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The Acidic Tumor Microenvironment Promotes the Reconversion of Nitrite into Nitric Oxide: Towards a New and Safe Radiosensitizing Strategy

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Abstract

Purpose: The biological status of nitrite recently evolved from an inactive end product of nitric oxide catabolism to the largest intravascular and tissue storage of nitric oxide (NO). Although low partial O_2 pressure favors enzymatic reconversion of nitrite into NO, low pH supports a nonenzymatic pathway. Because hypoxia and acidity are characteristics of the tumor microenvironment, we examined whether nitrite injection could preferentially lead to NO production in tumors and influence response to treatments.
Experimental Design: The effects of nitrite were evaluated on arteriole vasorelaxation, tumor cell respiration and tumor blood flow, oxygenation, and response to radiotherapy.
Results: We first showed that a small drop in pH (-0.6 pH unit) favored the production of bioactive NO from nitrite by documenting a higher cyclic guanosine 3',5'-monophosphate-dependent arteriole vasorelaxation. We then documented that an i.v. bolus injection of nitrite to tumor-bearing mice led to a transient increase in partial O_2 pressure in tumor but not in healthy tissues. Blood flow measurements failed to reveal an effect of nitrite on tumor perfusion, but we found that O_2 consumption by nitrite-exposed tumor cells was decreased at acidic pH. Finally, we showed that low dose of nitrite could sensitize tumors to radiotherapy, leading to a significant growth delay and an increase in mouse survival (versus irradiation alone).
Conclusions: This study identified low pH condition (encountered in many tumors) as an exquisite environment that favors tumor-selective production of NO in response to nitrite systemic injection. This work opens new perspectives for the use of nitrite as a safe and clinically applicable radiosensitizing modality.

Nitric oxide (NO), one of the smallest biologically active molecules, plays a major role in many key pathophysiologic processes including the control of vascular tone (1) and angiogenesis (2). Although nitrites (NO_2) have been described for a long time, with nitrates, as the inert end products of the NO oxidative metabolism (3), recent evidence indicates that under specific conditions, nitrite can be reconverted into biologically active NO (4). The nitrite anion is now considered as the largest intravascular and tissue storage of NO, which may be made available depending on the tissue need. Nitrite was, for instance, shown to contribute to hypoxic vasodilation, i.e., a conserved systemic physiologic response that matches blood flow and oxygen delivery to tissue metabolic demand (5-7). Accordingly, several investigators showed that nitrite infusion could protect several organs, including heart, liver, kidney, and brain from ischemia-reperfusion injuries (8-13).

Different enzymatic and nonenzymatic pathways are proposed to support the reductive reconversion of nitrite into NO. Heme-containing enzymes including hemoglobin (7, 14, 15) and xanthine oxidase (16, 17) may act as nitrite reductases and/or S-nitrosothiol synthases under hypoxia, thereby offering a salvage pathway to produce NO when the O_2-consuming NO synthases become inoperative; endothelial NO synthase itself in the absence of oxygen may behave as a nitrite reductase (18). The nonenzymatic pathway for nitrite reconversion to NO requires another peculiarity of the microenvironment, namely a reduced pH, to favor the acidic reduction (disproportionation) of nitrite species (19-21). This is best exemplified in the stomach where the very low pH promotes the conversion of the high concentrations of nitrite present in saliva (derived from dietary nitrate) to NO and other nitrogen oxides to provide protection from swallowed pathogens (22) and enhance blood flow in the gastric mucosa (23).

Because acidic pH is a specificity of many tumors (24-26), although to a much lesser extent than in stomach, one may hypothesize that nitrite could represent an important source of bioactive NO in tumors and/or that infusion of nitrite could preferentially produce a local burst of NO in tumors (versus host tissues at physiologic pH). The

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therapeutic potential of an acute production of NO in tumors is huge as, by driving local vasodilation and increasing perfusion, NO may transiently increase the delivery of drugs into the tumor and correct hypoxia, thereby improving the efficacy of ionizing radiations. Moreover, these latter effects might be exacerbated by the intrinsic radiosensitizing effects of NO (27) and its capacity to inhibit mitochondrial respiration, thereby further increasing intracellular O_2 levels (28, 29).

In this study, we provide the proof of concept that nitrite may selectively induce a transient increase in tumor partial O_2 pressure {pO_2), which may be exploited to improve the efficacy of radiotherapy. The effects of nitrite were identified to be attributable not to an increase in tumor blood flow but to a reduction in the O_2 consumption rate of tumor cells. Based on this work, nitrite can be viewed as a promising, safe, and inexpensive adjuvant modality to antitumor strategies, particularly radiotherapy.

Materials and Methods

Mice and tumor cells.

Male Rj:NMRI mice received an i.m. injection of 10^6 syngeneic (TLT) transplantable liver tumor hepatocarcinoma cells in the posterior right leg at the vicinity of the saphenous arteriole (i.e., the vessel used for *ex vivo* vasorelaxation assay; see below), as previously described (30, 31). The tumor diameters were tracked with an electronic caliper. When the tumor diameter reached 4.0 ± 0.5 mm, mice were randomly assigned to a treatment group. Nitrite ($NaNO_2$) or saline was injected through the catheterized tail vein of anesthetized mice. Each procedure was approved by the local authorities according to national animal care regulations. Transplantable liver tumor TLT carcinoma cells were maintained in culture in DMEM containing 10% FCS.

Videomotion analysis of vessel relaxation.

Saphenous and mesenteric arterioles were dissected under a stereoscopic microscope and processed as previously described (31, 32). Briefly, vessels were mounted in a Plexigas isolated organ chamber circulated with oxygenated physiologic saline solution (37°C) and placed on an inverted microscope (Axiovert S100; Zeiss) connected to a charge-coupled device camera. Vessels were then pressurized with a physiologic saline solution - filled burette manometer at 60 mmHg. Digitized imaging (Ionoptix) allowed continuous monitoring of vessel external diameter. Arterioles were precontracted with a KC1 solution and were then exposed, at the indicated level of pH (6.8 or 7.4), to increasing concentrations of nitrite, in the presence (or not) of 1H-[1,2,4]Oxadiazolo[4,3-a]quinox-alin-1-one (ODQ), L-NAME, or allopurinol; all the treatments were added in the bathing solution.

Tumor oxygenation monitoring.

Electronic paramagnetic resonance (EPR) oximetry, using charcoal (CX0670-1; EM Science) as the oxygen-sensitive probe, was used to evaluate tumor and muscle oxygenation as previously described (33). EPR spectra were recorded using an EPR spectrometer (Magnettech) with a low frequence microwave bridge operating at 1.2 GHz and extended loop resonator. Data acquisition was started before nitrite administration; each mouse was used as its own control.

Tumor blood flow monitoring.

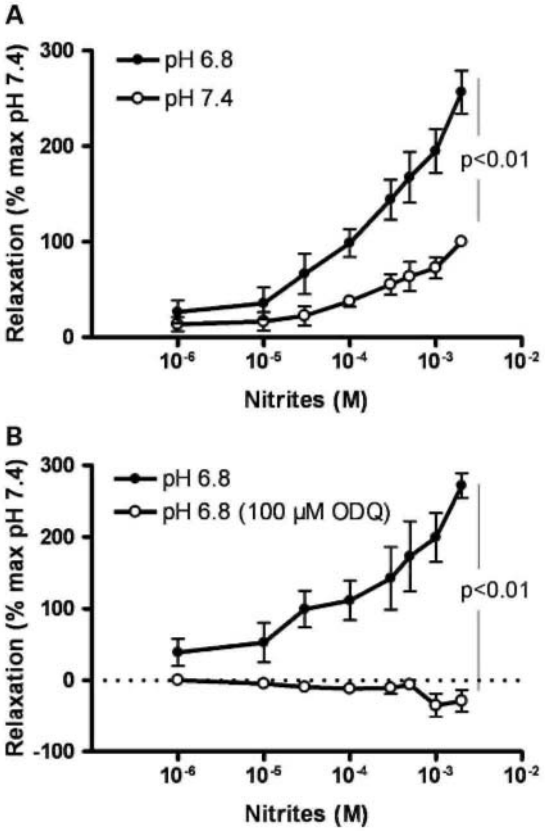
Tumor blood flow was measured with a Laser Doppler imager (Moor Instruments), which maps cortical tumor perfusion (with a tissue penetration of ~ 2 mm), and with Laser Doppler microprobes (OxyFlo; Oxford Optronix). For the Laser Doppler measurements, mice were anesthetized and fur was removed from the limbs using a depilatory cream. Perfusion of the tumor-bearing and control legs was evaluated on the basis of colored histogram pixels. For OxyFlo measurements, fiberoptic microprobes were inserted into the tumor and into the opposite (healthy) leg. Data were collected continuously at a sampling frequency of 20 Hz. For both assays, the animals were placed on a heating pad (37°C) to minimize variations in temperature and a 10-min stable recording baseline was acquired before treatment administration through the catheterized tail vein (to validate the absence of movement artifacts).

Oxygen consumption rate evaluation.

Tumor cells were trypsinized, centrifuged, and resuspended in buffered saline solution at pH 7.4 or 6.7. An aliquot of 2.10^6 cells was incubated for 15 min in the presence of 100 μmol/L nitrite before the addition of 20%

dextran and neutral nitroxide (an oxygen sensitive probe), ^{15}N 4-oxo-2,2,6,6-tetramethylpi-peridine-d_{16}-^{15}N-1-oxyl at 0.2 mmol/L (CDN Isotopes). The suspension was drawn into glass capillary tubes; cytotoxicity was concomitantly evaluated with a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (34). All spectra were recorded on a Bruker EMX EPR spectrometer operating at 9 GHz, as previously described (33). The probe was calibrated at various O_2 concentrations between 100% nitrogen and air so that the line width measurements were related to O_2 concentration at any value. Nitrogen and air were mixed in an Aalborg gas mixer, and the oxygen concentration was analyzed using a Servomex oxygen analyzer OA540. The sealed tubes were placed into quartz EPR tubes maintained at 37°C, and the O_2 levels were determined over time.

Fig. 1: *Nitrite induces vasodilation in a pH- and NO-dependent manner. A, arterioles were mounted on a pressure myograph, precontracted to restore the vascular tone, and exposed to increasing doses of nitrite. A, vasodilating response to nitrite obtained at pH 7.4 (○) or 6.8 (●; n = 6). B, effect of the guanyl cyclase inhibitor ODQ (100 µmol/L; ○) on the vasodilating response to nitrite at pH 6.8 (n = 3); the effect of the vehicle treatment is also shown (●; n = 3). Vasorelaxation is expressed as % (mean ± SE) of the maximal response observed at pH 7.4 (P < 0.01; n, number of arterioles per condition).*



Irradiation and tumor growth delay assay.

Anesthetized tumor-bearing mice were i.v. injected with saline or nitrite solutions 10 min before being locally irradiated using a RT-250 device (Philips Medical Systems) with a dose delivery of 0.76 Gy/min. The tumor was centered in a circular irradiation field, and healthy tissues were protected by a lead mask. After treatment, tumor diameters and mouse survival were tracked daily.

Statistical analyses.

Data are reported as mean ± SE, and statistical analyses were done using Student's *t* test, two-way ANOVA analyses, or Log-rank test where appropriate.

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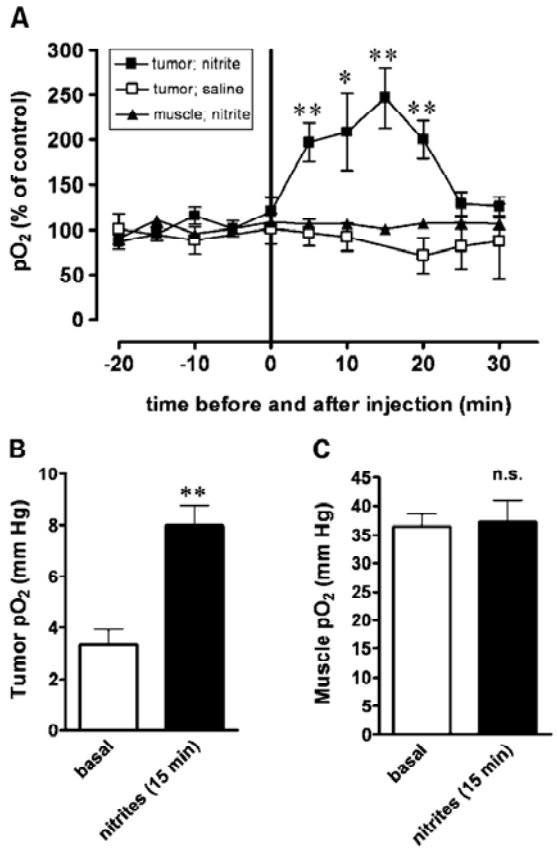
Fig. 2: *Nitrite i.v. injection induces a robust, transient, and tumor-specific increase in oxygenation. Tumor-bearing mice were i.v. injected with a 5 mmol/L nitrite solution (final blood concentration, ~100 μmol/L), and pO_2 was determined by EPR oximetry in the tumor (implanted in the right leg) and in the muscle (of the contralateral leg). A, tumor (■; n=8) and muscle (▲; n = 5) pO_2 before and after the injection (at t = 0); the effect of a saline injection on the tumor pO_2 is also shown as control (□; n= 4). Results are expressed as % (mean ± SE) of basal pO_2 levels (*, P < 0.05; **, P < 0.01; n, number of mice per condition). B to C mean (±SE) pO_2 (expressed in mmHg) as determined in tumor (B) and muscle (C), before (white bars) and 15 min after i.v. injection of nitrite (blackbars; **, P < 0.01; n.s., nonsignificant; n = 4-8 mice per condition); note that different Y-axis scales are used in B and C.*



Results

Nitrite induces arteriole dilation in a pH- and NO-dependent manner. We first aimed to determine whether bioactive NO could be produced from nitrite in response to a small drop in pH (as observed in tumors). For that purpose, we examined the response of arterioles (from the vascular bed wherein tumors are established in our mouse model) to nitrite administration. Arterioles (mean diameter, ~ 250 μm) were microdissected and mounted in a pressure myograph. Figure 1A shows that nitrite dose-dependently induced the relaxation of the arterioles both at physiologic and acidic pH (7.4 and 6.8, respectively). Interestingly, the maximal relaxation observed at pH 6.8 amounted to 2.5-fold to that obtained at pH 7.4. To further examine whether nitrite-driven vasodilation was due to the activation of the NO/cyclic guanosine 3',5'-monophosphate pathway, we repeated the experiments at pH 6.8 in the presence of 100 μmol/L ODQ, a pharmacologic inhibitor of the soluble guanyl cyclase. Figure 1B shows that ODQ completely prevented the nitrite-induced relaxation of the arteriole, confirming implication of NO. To address the role of NO synthase and xanthine oxidase as possible enzymatic sources of nitrite reductase, these experiments were repeated in the presence of the specific pharmacologic inhibitors, L-NAME and allopurinol, respectively. These inhibitors failed to alter nitrite-induced vasorelaxation at both pH values (data not shown).

Nitrite i.v. administration induces a robust, transient, and tumor-selective increase in oxygenation.

To evaluate a possible role of nitrite *in vivo,* we used EPR to determine changes in local pO_2 in a highly glycolytic mouse tumor model. Figure 2A shows that tail vein injection of nitrite (final blood concentration, ~100 μmol/L) led to a net increase in tumor pO_2 but failed to induce any changes in the (tumor free) contralateral limb muscle. Saline injection to tumor-bearing mice was also used as control and did not reveal any alteration in

the tumor pO_2 (Fig. 2A).

The effect of nitrite on tumor pO_2 was transient, peaking at 2.5-fold of the basal level after 15 minutes and back to normal after 30 minutes. The absolute numbers (see Fig. 2B) indicate that the temporary shift in pO_2 from 3.3 ± 0.6 to 8.0 ± 0.8 mmHg is in the range generally admitted to lead to radio-sensitizing effects (35). In the muscle, the pO_2 was much higher (~35 mmHg) and not influenced by nitrite administration (Fig. 2C).

Fig. 3: *Nitrite injection does not alter tumor blood flow*. *Tumor-bearing mice were i.v. injected with nitrite or saline (as described in Fig. 2), and blood flow was measured in tumor and healthy muscle. A, representative pictures from laser Doppler imaging obtained 15 min after nitrite injection; the zone corresponding to the tumor is surrounded by a dotted line. B, evolution of blood flow determined by laser Doppler imaging, simultaneously, in tumor (●) and muscle (○) before and after the nitrite injection (at t = 0). Data are expressed as % (mean ± SE) of the basal blood flow measured after saline injection (n = 6). C blood flow in tumor and muscle determined by Oxyflo microprobes 15 min after the i.v. injection of nitrite. Data are expressed as % (mean ± SE) of the basal blood flow measured in the muscle (n = 5 mice per condition).*

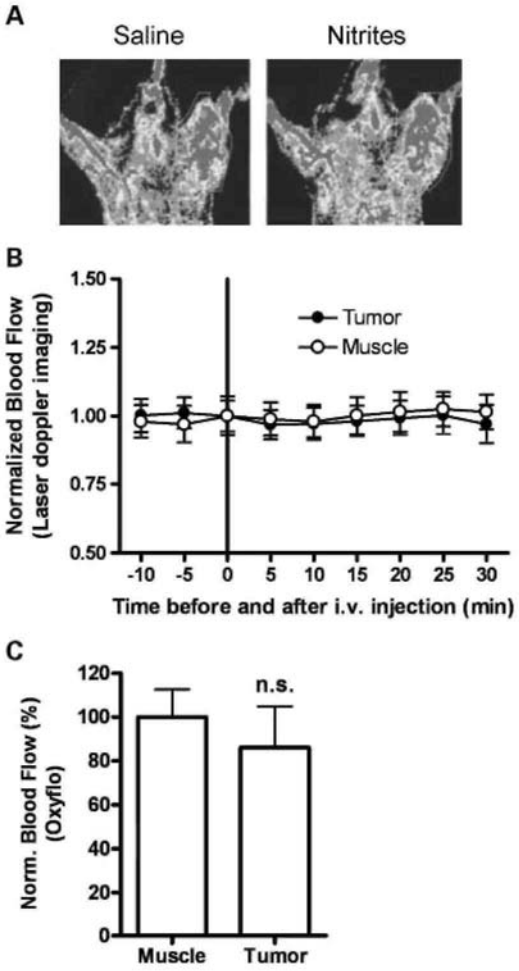
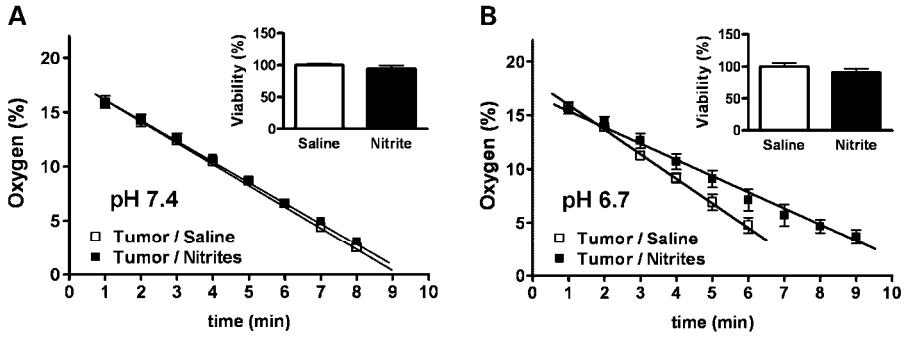


Fig. 4: *Nitrite reduces tumor cell oxygen consumption at acidic pH. Graphs represent the tumor cell O_2 consumption rate after exposure to a 100 µmol/L nitrite solution (■) ora saline solution (□) at pH 7.4 (A) and 6.7 (B), as measured by EPR oximetry. Data (mean ± SE) are expressed as % of O_2 detected in the sealing tubes (n = 3-5; different cell pools); lack of overall cytotoxicity was verified in MTTassays (inset). C slope values (mean ± SE) of linear regressions presented in A and B are presented (**, P < 0.01).*



C

pH	Saline	Nitrites 100 µM	P values
7.4	-1.96 ± 0.04	-1.86 ± 0.05	n.s.
6.7	-2.20 ± 0.12	-1.59 ± 0.09	P < 0.01

Nitrite administration does not alter tumor blood flow.

We then sought to verify whether the effects of nitrite on pO_2 could be attributed to an increase in tumor perfusion. We first used laser Doppler imaging to monitor blood flow at the surface of the tumor, where the microcirculation is the most developed in this tumor model (data not shown). Figure 3A shows that administration of nitrite (at the same concentration as used in Fig. 2) failed to induce any change in tumor (and muscle) perfusion. Normalization of the blood flow values confirmed that nitrite did not alter perfusion neither in the tumor, nor in the muscle when compared with a saline injection (Fig. 3B). We also used Oxyflo microprobes to monitor blood flow deeper in the tumor. Again, this more invasive technique failed to reveal significant alterations in the tumor (and muscle) perfusion in response to nitrite administration (Fig. 3C).

Nitrite exposure decreases tumor cell oxygen consumption rate at acidic pH.

As a change in tumor blood flow could not account for the observed increase in tumor pO_2, we then examined whether the consumption of O_2 could differ in tumor cells exposed to nitrite. Tumor cells were isolated and placed in a sealed tube with nitrite, and respiration was monitored with a EPR oxygen - sensitive probe. We found that the rate of oxygen consumption was unaltered by the presence of nitrite when cells were bathed in a medium at pH 7.4 (Fig. 4A), whereas the respiration was significantly slowed down when the medium was buffered at pH 6.7 (Fig. 4B). Slope analysis revealed that the oxygen consumption rate under these mild acidic conditions was 38% smaller in the presence of nitrite (Fig. 4C). We also verified that in these experimental conditions, cell viability was not altered by the addition of nitrite (Fig. 4A and B, *insets).*

A single nitrite administration radiosensitizes tumor.

Finally, to validate the therapeutic effect of the reduction in O_2 consumption (i.e., the local increase in tumor pO_2), we locally irradiated tumor-bearing mice 15 minutes after i.v. administration of nitrite. Figure 5A shows that the combination of nitrite and a 6 Gy radiation dose significantly improved mouse survival. Importantly, nitrite alone did not effect tumor growth, even when administered at a 10-fold higher concentration. Determination of the tumor growth doubling time revealed that whereas radiotherapy alone delayed this variable by 2 days, the combination of irradiation and nitrite extended this time to 5.5 days, increasing by almost 2-fold the absolute doubling time value determined in saline-treated mice (Fig. 5B).

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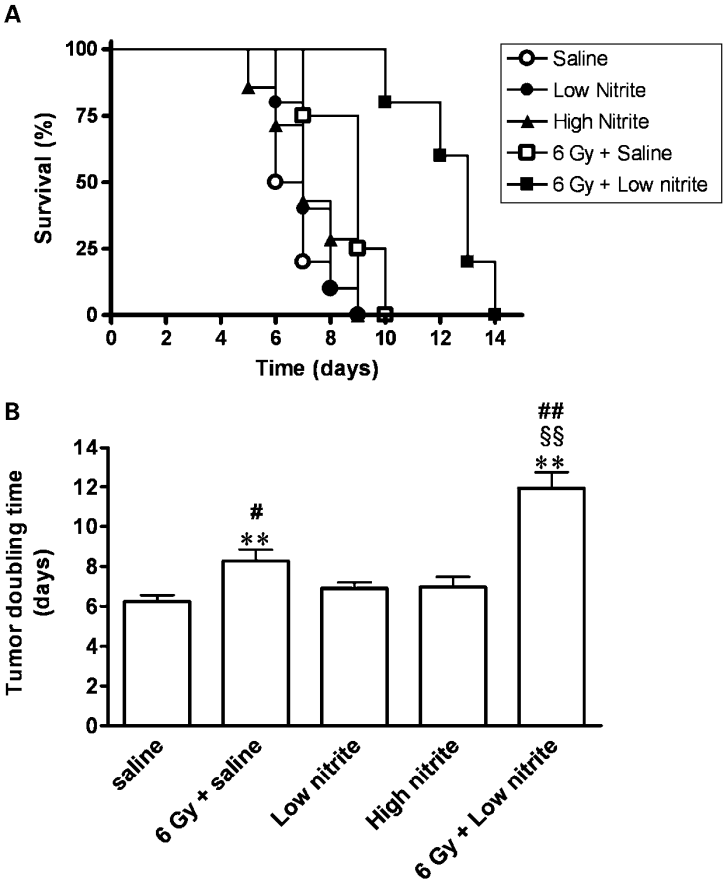
Discussion

The major findings of this study are the demonstration of a tumor-selective increase in pO_2 in response to a bolus systemic administration of nitrite and the proof of concept that such a procedure is therapeutically exploitable to radiosensitize tumors. Moreover, we have identified tumor cell respiration but not tumor perfusion as a critical target of nitrite, and low pH, as encountered in many tumor types, as a necessary condition to promote the nitrite conversion into bioactive NO.

We found that both vascular and tumor cells may be influenced by nitrite exposure to a larger extent under acidic conditions than at physiologic pH. The same dose of nitrite (100 μmol/L) showed, for instance, a 3-fold higher capacity to dilate isolated arterioles (Fig. 1A) and a reduction in tumor cell O_2 consumption by ~ 40% (Fig. 4B) when the pH was set at 6.7 to 6.8 (versus pH 7.4). Together with the observation that a blocker of the NO/cyclic guanosine 3',5'-monophosphate pathway prevented these effects, our results authenticate the acidic environment as a key variable to favor nitrite reconversion into NO. Interestingly, however, although we observed an increase in tumor pO_2 after a bolus i.v. administration of nitrite *in vivo,* we failed to detect any effects of nitrite on the tumor blood flow using superficial and invasive methods of measurements. The balance between oxygen delivery and oxygen consumption implies that the nitrite-driven increase in tumor pO_2 is attributable to the consumption arm of the equation, i.e., to the inhibition of mitochondrial respiration (as observed *in vitro).* Inhibitory effects of NO on cell respiration were previously reported to mainly arise from the nitrosylation and consecutive inhibition of the cytochrome *c* oxidase and complex I within the mitochondrial respiratory chain (28).

Fig. 5: *Nitrite i.v. injection radiosensitizes tumor. A, Kaplan-Meier survival curves depicting the effects of low and high doses of nitrite [100 μM (●; n = 10) and 2 mmol/L (▲; n = 5), respectively], radiotherapy (6 Gy) + saline (□; n = 5) or a combination of radiotherapy and low dose of nitrite (administered 15 min before irradiation; ■ ; n = 5); the effect of saline injection (○; n = 10) is also shown as control. Data are expressed as % of mouse survival (*, P < 0.05 and **, P < 0.01 versus saline; #, P < 0.05 and ##, P < 0.01 versus low dose of nitrite; §§, P < 0.01 versus radiotherapy + saline; n = number of mice per condition). B, tumor doubling time (mean ± SE) in the same groups as in (A ; **, P < 0.01 versus saline; #, P < 0.05 and ##, P < 0.01 versus low dose of nitrite; §§, P < 0.01 versus radiotherapy + saline).*



The absence of alterations in the tumor cell respiration measured *in vitro* at physiologic pH emphasizes the critical role of acidity in driving the nitrite effects. Although blood flow removes waste products of the tumor cell metabolism, including lactic acid, thereby creating *in vivo* an acidity gradient from the blood vessels toward the surrounding tumor mass, such gradient is buffered *in vitro* by "normal" culture medium. It should be noted however that the small drop in pH (less than one pH unit; refs. 24-26) generally observed in tumors (including in highly glycolytic tumors as used in this study) is by far less pronounced than in the stomach where the continuous delivery of saliva-containing nitrite allows the production of bacteriostatic NO. Reducing equivalents particularly abundant in tumor cells (36) are therefore likely to play an important role in the local production of NO from nitrite. Interestingly, Zweier and colleagues (37) reported that the addition of an aliquot of ischemic tissue homogenates to nitrite in the presence of a low pH led to a dramatic increase in the rate of NO generation.

Hemoglobin was identified by several authors to act as a transporter and provider of NO/NO derivatives through either direct S-nitrosylation (including by nitrite) and consecutive release of nitrosothiols (38) or through a direct interaction of nitrite with deoxyhemoglobin, which may release NO in regions of poor oxygenation (39, 40). These effects of hemoglobin were documented to favor vasorelaxation and thereby to help redistributing blood flow to ischemic regions of greatest need. In our experiments, the absence of changes in tumor and muscle blood flow indicates that such role of hemoglobin in driving the blood conversion of nitrite into NO is limited or at least counterbalanced by the NO-scavenging capacity of oxyhemoglobin and deoxyhemoglobin (41). A role of NO in modifying the affinity of hemoglobin for O_2 (7) can however not be excluded in our experimental conditions and certainly warrants further investigation. By contrast, the incapacity of pharmacologic inhibitors of xanthine oxidase and NO synthase (allopurinol and L-NAME, respectively) to prevent nitrite effects allows us to reasonably rule out a major role of these two main tissue enzymes endowed with a nitrite reductase activity.

The clinical potential of using nitrite as a strategy to sensitize tumor cells to radiotherapy is huge. First, the acidic microenvironment is a hallmark of many tumors, making this treatment modality applicable to a large variety of cancers. Furthermore, the effects of a bolus administration of nitrite are transient and occur rapidly. The 30-minute window of increased pO_2 with the peaking value around 15 minutes may be conveniently anticipated before irradiation. Second, the strategy is inexpensive and if validated in clinical trials, could be applied as a standard clinical procedure. Third, and not least, the administration of nitrite seems as a safe approach with a favorable hazard/benefit ratio. As indicated above, the transient and tumor-selective nature of the NO burst needs to be distinguished from a sustained production of high amounts NO and the associated mutagenic effects (as observed in response to inducible NOS expression during chronic inflammation; ref. 42). In fact, considering the rate of nitrite reconversion into NO at pH 6.7 (19), the maximum concentration of NO that could be generated in our experimental conditions remains in the nanomolar range. It is also worthy to note that different authors have reported the safe administration of nitrite in mice and rats suffering from cardiovascular diseases. Data in non-rodent animals and humans are also available. For instance, Pluta and colleagues (43) reported that sodium nitrite infusion prevented delayed cerebral vasospasm in a primate model of subarachnoid hemorrhage without clinical or pathologic evidence of toxicity. Cosby and colleagues (39) reported that nitrite infusion into the human forearm brachial artery resulted in increased blood flow before and during exercise. It should also be mentioned that nitrite is historically used as a treatment for cyanide poisoning (44).

In conclusion, our study shows that the small reduction in pH values (versus physiologic pH) as encountered in the extracellular medium of many tumors is sufficient to produce large amounts of bioactive NO in response to a systemic bolus nitrite injection. The consecutive increase in tumor pO_2 may translate in a tumor-selective, safe, and inexpensive therapeutic strategy to sensitize tumors to radiotherapy.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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