Effect of the Recreational Agent Isobutyl Nitrite on Human Peripheral Blood Leukocytes and on in Vitro Interferon Production

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ABSTRACT

The effects of the isobutyl nitrite sold as incense and the chemically pure compound on various in vitro parameters of leukocyte function were studied. This was done because of the potential relationship of isobutyl nitrite use to the opportunistic infection and Kaposi’s sarcoma seen in homosexual men. Various concentrations of isobutyl nitrite dissolved in ethyl alcohol were added to various leukocyte cultures. The final added concentrations were 0.001 to 1.0%. Because of the poor solubility of the agent, the fluid concentrations were quite low after addition. Thus, the measured concentrations in the medium after preparation of 1% (v/v) solution were 0.45 mM at 1 hr, 0.04 mM at 24 hr, and 0.04 mM at 48 hr of incubation at 37°C in 5% CO₂ in air. At the 1% added concentration, the agent lysed leukocytes and reduced viability from 95% to 21% in 24 hr. At an added concentration of 0.5% or below, cell count and viability were unaffected, but the agent inhibited in vitro lymphocyte blastogenic responses to phytohemagglutinin, pokeweed mitogen, and concanavalin A. It also inhibited natural killer cell activity to the K562 cell line, lymphocyte-mediated antibody-dependent cellular cytotoxicity to the CEM cell line, monocyte-mediated antibody-dependent cellular cytotoxicity to human red blood cells, and in vitro adherence and transformation of monocytes to macrophages. Inhibitory effects were greater than 90% at the 0.5% concentration and were still detectable at 0.01%. Chemically pure isobutyl nitrite and the form sold as incense had identical effects. The agent volatilized from the tissue culture medium at 37°C so that its effect on cell viability was reduced about one-third after 24 hr and was gone by 48 hr. The agent inhibited leucine, uridine, and thymidine incorporation approximately equally. Within 2 hr of exposure to isobutyl nitrite, uridine and leucine incorporation were markedly inhibited. After 24 hr of exposure to the agent, the effects on various lymphocyte function parameters were not reversible. The thymidine incorporation of myeloid and solid tumor cell lines was also inhibited by the same concentrations of isobutyl nitrite which inhibited leukocyte functions. Induction of αβ-interferon by polyriboinosinic-polyribocytidylic acid in mouse embryo fibroblasts was inhibited by pretreatment of the cells with isobutyl nitrite. These data suggest that isobutyl nitrite has nonspecific cytotoxic activity for various cells in vitro and could have immunosuppressive effects on tissues exposed in vivo during its recreational use. We speculate that these immunosuppressive effects, combined with the ability of nitrates to convert amines to nitrosamines, may be related to the development of opportunistic infections and Kaposi’s sarcoma in homosexuals who use this agent.

INTRODUCTION

During the last year, there have been several reports of opportunistic infection and Kaposi’s sarcoma occurring either alone or concurrently in previously healthy young adult males, mainly homosexuals (12, 18, 22, 28). Patients in these reports, who had severe infections such as pneumocystis carinii pneumonia, were severely immunodeficient, with skin test anergy, impaired lymphocyte-proliferative responses, and low-helper or high-suppressor T-cell subsets resulting in an inverted helper:suppressor ratio (12, 18, 22, 28). The etiology of the apparent new syndrome is obscure, but it has been attributed to some component or combination of components of the homosexual life style. Possible factors include multiple sexual partners; frequent direct or indirect oral-anal contact; multiple sexually transmitted diseases, particularly viral infection such as cytomegalovirus and hepatitis; and the use of so-called recreational drugs (5). In the latter category, attention has been focused on the nitrates because they have been widely used as aphrodisiacs only since the 1970s and because it is known that nitrates convert amines and amides to carcinogenic nitrosamines in vivo (14). It is speculated that the development of the opportunistic infection and Kaposi’s sarcoma syndrome in homosexuals must involve some new element since homosexual activity itself is not new. Thus, the focus is on drug usage. In the current report, we outline the results of studies of some in vitro effect of isobutyl nitrite (both that sold for recreational use and that which is chemically pure) on various peripheral blood leukocyte parameters. At high concentrations, the drug is cytotoxic; while at lower concentrations, at which viability is preserved, it still inhibits such functions as lymphocyte blastogenesis and NK3 cell activity. The effect is not lymphocyte specific, however, in that the proliferation of other cell types is also inhibited in vitro. Thus, interferon production is also inhibited by agent treatment of fibroblasts. The data suggest that isobutyl nitrite does have the capacity to inhibit host defense parameters in vitro and therefore may also do so in vivo.

MATERIALS AND METHODS

Isobutyl Nitrite Preparations. Isobutyl nitrite for inhalant use is...
commercially available in a variety of brands. One of these, Rush, is sold as liquid incense by Pacific Western Distributing Corporation, San Francisco, Calif. Rush contains greater than 90% nitrates, with small quantities of alcohol or vegetable oil added to reduce volatilization (31). Chemically pure isobutyl nitrite is prepared by the action of nitrous acid on butyl alcohol and is available from Alpha Products, Danvers, Mass. Isobutyl nitrite is not soluble in water, but it is miscible with alcohol. Rush, or chemically pure isobutyl nitrite, was diluted 1:1 with 95% ethanol. The Rush-ethanol, or chemically pure isobutyl nitrite:ethanol, or 95% ethanol were then diluted with RPMI 1640 to 10, 5, 1, 0.1, and 0.01% stock solutions (v/v). The stock solutions were added in required amounts to cultures to bring about another 1:10 dilution. The alcohol-dissolved Rush, pure isobutyl nitrite, or ethanol controls had calculated final culture concentrations of 1, 0.5, 0.1, 0.05, 0.005, and 0.001% (v/v).

The true concentration of isobutyl nitrite in the cultures was determined by high-performance liquid chromatography. Cultures were set up with 1% (v/v) isobutyl nitrite in alcohol added to complete tissue culture medium. The fluid was evaluated for isobutyl nitrite content compared to a standard at 1, 2, 4, 24, and 48 hr of incubation at 37°.

The nitrite analyses were performed with a Waters Associates liquid chromatograph consisting of a Model 710B sample processor, a Model M6000A pump, a Model 450 variable-wavelength UV detector, and a data module. A Waters analytical reverse-phase octadeclisilane column (30 x 4.5 cm; 10-µm particle size) was used for all analyses. The solvent consisted of 40% methanol (Burdick and Jackson Laboratories, Muskegon, Mich.) in water delivered at a flow rate of 2 ml/min. The column eluate was monitored for UV absorbance at 356 nm. The retention time of an isobutyl nitrite standard in this system was 2.8 min.

Lymphocyte Metabolism. Incorporation of [3H]thyminde (specific activity, 1.9 Ci/mmol), [3H]uridine (specific activity, 20 Ci/mmol), and [3H]leucine (specific activity, 61 Ci/mmol) was assayed in lymphocyte microcultures as described above. The cultures assaying [3H]leucine incorporation were set up using leucine-free Eagle’s minimal essential medium. The agent:ethanol mixture and ethanol were added as described above. The cultures were pulsed with 1 µCi of isotope at 24, 48, and 72 hr. After an additional 8-hr incubation, the cultures were harvested and counted as described above.

Cell Line Proliferation. The effect of the agent on tumor cell line proliferation was assayed in microcultures as described above. The cell lines used were: CEM (a T-cell lymphoma cell line); K562 (a human myeloid cell line); MB453 (a human breast cancer cell line); and H4534 (a human fibroblast cell line). Cells of each of the cell lines were plated at concentrations of 2 x 10⁵ cells/well in a 0.2-ml volume of RPMI 1640 with 20% fetal calf serum in microtiter plates. Cells were incubated for 24 hr before the addition of the agent. Agent and controls were then added, and 48 hr later 1 µCi [3H]thymidine was added. After an additional 24 hr, the cultures were harvested. CEM and K562 cultures were harvested using the method described for harvesting lymphocyte cultures. Cell lines MB453 and H4534 were harvested after washing the wells by adding 50 µl of 0.1 M NaOH to each well, which were then absorbed with 2 cotton swabs. The swabs were dried, placed in scintillation vials, and counted in a liquid scintillation counter.

ADCC and NK Cell Assays. The ADCC to HRBC and the CEM line and NK cell activity to the K562 cell line were set up as a modification of the method of Poplack et al. (27). The target cells HRBC (50 µCi) and CEM and K562 (100 µCi) were labeled with sodium [51Cr]chromate, by incubation for 40 min and washed 3 times to remove unbound [51Cr]. The antiserum used for ADCC to HRBC was a 1:1 dilution of Dade anti-B antiserum (Dade-Behring Diagnostics, Miami, Fla.). For ADCC to CEM, the antiserum was a 1:1000 dilution of rabbit antiserum produced by weekly immunization of rabbits with viable CEM cells for 3 weeks. The effector:target ratios used were 1:1 and 2:1 for HRBC, 20:1 for CEM, and 20:1 and 40:1 for K562. Mononuclear cells were adjusted to 2 x 10⁵ cells/ml in RPMI 1640. The cell suspension (0.1 ml) and 0.1 ml autologous serum were added to microwells in microtiter plates. The agents were added to triplicate cultures for each concentration. After 7 days of incubation at 37° in moist 5% CO₂ in air, all wells were washed with prewarmed HBSS to remove nonadherent cells and debris. Then 50 µl of WBC-lysing agent (Coulter) was added to each well and incubated for 30 sec. The number of adherent monocytes per ml of original whole blood was calculated as described previously (4) from Coulter cell counts of the washed mononuclear cell fraction.

Interferon Production. Mouse α,β (type I) interferon was produced in mouse embryo fibroblasts as described previously (1). Poly(I)-poly(C) was prepared by annealing polyriboinosinic acid and polyribocytidylic acid (P-L Biochemicals, Milwaukee, Wis.) by heating for 1 hr at 45°. α,β-Interferon was induced in cells by adding 50 µg of poly(I)-poly(C)
to tissue cultures for 60 min and then adding additional fresh tissue culture medium. DEAE-dextran was included to ensure maximum interferon induction, and tissue culture supernatants were harvested at 24 hr. Interferon antiviral titers were determined by plaque reduction on murine L-929 cells using the Indiana strain of vesicular stomatitis virus (1). The interferon titer corresponded to the reciprocal of the greatest dilution of test sample that reduced virus plaques by 50%. One interferon unit in this assay was equivalent to 0.88 NIH G-002-904-511 reference units.

**Statistical Considerations.** The leukocyte cultures described here showed significant depression at the 5% level if counts were reduced 25%, at the 1% level if counts were reduced 50%, and at the 0.1% level if counts were reduced 75% or greater (15).

**RESULTS**

High-performance liquid chromatography determinations of the 1% added concentration showed 46.14 µg/ml (0.45 mm) at 1 hr of incubation in the media, 37 µg/ml (0.36 mm) at 4 hr, 3.8 µg/ml (0.036 mm) at 24 hr, and 4.1 µg/ml at 48 hr. Thus, the amount added and the amount in solution are quite different. At 0 hr and at 20°, solubility was poor, and for this reason 1-hr incubation data are given.

At a 1% added concentration in complete media, commercial isobutyl nitrite was highly toxic to peripheral blood leukocytes. We examined the WBC, differential counts, and viabilities of Ficoll-Hypaque density solution centrifugation-separated leukocytes (originally set up at 4.5-cu mm x 10⁵) incubated with the agent for 24 hr compared to an untreated control and to ethanol controls. The 1% added concentration of the agent reduced the viability from approximately 95% to 21%, and the cell count was reduced from approximately 4000/cu mm to 1600/cu mm. The 0.5% concentration did not reduce the count or viability at all. The ethanol concentrations from 1% to 0.01% showed no effect on viability or count. This experiment was repeated 3 times with identical results. No major changes in surface markers (erythrocyte rosettes, surface immunoglobulin, or OKT 3, 4, and 8) were noted except at the highest concentration where toxicity was also seen (data not shown).

Table 1 shows experiments designed to test whether commercial isobutyl nitrite had an effect on some of the host defense parameters which we regularly use to test immunocompetence in cancer patients. The cultures were set up with the agent in culture from the beginning of the culture period. The blastogenic responses were reduced by concentrations as low as 0.1%. NK cell activity and ADCC to HRBC and CEM, as well as monocyte adherence, were reduced by concentrations as low as 0.01%. In 3 experiments of this type, the minimal inhibitory concentration was either 0.1 or 0.01% added. Thus, there was considerable variability in sensitivity from subject to subject. The alcohol control cultures showed that only a 1% concentration had a slight inhibitory effect on only some of the parameters (NK cell activity and monocyte adherence).

Experiments were conducted to characterize the effects of isobutyl nitrite on the DNA, RNA, and protein synthesis of peripheral blood lymphocytes stimulated with mitogens over the course of the culture period. The data are shown in Chart 1. It can be seen that at the 0.01% concentration, in PHA-stimulated cultures only, leucine incorporation was inhibited. In PWM-stimulated cultures, both leucine and uridine incorporations were inhibited. Not shown are data on Con A-stimulated cultures in which the effects of the agent were essentially identical to those seen in the PWM-stimulated cultures. These data indicate that protein and RNA synthesis are more inhibited than DNA synthesis in several types of mitogen-stimulated cultures. At the next higher added concentration (0.1%), DNA, RNA, and protein synthesis were all markedly inhibited. This experiment was repeated 3 times with identical results.

We next asked whether the effects of the agent were reversible if it was washed out of the cultures. This was approached using cell-mediated cytotoxicity (Chart 2). Cells were incubated with commercial isobutyl nitrite for 24 hr and washed 3 times, and then target cells were added for the standard 4-hr period. The effects of the agent were generally not reversible by washing it out of the cultures. Therefore, once exposed, leukocytes can be permanently damaged by isobutyl nitrite.

It was of interest to determine whether isobutyl nitrite had similar effects on cells other than peripheral blood leukocytes. We therefore evaluated the effects of the agents on a T-leukemia cell line (C), a myeloid leukemia cell line (K562), a breast cancer cell line (MB453), and a fibroblast cell line (H4534) (Chart 3). The breast cancer cell line was sensitive to the 0.01% concentration of the agent, and all lines were sensitive to the 0.1% concentration in terms of reduction of thymidine...
Chart 1. Effect of isobutyl nitrite on leucine (LEU), uridine (UR), and thymidine (THY) incorporation of PHA- and PWM-stimulated lymphocyte cultures at 24 and 48 hr at isobutyl nitrite concentrations of 0.01 % (A) and 0.1 % (B). Cultures with drug: •, leucine; ▲, uridine; △, thymidine. At all time points, the treated and control cultures were different (p < 0.01) in the 0.1 % added isobutyl nitrite cultures. At 0.01 %, only the leucine incorporation was suppressed (p < 0.05).

Chart 2. Effect of isobutyl nitrite on cell-mediated cytotoxicity. Persistence of effect after removal of drug from culture. Leukocytes were cultured for 4 hr with isobutyl nitrite and target cells or were cultured for 24 hr with isobutyl nitrite after which they were washed 3 times and incubated with target cells for 4 hr. After target cell incubation, all cultures were harvested. •, 24-hr washed cultures; ▲, ADCC to CEM; ○, ADCC to HRBC; △, NK cell activity.

Chart 3. Effect of isobutyl nitrite on [3H]thymidine incorporation of various cell lines. Microwells were plated with 2 x 10^5 cells and were incubated in complete medium for 24 hr. Then, various concentrations of isobutyl nitrite were added for 18 hr, after which [3H]thymidine was added for 8 hr, and the cultures were harvested. Data are expressed as percentage of untreated control. A, CEM; B, K562; C, MB453; D, H4534.

incorporation. Thus, the inhibitory effects of these nitrites appear to be nonspecific relevant to the immune system.

Since the commercial isobutyl nitrite has not been fully characterized and may have other agents as well as various nitrites admixed, we obtained and tested chemically pure isobutyl nitrite dissolved in ethanol (Table 2). The effects on lymphocyte blastogenic responses were essentially identical to those produced by the commercial material. The minimum inhibitory concentration was between 0.05 and 0.10%. Thus, we conclude that the effects of the commercial material are due to the isobutyl nitrite itself.

Because the isobutyl nitrite is highly volatile and is reduced in concentration 90% over 24 hr in culture, we evaluated the persistence of its activity in culture. To do this, we incubated the agent at a 1% added concentration in media for up to 72 hr (Table 3). Leukocytes were added at 0, 24, or 48 hr and incubated for the subsequent 24 hr, after which viability was
incubated for the subsequent 24 hr, after which viability was evaluated. The cytotoxicity of the agent reduced at 24 hr and was completely gone by 48 hr. Thus, in longer-term cultures, such as blastogenesis and monocyte adherence, we assume that the effects of the agent would be greater if the agent did not volatilize out of the solution within a short period of time.

We next evaluated the onset of the isobutyl nitrite effect on cell proliferation in culture. Chart 4 shows the effects of various concentrations of the agent on [3H]uridine incorporation by K562 cells. Isotope was added at various times during the culture period. The onset of action was rapid, and maximum inhibitory effects were already present during the first 2 hr of the culture period. Similar experiments were done with PHA-,
PWM-, and Con A-stimulated lymphocyte cultures and with the CEM cell line. Leucine and thymidine incorporation were also evaluated. The results in all of the above were identical to those shown in Chart 4. All showed a very rapid onset of inhibitory activity.

Finally, we determined the effects of isobutyl nitrite on α,β-interferon production. Early-passage C3H/HeJ-derived mouse embryo fibroblasts were allowed to grow into a confluent monolayer. Different cultures were then treated for 24 hr with various concentrations of commercial or pure isobutyl nitrite. Control cultures were treated with equivalent volumes of ethanol, the solvent for isobutyl nitrite. Chemicals were then removed from the cells, and cells were washed with minimal essential medium containing 10% fetal bovine serum and serum-free medium to more fully remove the chemicals. α,β-Interferon was then induced with poly(I)-poly(C). Viability of the cell cultures was determined by trypan blue dye exclusion. Concentrations of isobutyl nitrite added of 0.1% or greater were toxic to the cells. Concentrations of isobutyl nitrite below 0.1% had no apparent toxic effects on the cells, and total cell viability was greater.
Twenty-four hr later, the culture supernatants were harvested, than 95%. Ethanol treatment had no effect on cell viability. Ethanol treatment had no effect on cell viability. E. M. Hersh et al. and purified isobutyl nitrite severely inhibited interferon production at concentrations of 0.05 and 0.01%, when cell viability was not apparently affected (Table 5). Since the interferon assay is a titration, a decrease of 50% or greater was required to have a reliable effect on interferon induction. Concentrations of isobutyl nitrite below 0.01% had no effect on interferon induction.

**DISCUSSION**

The syndrome of opportunistic infection and Kaposi’s sarcoma in homosexual men is one of the most distressing and potentially serious health problems of modern American life (12, 18, 22, 28). Hundreds of cases of this syndrome are coming to medical attention each year, and the magnitude of the problem appears to be growing and spreading with time (2). The mortality of the syndrome is as high as 60%. Extensive speculations on the causes of this syndrome have been made. They include the immunosuppressive and potential carcinogenic effects of the herpesvirus group including cytomegalovirus (9), the potential immunosuppressive properties of rectally or orally ingested components of semen such as polyamines and prostaglandins (23), the extensive use of glucocorticoid-containing skin creams by these patients (24), and the use of so-called recreational drugs including marijuana, cocaine, heroin, and nitrates (11).

Volatile alkyl nitrates, such as amyl nitrite and isobutyl nitrite, have been used extensively in the homosexual community since approximately 1969 and are now being used more frequently also among heterosexuals (25). The acute transient vasodilation and tachycardia produced by these volatile nitrates apparently have aphrodisiac qualities related to the intensification of orgasm and relaxation of the anal sphincter (26). The various commercial products such as Rush, Lockeroom, Aroma of Men, and Gatorade are unregulated by the Food and Drug Administration since they are not sold as drugs but rather as incense or room odorizers. The extent of the use of these products is not known since they are also not regulated by the Consumer Products Safety Commission or the Environmental Protection Agency. However, it has been suggested that one brand has sold about 12 million 0.25-oz. bottles since 1974 (31) and that 11.1% of high school seniors report having used them as drugs (19).

Concern about these nitrates has been mainly related to their carcinogenic potential. However, they also have other acute or subacute toxicities including the production of methemoglobinemia (17), Heinz body hemolytic anemia (13), splenomegaly, skin rash (7), and death after acute overcongestion causing methemoglobinemia and hypokinetic anoxia (6).

The carcinogenic potential of the nitrates and nitrates relates to their ability to nitrosate various amines and amides forming n-nitroso compounds such as dimethylnitrosamine (14). The carcinogenicity of dimethylnitrosamine was first described by Magee and Barnes (21) in 1956. Nitrosamines are both toxic and carcinogenic to a variety of organs including liver, lung, kidney, bladder, and the upper respiratory and gastrointestinal tracts. A variety of natural food compounds and drugs undergo nitrosation when exposed to nitrates in aqueous solution. Cancer can be induced in experimental animals by the cofeeding of natural amines or amides and nitrates (8). Some drugs can be substrates and can be nitrosated by nitrates. They include aminophenzone, disulfiram, methadone, propoxyphene, and phenacetin (3). It is to be emphasized that the carcinogenic potential of butyl nitrite is hypothetical at present and is extrapolated from other studies.

Furthermore, we can only speculate as to the role, if any, of these recreational nitrates in the development of opportunistic infection and Kaposi’s sarcoma in homosexual men. It appears that many of the risk factors such as viral infection, sexual promiscuity, drug abuse, and the resultant or associated diseases go hand in hand. Goedert et al. (10) have demonstrated that the helper:suppressor ratio of peripheral blood lymphocytes is more inverted among nitrates users than among nonusers. This was the first indication of the potential immunological relationship to the nitrates. The concentration of nitrates in local lymphoid tissues such as in nasopharyngeal tissue could conceivably be high enough to produce both immunosuppressive and carcinogenic affects.

In the current paper, we have demonstrated that, in vitro, both "commercial" isobutyl nitrite and purified isobutyl nitrite dissolved in ethanol depress various leukocyte function parameters associated with host defense. Lymphocyte blastogenesis, cell-mediated cytotoxicity, and monocyte adherence were all suppressed by concentrations of isobutyl nitrite which were nontoxic in terms of cell viability. The data suggest but do not prove that the agents may be immunosuppressive in vivo. Preliminary data from our laboratory suggest that isobutyl nitrite does suppress the NK cell response of mice.4

These compounds are somewhat difficult to work with in that they readily and rapidly volatilize from in vitro tissue culture fluids. Therefore, cells are exposed to a progressive decrease in concentration with time. Furthermore, the effects on cellular functions were nonspecific in that the proliferation and metabolism of nonlymphoid and nonmyeloid cells were inhibited by the same concentrations which inhibit lymphocyte function. This is of some additional interest in terms of a possible relationship to the mild or moderate chronic myelosuppression which we have observed in the homosexual patients with opportunistic infection or Kaposi’s sarcoma.5 The induction of α,β-interferon was also inhibited by isobutyl nitrite treatment of fibroblast cell cultures. We have shown previously that many chemicals that are carcinogenic can inhibit interferon induction (15, 29, 30). Inhibition of interferon induction might contribute to infectious disease and cancer in isobutyl nitrite users. Further characterization and clarification of the potential immunotoxic role of the nitrates may be forthcoming through studies of noncytotoxic doses in vivo in experimental systems. However, even these in vitro studies strongly suggest that the inhalant nitrates may indeed be dangerous, and their use should be condemned by those physicians who treat patients who use these drugs regularly. This is particularly supported by the evidence that 0.001% (0.09 mm) added 3 times strongly inhibited the proliferation of lymphocytes.

The added concentration of the agent used in these studies, being 0.001 to 1.0%, ranged from 0.09 to 88 mm. However, when we measured the actual concentration within the culture

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4 E. Lotzova and E. M. Hersh, unpublished data.
5 P. W. A. Mansell et al., unpublished observations.
fluid (not on the surface to avoid possible effects of phase separation), we found that the 1% added concentration yielded a fluid concentration of 46 μg/ml (0.45 μM) at 1 hr of incubation. Furthermore, this fell by 90% upon incubation for 24 hr. It is unknown as to whether equivalent concentrations occur in the nasopharyngeal or other tissues of users.

These in vitro results are important, however, for the following reasons: (a) the potential for toxicity or carcinogenicity of a chemical upon chronic exposure can be assessed by single acute exposure at higher doses or concentrations. Since we observed suppression of leukocyte functions at noncytotoxic doses, this potential should be further explored for both in vivo and in vitro; (b) there already is some evidence that use of the agent by humans is associated with immunological abnormalities (10); (c) we have observed that the injection of nontoxic doses of the agent into mice significantly suppresses their NK cell activity. Finally, as noted above, an effect of isobutyl nitrite was evident after only 2 hr of exposure, suggesting that prolonged exposure may not be necessary. Thus, while the concentrations used in this study may or may not be achieved in a single in vivo use in humans, the observations are still relevant.

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