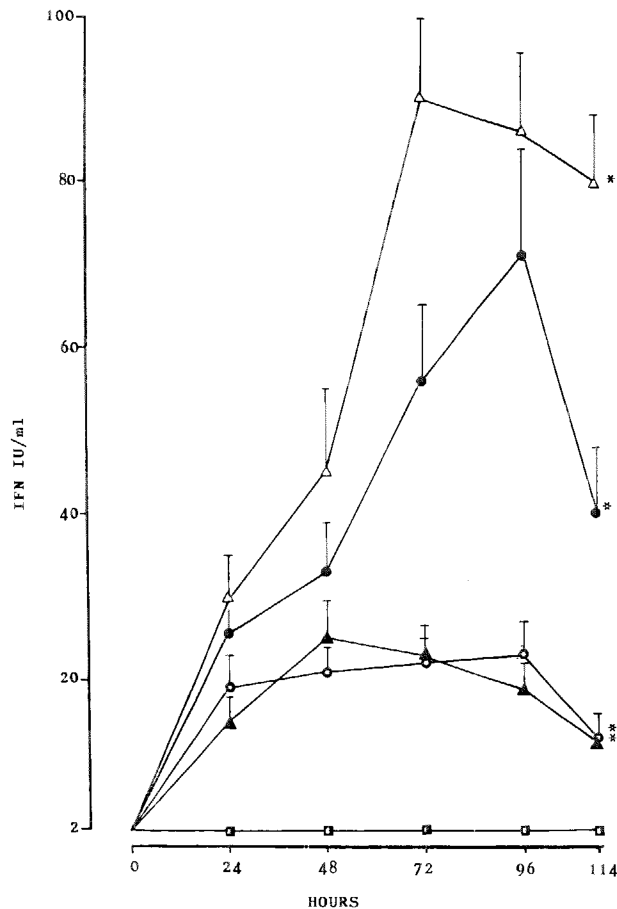

Fig. 1. - Kinetics of IFN production on whole blood insufflated with either different ozone concentrations: ■——■ 2.2, ▲——▲ 11.5, •——• 42, ○——○ 108 μg/ml or with air: □——□ control, △——△ SEB (9 μg/ml).
Values are reported as mean of 5 samples ± SD.
* AUC p values < O.001 are calculated in comparison to the control.

ozone concentration tested (108 μg/ml) depressed IFN production which, on the other hand, was well induced by SEB. The next obvious step was to evaluate IFN production on isolated PBMC.

In this case, owing to the lack of erythrocytes, reactive oxygen species produced upon ozonisation mainly acted on monocytes and lymphocytes. Fig. 3 shows that the lowest ozone concentration (2,2 μg/ml) now becomes the most effective IFN inducer, while 11.5 μg is far less effective and neither 42 nor 108 μg/ml cause the release of IFN. SEB proved to be a remarkable inducer of IFN, in that levels as high as 2,100 ± 660 IU/ml were reached at 96 h. In order to further clarify whether an ozone concentration as high as 108 μg/ml may be noxious to PBMC, we ozonised PBMC either 1 h before or after addition of SEB. In both cases production of IFN was about halved, suggesting that cell oxidation depresses IFN induction by SEB.

To know more about the optimal ozone concentration either versus time of exposure or cell concentration, we carried out some experiments the results of which are presented in Tables 1 and 2. The former table shows that using the ozone concentration of 2.2 μg/ml there is a fairly linear relationship between

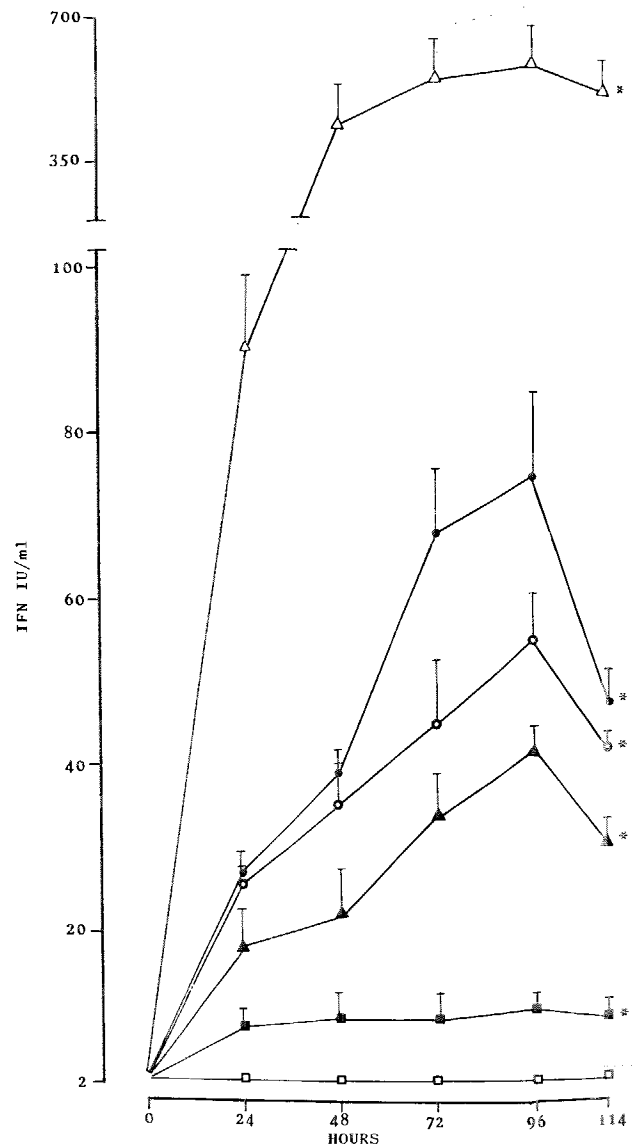

Fig. 2. - Kinetics of IFN production on resuspended buffy-coat insufflated with either different ozone concentrations: ■——■ 2,2, ▲——▲ 11.5, •——• 42, ○——○ 108 μg/ml or with air: □——□ control, △——△ SEB (9 μg/ml).
Values are reported as mean of 5 samples ± SD.
* AUC p values < 0.001 are calculated in comparison to the control.

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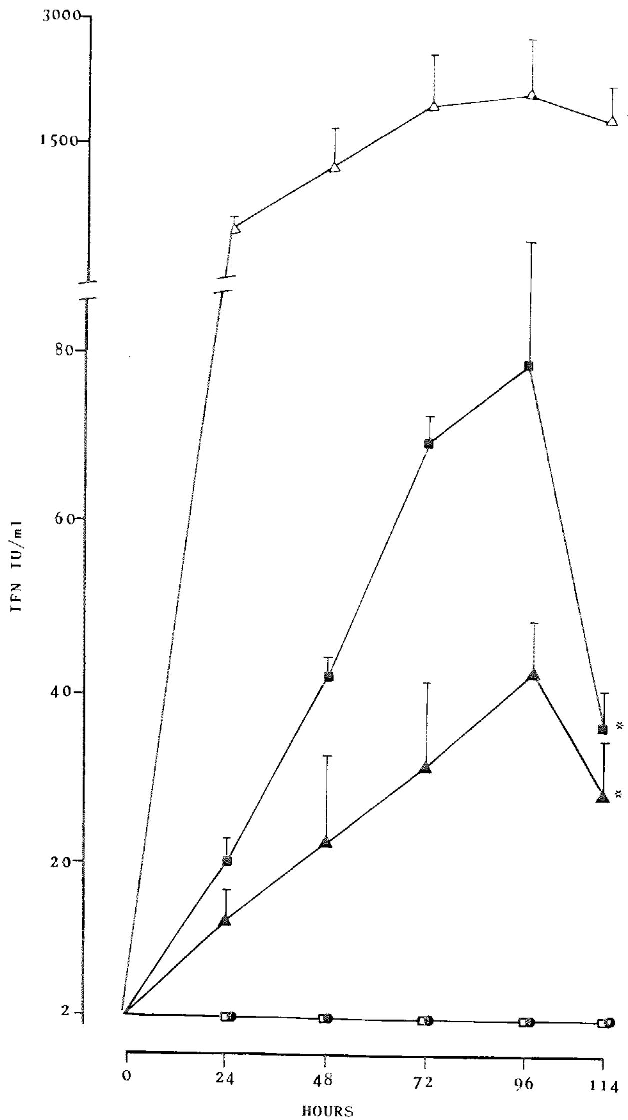

Fig. 3. - Kinetics of IFN production on Ficoll-purified mononuclear cells insufflated with either different ozone concentrations: ■——■ 2.2, ▲——▲ 11.5, •——• 42, ○——○ 108 μg/ml or with air: □——□ control, △——△ SEB (0.5 μg/ml).
Values are reported as mean of 5 samples ± SD.
* AUC p values < 0.001 are calculated in comparison to the control.

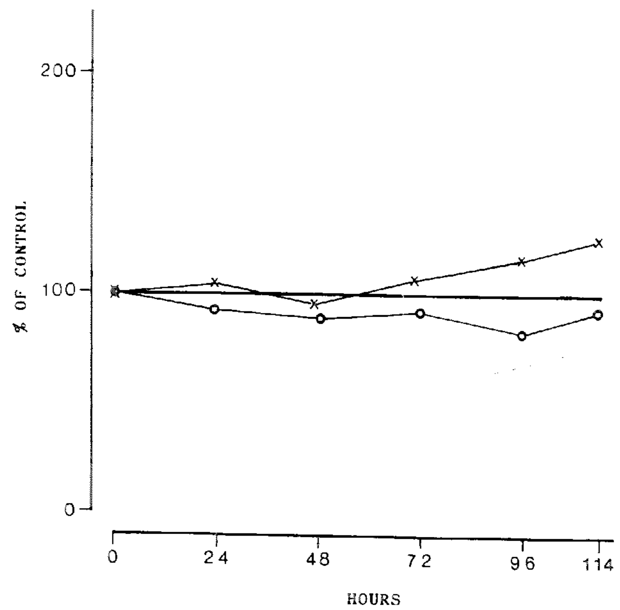

Fig. 4. - Evaluation of cell viability. Controls are expressed as the straight line at 100% value. Samples treated with O_3 at 2.2 μg/ml (x) and at 11.5 μg/ml (o) are indicated as a percentage of controls. Values are averages of two experiments.

Table 1. - *Influence of insufflation time either with ozone or air on interferon production by Ficoll purified mononuclear cells of three donors (means* ± SD; n=3).

Samples	Insufflation time (sec)		
	10	20	30
		IFN IU/ml	
Ozone (2.2 ug/ml)	33±8 [a]	48±8 [b]	78±8 [c]
Air	<2	<2	<2
Air plus SEB *			2,100±660

IFN production was measured on cells incubated for 96 hours.
c vs a $p < 0.002$; c vs b $p < 0.01$.
* 0.5 μg/ml.

time of exposure and maximal IFN release after 96 h incubation. It will be useful to investigate whether further ozone exposure increases or depresses IFN production, but clearly IFN yield also depends upon cell concentration (Table 2).

Finally, as far as cell viability is concerned, Fig. 4 shows that ozone treatment of PBMC at a concentration of 11.5 μg/ml depressed cell respiration somewhat. On the other hand, the concentration of 2.2 μg/ml does not seem deleterious and, actually, there is a definite increase of formazan formation after 72 h of incubation.

In order to define the type of IFN, we characterised the antiviral activity present in plasma and in the medium of PBMC treated with either ozone or with SEB. The activity was referable to a pH 2 labile protein, sensitive to trypsin, not sedimentable at 110,000×g and not active on rabbit (RK13) cells. Moreover, while the activity was completely neutralised after the addition of and incubation with goat antiserum against human IFN-γ, antisera against hu-

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Table 2. - *Influence of cell density of interferon production by Ficoll-purified mononuclear cells of three donors (means ± SD; n=3).*

Samples	Cell density/ml		
	5.10 [5]	1.5.10 [6]	3.10 [6]
		IFN IU/ml	
Ozone (2.2 ug/ml)	42±8 [a]	78±7 [b]	74±8[c]
Air	<2	<2	7
Air plus SEB *	1,500±128	2,100±660	2,100±370

IFN production was measured on cells incubated for 96 hours.
b vs a $p < 0.004$; e vs a $p < 0.008$.
* 0.5 μg/ml.

man IFN-α and IFN-β were either uneffective or barely effective, respectively.

Discussion

Particularly in the last decade it has been claimed that a brief ozonisation of human blood followed by reinfusion in the donor can represent a curative procedure for acute and chronic viral diseases. Since it is known that oxidising agents can induce IFN and probably other cytokines [11-15], it appeared to us reasonable to hypothesize that ozone or its reactive oxygen species may act mainly via the stimulation of PBMC and release of lymphokines. IFN and other mediators may then operate either directly as antiviral, or indirectly by activation of immune effector cells and by enhancing the expression of MHC antigens coupled to viral antigens [21].

The results presented in this paper show that under strictly defined conditions of ozonisation, PBMC either in whole blood or after isolation can be induced to produce significant amounts of IFN. At first we tried to repeat in vitro the procedure that is routinely used in clinical practice, but working with isolated PBMC obviously yields more clear-cut results. However, it is interesting to note that the presence of a high number of erythrocytes and plasma proteins apparently quenches the oxidising effect of free radicals on PBMC, so that effective ozone concentrations (in terms of IFN induction) are 2.2 and 42 μg/ml for either isolated PBMC or whole blood, respectively. Throughout the experimentation we got indications that ozone at high concentrations can be noxious to cell components: indeed, by increasing the ozone concentrations we and others [22] have noted a consistent increase of hemolysis and a reduced production of IFN and IL-2 in conjunction with reduced PBMC respiration. The evaluation of mitogenesis, as well as the release of other cytokines, such as TNF and IL-2, are being carried out and will be reported in the near future. Reactive oxygen species are probably not ideal lymphokine inducers because their generation and quantitation are not easily controllable, their site of action is very broad and the biological response may vary because of the variability of blood composition. However, because ozone haemotherapy is a diffused treatment modality, we feel that the exploration of the effects of ozone on PBMC has opened a new field of investigation that may yield useful results both in biological and practical terms.

STUDI SUGLI EFFETTI DELL'OZONO 1. INDUZIONE DI INTERFERONE γ SUI LEUCOCITI UMANI

In questo lavoro abbiamo esaminato l'effetto dell'ozono sul sangue umano, su sospensioni eritroleucocitarie e su cellule mononucleari purificate con Ficoll. I campioni venivano esposti a differenti concentrazioni di ozono (da 2,2 a 108 μg/ml) per 30 sec e poi incubati per tempi diversi a 37° C in una atmosfera umidificata contenente 95% di aria e 5% di CO_2. I supernatanti erano raccolti e congelati a -20°C fino al dosaggio dell'interferone (IFN). Abbiamo dimostrato che la concentrazione di ozono è critica per l'induzione di linfochine. Infatti, mentre basse concentrazioni (2.2 μg/ml) sono efficaci sui linfociti, esse non inducono IFN sul sangue intero, diluito (1:1) e sulle sospensioni eritroleucocitarie. In tali casi, venivano richiesti livelli più alti come 42 μg/ml. D'altronde, concentrazioni molto alte di ozono (108 μg/ml) sono meno efficaci. La massima produzione di IFN avviene a 72-96 h dopo l'esposizione all'ozono e la cinetica di rilascio di IFN è simile a quella ottenuta dopo aggiunta di Enterotossina Stafilococcica di tipo B. Poiché l'ozonizzazione del sangue è una pratica medica eseguita in diversi paesi per il trattamento delle malattie virali, noi riteniamo che questo studio abbia aperto un nuovo campo di ricerca che possa dare utili risultati sia sul piano biologico che pratico.

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