



# Rat Pial Microvascular Changes During Cerebral Blood Flow Decrease and Recovery: Effects of Cyanidin Administration

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The reactive oxygen species (ROS) are known to play a major role in many pathophysiological conditions, such as ischemia and reperfusion injury. The present study was aimed to evaluate the *in vivo* cyanidin (anthocyanin) effects on damages induced by rat pial microvascular hypoperfusion-reperfusion injury by cerebral blood flow decrease (CBFD) and subsequent cerebral blood flow recovery (CBFR). In particular, the main purpose was to detect changes in ROS production after cyanidin administration. Rat pial microvasculature was investigated using fluorescence microscopy through a cranial window (closed); Strahler's method was utilized to define the geometric features of pial vessels. ROS production was investigated *in vivo* by 2'-7'-dichlorofluorescein-diacetate assay and neuronal damage was measured on isolated brain sections by 2,3,5-triphenyltetrazolium chloride staining. After 30 min of CBFD, induced by bilateral common carotid artery occlusion, and 60 min of CBFR, rats showed decrease of arteriolar diameter and capillary perfusion; furthermore, increase in microvascular leakage and leukocyte adhesion was observed. Conversely, cyanidin administration induced dose-related arteriolar dilation, reduction in microvascular permeability as well as leukocyte adhesion when compared to animals subjected to restriction of cerebral blood flow; moreover, capillary perfusion was protected. ROS generation increase and marked neuronal damage were detected in animals subjected to CBFD and CBFR. On the other hand, cyanidin was able to reduce ROS generation and neuronal damage. In conclusion, cyanidin treatment showed dose-related protective effects on rat pial microcirculation during CBFD and subsequent CBFR, inducing arteriolar dilation by nitric oxide release and inhibiting ROS formation, consequently preserving the blood brain barrier integrity.

**Keywords:** cyanidin, cerebral blood flow reduction, reperfusion, pial microcirculation, reactive oxygen species, neuronal damage

## INTRODUCTION

Many evidences indicate that a diet rich in antioxidants is associated to a decreased incidence of cardiovascular diseases, such as stroke, acute myocardial disease or cancer (Galvano et al., 2004; Lapi et al., 2016). Anthocyanins, belonging to polyphenol family, are one of the natural antioxidants responsible of fruit and flower colors (red, orange and blue) and play an important role in counteracting the oxidative stress induced by reactive oxygen species (ROS). These radicals have been related to different pathophysiological conditions (Serraino et al., 2003; Accetta et al., 2016; Mondola et al., 2016). Higher ROS production, indeed, appears to be associated to the pathogenesis of ischemia/reperfusion injury (Tsuda et al., 1999). On the other hand, the protective role of natural anthocyanins has been demonstrated to be effective against ischemia/reperfusion injury in different organs, such as kidney, heart and intestine (Jaksevic et al., 2013; Quintieri et al., 2013; Isaak et al., 2017). Moreover, we previously demonstrated the protective effects of *Vaccinium myrtillus* extract supplementation to the diet (containing 34.7% of anthocyanins) on hamster pial microcirculation during brain hypoperfusion-reperfusion. In particular, after 2, 4, and 6 months of oral supplementation, anthocyanins were able to counteract microvascular changes such as arteriolar vasoconstriction, increase of microvascular permeability and leukocyte adhesion (Mastantuono et al., 2016).

Cyanidin, belonging to the anthocyanin family, has been widely studied in *in vivo* and *in vitro* models (Galvano et al., 2004). In particular, Aguirre et al. and Fratantonio et al. observed that Cyanidin-3-O-glucoside presents many properties, such as anti-inflammatory and anti-tumor effects (Fratantonio et al., 2016; Olivas-Aguirre et al., 2016). Furthermore, this substance appears to enhance the release of nitric oxide (NO) and other vasodilating factors, thereby improving endothelial-dependent vasodilation (Sivasinprasasn et al., 2016). It is worth noting that ORAC (oxygen radical absorbance capacity) activity is highest for cyanidin compared to other anthocyanins (Zheng and Wang, 2003). Tsuda et al. have demonstrated that rats, treated with an orally administered Cyanidin-3-O-glucoside for 14 days, were preserved from hepatic I/R damage (Tsuda et al., 1999).

The aim of this study was to investigate the *in vivo* effects of cyanidin on oxidative stress and changes in rat pial microvasculature determined by 30 min of cerebral blood flow decrease (CBFD) and 60 min of cerebral blood flow recovery (CBFR). To do this, we evaluated ROS production during CBFD and CBFR, respectively, and assessed the antioxidant properties of cyanidin using 2'-7'-dichlorofluorescein-diacetate (DCFH-DA) assay. Finally, the neuronal damage was quantified by 2,3,5-triphenyltetrazolium chloride (TTC) staining.

## MATERIALS AND METHODS

### Experimental Groups

Experiments were carried out utilizing male Wistar rats, 250–300g (Harlan, Italy), randomly assigned to three groups, as reported in **Table 1**: (1) sham group (SO group), subjected to the same surgical procedure of the other experimental groups

without changes in cerebral blood flow; (2) reduced blood flow group (RF group) and (3) cyanidin-treated group (Cy group) underwent 30 min CBFD plus 60 min CBFR.

SO animals were divided in subgroups: (a) SO-Na subgroup ( $n = 14$ ) was injected with intravenous (i.v.) saline solution (0.9% NaCl); (b) SO-Cy subgroup ( $n = 10$ ), successively divided in SO-Cy<sub>1</sub> ( $n = 5$ ) and SO-Cy<sub>2</sub> ( $n = 5$ ) subgroups, received i.v. cyanidin, 10 mg/kg body weight (b.w.) or 20 mg/kg b.w., respectively; (c) SO-L subgroup ( $n = 5$ ) was infused with i.v. N5-(1-iminoethyl)-L-ornithine (L-NIO), 10 mg/kg b.w. The animals of each subgroup received the substances twice within 40 min interval.

RF group ( $n = 14$ ) was injected with i.v. saline solution (0.9% NaCl), 10 min before the CBFD and at the beginning of CBFR.

Cy group was differentiated in the following subgroups: (a) Cy<sub>1</sub> ( $n = 14$ ) and Cy<sub>2</sub> ( $n = 14$ ), administered with i.v. cyanidin, 10 mg/kg b.w. or 20 mg/kg b.w., respectively, 10 min before the CBFD and at the beginning of CBFR; (b) L/Cy<sub>2</sub> subgroup ( $n = 14$ ) was administered with i.v. L-NIO, 10 mg/kg b.w., prior to i.v. higher dosage cyanidin (20 mg/kg b.w.).

Five animals for SO-Na subgroup, RF and Cy groups were investigated by *in vivo* fluorescence microscopy, to detect microcirculation damage; six rats were utilized to assess oxidative stress by DCFH-DA assay after CBFD ( $n = 3$ ) and after CBFR ( $n = 3$ ); in three animals tissue damage was evaluated by TTC staining. The rats belonging to the SO-Cy<sub>2</sub> and SO-L subgroups were utilized only for microcirculation investigations.

### Drug Administration

Each utilized drug (cyanidin or L-NIO) was dissolved in 0.5 mL saline solution and, successively, i.v. injected to rats within 3 min, 10 min before CBFD and at the beginning of CBFR.

We tested the effects of two cyanidin doses: 10 or 20 mg/kg b.w. Pilot experiments indicated that cyanidin dosages below 10 mg/kg b.w. were ineffective on the pial microvasculature; on the other hand, dosages higher than 20 mg/kg b.w. did not improve microvascular protection detected in the animals administered with 20 mg/kg b.w. cyanidin before and after CBFD. In **Table 2** we reported the data about animals treated with cyanidin at a dosage of 5 mg/kg or 30 mg/kg b.w. (chosen as reference values) administered 10 min before CBFD and at the beginning of CBFR.

Moreover, L-NIO, known to inhibit the NO release (Moreau et al., 1995; Lapi et al., 2012), was administered at the dosage of 10 mg/kg b.w., 10 min before i.v. infusion of higher dosage cyanidin (20 mg/kg b.w.). In pilot experiments L-NIO, 10 mg/kg b.w., was effective in blunting arteriolar dilation determined by i.v. injection of 10 mg/4 min L-arginine (diameter increase by  $22.8 \pm 2.0\%$ , compared to basal values) or in abolishing vasodilation due to topical administration of 100  $\mu$ M acetylcholine (diameter increase by  $5.0 \pm 1.5\%$ , compared to basal values).

The protocol of drug administration was previously described (Lapi et al., 2016). Appropriately mixing 2'-7'-dichlorofluorescein-diacetate (DCFH-DA) and artificial cerebrospinal fluid (aCSF) allowed us to superfuse the pial layer with 250 mM DCFH-DA solution (Watanabe, 1998) for 30 min after CBFD. Sigma Chemical, St. Louis, MO, USA supplied all drugs.

**TABLE 1** | Number of animals (N), experimental protocol and treatment of each group.

Group	Subgroup	N	Experimental protocol	Treatment
SO	SO-Na	14	Same surgical procedure as in the other experimental groups without changes in cerebral blood flow	saline solution, twice within 40 min interval
	SO-Cy <sub>1</sub>	5	Same surgical procedure as in the other experimental groups without changes in cerebral blood flow	10 mg/kg b.w. cyanidin, twice within 40 min interval
	SO-Cy <sub>2</sub>	5	Same surgical procedure as in the other experimental groups without changes in cerebral blood flow	20 mg/kg b.w. cyanidin, twice within 40 min interval
	SO-L	5	Same surgical procedure as in the other experimental groups without changes in cerebral blood flow	10 mg/kg b.w. L-NIO, twice within 40 min interval
RF		14	30 min CBFDF plus 60 min CBFR	saline solution, 10 min before CBFDF and CBFR
Cy	Cy <sub>1</sub>	14	30 min CBFDF plus 60 min CBFR	10 mg/kg b.w. cyanidin, 10 min before CBFDF and CBFR
	Cy <sub>2</sub>	14	30 min CBFDF plus 60 min CBFR	20 mg/kg b.w. cyanidin, 10 min before CBFDF and CBFR
	L/Cy <sub>2</sub>	14	30 min CBFDF plus 60 min CBFR	10 mg/kg b.w. L-NIO, prior to higher dosage cyanidin

**TABLE 2** | Variations of the main parameters in the two pilot groups: Cy<sub>A</sub> and Cy<sub>B</sub> subgroup (rats treated with cyanidin at the doses of 5 mg/kg b.w. or 30 mg/kg b.w.) and subjected to 30 min CBFR and 60 min CBFR, compared with RF group and Cy<sub>2</sub> subgroup.

Groups	Number of animals/arterioles (n)	Percent diameter changes (%)		Microvascular leakage (NGL)	Leukocyte adhesion (number of leukocyte/100 μm of venular length/30s)	Capillary perfusion (BFCL) (% reduction compared to baseline)
		After 30 min CBFDF	After 60 min CBFR			
RF group	5/25	85.0 ± 2.5 <sup>#</sup>	75 ± 3 <sup>#</sup>	0.48 ± 0.03 <sup>#</sup>	10 ± 2 <sup>#</sup>	48 ± 4 <sup>#</sup>
Cy <sub>A</sub> subgroup	5/25	90.5 ± 3.0 <sup>#</sup>	80.0 ± 2.5 <sup>#</sup>	0.40 ± 0.02 <sup>#</sup>	8 ± 2 <sup>#</sup>	40 ± 5 <sup>#</sup>
Cy <sub>B</sub> subgroup	5/25	134 ± 3.5 <sup>^</sup>	140 ± 4 <sup>^</sup>	0.24 ± 0.02 <sup>^</sup>	4 ± 1 <sup>^</sup>	12 ± 2 <sup>^</sup>
Cy <sub>2</sub> subgroup	5/25	130 ± 3 <sup>^</sup>	138.0 ± 3.5 <sup>^</sup>	0.25 ± 0.03 <sup>^</sup>	5 ± 2 <sup>^</sup>	14 ± 3 <sup>^</sup>

Data are reported as Mean ± SEM; <sup>#</sup>*p* < 0.01 vs. Cy<sub>A</sub> and Cy<sub>2</sub> subgroup, <sup>^</sup>*p* < 0.01 vs. RF group and Cy<sub>B</sub> subgroup.

## Rat Preparation

All experiments conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and to institutional rules for the care and handling of experimental animals, as previously reported (Lapi et al., 2012). The protocol was approved by the “Federico II” University Medical School of Naples, Ethical Committee (n° 2011/0059997, 24/05/2011).

Rats were anesthetized with intra peritoneal (i.p.) injection of α-chloralose, (60 mg/kg b.w. for induction; afterward 30 mg/kg b.w.) and mechanically ventilated after tracheotomy, according to the protocol previously reported (Lapi et al., 2012). Briefly, two catheters were placed, one in the right femoral artery and the other in the left femoral vein, respectively, for the measurement of arterial blood pressure and to inject the fluorescent tracers [fluorescein isothiocyanate bound to dextran, molecular weight 70 kDa (FD 70), 50 mg/100 g b.w., as 5% wt/vol solution in 3 min just once at the start of experiment after 30 min of the preparation stabilization; rhodamine 6G, 1 mg/100 g b.w. in 0.3 mL, as a bolus with supplemental injection throughout CBFDF and CBFR (final volume 0.3 mL·100 g<sup>-1</sup>·h<sup>-1</sup>) to label leukocytes for adhesion evaluation]. Both carotid arteries were prepared for clamping.

Blood gases were measured on arterial blood samples at 30 min intervals (ABL5; Radiometer, Copenhagen, Denmark). The parameters monitored in all animals were: heart rate, mean arterial blood pressure, respiratory CO<sub>2</sub> and blood gases values.

They were stable within physiological ranges. Rectal temperature was recorded and maintained at 37.0 ± 0.5°C, as previously reported (Lapi et al., 2016).

The visualization of pial microvasculature was carried out as previously reported (Morii et al., 1986; Ngai et al., 1988; Lapi et al., 2012). Briefly, a closed cranial window was positioned at the level of the left frontoparietal cortex through an incision in the skin to operate a craniotomy. Cerebral cortex was preserved by overheating caused by drilling with saline solution superfusion of the skull. The dura mater was gently cut and displayed on the corner; a quartz microscope coverglass was bound to the skull bone. Artificial cerebrospinal fluid was superfused the cerebral surface with a rate of 0.5 mL/min. The composition of the aCSF was 119.0 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0 mM NaH<sub>2</sub>PO<sub>4</sub>, 26.2 mM NaHCO<sub>3</sub>, 2.5 mM CaCl<sub>2</sub> and 11.0 mM glucose (equilibrated with 10.0% O<sub>2</sub>, 6.0% CO<sub>2</sub> and 84.0% N<sub>2</sub>; pH 7.38 ± 0.02).

The decrease in cerebral blood flow (CBFD) was produced by clamping both common carotid arteries, previously prepared. The clamping was removed after 30 min; thereafter the pial microvasculature was investigated during the recovery of cerebral blood flow (CBFR), lasting 60 min (Hudetz et al., 1985).

## Fluorescence Microscopy

A fluorescence microscope was utilized to study pial microvascular networks as previously described

(Lapi et al., 2012). In brief, the microscope (Leitz Orthoplan, Wetzlar, Germany) was equipped with long-distance objectives (2.5 x, numerical aperture (NA) 0.08; 10 x, NA 0.20; 20 x, NA 0.25; 32 x, NA 0.40) a 10x eyepiece. Moreover, a x10 eyepiece and a filter block (Ploemopak, Leitz) were used. A 100-Watt mercury lamp was used for epiillumination with the corresponding filters for FITC and rhodamine 6G. A heat filter prevented overheating of the preparations (Leitz KG1). Pial microvascular networks were televised with a DAGE MTI 300 low-light level camera and stored through a computer-based frame grabber (Pinnacle DC 10 plus, Avid Technology, Burlington, MA, USA).

## Geometric Detection of Microvascular Network

In each animal, first we characterized the arteriolar network by stop-frame images and pial arterioles were assigned order according to Strahler's method, starting from capillaries to the largest arterioles (centripetal method), as previously reported (Kassab et al., 1993; Lapi et al., 2008). In each experiment we studied one order 4 arteriole, two order 3 and two order 2 arterioles. Furthermore, we assessed the functional changes of each arteriolar order under the experimental conditions. We report, however, the results detected in order 2 arterioles.

## Microvascular Parameter Assessment

Microvascular parameters were measured off-line utilizing a computerized imaging technique, previously described in details by Lapi et al. (2012) and Lapi et al. (2016). Concisely, arteriolar diameters were measured with a computerized method, Microvascular Imaging Program (MIP), frame by frame. The increase in permeability was measured by evaluating fluorescent dextran extravasation from venules and expressed as normalized gray levels (NGL):  $NGL = (I - I_r)/I_r$ , where  $I_r$  is the baseline gray level at the microvasculature filling with fluorescence, and  $I$  is the value at the end of CBF/D or CBF/R. Gray levels were obtained using the MIP image program by average of 5 windows, measuring  $50 \times 50 \mu\text{m}$  (10x objective) and located outside the venules. During recordings the same regions of interest were localized by a computer-assisted device for XY movement of the microscope table.

Leukocytes sticking to the vessel walls (45 venules for every group) over a 30-s time-period were reported as number of adherent cells/100  $\mu\text{m}$  of venular length (v.l.)/30 s, utilizing appropriate magnification (20 x and 32 x, objectives) (Lapi et al., 2012). Perfused capillaries were evaluated as the length of the capillaries showing blood flow (BFCL), assessed by MIP image in an area of  $150 \times 150 \mu\text{m}$  (Lapi et al., 2016).

A Gould Windograf recorder (model 13-6615-10S, Gould, OH, USA) was utilized to record arterial blood pressure (mean), by Viggo-Spectramed P10E2 transducer; Oxnard, CA, USA, linked to catheterized femoral artery, and heart rate, as previously reported (Lapi et al., 2012). We measured the arterial blood gases (ABL5; Radiometer, Copenhagen, Denmark) at 30 min intervals, as previously reported (Lapi et al., 2012), as well as the hematocrit in basal conditions, at the end of CBF/D and CBF/R.

## ROS Production Evaluation

Superfusion of the pial layer with artificial cerebrospinal fluid, containing 250 mM 2'-7'-dichlorofluorescein-diacetate (DCFH-DA) at  $37.0 \pm 0.5^\circ$ , was carried out after 30 min CBF/D ( $n = 3$ ) or 60 min CBF/R ( $n = 3$ ), as previously reported (Lapi et al., 2013). DCFH-DA is widely used as a marker for oxidative stress of the cells and tissues (Wang and Joseph, 1999). DCF fluorescence intensity, related to the intracellular ROS level, was assessed using an appropriate filter (522 nm) and measured by NGL (Watanabe, 1998).

## Tissue Damage Estimation

At the end of CBF/R, rats were sacrificed to evaluate tissue damage. The brains were isolated and rostro-caudally cut into coronal sections (1 mm) with a vibratome (Campden Instrument, 752 M; Lafayette, IN, USA). Slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (20 min) at  $37^\circ\text{C}$  and in 10% formalin overnight, as previously reported (Lapi et al., 2013). TTC, a white salt, is reduced to red 1,3,5-triphenylformazan by dehydrogenases in living cells. The location and extent of necrotic areas were assessed by computerized image analysis (Image-Pro Plus; Rockville, MD, USA). Moreover, the infarct size was quantified by manual measurements, according to the following formula:  $[(\text{area of nonhypoperfused, or area not subjected to cerebral blood flow decrease, cortex or striatum} - \text{area of remaining hypoperfused, or area subjected to cerebral blood flow decrease, cortex or striatum})/\text{area of nonhypoperfused cortex or striatum}] \times 100$  (Bederson et al., 1986).

## Statistical Analysis

All data were reported as mean  $\pm$  SEM. Normal distribution of data was assessed with the Kolmogorov-Smirnov test. Parametric (Student's *t*-tests, ANOVA and Bonferroni post hoc test) or nonparametric tests (Wilcoxon, Mann-Whitney and Kruskal-Wallis tests) were utilized, according to data distribution; diameter and length data among experimental groups were compared with nonparametric tests, as previously reported (Lapi et al., 2016). Data derived from DCFH-DA treated rats were analyzed with non-parametric tests. SPSS 14.0 statistical package (IBM Italia, Segrate, MI, Italy) was used. Statistical significance was set at  $p < 0.05$ .

## RESULTS

Under baseline conditions, Strahler's method was used to differentiate arterioles in pial microvascular networks of all animals according to a centripetal scheme (Lapi et al., 2008). In particular, five orders of arterioles were observed, assigning order 5 to the largest vessels (mean diameter  $62.6 \pm 4.5 \mu\text{m}$ ) up to the smallest ones, identified as order 1 (mean diameter:  $15.8 \pm 2.0 \mu\text{m}$ ). Order 0 was assigned to the capillaries, sprouting from order 1 arterioles.

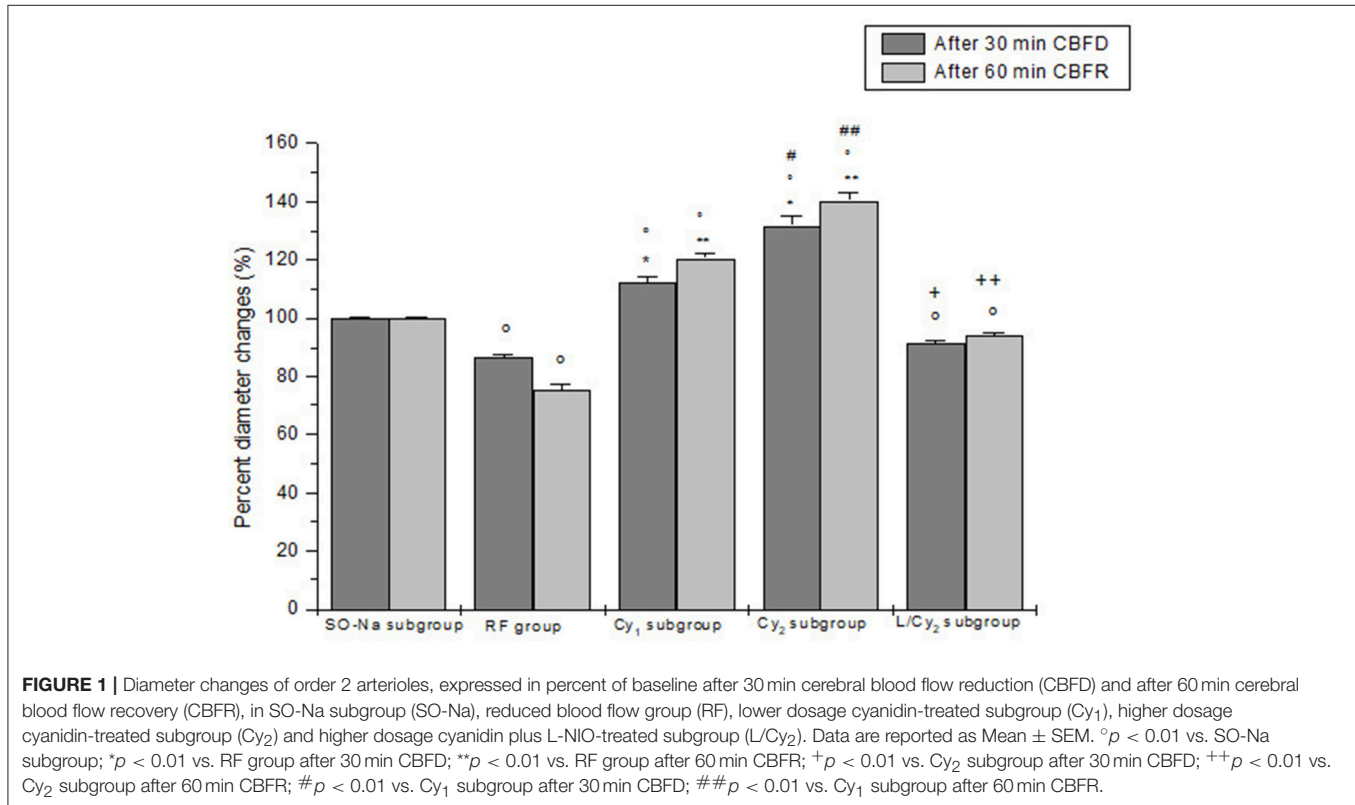
## SO Group

After the investigation period, no differences in microvascular parameters were detected in SO-Na subgroup, as reported in **Table 3** and **Figure 1**. Furthermore, DCF fluorescence intensity

**TABLE 3** | Variations of the main parameters at the end of reperfusion in SO-Na subgroup (SO-Na), reduced blood flow group (RF), lower dosage cyanidin-treated subgroup (Cy<sub>1</sub>), higher dosage cyanidin-treated subgroup (Cy<sub>2</sub>) and higher dosage cyanidin plus L-NIO-treated subgroup (L/Cy<sub>2</sub>).

Groups	Number of animals/arterioles (n)	Microvascular leakage (NGL)	Leukocyte adhesion (number of leukocyte/100 μm of venular length/30 s)	Capillary perfusion (BFCL) (% reduction compared to baseline)
SO-Na subgroup	5/25	0.02 ± 0.01	2 ± 1	0 ± 5
RF group	5/25	0.48 ± 0.03 <sup>§◊</sup>	10 ± 2 <sup>§◊</sup>	48 ± 4 <sup>§◊</sup>
Cy <sub>1</sub> subgroup	5/25	0.36 ± 0.02 <sup>§◊*</sup>	7 ± 1 <sup>§◊*</sup>	22 ± 6 <sup>§◊*</sup>
Cy <sub>2</sub> subgroup	5/25	0.25 ± 0.03 <sup>§◊*</sup>	5 ± 2 <sup>*</sup>	14 ± 3 <sup>§◊*</sup>
L/Cy <sub>2</sub> subgroup	5/25	0.27 ± 0.02 <sup>§◊*</sup>	6 ± 1 <sup>*</sup>	17 ± 5 <sup>§◊*</sup>

Leukocyte adhesion: n = 45 venules for each entry. Data are reported as Mean ± SEM; <sup>§</sup>p < 0.01 vs. baseline; <sup>◊</sup>p < 0.01 vs. SO-Na subgroup; <sup>\*</sup>p < 0.01 vs. RF group.



**FIGURE 1** | Diameter changes of order 2 arterioles, expressed in percent of baseline after 30 min cerebral blood flow reduction (CBFD) and after 60 min cerebral blood flow recovery (CBFR), in SO-Na subgroup (SO-Na), reduced blood flow group (RF), lower dosage cyanidin-treated subgroup (Cy<sub>1</sub>), higher dosage cyanidin-treated subgroup (Cy<sub>2</sub>) and higher dosage cyanidin plus L-NIO-treated subgroup (L/Cy<sub>2</sub>). Data are reported as Mean ± SEM. <sup>◊</sup>p < 0.01 vs. SO-Na subgroup; <sup>\*</sup>p < 0.01 vs. RF group after 30 min CBFD; <sup>\*\*</sup>p < 0.01 vs. RF group after 60 min CBFR; <sup>+</sup>p < 0.01 vs. Cy<sub>2</sub> subgroup after 30 min CBFD; <sup>++</sup>p < 0.01 vs. Cy<sub>2</sub> subgroup after 60 min CBFR; <sup>#</sup>p < 0.01 vs. Cy<sub>1</sub> subgroup after 30 min CBFD; <sup>##</sup>p < 0.01 vs. Cy<sub>1</sub> subgroup after 60 min CBFR.

did not change ( $0.04 \pm 0.02$  NGL) in the animals belonging to the SO-Na subgroup, superfused with DCFH-DA (**Figure 3A**).

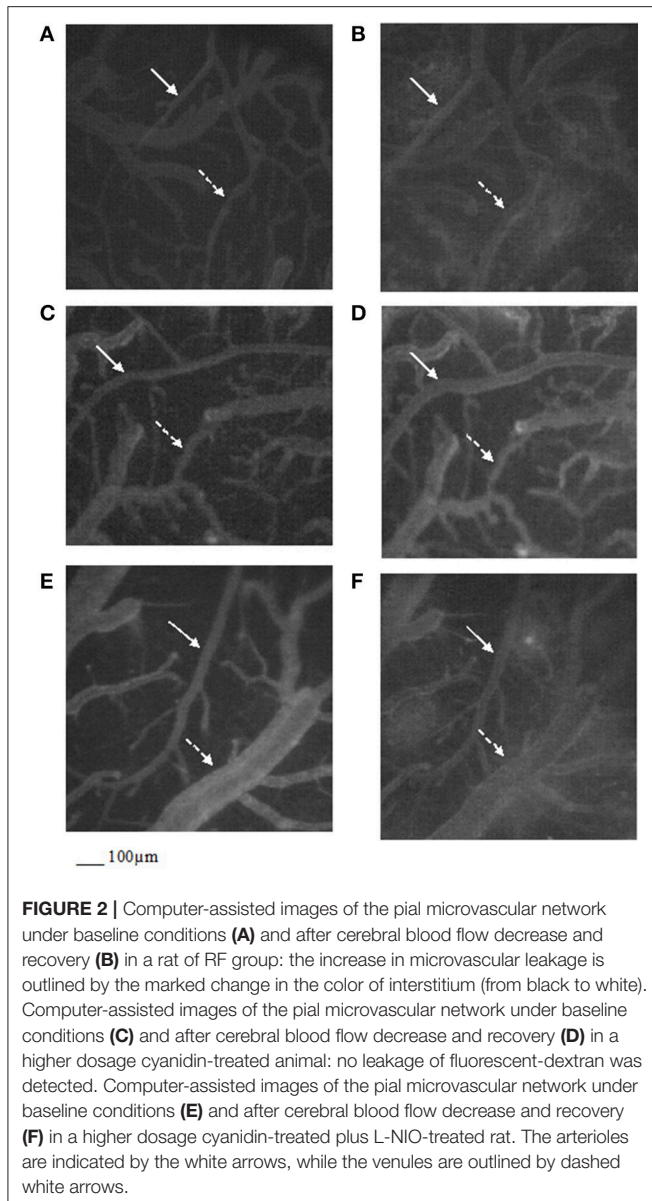
Cyanidin injection caused arteriolar dilation in dose-related manner: order 2 arteriole diameter increased by  $15.1 \pm 1.8$  and  $34.6 \pm 2.4\%$  of baseline ( $p < 0.01$  vs. baseline) in the rats of SO-Cy<sub>1</sub> and SO-Cy<sub>2</sub> subgroups, respectively. However, no significant changes in the other microvascular parameters and DCF fluorescence intensity were observed. Furthermore, after L-NIO injection, no significant variations of all parameters were detected in the animals of the SO-L subgroup.

## RF Group

The decrease in cerebral blood flow for 30 min was accompanied by a reduction in diameter of all arteriolar orders with a decrease by  $13.8 \pm 1.5\%$  of baseline in order 2 arterioles ( $p < 0.01$  vs.

baseline: mean diameter  $25.6 \pm 2.2 \mu\text{m}$ , and SO-Na subgroup; **Figure 1**). Leakage of fluorescent dextran was detected along the venules, indicating increased microvascular permeability ( $0.27 \pm 0.02$  NGL;  $p < 0.01$  vs. baseline and SO-Na subgroup). Furthermore, fluorescence intensity increase was observed in animals subjected to DCFH-DA superfusion, demonstrating an increased ROS generation (**Figure 3A**).

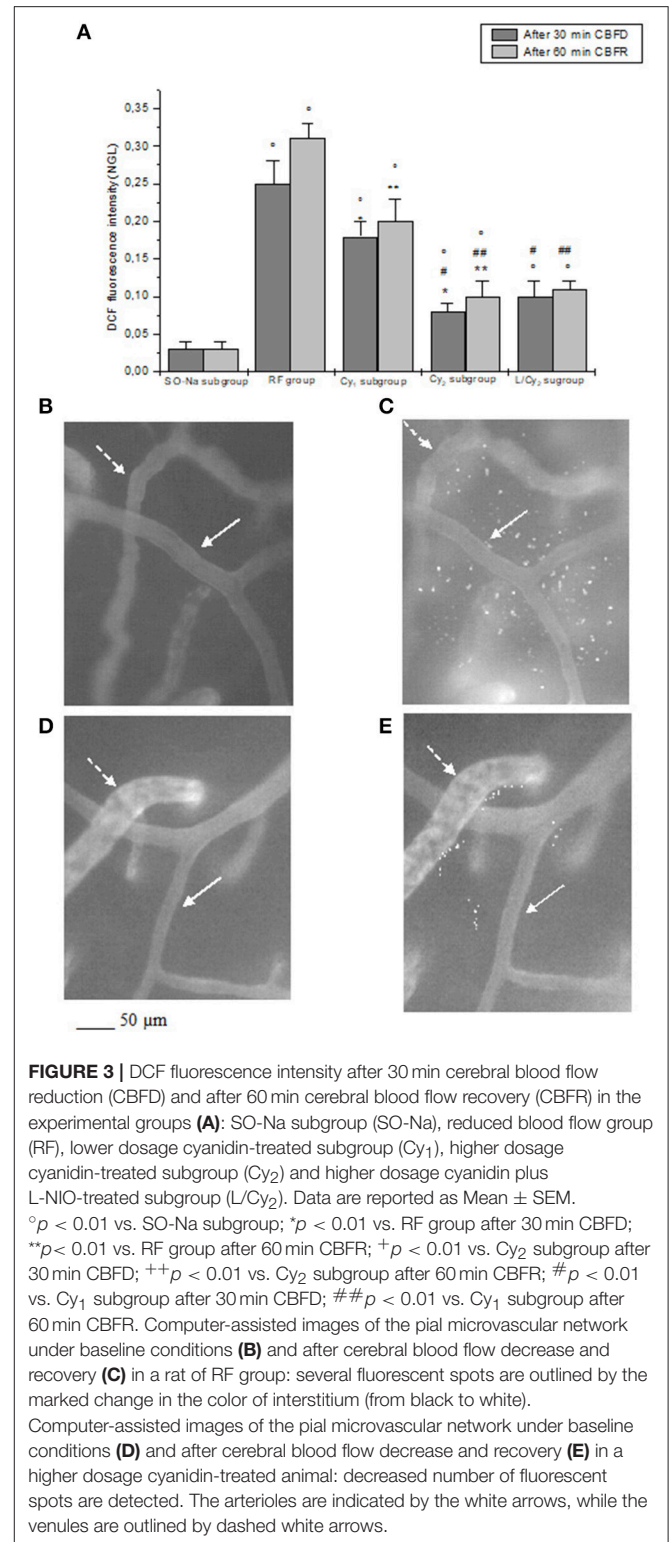
At the end of CBFR, all arteriolar orders presented a decrease in diameter when compared to baseline. Order 2 arteriole diameter diminished by  $24.5 \pm 2.0\%$  of baseline ( $p < 0.01$  vs. baseline and SO-Na subgroup; **Figure 1**). Moreover, dextran leakage significantly increased indicating marked microvascular permeability ( $0.48 \pm 0.03$  NGL;  $p < 0.01$  vs. baseline and SO-Na subgroup); there was also increase in leukocytes adhesion ( $10 \pm 2/100 \mu\text{m v.l./30 s}$ ;  $p < 0.01$  vs. baseline and SO-Na subgroup; **Figures 2A,B**). BFCL was reduced by  $48.0 \pm 4.0\%$  of baseline



( $p < 0.01$  vs. baseline and SO-Na subgroup) (Table 3). Finally, DCF fluorescence intensity was marked in the rats subjected to DCFH-DA perfusion, indicating a further increase in ROS formation: NGL were  $0.31 \pm 0.02$  ( $p < 0.01$  vs. SO-Na subgroup; Figures 3A–C).

### Cy Group

Cyanidin, at 10 mg/kg b.w. dosage (Cy<sub>1</sub> subgroup), prevented damage of the microvascular networks, inducing an increase in arteriolar diameter at the end of CBFD. In particular, order 2 arterioles dilated by  $12.5 \pm 1.8\%$  of baseline ( $p < 0.01$  vs. baseline: mean diameter  $25.2 \pm 2.3 \mu\text{m}$ , SO-Na subgroup and RF group; Figure 1). Microvascular permeability significantly decreased compared to RF group ( $0.22 \pm 0.03$  NGL;  $p < 0.01$  vs. baseline, SO-Na subgroup and RF group) as well as DCF



fluorescence intensity ( $0.18 \pm 0.02$  NGL; Figure 3A) ( $p < 0.01$  vs. baseline, SO-Na subgroup and RF group).

After 60 min CBFR, all pial arterioles dilated: order 2 arteriole diameter increased by  $20.5 \pm 1.5\%$  of baseline ( $p < 0.01$  vs. baseline, SO-Na subgroup and RF group; Figure 1).

Furthermore, fluorescent dextran leakage was significantly reduced compared to RF group ( $0.36 \pm 0.02$  NGL;  $p < 0.01$  vs. baseline, SO-Na subgroup and RF group) as well as leukocytes adhering to venular walls ( $7 \pm 1/100 \mu\text{m v.l./30 s}$ ;  $p < 0.01$  vs. baseline, SO-Na subgroup and RF group). BFCL decreased by  $22.0 \pm 2.2\%$  of baseline ( $p < 0.01$  vs. baseline, SO-Na subgroup and RF group) (Table 3). Finally, a slight increase in DCF fluorescence intensity was observed in rats belonging to the Cy<sub>1</sub> subgroup, after DCFH-DA superfusion: NGL were  $0.20 \pm 0.03$  ( $p < 0.01$  vs. baseline, SO-Na subgroup and RF group) (Figure 3A).

Cyanidin, at the dosage 20 mg/kg b.w. (Cy<sub>2</sub> subgroup), determined a marked dilation in all arterioles compared to Cy<sub>1</sub> subgroup at the end of CBF<sub>D</sub>. There was an increase in order 2 arteriole diameter by  $31.7 \pm 3.0\%$  of baseline ( $p < 0.01$  vs. baseline: mean diameter  $27.0 \pm 1.5 \mu\text{m}$ , SO-Na subgroup, RF group and Cy<sub>1</sub> subgroup; Figure 1). Microvascular leakage appeared to be blunted compared to animals of RF and Cy<sub>1</sub> subgroup ( $0.16 \pm 0.02$  NGL;  $p < 0.01$  vs. baseline, SO-Na subgroup, RF group and Cy<sub>1</sub> subgroup). Intensity in DCF fluorescence appeared to be slight ( $0.08 \pm 0.01$  NGL;  $p < 0.01$  vs. baseline, SO-Na subgroup, RF group and Cy<sub>1</sub> subgroup) (Figure 3A).

After 60 min CBF<sub>R</sub>, order 2 arteriole diameter increased up to  $40.5 \pm 2.8\%$  of baseline ( $p < 0.01$  vs. baseline, SO-Na subgroup,

RF group and Cy<sub>1</sub> subgroup; Figure 1). Additionally, there was a significant decrease in fluorescent dextran leakage when compared to RF and Cy<sub>1</sub> subgroup ( $0.25 \pm 0.03$  NGL;  $p < 0.01$  vs. baseline, SO-Na subgroup, RF group and Cy<sub>1</sub> subgroup). Leukocyte adherent to venules were  $5 \pm 2/100 \mu\text{m v.l./30 s}$ , while BFCL decreased by  $14.0 \pm 2.0\%$  of baseline ( $p < 0.01$  vs. baseline, SO-Na subgroup, RF group and Cy<sub>1</sub> subgroup) (Table 3 and Figures 2C,D). Finally, ROS production was completely counteracted by higher dosage cyanidin administration, as detected by reduction in fluorescence intensity in Cy<sub>2</sub> subgroup, superfused with DCFH-DA: NGL were  $0.10 \pm 0.02$  ( $p < 0.01$  vs. baseline, SO-Na subgroup, RF group and Cy<sub>1</sub> subgroup) (Figures 3A,D,E).

In L/Cy<sub>2</sub> subgroup, L-NIO injection (10 mg/kg b.w.) 10 min before cyanidin treatment at the higher dose, blunted cyanidin effects on arteriolar diameter within 30 min of CBF<sub>D</sub>. In particular, diameter of order 2 arterioles was reduced by  $8.7 \pm 1.2\%$  of baseline (mean diameter:  $24.3 \pm 1.7 \mu\text{m}$ ;  $p < 0.01$  vs. Cy<sub>2</sub> subgroup; Figure 1). Conversely, L-NIO administration did not alter cyanidin effects on microvascular permeability (Table 3).

At the end of CBF<sub>R</sub>, all order 2 pial arterioles constricted with a decrease by  $6.2 \pm 1.0\%$  of baseline diameter ( $p < 0.01$  vs. Cy<sub>2</sub> subgroup; Figure 1). However, microvascular permeability and adhesion of leukocytes were not influenced by L-NIO administration (Figures 2E,F). BFCL decreased by  $17.0 \pm 1.5\%$

**TABLE 4 |** (A) Mean arterial blood pressure (MABP), heart rate, respiratory CO<sub>2</sub> and (B) blood gases (pCO<sub>2</sub> and pO<sub>2</sub>) under baseline conditions, at the end of CBF<sub>D</sub> and CBF<sub>R</sub> in all subgroups.

Groups	MABP (mmHg)			Heart rate (bpm)			Respiratory CO <sub>2</sub> (mmHg)		
	Baseline	CBFD	CBFR	Baseline	CBFD	CBFR	Baseline	CBFD	CBFR
<b>A</b>									
SO-Na	100.0 ± 1.5	–	–	328.5 ± 1.8	–	–	30.5 ± 1.5	–	–
SO-Cy <sub>1</sub>	101.0 ± 1.6	–	–	330.0 ± 1.5	–	–	30.8 ± 1.6	–	–
SO-Cy <sub>2</sub>	101.0 ± 1.4	–	–	325.0 ± 1.3	–	–	30.6 ± 1.8	–	–
SO-L	102.0 ± 1.5	–	–	327.0 ± 1.9	–	–	29.8 ± 2.0	–	–
RF	101.0 ± 1.7	92.5 ± 1.6*	97.5 ± 1.8	320.0 ± 1.5	315.5 ± 1.7	324.0 ± 2.0	30.2 ± 1.8	29.3 ± 1.5	30.8 ± 1.4
Cy <sub>1</sub>	102.0 ± 1.8	93.7 ± 1.5*	99.7 ± 1.6	321.5 ± 1.7	318.0 ± 1.5	325.0 ± 2.0	30.5 ± 1.5	30.5 ± 1.4	31.0 ± 1.6
Cy <sub>2</sub>	104.0 ± 2.8	95.8 ± 1.4*	100.0 ± 2.5	328.0 ± 1.6	320.5 ± 1.8	330.0 ± 1.5	30.6 ± 1.8	30.5 ± 1.7	30.9 ± 1.5
L/Cy <sub>2</sub>	106.0 ± 2.5	97.5 ± 1.6*	101.5 ± 2.0	320.8 ± 1.8	318.0 ± 1.4	320 ± 2.5	30.8 ± 2.0	31.0 ± 1.7	30.8 ± 1.8
Groups	pCO <sub>2</sub> (mmHg)			pO <sub>2</sub> (mmHg)					
	Baseline	CBFD	CBFR	Baseline	CBFD	CBFR			
<b>B</b>									
SO-Na	41.5 ± 2.0	–	–	96.5 ± 2.0	–	–			
SO-Cy <sub>1</sub>	41.8 ± 1.8	–	–	97.0 ± 1.8	–	–			
SO-Cy <sub>2</sub>	41.0 ± 1.5	–	–	97.2 ± 1.7	–	–			
SO-L	42.0 ± 1.6	–	–	96.0 ± 2.2	–	–			
RF	40.2 ± 2.1	40.1 ± 1.8	40.1 ± 2.1	96.8 ± 2.0	96.1 ± 1.8	96.0 ± 1.8			
Cy <sub>1</sub>	40.8 ± 1.7	40.8 ± 1.6	40.5 ± 1.5	97.1 ± 1.7	98.0 ± 1.8	98.0 ± 1.5			
Cy <sub>2</sub>	40.6 ± 2.0	41.0 ± 1.9	40.9 ± 1.7	97.0 ± 1.8	97.5 ± 1.7	97.0 ± 2.0			
L/Cy <sub>2</sub>	40.8 ± 2.2	40.5 ± 2.0	40.4 ± 1.9	96.4 ± 2.1	96.0 ± 1.9	96.0 ± 1.7			

Data are reported as Mean ± SEM; \* $p < 0.01$  vs. baseline.





particular, the highest ORAC (oxygen radical absorbance capacity) activity has been demonstrated for cyanidin, compared to other anthocyanins (Zheng and Wang, 2003). Moreover, this polyphenol down-regulates plasminogen activator inhibitor-1 and pro-inflammatory cytokine IL-6, ameliorating human adipocytokine profile (Tsuda et al., 2006). It is interesting to note that isolated rat hearts are protected by oxidative stress, increased in several cardiovascular diseases, after treatment with cyanidin (Ziberna et al., 2012) as well as rat hepatic I/R damage is decreased by cyanidin (Tsuda et al., 1999). In the present study, cyanidin showed a strong antioxidant activity reducing ROS production, but at the same time was able to decrease adhesion of leukocytes to vessel walls blunting the damage induced by leukocyte activation, a mechanism effective in promoting ROS generation. Therefore, the decrease in leukocyte sticking to vessels walls and the reduction in ROS generation merged and reduced the effects of ROS on vessel wall leakage. These effects were effective in preventing marked microvascular damage, avoiding the disruption of blood-brain barrier and tissue edema. All together these vasodilatory and antioxidant properties preserved cerebral perfusion and prevented neuronal loss at the end of blood flow recovery in cyanidin-treated animals. Consequently, there was a reduction of the infarct size in cortical and striatal zones, compared to the rats subjected to decrease in cerebral blood flow and subsequent recovery.

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- Our data are in agreement with previous observations by Di Giacomo et al. who have observed the effects of Cyanidin-3-O-glucoside injection before the bilateral common carotid artery occlusion and during reperfusion. Their data indicate that cyanidin is able to reduce the lipid hydroperoxides and the expression of neuronal and inducible NOS and to increase the expression in endothelial nitric oxide synthase (eNOS) (Di Giacomo et al., 2012).
- In conclusion, our data are the first evidence in an *in vivo* study that rat pial microcirculation was protected against different mechanisms of damage: cyanidin was able to induce arteriolar dilation, to reduce oxidative stress and prevent neuronal loss. All these activities resulted in the protection of cerebral perfusion, blood brain barrier integrity and brain function. Therefore, cyanidin appears to be useful in counteracting ROS generation in brain circulation and to protect cerebral tissues.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TM, AC, and DL. Performed the experiments and the animal treatments: TM, MD, MC, LB, NS, AC, and DL. Analyzed the data: TM, MD, MC, LB, EM, GN, AC, and DL. Wrote the paper: TM, MD, MC, AC, and DL.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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