Effects of oral administration of different doses of purified micronized flavonoid fraction on microvascular reactivity after ischaemia/reperfusion in the hamster cheek pouch

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1   The effects of a purified micronized flavonoid fraction (S5682) on mean internal diameter and blood flow of arterioles and venules, as well as the functional capillary density (FCD) were evaluated in the hamster cheek pouch microvasculature before and after 90 min of total ischaemia.

2   Male hamsters were treated for ten days, twice a day, with oral doses of S5682 (5, 20, 80 and 160 mg kg^{-1} day^{-1}) or placebo (10% lactose solution). The cheek pouch preparation was placed under an intravital microscope coupled to a closed circuit TV system. Local ischaemia was obtained by a cuff mounted around the neck of the everted pouch where it leaves the mouth of the hamster.

3   Measurements were performed before ischaemia, at the onset of reperfusion and 10, 20, 30, 45 and 60 min thereafter. Diameters were measured by means of an image shearing device. Red blood cell (RBC) velocity was analysed by use of the dual-slit photometric technique. Blood flow was calculated from diameters and RBC velocities. FCD, defined as the number of capillaries with flowing blood per field of observation, was also assessed.

4   During reperfusion, placebo-treated animals showed a significant vasodilatation, a decrease in blood flow and FCD and S5682-treated animals showed a clear trend, dose-dependent, towards maintaining these parameters closer to the value found before ischaemia.

5   In conclusion, our results indicate that S5682 improves the microvascular reactivity and FCD after ischaemia/reperfusion. These data suggest that S5682 could function as an antioxidant, which may explain its beneficial therapeutic effect in chronic venous insufficiency where oxidative stress is involved in the pathological mechanism.

Keywords: Hamster cheek pouch preparation; arteriolar diameter; venular diameter; arteriolar blood flow; venular blood flow; functional capillary density; microvascular reactivity

Introduction

S5682 (Daflon 500 mg), a purified micronized flavonoid fraction containing 90% diosmin (diosmetin-7-rhamnoglucoside) and 10% hesperidin (hesperitin-7-rhamnoglucoside) is clinically used in chronic venous insufficiency and haemorrhoidal attacks. Its anti-oedematous properties have been demonstrated in animals (Damon *et al*., 1987) and man (Gilly *et al*., 1994).

Recently, experimental studies have been conducted to elucidate the mechanism of action of the compound and more specifically its effects on the microcirculation by use of intravital microscopy. Thus, a reduction in macromolecular permeability increase induced by topical application of bradykinin for 5 min and 60 min ischaemia was elicited in the rat cremaster muscle after two oral administrations of S5682 (100 mg kg^{-1}, p.o.) (Stucker *et al*., 1989). In the hamster skinfold preparation, a single dose of S5682 (30 mg kg^{-1}, p.o.) decreased the number of sticking leukocytes in postcapillary venules after 4 h of ischaemia (Friesenecker *et al*., 1994a). Previous experiments performed in our laboratory with the hamster cheek pouch preparation have demonstrated that S5682, administered for 10 days (20 mg kg^{-1} day^{-1}, p.o.) inhibited the macromolecular permeability increase induced by histamine, bradykinin and leukotriene B$_4$ (LTB$_4$) (Bouskela *et*

al., 1995). By use of the same experimental conditions and dose of S5682, postischemic macromolecular permeability increase and leukocyte adhesion were also reduced (Bouskela & Donyo, 1997).

Hence, it appears that this compound mitigates ischaemia-reperfusion injury characterized by sequestration of leukocytes and an increase in macromolecular permeability. However, microvascular blood flow disturbances and capillary perfusion failure that also contribute to the ischaemia-reperfusion injury had not yet been investigated.

The aims of the present work were to evaluate the effects of S5682 on the microvascular reactivity assessed by diameters and blood flow in arterioles and venules, as well as the perfusion by capillary density after a 90 min period of ischaemia in the hamster cheek pouch. The compound was administered for 10 days in 4 different doses (5, 20, 80 or 160 mg kg^{-1} day^{-1}, p.o.) with the aim of exploring the existence of a dose-dependent relationship. The results were compared with those obtained from placebo-treated animals.

A preliminary account of this work was presented at the XII World Congress of the Union Internationale de Phlébologie (September 3–8, 1995, London, U.K.).

Methods

Male hamsters (Mesocricetus auratus, Engle Labs Farmersburg, Indianapolis, U.S.A.), aged 7 to 10 weeks, were placed randomly into ten groups of 6 animals in each. S5682 (in suspension in 10% lactose solution) or placebo (10% lactose

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solution) was administered orally for 10 days, twice per day, at 8h 00min and 17h 00 min. Five groups of 6 animals (groups I, III, V, VII and IX) received placebo, or S5682 5, 20, 80 or 160 mg kg^{-1} day^{-1}) and were evaluated for mean internal diameters and blood flow in arterioles as well as functional capillary density (FCD). Five other groups of 6 animals (groups II, IV, VI, VIII and X) received under the same conditions placebo or S5682 5, 20, 80 or 160 mg kg^{-1} day^{-1} and were evaluated for mean internal diameters and blood flow in venules.

The experiments were performed according to protocols approved by the Ethical Committees of the University of Lund, Lund, Sweden (M29/91) and State University of Rio de Janeiro, Rio de Janeiro, Brazil , H36/94, in a blind fashion by allocating code numbers to the animals.

Experimental protocol

On the day of the experiment, that is, on the 11th day after the onset of the treatment, anaesthesia was induced by an intra-peritoneal injection of 0.1–0.2 ml of sodium pentobarbitone (Mebumal vet., ACO, Solna, Sweden, 60 mg ml^{-1}), 30 min after the last oral dose of either placebo or S5682, and maintained with α-chloralose (1,2-O-(2,2,2-trichlooethylidene) α-D-glucofuranose, Merck, Darmstadt, Germany, 100 mg kg^{-1}) administered through the femoral vein. The femoral artery was also cannulated for pressure measurements. Throughout surgery and the subsequent experiment, the temperature of the animals was kept at 37.5°C with a heating pad controlled by a rectal thermistor. A tracheal tube was inserted to facilitate spontaneous breathing. The hamster was placed on a microscope stage similar to that described by Duling (1973) with minor modifications (Bouskela & Grampp, 1992). The cheek pouch was gently everted and pinned with four to five needles into a circular well filled with silicone rubber to provide a flat bottom layer, thus avoiding stretching of the tissue, but preventing shrinkage. In this position, the pouch was submerged in a superfusion solution that continuously flushed the pool of the microscope stage. Before the pouch was pinned, large arterioles and venules were located with the aid of a Zeiss binocular stereomicroscope. In order to produce a single-layer preparation, an incision was made in the upper layer so that a triangular flap could be displaced to one side. The exposed area was dissected at 10–16 × magnification under the stereomicroscope, and the fibrous, almost avascular, connective tissue covering the vessels was removed by using ophthalmic instruments. The dissected part of the pouch was 125 to 150 μm thick. Dissected pouches with petechial haemorrhages and those without blood flow in all vessels were discarded.

The superfusion solution was a HEPES-supported HCO$^{-}_{3}$-buffered saline solution (composition in mM: NaCl 110.0, KCl 4.7, CaCl$_2$ 2.0, MgSO$_4$ 1.2, NaHCO$_3$ 18.0, HEPES 15.39 and HEPES Na$^+$-salt 14.61); the temperature was maintained at 36.5°C and the superfusion rate was 4 ml min^{-1}. The pH was set to 7.40 by bubbling the solutions continuously with 5% CO$_2$ in 95% N$_2$.

For measurements of microvascular variables, the preparations were placed under an intravital microscope (optical magnification × 210; NA 0.22) coupled to a closed circuit TV system, where they were allowed to rest for 30 min. If after this time there was (1) an indication of a good vascular tone (which, in 15 separate control tests, implied that the arteriolar diameter could be increased by 56±3% through topical application of papaverine, 10 μg/ml^{-1}), (2) a brisk blood flow in all parts of the vascular bed including the larger veins (such that individual erythrocytes were not discernible in the image of the blood stream) and (3) no tendency for the leukocytes to adhere to the vessel wall, experiments were performed (Bouskela & Grampp, 1992).

Local ischaemia of the cheek pouch was produced by means of a cuff, made of thin latex tubing, which was mounted around the neck of the everted pouch where it leaves the mouth of the hamster (Persson *et al.*, 1985). The cuff could be put in place without any visible interference with the local blood flow. The intracuff pressure could be rapidly increased by air compression by using a syringe and could be just as rapidly decreased when required. An intracuff pressure of 200–220 mmHg resulted in a complete arrest of the microvascular blood flow within a few seconds. Throughout the 90 min occlusion period minor adjustments of blood movements could be seen in the larger vessels.

Measurements and calculations

Microvascular reactivity was assessed by the diameters and flows in arterioles and venules. Two to three arterioles and two or three venules were analysed per preparation. From the videotape recordings, continuous recordings of the internal diameter of the arterioles and venules were obtained by means of an image shearing monitor (IPM model 907). Time averages of the vessel diameter were then calculated from the whole length of each diameter registration. The image shearing device was calibrated against a Leitz stage micrometer; the standard deviation of the estimate was 0.5 μm, which is equivalent to the resolution of the microscope system. Red blood cell (RBC) velocity in arterioles and venules was measured continuously by a dual-slit photometric technique (Wayland & Johnson, 1967). Microvessel volume flow, F, was calculated from the recorded mean diameters, D, and RBC velocity, V, by an analogue circuit by means of the equation: $F = (\pi/4 \, D^2) \times V_{RBC}$. In this equation, the factor 1.6 was used to convert dual-slit velocity to whole-blood velocity according to Lipowsky and Zweifach (1978). These variables were recorded before ischaemia, at the onset of reperfusion, 10, 20, 30, 45 and 60 min thereafter on a six-channel stripchart recorder (Grass polygraph model RCS 7C8).

The functional capillary density (FCD), defined as the number of capillaries with flowing blood per field of observation, was assessed in one to three different sites (each site = 1 mm^2) within the preparation and determined in control conditions, at the onset of reperfusion, 10, 20, 30, 45 and 60 min thereafter.

Drugs

Suspensions of S5682 (Laboratoire Servier, Gidy, France) were freshly prepared for each dose and each experiment in 10% lactose solution. Each oral dose was given in a volume of 0.2 ml administered to each animal.

Statistics

The results are expressed as means±s.e.mean, unless otherwise noted. Statistical differences between groups were elicited by Student's *t* test adapted by Bonferroni's method for multiple comparisons. A value of $P < 0.05$ was considered to be significant.

Results

No significant changes in mean arterial pressure could be detected in any of the groups studied (placebo-treated animals: group I – 95.3±1.5 mmHg (before ischaemia) and 94.8±2.1 mmHg (after ischaemia) and group II – 93.4±1.1 mmHg (before ischaemia) and 94.1±1.8 mmHg (after ischaemia); 5 mg kg^{-1} body weight day^{-1} S5682: group III – 95.6±1.9 mmHg (before ischaemia) and 96.4±2.2 mmHg (after ischaemia) and group IV – 93.9±2.4 mmHg (before ischaemia) and 94.1±2.1 mmHg (after ischaemia); 20 mg kg^{-1} body weight day^{-1} S-5682: group V – 93.8±2.3 mmHg (before ischaemia) and 94.8±1.6 mmHg (after ischaemia) and group VI – 94.2±1.9 mmHg (before ischaemia) and 96.3±2.2 mmHg (after ischaemia); 80 mg kg^{-1} body weight day^{-1} S5682: group VII – 95.7±1.1 mmHg (before ischaemia) and 96.7±2.4 mmHg (after ischaemia) and group VIII –

94.3 ± 1.7 mmHg (before ischaemia) and 93.8 ± 2.1 mmHg (after ischaemia) and 160 mg kg^{-1} body weight day^{-1} S-5682: group IX – 95.4 ± 2.4 mmHg (before ischaemia) and 94.8 ± 2.5 mmHg (after ischaemia) and group X – 94.3 ± 2.1 mmHg (before ischaemia) and 96.2 ± 2.0 mmHg (after ischaemia)).

The results, presented in Figures 1 to 5, have been normalized to the respective preischaemic values, considered as 100% in order to facilitate the comparison between groups.

Mean internal diameters of arterioles and venules

At the onset of reperfusion, the mean internal diameter of venules (diameter ranges: group II – 28.7 to 65.3 μm; group IV – 29.5 to 59.2 μm; group VI – 28.5 to 56.3 μm; group VIII – 23.7 to 56.5 μm and group X – 23.6 to 54.1 μm) and arterioles (diameter ranges: group I – 16.9 to 51.4 μm; group III – 16.5 to 46.2 μm; group V – 15.1 to 56.3 μm; group VII – 23.7 to 43.5 μm and group IX – 17.8 to 47.3 μm) were significantly greater than preischaemic values (Figures 1 and 2). However, the analysis of the first hour after the onset of reperfusion showed that, only in the placebo-treated group,

arterioles and venules remained significantly dilated for the whole period, while in S5682-treated animals the mean internal diameter of arterioles and venules returned to values not significantly different from the ones found during the preischaemic period in at the most 10 min (venules) and 30 min (arterioles).

Arteriolar and venular mean blood flow

Postreperfusion, mean blood flow in arterioles and venules was significantly decreased in all groups compared with the preischaemic values (Figures 3 and 4). However, the reduction in blood flow was smaller in S5682-treated animals.
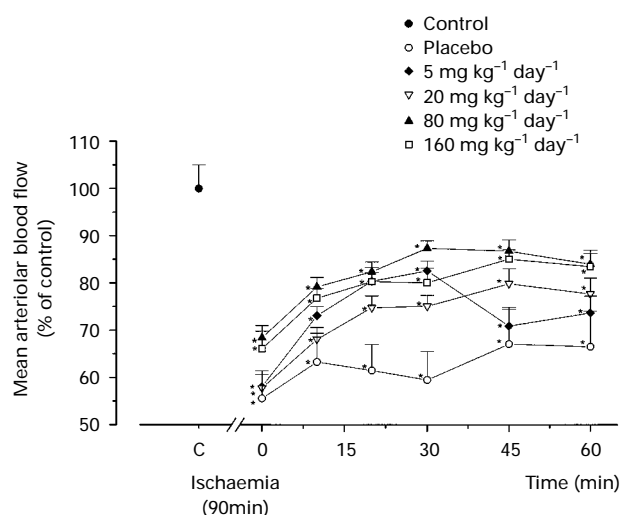
Functional capillary density

Postreperfusion, FCD was significantly decreased in all groups compared to preischaemic values (Figure 5). The decrement was maximal in the placebo group (approximately 50%) and again, the reduction in FCD was smaller in S5682-treated animals.
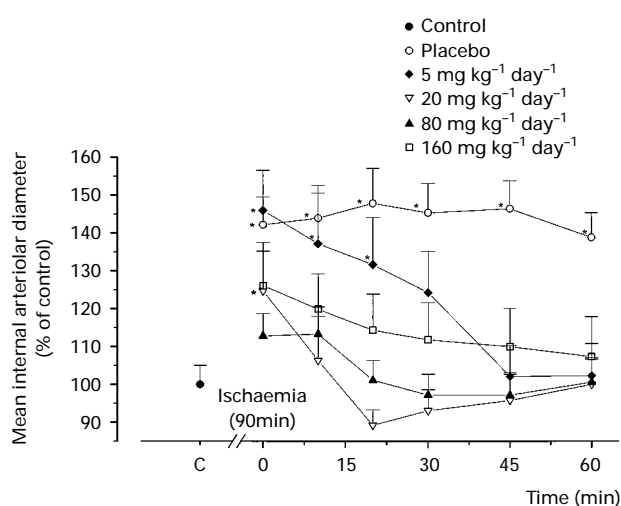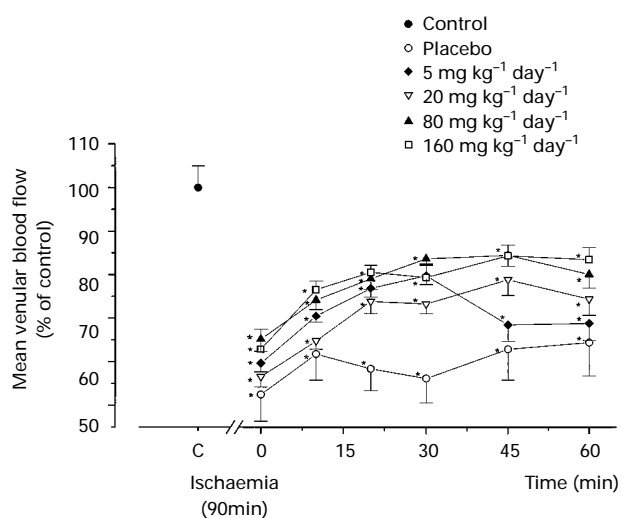

Figure 1 Effects of different doses of S 5682 on mean internal diameter of arterioles during reperfusion after 90 min in total of ischaemia. *Significantly different from control value, before ischaemia. Data are means and vertical lines show s.e.mean.

Figure 3 Effects of different doses of S5682 on mean arteriolar blood flow during reperfusion after 90 min in total of ischaemia. *Significantly different from control value, before ischaemia. Data are means and vertical lines show s.e.mean.
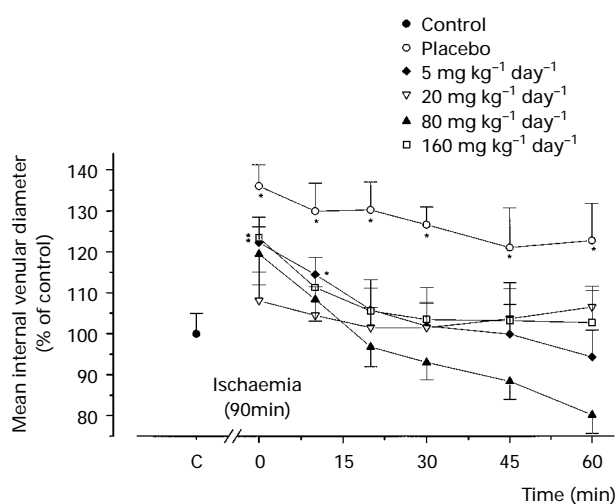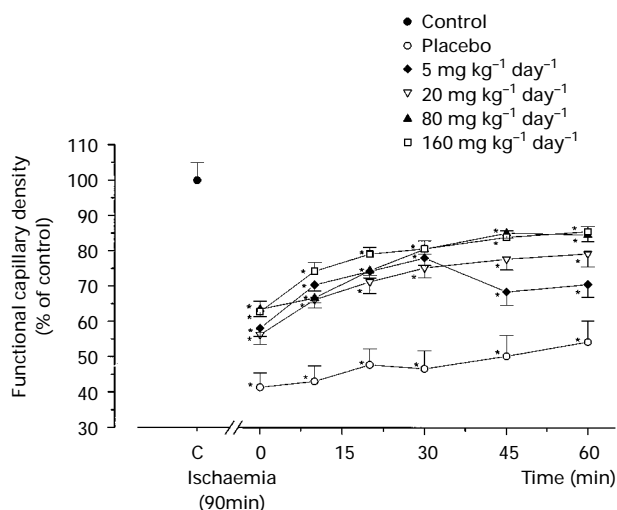

Figure 2 Effects of different doses of S5682 on mean internal diameter of venules during reperfusion after 90 min in total of ischaemia. *Significantly different from control value, before ischaemia. Data are means and vertical lines show s.e.mean.

Figure 4 Effects of different doses of S5682 on mean venular blood flow during reperfusion after 90 min in total of ischaemia. *Significantly different from control value, before ischaemia. Data are means and vertical lines show s.e.mean.

Figure 5 Effects of different doses of S5682 on functional capillary density during reperfusion after 90 min in total of ischaemia. *Significantly different from control value, before ischaemia. Data are means and vertical lines show s.e.mean.

Discussion

The main findings of our study are that during reperfusion, placebo-treated animals showed a significant vasodilatation, a decrease in blood flow and FCD (functional capillary density) compared with pre-ischaemic (control) values. S5682-treated animals showed a clear trend, dose-dependent, towards maintaining these parameters closer to the value found before ischaemia, i.e., the mean internal diameter of arterioles and venules returned to values not significantly different from the ones found during the control period (before the ischaemia) and the reduction in blood flow and FCD during reperfusion was smaller.

Ischaemia/reperfusion injury has been described in many different tissues (Kovacs *et al.*, 1966; Summers *et al.*, 1971; Kloner *et al.*, 1974; Wright *et al.*, 1988; Menger *et al.*, 1992; Friesenecker *et al.*, 1994b). Ischaemia and the reoxygenation of the tissue during reperfusion initiate a sequence of oxygen-dependent biochemical reactions that cause endothelial cell damage leading to capillary flow stoppage and a subsequent reduction in FCD (Menger *et al.*, 1992). This process, called capillary 'no reflow', leads to cell death and irreversible tissue injury.

Although tissue damage due to ischaemia is augmented during reperfusion because oxygen transported into a previously ischaemic tissue promotes the generation of oxygen-derived free radicals (Korthuis *et al.*, 1985), the restoration of blood flow has a beneficial effect, since it causes the washout of toxic metabolites accumulated during anaerobic metabolism. The level of blood flow during reperfusion after ischaemia might be a critical factor in determining tissue survival (Friesenecker *et al.*, 1994b). The extent to which blood flow is re-established during reperfusion is not known, and the available information is controversial, with different experimental methods for the same tissue yielding contrasting results. According to some studies, too little flow prolongs the process, leading to the generation of free radicals during reperfusion. Thus, flow restoration to resting or baseline levels may mitigate ischaemia/reperfusion injury through reduction of the reoxygenation rate (Sheehan & Epstein, 1982; Jolly *et al.*, 1984; Walker *et al.*, 1987) and oedema formation (Wright *et al.*, 1989), while significantly increased flow is hypothesized to promote oedema, extravascular osmolarity, and an overload of intracellular scavanger systems favouring accumulation of toxic metabolites (Wright *et al.*, 1989).

Direct data from *in vivo* experiments on the extent of capillary tissue reperfusion have not been uniform. FCD after tourniquet ischaemia ranged from 35% (Menger *et al.*, 1992) to 92% of control in a study of pressure-induced ischaemia/reperfusion injury (Friesenecker *et al.*, 1994b) in the subcutaneous tissue and muscle, both in the hamster skinfold model. The decreased FCD was also associated with significant capillary flow reduction leading to substantial impairment of tissue perfusion. In contrast, other investigators have shown the occurrence of prolonged hyperaemia in comparable models of ischaemia (Suval *et al.*, 1987; Wright *et al.*, 1989; Potter *et al.*, 1993).

In placebo-treated animals, the value we obtained for FCD during reperfusion was between the ones found by Menger and co-workers (1992) and Nolte and co-workers (1992) in the hamster skinfold preparation. The same level of dilatation found in arterioles and venules in our study was also obtained by Friesenecker and co-workers (1994b) after 4 h ischaemia in the hamster skinfold preparation. In our experiments, blood flow was reduced, even in the presence of vasodilatation, due to a significant decrease in red blood cell velocity in the microvessels studied. Previous experiments in our laboratory (Bouskela *et al*, personal observations) have shown that when the duration of the ischemic period is longer than 30 min the blood flow does not return to control (preischaemic levels) within the first hour of reperfusion, probably due to endothelial cell swelling and/or tissue injury. The fact that we observed vasodilatation with flow reduction was consistent with the finding that the FCD was also significantly reduced. To our knowledge, there are no data in the literature on microvascular blood flow during early stages of reperfusion. The capillary no-reflow and lower capillary red blood cell velocity we observed during reperfusion may be due to effects on the endothelium after prolonged hypoxia. Indeed, Nolte *et al.* (1992) pointed to endothelium swelling as the cause of luminal narrowing, leading to 45% reduction in capillary diameter and flow cessation. Furthermore, the observed reduced venular flow velocity may increase leukocyte margination due to decreased wall shear stress (Firrel & Lipowsky, 1989). In fact, a 20% decrease in flow velocity in the venules was enough to triple the number of rolling leukocytes (Firrel & Lipowsky, 1989).

The differences found in our study between placebo- and S5682-treated animals cannot be explained by differences in mean arterial pressure since no significant changes could be detected between the groups treated with lactose solution (placebo) or different doses of S5682. In addition, in a separate set of experiments in animals treated orally with S5682 for 10 days, at a dose of 20 mg kg^{-1} day^{-1} given twice a day and in the same preparation, we found no significant differences in mean internal diameter or blood flow in arterioles or venules (Bouskela *et al*, personal observations). On the other hand, ischaemia/reperfusion-induced increase in microvascular permeability and leukocyte adhesion was inhibited by S5682, given at the same dose and over the same period of time (Bouskela & Donyo, 1997), which is consistent with improvement in blood flow through the microvascular network (Sheehan & Epstein, 1982; Jolly *et al.*, 1984; Walker *et al.*, 1987). In fact, the increase in blood flow observed could be a mechanism for the prevention or diminution of leukocyte sticking during reperfusion.

The observation that the S5682-treated animals showed a clear trend, dose-dependent, towards maintaining mean arteriolar and venular diameter, mean arteriolar and venular blood flow and FCD could be due to a protective effect of S5682, as suggested by Potter and co-workers (1993) for hypothermia. This protective effect might include a decrease in endothelial cell swelling with a consequent decrease in flow resistance. Potter and co-workers (1993) demonstrated significantly increased flow and maintained FCD after 4 h of ischaemia when the tissue was allowed to cool to 24°C during the no-flow period.

However, another possibility for the observed effects of S5682 is an antioxidant effect. More than a decade ago it was

proposed that oxygen free radicals could contribute to the ischaemia/reperfusion induced injuries (Demopoulos *et al.*, 1977; Flamm *et al.*, 1978). Since then several studies have produced support for this view (McCord, 1985; Parks & Granger, 1988) and in experimental models such as the rat intestine (Granger et al., 1981), the hamster cheek pouch (Erlansson *et al.*, 1990) and the hamster skinfold (Menger *et al.*, 1992), superoxide dismutase (SOD) was able to inhibit the postischaemic increase in vascular permeability. Oxygen derived free radicals are also known to facilitate the rapid inactivation of endothelium-derived relaxing factor, mediate abnormal vasomotor tone (Bucala *et al.*, 1991) and damage cells by causing a multitude of biochemical and structural lesions, such as lipid peroxidation, which determines changes in the endothelium barrier (Bertuglia *et al.*, 1995; Wolin, 1996). It has been pointed out that flavonoids such as flavones, isoflavones and flavanones, which includes S5682, acted as antioxidants against peroxyl and hydroxyl radicals and served as

prooxidants in the presence of Cu^{2+} (Cao *et al.*, 1997). *In vitro*, flavonoids inhibited the oxidation of low-density lipoproteins (LDL), but the biochemical mechanisms responsible for this effect remain to be addressed (Catapano, 1997).

In view of these results it is reasonable to propose that S5682, a micronized flavonoid fraction, may protect the endothelial cells during ischaemia/reperfusion by decreasing the endothelial swelling and/or decreasing/changing the number of reactive O_2 produced species and/or the activity of the xanthine oxidase. Further studies are necessary to test these hypotheses.

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