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Analytical method for determination of nitric oxide in zebrafish larvae: Toxicological and pharmacological applications

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ABSTRACT

Zebrafish are currently used at various stages of the drug discovery process and can be a useful and cost-effective alternative to some mammalian models. Nitric oxide (NO) plays an important role in physiology of zebrafish. The availability of appropriate analytical techniques to quantify the NO is crucial for studying its role in physiological and pathological conditions. This work aimed at establishing a high-performance liquid chromatography method for determination of NO levels in zebrafish larvae. Attempts were also made to assess the normal levels of NO at the first days postfertilization and the possible changes under pathological conditions. The method validation was quantitatively evaluated in terms of sensitivity, specificity, precision, accuracy, linearity, and recovery. NO levels from zebrafish larvae at the first days postfertilization and larvae challenged to N(G)-nitro-L-arginine methyl ester, sodium nitroprusside, *Escherichia coli* lipopolysaccharide, and copper sulfate were analyzed. The samples were derivatized with 2,3-diaminonaphthalene, and fluorescence detection was used for the indirect determination of NO. The method showed a good performance for all validation parameters evaluated and was efficient to monitor changes in NO concentration under physiological and pathophysiological conditions. This method might represent a powerful tool to be applied in NO studies with zebrafish larvae.

Drug discovery involves a complex interactive process of biochemical and cellular assays, with final validation in animal models and ultimately in humans. Zebrafish (*Danio rerio*) is currently used at various stages of the drug discovery process and has several advantages when compared with other experimental models such as cell culture and rodents [1,2]. Advantages of zebrafish as a model organism include the small size of embryos and larvae, the high fecundity of adults, the optical transparency of embryos and larvae, the speed at which they develop, and the possibility of simply adding compounds to water for screening of biomarkers, drugs, and toxic agents [3,4].

The teleost zebrafish is a small tropical fish that produces optically transparent embryos that develop outside of the mother's body [5–7]. At 6 days postfertilization (dpf),[1] a complex circulatory system and counterparts of most mammalian organs are developed. Although zebrafish proteins display less than 70% identity to their human orthologues, the conservation of functional domains, such as the substrate binding regions (often the drug-binding targets), is considerably higher, with approaching values of 100% similarity [5].

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[1] *Abbreviations used:* dpf, days postfertilization; NO, nitric oxide; NOS, nitric oxide synthase; NO_2^-, nitrite; NO_3^-, nitrate; HPLC, high-performance liquid chromatography; HCl, chloride acid; SNP, sodium nitroprusside; $CuSO_4$, copper sulfate; $NaNO_2$, sodium nitrite; NaOH, sodium hydroxide; DAN, 2,3-diaminonaphthalene; LPS, lipopolysaccharide; L-NAME, N(G)-nitro-L-arginine methyl ester; dd-water, distilled/deionized water; PBS, phosphate-buffered saline; ANOVA, analysis of variance; NAT, 2,3-naphthotriazole; CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification; DAF-2, 4,5-diaminofluorescein.

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Nitric oxide (NO), a free radical and a signaling molecule, plays an important role in regulating vascular tone, neurotransmission, host immunity, nutrient metabolism, whole-body homeostasis, macrophage activity and cell proliferation, differentiation, and apoptosis [1,8,9]. NO is formed in biological tissues from L-arginine by three major nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). All three major NOS isoforms are expressed during early development of zebrafish [10].

During the past few years, several additional tools that greatly increased the utility of zebrafish as an experimental organism have been developed [6]. The availability of appropriate analytical techniques to quantify the NO is crucial for studying its role in physiological and pathological conditions. However, because of its very short half-life in circulation and in cells (<5 s), the direct measurement of NO is extremely difficult. For this reason, the cellular generation of NO is most often assessed by measuring the nitrite (NO_2^-) and nitrate (NO_3^-) contents in biological fluids [8].

The aim of this study was to develop a simple and rapid routine method using high-performance liquid chromatography (HPLC) for determination of NO in nanomolar concentrations. The method validity was further confirmed by evaluating the basal levels of NO during the first days after fertilization in addition to the changes of NO levels under inflammatory conditions.

Materials and methods

Chemicals

All chemicals were of HPLC grade. Methanol, chloride acid (HCl), sodium nitroprusside (SNP), and copper sulfate ($CuSO_4$) were purchased from Merck (Darmstadt, Hessen, Germany). Sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), 2,3-diaminonaphthalene (DAN), lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS), and N(G)-nitro-L-arginine methyl ester (L-NAME) were acquired from Sigma Chemical (St. Louis, MO, USA).

Standard solutions

Stock solutions of SNP, $CuSO_4$, and LPS for larvae treatment were prepared in distilled/deionized water (dd-water). Stock standard solution of $NaNO_2$ (1 mM), for the HPLC assay, was prepared and added in dd-water to produce final concentrations of 20, 30, 50, 100, 250, 500, 1000, and 1500 nM. DAN solution (0.05 mg/ml) was prepared in HCl (0.65 M). These solutions were made on the day of the experiments.

Zebrafish larvae and treatments

Zebrafish larvae were generated by natural pairwise mating in aquariums with a filtration system in series (ZebTEC, Tecniplast, Italy). They were staged and reared according to standard procedures [11]. Because the embryo receives nourishment from an attached yolk sac, no feeding was required until 7 dpf [12]. All protocols were approved by the institutional animal care committee (09/00135, CEUA–PUCRS) and followed the Canadian Council for Animal Care (CCAC) guide on the care and use of fish in research, teaching, and testing.

All experiments using animals were conducted in triplicates containing a pool of 20 larvae each. The concentration of SNP was selected on the basis of the previous publication of Pelster and coworkers [9]. Survival curves for LPS and $CuSO_4$ were performed to choose a sublethal concentration to induce inflammation. For this purpose, two replicates were made with 15 larvae for each concentration. The treatments were performed in 6-well culture plates.

NO_2^- levels were determined in zebrafish larvae at 0 dpf (1 h postfertilization) and at 1, 3, 5, and 7 dpf. In a separate series of experiments, the larvae with 7 dpf were incubated for 1 h with the nonselective NOS inhibitor, L-NAME (1 and 5 μM), to confirm that NO_2^- detected in our protocol is derived from NOS.

To determine whether NO_2^- assay could be used to detect changes in NO production associated with stress situations, we performed three additional sets of experiments. Larvae with 7 dpf were treated with SNP (1 mM), an NO donor used as positive control, $CuSO_4$ (10 μM), a chemical inducer of inflammation, and LPS (150 μg/ml), an infectious stimulus. The agents used to stimulate NO release were directly added to water, and NO_2^- was evaluated at 4 h for SNP and at 4 and 24 h for LPS and $CuSO_4$.

Sample preparation

Zebrafish larvae stored at –80 °C in 500 μl of phosphate-buffered saline (PBS, pH 7.3) were thawed and washed four times with 1 ml of PBS to eliminate any environmental contamination of NO_2^-. After cleaning procedures, the samples were homogenized in 500 μl of cold PBS using a glass–Teflon homogenizer immersed in ice. The samples were centrifuged at 13,500*g* for 5 min at 4 °C in 1.5-ml tubes, and the supernatants were collected for NO_2^- analysis.

The samples were placed in 0.6-ml polypropylene tubes. DAN working solution (10 μl) was added to each polypropylene tube with 50 μl of dd-water and 50-μl samples, either standards or blank (dd-water/PBS, 1:1). The tubes were capped, protected from light, mixed, and incubated for 15 min at room temperature. The reaction was stopped by adding 15 μl of NaOH (1 M). This reaction mixture was maintained at 4 °C in the dark and used for the chromatographic separation within 6 h. Then 20 μl of this solution was injected into the HPLC system.

The total protein concentration of zebrafish larvae homogenates was determined according to the method of Bradford [13]. The total weight of larvae was not used to correct the results because the concentration of the protein has a better correlation with the quality of the homogenate and, consequently, with the release of the analytes for the material used for quantification (supernatant).

Chromatographic conditions and equipment

An HPLC system equipped with an isocratic pump, a fluorescence detector, a degasser, and a manual injection system was used (all HPLC components and software ChemStation were obtained from Agilent Technologies, Santa Clara, CA, USA). Chromatographic separations were performed using a reverse-phase column (150 × 4 mm, 5 μm, Agilent 100 RP-18 EC). The column was protected by a guard column (4 × 4 mm, 5 μm, Agilent 100 RP-18 EC) and was maintained at 22 ± 2 °C. The flow rate of the acetonitrile–phosphate (35:65, v/v) buffer (50 mM, pH 8.2) mobile phase was 0.8 ml/min, and the fluorescence was monitored with excitation at 355 nm and emission at 460 nm.

To confirm the specificity and accuracy of the method, an NO analyzer (model 270B NOA, Sievers Instruments, Boulder, CO, USA) was used [14].

Validation of HPLC method and quality control procedures

The HPLC method was quantitatively evaluated in terms of sensitivity, specificity, precision, accuracy, linearity, and recovery. We tested the levels of NO_2^- in dd-water obtained from Deionizator Permution and water obtained from a Millipore system to choose the one with the lowest levels of NO_2^- for cleaning materials and

the preparation of solutions used in the tests. The solutions for the preparation of the homogenate were also tested (saline and PBS). Six aliquots of each solvent were tested and were analyzed as the samples.

Statistical analysis

The results of validation are presented as means ± standard deviations, and precision and accuracy are expressed as percentages. The linear relationship between concentrations of NO_2^- obtained by HPLC and NO analyzer was obtained using the Pearson correlation coefficient. The matching (between methods) was analyzed with the Bland–Altman method. Survival curves were estimated using the Kaplan–Meier analysis. Statistical comparison of the data from NO_2^- determination in zebrafish larvae was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Values of $P < 0.05$ were considered as significant.

Results

Chromatographic behavior and specificity

Laboratory plasticware and glassware and buffer solutions are common sources of contamination, and considerable attention needs to be paid to the water quality. Problems may also be encountered regarding the procedures for the extraction of samples and assay reproducibility, again often arising from NO_2^- and NO_3 contamination [1]. In the preparation of solutions and curves of NO_2^-, dd-water was used by presenting basal levels of NO_2^- 45% lower than Milli-Q ultrapure water. For the preparation of homogenates, PBS was used and showed basal levels of NO_2^- 59% lower than sterile saline solution.

The fluorometric assay for NO_2^- detection is based on the reaction of NO_2^- with DAN under acid conditions to form fluorescent 2,3-naphthotriazole (NAT). For specific analysis of NAT, the reverse-phase HPLC method has been used to separate NAT from DAN and other fluorescent substances present in biological samples [15,16]. The retention times of DAN and NAT were 3.0 and 5.9 min, respectively. Reliable separation of the analysis was achieved with adequate retention times for routine analysis, with runs less than 7 min. Fig. 1 shows representative chromatograms of NAT from blank (Fig. 1A) and zebrafish samples with 247 nM NO_2^- (Fig. 1B).

Specificity was evaluated by determining the retention time of DAN and NAT and also by analyzing six homogenates ($n = 6$) from different reproductions to check endogenous interference components. All tested zebrafish larvae homogenates were free for endogenous interference in this assay. Moreover, to confirm the specificity of our method for NO_2^-, three different curves were made and analyzed using the method described in this article and in an NO analyzer. Fig. 2 shows a high correlation between NO_2^- measured by HPLC and NO_2^- measured by NO analyzer (Pearson correlation coefficient = 0.9619, $P < 0.0001$). The slope of the regression lines between the two assays was 1.028. Moreover, Bland–Altman analysis (Fig. 3) showed that the concentrations of NO_2^- measured by two methods are not significantly different. Applying the Bland–Altman plot to measure the difference between NO_2^- by HPLC and NO Analyzer, we found a bias mean difference of 7.07 nM and a 95% limit of agreement from –98.85 to 112.98 nM.

Precision and accuracy

The precision and accuracy were determined by analysis of four different concentrations, covering the low, medium, and higher ranges of the calibration curves. The intra- and interday precision and accuracy data are shown in Table 1. Precision is expressed as a percentage coefficient of variation (CV), and accuracy is expressed as a percentage of the added concentration (found value × 100/added value). The interday precision and accuracy were determined over a period of 1 week.

Linearity

The calibration curves were linear using different calibrating standards containing known amounts of NO_2^- in the range of 30 to 1500 nM. The calibration equation was $y = 0.04543138x + 0.1075868$ (where y is the peak area and x is the concentration in nM) with a correlation coefficient of $r = 0.9996$.

Sensitivity

The limit of detection (LOD) was determined as being the detected concentration with an area greater than that presented in the blank prepared with dd-water (8.0 nM). The limit of quantification (LOQ) is the lowest analyzed amount, which can be measured with defined precision and accuracy and reproducible with CV less than 20% and accuracy of 80% to 120% [17]. The LOQ were found to be 21 ± 4 nM with CV of 18.58% and accuracy of 103.98% for NO_2.

NO_2 recovery

The recovery for NO_2^- was determined by spiking known quantities of standards into 7 dpf larvae homogenate to obtain low, medium, and high ranges of the calibration curve. The results were obtained by calculating the difference between NO_2^- before and after spiking standards in samples. Results of samples were divided by added concentrations, and the results were multiplied by 100. The recoveries of NO_2^- to 30, 250, 1000, and 1500 nM are expressed as means ± standard deviations, revealing results of 90 ± 4, 93 ± 2, 95 ± 2, and 97 ± 1, respectively.

NO levels, blockade with L-NAME, and stimulation with SNP, LPS, and $CuSO_4$

First, zebrafish larvae of 0, 1, 3, 5, and 7 dpf were evaluated to determine the NO_2^- levels during the early days of development. The results show that the concentration of NO_2^- increased gradually after fertilization, as shown in Fig. 4. In the inset of Fig. 4, it is shown that L-NAME treatment decreased the basal concentrations of NO_2^-, demonstrating that the NO increase until 7 dpf is likely the result of a gradual augmentation in the activity of NOS.

The survival curves to choose sublethal concentrations of LPS and $CuSO_4$ to induce inflammation are shown in Fig. 5. The concentration chosen was the one that induced mortality between 10% and 20% (150 μg/ml and 10 μM induced 16.7% and 11.5% of mortality for LPS and $CuSO_4$, respectively). The SNP was assessed only at 4 h because this agent is a short-acting NO donor [18]. For LPS and $CuSO_4$ groups, the time points of 4 and 24 h were used to determine the best periods for the establishment of an acute inflammatory response. The zebrafish larvae challenged to SNP for 4 h (5200 ± 340 nmol/mg protein), to LPS for 4 h (1040 ± 50 nmol/mg protein), and to $CuSO_4$ for 24 h (1300 ± 160 nmol/mg protein) showed a significant increase of NO_2^- levels when compared with the control groups (790 ± 50, 790 ± 90, and 760 ± 90 nmol/mg protein, respectively), with significance levels of $P < 0.001$, $P < 0.01$, and $P < 0.01$, respectively, as shown in Fig. 6.

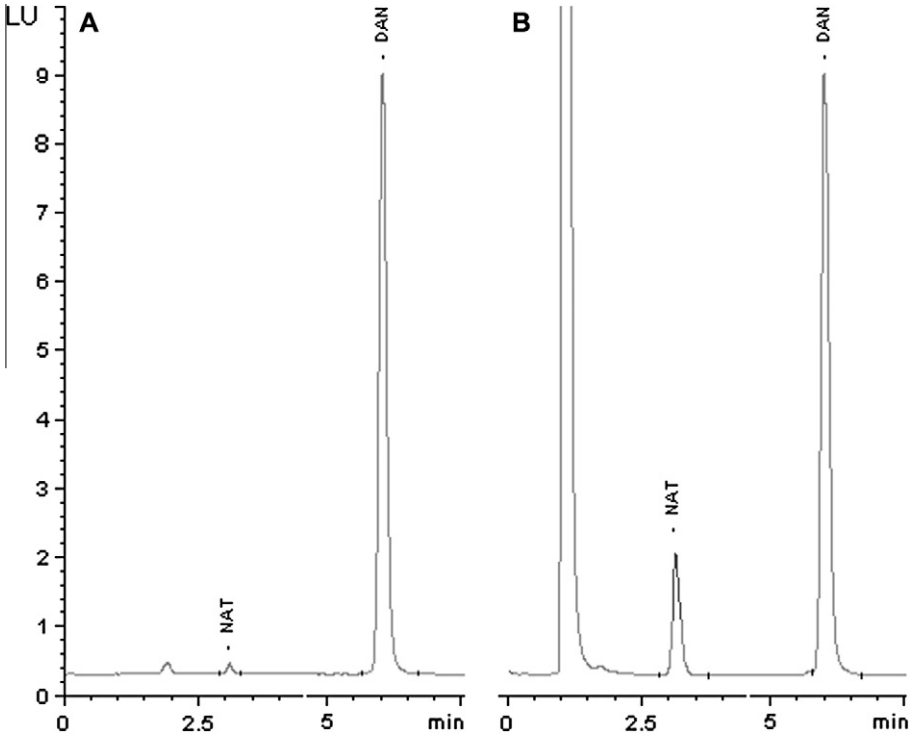

Fig.1. Typical chromatograms of NAT from blank (A) and zebrafish sample with 247 nM (B) of NO_2^-.

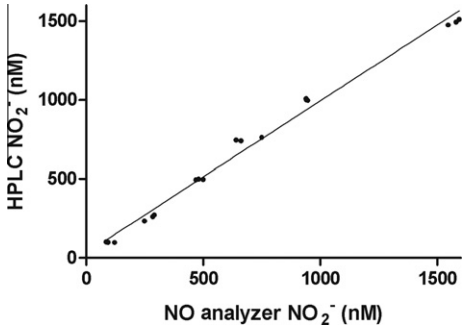

Fig.2. Correlation between the concentrations of NO_2^- obtained by HPLC and those obtained by NO analyzer. The linear relationship between concentrations was obtained using the Pearson correlation coefficient.

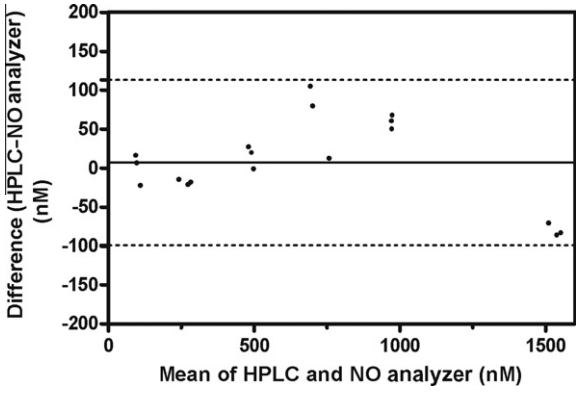

Fig.3. Bland–Altman bias plot of the difference between the NO_2^- concentrations obtained by HPLC and NO analyzer and the mean of the two measurements. The thick solid line represents the bias between the assays, and the dashed lines represent 95% limits of agreement.

Discussion

Several animal models have been used in toxicological and pharmacological studies. Zebrafish has emerged as a promising experimental model, and developing methods for the quantification of different biochemical and physiological markers are extremely important for its consolidation [19].

In this study, we presented a new, simple, rapid, and suitable HPLC method with fluorescence detection for determination of NO_2^- in homogenates of zebrafish larvae. The method presented here employs an extremely simple sample preparation that does not need internal standard, using a small sample volume. Furthermore, we achieved satisfactory separation under isocratic conditions. These characteristics are easily found in many laboratories. Moreover, because we were working with low levels of protein and a very sensitive method, a simple dilution was able to prevent any interference from chlorine or protein. Thus, it was not necessary to use processes of precipitation or ultrafiltration of proteins and/or removal of chlorine from samples using cation exchange chromatography, precipitation with silver reagents, or silver-based solid-phase extraction C18 [1,15].

When we compared the data quantified by HPLC and by NO analyzer, no significant differences were found in the results, confirming the specificity and accuracy of the method developed. Moreover, the curve determined by the analyzer showed sensitivity only above 100 nM, making the new method described here more sensitive. Other methods for the determination of NO in zebrafish larvae employ procedures based on the use of chemical probes or molecular biology assays [19,20]. Although these methods are efficient, reagents and equipment are not always available in laboratories. Thus, we provide a new sensitive, safe, and fast methodology for quantifying NO_2^-. A disadvantage of the proposed method is the impossibility of directly assessing NO_3^- levels, which might be reached through NO_3^- reduction, and the subsequent determination according to the technique described here. During inflammatory challenges, not only is NO formation

Table 1
Intra- and interday precision and accuracy for NO_2^- quantification by HPLC.

NO_2^- added (nM)	Intraday ($n = 6$)			NO_2^- added (nM)	Interday ($n = 6$)		
	Found (nM, mean ± SD)	Precision (CV, %)	Accuracy (%)		Found (nM, mean ± SD)	Precision (CV, %)	Accuracy (%)
30	32 ± 2	5.12	106.1	30	32 ± 2	7.14	108.3
250	249 ± 15	5.98	99.73	250	251 ± 12	4.88	100.34
1000	998 ± 21	2.08	99.76	1000	1003 ± 25	2.53	100.28
1500	1495 ± 23	1.51	99.68	1500	1499 ± 33	2.17	99.91

Note. SD, standard deviation; CV, coefficient of variation.

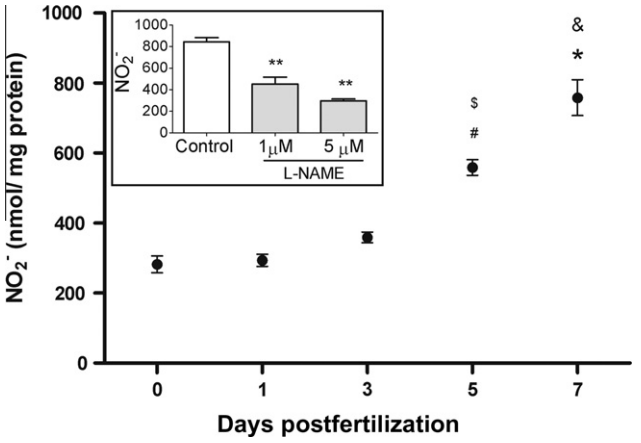

Fig.4. NO_2^- levels during the early days of development. Each point represents the mean of three homogenates (20 zebrafish larvae each), and the vertical lines show the standard deviations. An asterisk (∗) indicates $P < 0.001$ when compared with 0, 1, and 3 dpf; an ampersand (&) indicates $P < 0.01$ when compared with 5 dpf; a pound sign (#) indicates $P < 0.001$ when compared with 0 and 1 dpf; a dollar sign ($) indicates $P < 0.01$ when compared with 3 dpf. The inset shows the effect of L-NAME on the NO_2^- concentrations in zebrafish larvae of 7 dpf. Asterisks denote the significance levels in comparison with control values: $^*P < 0.05$; $^{**}P < 0.01$. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

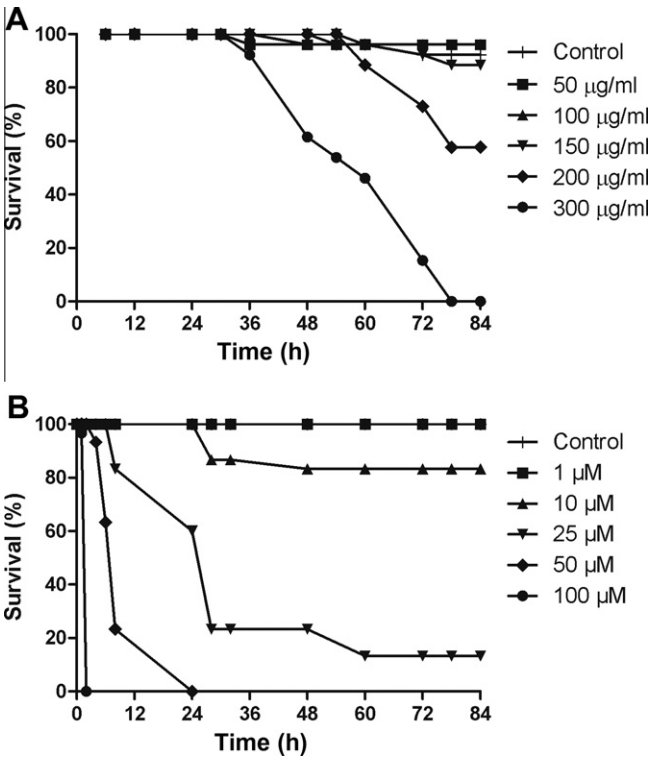

Fig.5. Kaplan–Meier survival curves from zebrafish larvae challenged with different concentrations of LPS (A) and $CuSO_4$ (B).

increased, but also the level of superoxide and other reactive oxygen species is augmented, providing multiple routes for oxidative breakdown of NO (e.g., by peroxynitrite formation or myeloperoxidase-catalyzed nitrogen dioxide formation in the presence of nitrite and hydrogen peroxide), leading to NO_3^- formation. Therefore, a limitation of the current technique is that NO ending up as NO_3^- is not detected, which could lead to underestimation of NO under inflammatory conditions (e.g., LPS treatment). Among the possible techniques used to avoid NO_3^- reduction are fluorescence probes such as 4,5-diaminofluorescein (DAF-2). The fluorescein reacts with an oxidation product of NO to the highly fluorescent DAF-2T triazol fluorescein by the known reaction mechanism. This probe is used to measure NO extracellularly and could be adapted to other experimental models [21].

Rico and coworkers recently suggested further investigations about the functional role of NO in zebrafish to evaluate changes in this system induced by pharmacological approaches and toxic agents [22]. In addition to validation of an NO_2^- determination method, a series of biological assays, including unpublished results, demonstrates the efficiency and applicability of the new method. The determination NO_2^- in zebrafish larvae of different days postfertilization showed the difference in NO concentrations according to the stage of development. These findings are consistent with the fast development of these animals, as demonstrated by Lepiller and coworkers [20]. One proposed mechanism for the effects of NO in developmental processes is a suppressive influence on DNA synthesis, whereby NO acts as a negative regulator on precursor cells, affecting the balance of cell proliferation, differentiation, and apoptosis [10,23]. The differentiated expression of NOS isoforms in certain tissues at different developmental stages indicates that temporal and spatial NO-mediated activities may be regulated by distinct NOS-producing systems [10]. For example, the expression of NOS in zebrafish embryos was detected at 16 hours postfertilization (hpf) in the hypothalamus, and it was present in discrete central nervous system locations after 3 dpf [22]. Additional studies are still required to better characterize the NOS isoforms related to zebrafish postfertilization NO production.

Several studies have shown the multifunctional properties of NO in various normal and pathophysiological events in early life processes [10,24–26]. NO production is increased in a variety of diseases, and several classic signs of inflammation are reversed by NOS inhibitors. Zebrafish is a good model for studying inflammation and infectious diseases; for instance, Watzke and coworkers and Novoa and coworkers showed that LPS was able to induce the expression of proinflammatory cytokine genes as part of the innate immune responses in zebrafish embryos and larvae by immersion in bacteria or bacterial LPS [2,27]. D'Alençon and coworkers showed that exposure of fish larvae to sublethal concentrations of $CuSO_4$ selectively damages the sensory hair cell population, inducing infiltration of leukocytes, release of reactive oxygen species, and a robust acute inflammatory response [28–30]. The method proposed here was efficient at monitoring changes in NO production in zebrafish

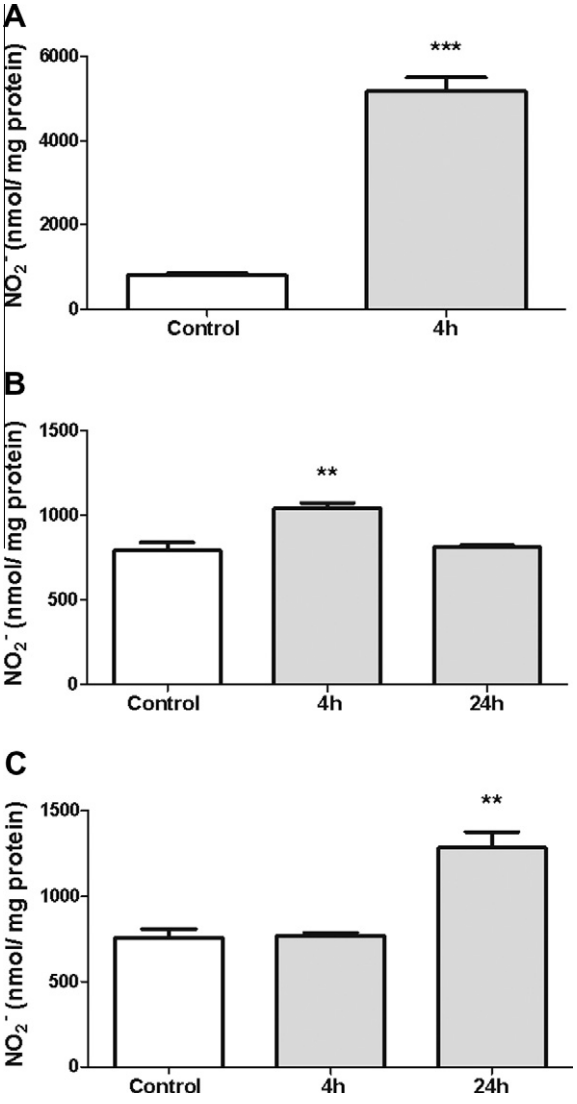

Fig.6. Zebrafish larvae were treated with 1 mM SNP (A), 150 μg/ml LPS (B), and 10 μM $CuSO_4$ (C). Each column represents the mean of three homogenates (20 zebrafish larvae each), and the vertical lines show the standard deviations. Asterisks denote the significance levels in comparison with control values: $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

larvae at first days postfertilization when stimulated with NO donor or under inflammation induced by infection and chemical agents. NO_2^- levels were found to be significantly increased in LPS-induced inflammation at 4 h, whereas inflammation induced by $CuSO_4$ showed a significant increase at 24 h. This probably occurred because the inflammation induced by LPS involves an infectious process and NO formation is one of the first-line defenses against invading microbial organisms, including parasites, bacteria, and viruses [31]. In addition, the later release of NO induced by $CuSO_4$, compared with LPS, might likely occur because the copper is taken up from water and accumulates in zebrafish in a time- and concentration-dependent manner [32]. Accordingly, Craig and coworkers showed that the highest concentrations of copper in the liver and gills of zebrafish are found after 24 h of exposure to this metal [33]. The low response using LPS might be explained by differences in the functionality of TLR-4 in this experimental organism, which has a known weak responsiveness to LPS [34].

In summary, NO_2^- was determined with high sensitivity, accuracy, and precision using a very simple, quick, and inexpensive method. This method was shown to be efficient at monitoring changes in physiological NO production during the development phase and pathophysiological conditions such as inflammation. It is tempting to propose that this method is a powerful tool to be applied for NO studies in zebrafish larvae. In addition, NO levels during the early stages of development showed a gradual increase from fertilization until day 7 of life, and the larvae exposed to inflammatory agents and NO donor showed a significant release of NO.

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