

Topotecan hydrochloride effects on retinal vessels in newborn rats

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Summary. A physiological system, i.e. rodent retina during vessel formation and hierarchical organization, was utilised for assaying antiangiogenic properties of Topotecan, a topoisomerase I inhibitor, capable of inhibiting tumoral growth in animal models of retinoblastoma. In particular we analysed possible differences in effectiveness and side effects among different drug dosages and ways of administration. In the present research only qualitative analyses were undertaken. After preliminary experiments, in which suckling animals subcutaneously treated with Topotecan dosages comprised between 9 and 3 mg/kg underwent high lethality and extremely severe systemic damages, 7 day-old rats were subcutaneously, intravenously or peribulbar injected with a single dose of 1 mg/kg; retinal vessels were visualized in retinal fluorangiographies taken 1 and 2 weeks after treatment. The most important and frequent alterations were found to affect radial vessels, which showed non-perfused and/or regionally mislocated segments, together with abnormal branching and enlargements in retinal periphery; persistence of capillary-free periarteriolar regions, non-vascularised regions and spots of extravascular FITC were also detected. Despite the high individual variability the alterations were substantially similar among the different ways of drug administration, while they appeared milder in 21 day-old rats, with respect to younger ones. The extensive vascular remodelling found after Topotecan administration, besides demonstrating the antiangiogenic properties of this chemioterapeutic drug, confirms the rodent retina as a highly valuable model system for studying angiogenesis modulation.

Key words: Topoisomerase inhibitor, Peribulbar injection, Retinal fluorangiography, Angiogenesis modulation, Confocal microscopy

Introduction

Angiogenesis is a process that takes place at any time and at any site where tissue mass increases, from embryogenesis to postnatal life (Risau, 1997; Carmeliet, 2003). Angiogenesis constitutes a key step in tumour growth. Therefore, its modulation constitutes an important therapeutic goal. Gene knockout studies have succeeded in identifying many molecules implicated in embryonic angiogenesis, from the generation of cell populations to the formation of higher-order architecture (Yancopoulos et al., 2000; Rossant and Howard, 2002). However, mortality of mutant embryos and manipulation difficulty have frequently precluded sequential observations.

Being “an approachable part of the brain” the retina provides a highly valuable system for investigating not only neurosensory processes, but also relationships between angiogenesis and neuronal and glial development. In the retina, in fact, planar vascular plexuses align precisely with horizontal neuronal and astrocytic laminae and timing and direction of vessel maturation coincide with developmental processes that presumably determine local oxygen tension. In early stages of retinal vascularisation, for example, the selective pruning of capillaries adjacent to the newly formed, still immature, arterioles is presumed to be due to the relative local hyperoxia, which negatively regulates vascular endothelial growth factor (VEGF) production and endothelial cell survival (Claxton and

Abbreviations: VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; DEHP: bis (2-ethylhexyl) phthalate; PVC: poly chloroethanediyl; ECF: extracellular fluid; CNS: central nervous system; FITC: fluorescein-isothio-cyanate; PBS: phosphate-buffered saline; DMSO: dimethyl sulfoxide; BSA: PBS containing 1% bovine serum albumin; alpha-SMA: alpha isoform of smooth muscle actin; bFGF: basic fibroblast growth factor; PI3K-Akt: phosphatidylinositol-3-OH kinase; MAP: mitogen-activated protein; eNOS: endothelial nitric oxide synthase.

Fruttiger, 2003). Moreover, in normal retina vessel maturation results from sequential reciprocal cellular interactions: i) neuron-derived PDGF (platelet-derived growth factor) stimulates and patterns astrocyte invasion, which in turn induces VEGF-dependent and cadherin-dependent vascular growth and guidance; ii) vessel growth, raising tissue oxygen tension, promotes astrocytic maturation; iii) developing retinal vessels are stabilized through association of pericytes and smooth muscle cells (Gariano and Gardner, 2005).

Angiogenesis occurring in postnatal life provides the advantages of visibility and manipulability. For rodent retina, in particular, in which vessel maturation starts at birth and eventually develops into a highly organized three-layered architecture, flat-mount preparations, where the vascular network can be seen as a whole, constitute an excellent model for describing endothelial sprouting and vessel hierarchical organization (Uemura et al., 2002; Gerhardt et al., 2003; Lu et al., 2004), during experimentally induced neovascularisation. Recently, for example, we described the extensive vascular remodelling occurring in retina of neonatal rats after bis (2-ethylhexyl) phthalate (DEHP) administration to pregnant and lactating females (Zei et al., 2009). These results, together with those previously obtained in lung, where DEHP treatment caused severe impairment of alveolarization (Magliozzi et al., 2003; Rosicarelli and Stefanini, 2009) suggested that, in preterm babies submitted to endotracheal intubation, the phthalate released by poly chloroethanediyl (PVC) devices remarkably affects perinatal development of several tissues in different body districts. Finally, developing retinal vessels can be utilised for investigating pro- and antiangiogenic properties of different substances (Uemura et al., 2006). In fact, the superficial vascular plexus is particularly useful for analyzing the cellular and molecular events involved in the establishment of a hierarchical architecture consisting of arteries, veins, and capillaries. New vessel formation is a key step in tumour growth and invasivity. Therefore, identification of chemioterapeutic drugs with antiangiogenic properties constitutes a very important component in antitumoral strategy.

For this reason, we decided to investigate the effects exerted on retinal angiogenesis by the camptothecin-derived Topotecan (10-Hidroxy-9-dimethylamino-methyl-20(s)-camptothecin), a topoisomerase I inhibitor (Staker et al., 2002; Streltsov et al., 2003; Pommier, 2006). The lactone form of Topotecan interacts with DNA and shows a relatively high brain extracellular fluid (ECF) to plasma distribution ratio (El-Gizawy and Hedaya, 1999), a property that makes it a potential candidate for first-line treatment of central nervous system (CNS) tumours, and especially of retinoblastoma; consequently, several studies were carried out to determine how different administration schedules influence the extent and the mechanism by which topotecan gains access to the vitreous.

In particular, rabbits which had received 1 mg

topotecan by periocular injection or as a 30-minute intravenous infusion showed potentially active lactone topotecan intravitreal levels, and all results (high topotecan level in plasma after both administration schedules, similar vitreal concentrations in both eyes after periocular injection, drug absence in controlateral eye of postmortem-injected animals) confirmed the systemic delivery of the drug (Carcaboso et al., 2007). In LH beta-Tag transgenic mice (retinoblastoma animal model) the subconjunctival injection of 0.1 mg Topotecan caused a significant bilateral reduction in tumor burden without a significant difference in treated versus controlateral eyes; in this system the major route of drug delivery also appeared hematogenous rather than transscleral (Tsui et al., 2008).

In the present research Topotecan was given, to 7 day-old rats, by means of subcutaneous, intravenous and periocular injection, and the morphology of actually perfused retinal vessels was qualitatively described in retinal fluorangiographies obtained at 14 and 21 days of life.

Materials and methods

Animals and treatment

Albino Wistar rats (from Harlan spa, Italy) were kept at 20-22°C, with a dark/light cycle of 12 h/12 h and were fed ad libitum with standard diet. Females were placed with males overnight and examined the following morning for presence of sperm in the vaginal smear. In preliminary experiments 5 suckling rats were submitted to three subcutaneous injections with Topotecan 3 mg/kg, while another 5 animals were submitted to three injections with Topotecan 1 mg/kg. Each animal was treated at 5, 8 and 11 days of life and for every injection Topotecan was diluted in 200 µl of PBS. Treated rats were sacrificed at 14 days of life and 6 retinal fluorangiographies were extensively examined. Due to the high lethality (about 30%) and extremely severe systemic damage found in treated animals, in following experiments 7 day-old rats were subcutaneously (10 animals) or intravenously (10 animals) injected with 80 µl of a solution containing 0,2 mg Topotecan in 1 ml PBS (approximately Topotecan 1 mg/kg). From these animals, sacrificed at 14 days of life, a total number of 16 (8+8) retinal fluorangiographies were extensively examined. With the aim of reducing systemic damage, 24 (7 day-old) pups were injected into the peribulbar space of the right eye with 20 µl of a solution containing 1 mg Topotecan in 100 µl dimethyl sulfoxide (DMSO) and 900 µl PBS (approximately Topotecan 1 mg/kg). From these animals, sacrificed at 14 days of life, 20 retinal fluorangiographies of injected eye and 12 of controlateral eye were extensively examined. In order to evaluate the possible involvement of DMSO in Topotecan-induced vascular alterations, 5 rats were peribulbar injected with 20 µl of DMSO 10% in PBS and 3 retinal fluorangiographies of injected eye and 2 of

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contralateral eye were extensively examined. The presence of long lasting effects was investigated in subcutaneously (10) and peribulbary (16) injected rats with Topotecan 1 mg/kg at 7 days of life and sacrificed at 21 days. From these animals, a total number of 20 (8+12) retinal fluorangiographies were extensively examined. Finally, a total number of 24 retinal fluorangiographies (14 at 14 days and 10 at 21 days of life) were obtained from untreated animals.

Before sacrifice, all treated and control pups were weighed and carefully inspected to detect the presence and severity of alopecia, defects in walking ability, shivering; afterwards they were anaesthetized with subcutaneously injected Farmotal (100 mg/kg) and injected through the left ventricle with 500 μ l of 5% FITC-conjugated dextran (FD 2000S) in PBS. Following heart resection, eyes were enucleated and prefixed in 4% paraformaldehyde for 2 hours; liver was excised and weighed and the relative liver weight (g/100 g body weight) was calculated. After careful dissection of cornea, lens, hyaloid vessels and vitreous humour from the inner surface, sclera and choroid from the outer surface, isolated retinas underwent radial incision, by which 4 or 5 triangular regions were obtained, and overnight postfixation. The majority of specimens were then directly observed and photographed.

Some of them were incubated as follows:

- PBS containing 1% bovine serum albumin (BSA) and 0.5% Triton X-100, overnight at 4°C;
- 1:100 mouse monoclonal antibody to the alpha isoform of smooth muscle actin (alpha-SMA) in the medium described above, 5-6 hours at RT;
- 1:200 rhodamine-conjugated goat anti-mouse IgG in PBS, 30 min at RT in the dark.

All fluorangiographies were examined by fluorescence (Zeiss Axioskop) microscopy.

For each fluorangiography, the morphology of a

radial arteriole and a radial venule was examined by confocal (Leica TCS SP5) microscopy (20 x and 50x magnifications); in these areas, the thickness of vascularised retinal tissue was determined by counting the 3 μ m-spaced virtual sections containing labelled vessels. In selected fields, finally, the captured volumes were virtually rotated with respect to x and z axes and further elaborated through artificial colouring of the different focal planes (blue superficial plexus, green connecting capillaries and orange-red deep plexus); by this method it was possible to examine the position of individual vessels with respect to the retinal layers.

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Italian Health Ministry guidelines.

Chemicals

Topotecan was a generous gift of Sigma-Tau Industrie Farmaceutiche Riunite (Pomezia, Italy); Farmotal was from Amersham Pharmacia Biotech Italia (Cologno Monzese, Milan, Italy); FITC-conjugated dextran and mouse monoclonal anti alpha-SMA antibody were from Sigma Chemical Co. (Schnelldorf, Germany); rhodamine-conjugated goat anti mouse IgG antibody was from Molecular Probes (Eugene, USA).

Results

In suckling rats submitted to three subcutaneous injections with 3 mg/kg Topotecan, as well as in those submitted to three injections with 1 mg/kg, lethality was approximately 30%; moreover, at 14 days body weight was very much lower than in age-matched controls (15.04 \pm 2.16 g vs. 29.57 \pm 4.04 g) and severe systemic damage (uneven and shorter hair on the whole body

Table 1. Description of normal vessels and alterations of vascular morphology induced by Topotecan 1 mg/kg.

14 day-old	Superficial plexus, main vessels	Superficial plexus, capillaries	Deep plexus and connecting layer
Control	Arterioles and venules reach the ora serrata; their size does not change along length.	Capillary net is regularly arranged and reaches retinal outermost periphery. Capillary free regions around radial arterioles are no longer present.	Deep net capillaries are regularly arranged and reach retinal outermost-periphery.
Systemic treatment	Arterioles and venules reach the ora serrata but their size remarkably changes along length; several radial vessels are non-perfused near the optic nerve head and undergo branching and enlargement in retinal periphery. Spots of leaked FITC are present.	Capillary net is coarsely arranged, showing non-vascularised or hypovascularised regions. Capillary-free regions around radial arterioles are occasionally still present.	Vessels are irregularly arranged. In outermost periphery deep capillaries are absent or abnormally large, while connecting capillaries are extremely few or absent.
Peribulbar treatment	Arterioles and venules reach the ora serrata but their size changes along length; some radial vessels are non-perfused near the optic nerve head and undergo branching and enlargement in retinal periphery. Spots of leaked FITC are occasionally present.	Non-vascularised or hypovascularised regions, as well as periarteriolar capillary-free regions are present.	Vessels are irregularly arranged. Abnormally large capillaries are found, especially in mid and extreme periphery.

FITC: fluorescein-isothiocyanate.

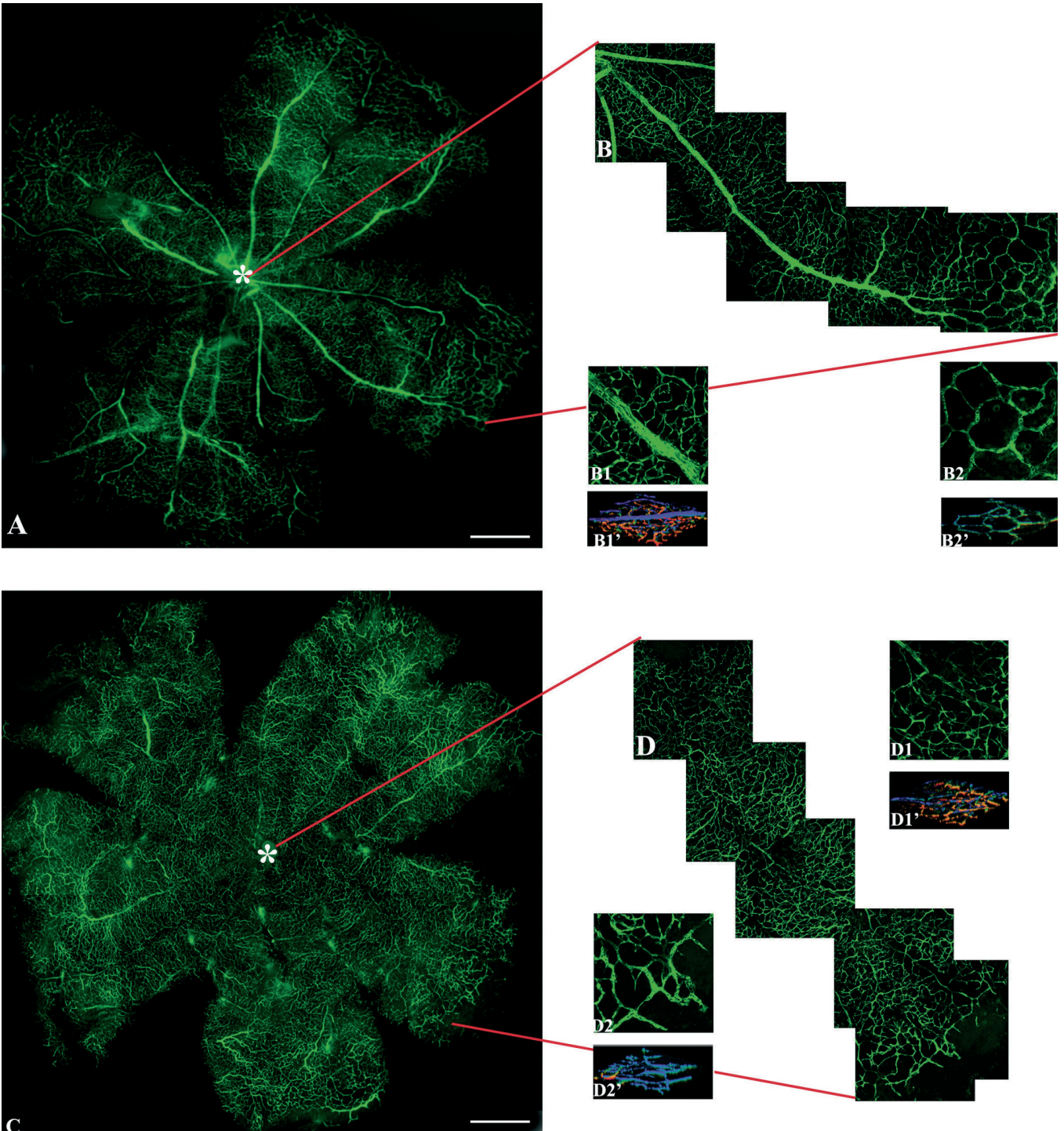


Fig. 1. Retinal fluorangiographies from 14 day-old rats; subcutaneous injection with Topotecan 9 mg/kg (**A and B**) and 3 mg/kg (**C and D**). **A, C:** Fluorescence microscopy, 2.5 x objective, focused on the superficial vascular plexus. **B, D:** Confocal microscopy. **B and D** are collages of 3D images, each one obtained from virtual sections taken at 20x magnification. B1, B2, D1 and D2 were obtained from virtual sections taken at 50x magnification. In B1', B2', D1' and D2' the captured volumes were virtually rotated with respect to x and z axes and further elaborated through artificial colouring of the different focal planes (blue superficial plexus, green connecting capillaries and orange-red deep plexus). **A.** The majority of radial vessels are strongly dilated and show non-perfused segments; large spots of leaked FITC are present, especially in retinal periphery. Capillary net is rarefied and irregularly shaped, several large non-vascularised regions are present. **B.** A remarkably dilated radial vein is shown from the optic nerve head till the outermost retinal periphery. In its proximal part the vein is strongly dilated and deep capillary plexus is abnormally thick (see the zoomed image B1 and the zoomed and rotated image B1'). In the outermost periphery both superficial and deep plexuses are absent (see the zoomed image B2 and the zoomed and rotated image B2'), whereas in the connecting layer abnormally large vessels form a rough net (green in the B2' image); in this region, vascularised tissue is extremely thin (24 μm , with respect to control mean value $43.13 \pm 3.18 \mu\text{m}$). **C.** The majority of radial vessels are non-perfused near the optic nerve head and dilated and branching in retinal periphery; other radial vessels appear non-perfused in the entire length; several small spots of leaked FITC are present. **D.** A severely hypoperfused radial vessel is shown from the optic nerve head till the outermost retinal periphery. In the mid periphery the radial vessel is non-perfused and superficial capillaries are extremely rarefied (see the zoomed and rotated image D1'); in the outermost periphery extremely dilated superficial vessels can be seen (blue in the D2' image). *: optic nerve head. Bar: 1 mm

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surface, defective or absent walking ability, shivering) were present. In retinal fluorangiographies the majority of radial vessels appeared non-perfused for long segments or even in their entire length, and several spots of leaked FITC were present. Occasionally, the vascularised tissue was abnormally thin with large vessels situated in the connecting layer (Fig. 1). In animals submitted to systemic (intravenous or subcutaneous) treatment with a single dose of Topotecan 1 mg/kg at 7 days and sacrificed at 14 and 21 days, lethality was absent and systemic damage was milder than after higher dosages. However, body weight was significantly decreased (Fig. 2) and relative liver weight significantly increased with respect to controls (at 14 days 2.67 ± 0.42 vs. 1.90 ± 0.42 , $p < 0.01$).

Following peribulbar injection of a single dose of Topotecan 1 mg/kg, lethality was absent and the severity of systemic damage varied among the different pups, and with age of sacrifice. In fact, at 14 days of life body weight (Fig. 2) was significantly ($p < 0.01$) lower than in control but higher than after systemic treatment, whereas relative liver weight was significantly higher than in control (2.30 ± 0.49 vs. 1.90 ± 0.42 , $p < 0.01$) but significantly ($p < 0.05$) lower than after systemic treatment. Moreover, the majority of pups showed whole body alopecia and shivering, while in a few of them alopecia was restricted to the periocular region and trembling was absent. By contrast, in animals sacrificed at 21 days body weight was identical to control and in the majority of pups alopecia was restricted to periocular region and tremors were absent.

In retinal fluorangiographies of the injected eye, at 14 days of life (one week after treatment), Topotecan-induced alterations mainly consisted of non-perfusion, hypoperfusion, enlargement and abnormal branching of radial vessels, capillary absence in several retinal regions, (besides that in periarteriolar regions), FITC leakage and irregularity in deep vascular plexus; the severity of alterations sensibly changed among the examined animals (Figs. 3, 4). Confocal microscopy showed that segments of large vessels were often

mislocated in the connecting or even in the deep vascular plexus, instead of the superficial one. In particular, in fluorangiographies taken from peribulbar injected animals the thickness of vascularised tissue, estimated by counting 3 μm -spaced confocal microscopic virtual sections obtained in 5 microscopic fields/specimen, was found to be significantly higher than in normal counterparts ($49.71 \pm 7.5 \mu\text{m}$ vs. $43.13 \pm 3.18 \mu\text{m}$, $p < 0.05$). In fluorangiographies obtained

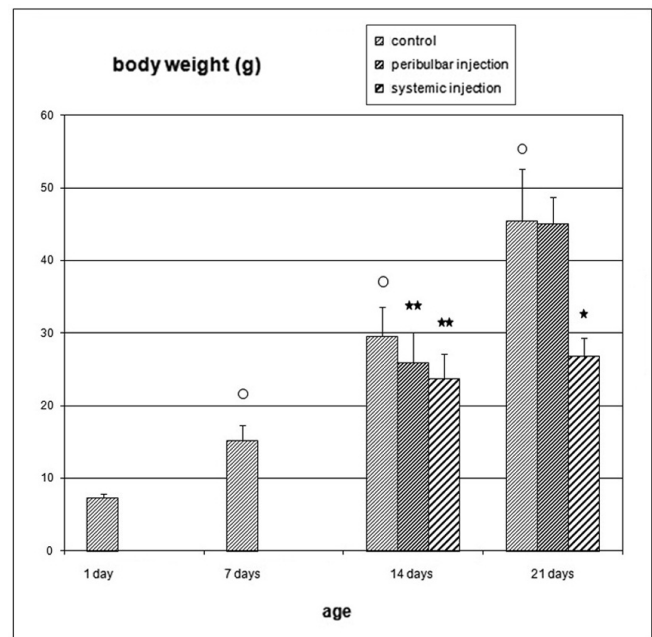


Fig. 2. Body weight of normal and Topotecan-treated suckling rats. For each experimental condition, data are mean \pm SD. **: Significantly different from the corresponding control ($P < 0.01$). *: Significantly different from the corresponding control ($P < 0.05$). °: Significantly different from the previous stage ($P < 0.01$). The relatively low P value found at 21 days is due to the low number of individuals sacrificed at this age (see Materials and Methods).

Table 2. Description of normal vessels and alterations of vascular morphology induced by Topotecan 1 mg/kg.

21 day-old	Superficial plexus, main vessels	Superficial plexus, capillaries	Deep plexus and connecting layer
Control	Arterioles and venules reach the ora serrata; their size only mildly changes along length.	Capillary net is thick and regularly arranged and reaches retinal outermost periphery.	Capillary net is regularly arranged and reaches retinal outermost-periphery.
Systemic treatment	Some radial vessels show enlargement and branching in the retinal periphery. Some spots of leaked FITC are present.	Capillary net is rarefied in several small regions.	Regularly arranged capillaries reach retinal outermost-periphery.
Peribulbar treatment	Few radial vessels show size alterations and short hypoperfused segments. Spots of leaked FITC are only occasionally present.	Non-vascularised or hypovascularised regions are occasionally present.	Regularly arranged capillaries reach retinal outermost-periphery.

FITC: fluorescein-isothiocyanate.

from the contralateral eyes the alterations were milder and the individual variability higher than in test. In retinal fluorangiographies taken from 21 day-old animals

Topotecan-induced alterations were qualitatively similar to those found in younger pups (Fig. 5), but their severity and numerical occurrence appeared lower. In

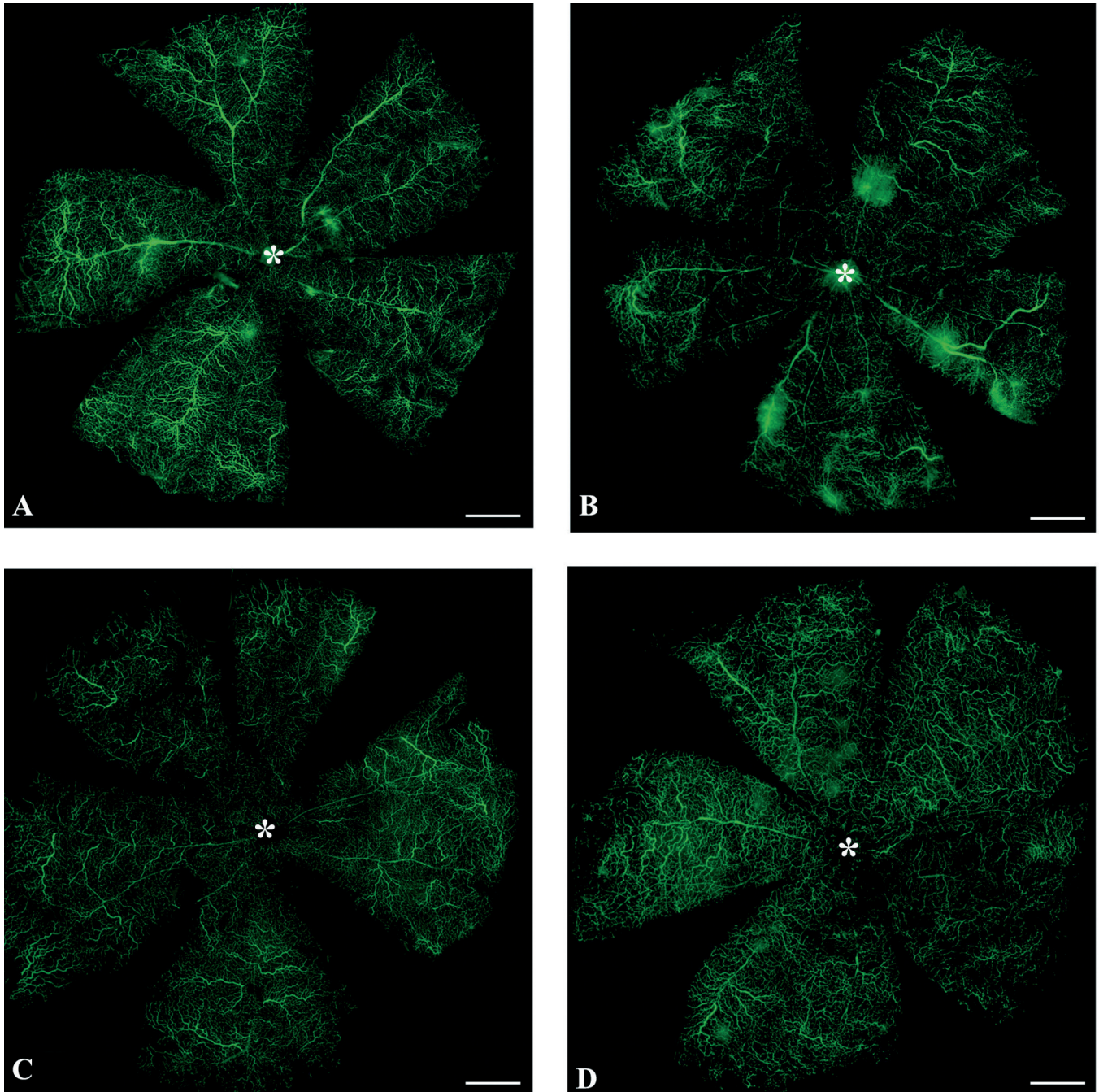


Fig. 3. Retinal fluorangiographies from 14 day-old rats; subcutaneous (A), intravenous (B) and peribulbar (C) injection with Topotecan 1 mg/kg. D. control. Fluorescence microscopy, 2.5 x objective, focused on the superficial vascular plexus. A. Several spots of leaked FITC are evident. Capillary net is irregularly shaped, some regions being hypovascularised. B. The majority of radial vessels are non-perfused near the optic nerve head and dilated and branching in retinal periphery; some very large spots of leaked FITC are evident. Capillary net is extremely irregular, several large non-vascularised regions are present. C. The majority of radial vessels are non-perfused or hypoperfused near the optic nerve head. Capillary net is irregularly shaped, some regions are hypovascularised. D. Radial vessels show normal size for the whole length; capillary net is thick and regular; no FITC leakage can be seen. *: optic nerve head. Bar: 1 mm

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both the examined stages, intra-vitreous vascular branching was never observed and alpha-SMA immunolocalization failed to reveal alterations in vessel mural cells (Figs. 3-5).

In animals peribulbar injected with DMSO, at 14 days of life body weight was normal, systemic damage was absent and retinal fluorangiographies were similar to control. Alterations of vascular morphology induced

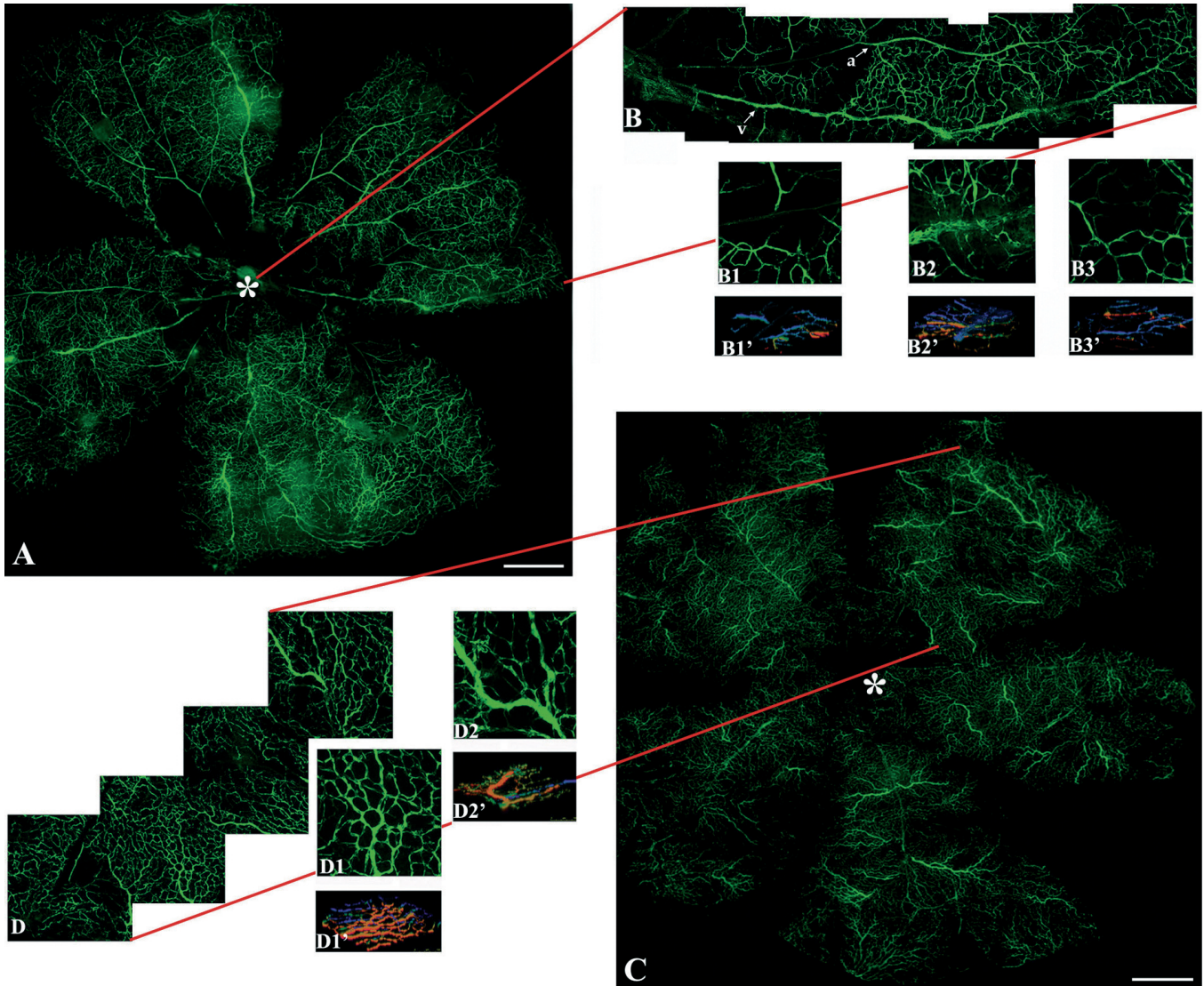


Fig. 4. Retinal fluorangiographies from 14 day-old rats; peribulbar injection with Topotecan 1 mg/kg. **A and C.** Fluorescence microscopy, 2.5 x objective, focussed on the superficial vascular plexus. **B and D:** confocal microscopy. **B and D** are collages of 3D images, each one obtained from virtual sections taken at 20x magnification. B1, B2, D1 and D2 were obtained from virtual sections taken at 50x magnification. In B1', B2', D1' and D2' the captured volumes were virtually rotated with respect to x and z axes and further elaborated through artificial colouring of the different focal planes (blue superficial plexus, green connecting capillaries and orange-red deep plexus). **A.** Radial vessels are non-perfused near the optic nerve head and enlarged and branching in the retinal periphery; capillaries are absent in the whole proximal retina; some spots of leaked FITC are present. **B.** A radial vein (v) and a radial artery (a) are shown from the optic nerve head till the outermost retinal periphery. Long segments of radial artery are non-perfused, both in proximal (see B1 and B1') and in peripheral (see B3 and B3') retina; in the same regions superficial capillary plexus is extremely rarefied or absent. The radial vein is dilated and leaking in some segments (see B2 and B2'). The thickness of vascularised tissue is $45.9 \pm 5.7 \mu\text{m}$. In mid periphery, deep plexus shows abnormally large capillaries (see B2'), and vascularised tissue is thicker ($51 \mu\text{m}$), than in other regions of the same specimen. **C.** Several radial vessels are non-perfused in their proximal part and branching and dilated in periphery; some regions are capillary-devoid. **D.** A severely hypoperfused radial vessel is shown from the optic nerve head till the outermost retinal periphery. The superficial vascular net is very rarefied in the whole specimen; the deep plexus shows abnormally numerous capillaries near the optic nerve head (red in the D1' image), and extremely large vessels in outermost periphery (red in the D2' image). The thickness of vascularised tissue is $54.8 \pm 13.0 \mu\text{m}$ in the whole specimen and $72 \mu\text{m}$ in outermost periphery. *: optic nerve head. Bar: 1 mm

by Topotecan 1 mg/kg are scheduled in Tables 1 and 2, which also contains a description of normal vessels.

Discussion

Until now antiangiogenic properties of Topotecan were only demonstrated *in vivo* in an experimental model of basic fibroblast growth factor (bFGF)-induced corneal vascularisation (O'Leary et al., 1999) and in

cultured endothelial cells from human umbilical vein (HUVEC) (Nakashio et al., 2002). *In vitro* the decreased endothelial proliferation was found to be caused by the decreased expression of genes codifying for HIF-1 α (Rapisarda et al., 2002, 2004), with a consequent decrease in VEGF expression (Puppo et al., 2008), while the down regulation of signalling pathways involving phosphatidylinositol-3-OH kinase (PI3K-Akt) and mitogen-activated protein (MAP) kinases was found to

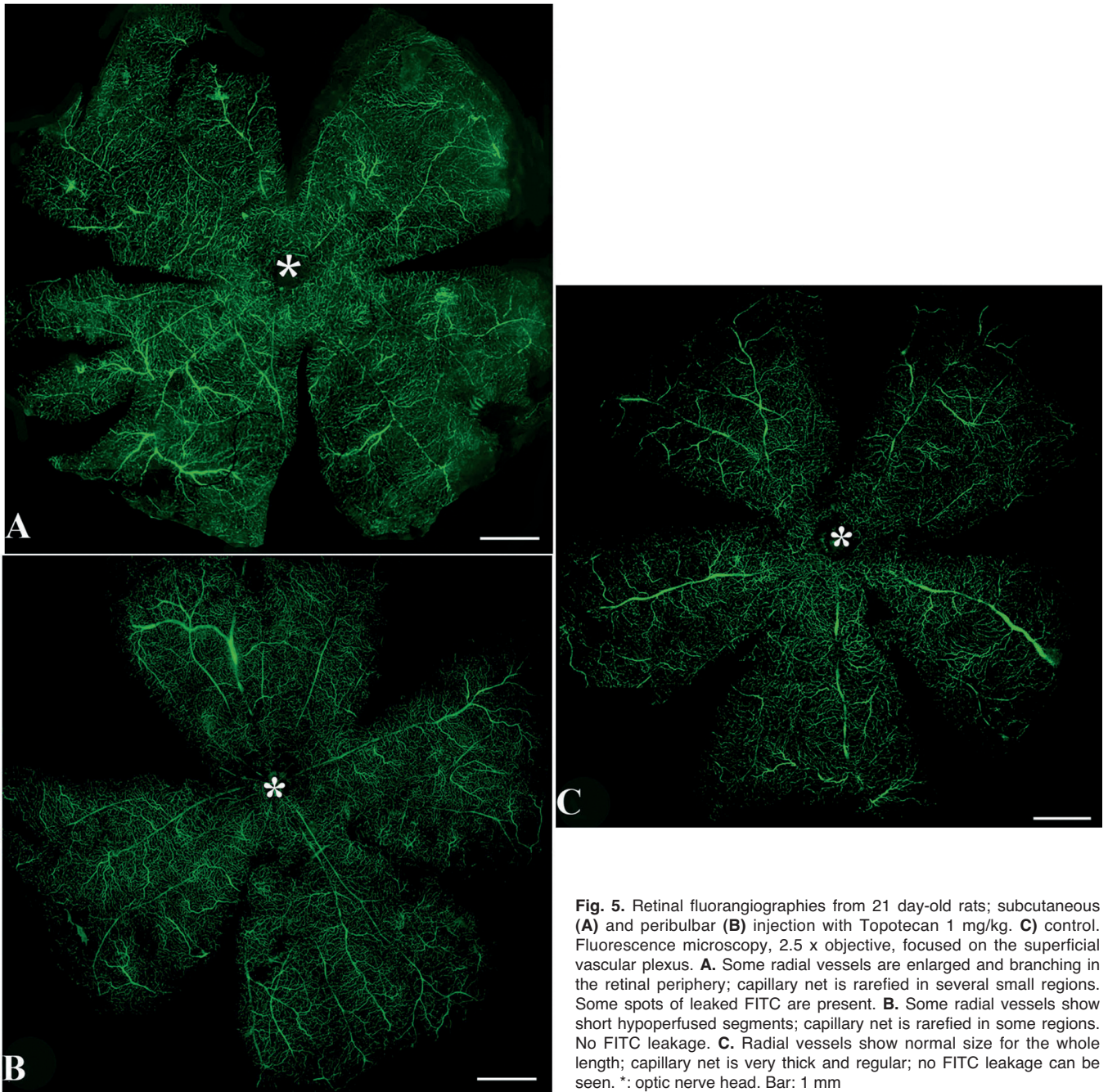


Fig. 5. Retinal fluorangiographies from 21 day-old rats; subcutaneous (A) and peribulbar (B) injection with Topotecan 1 mg/kg. C) control. Fluorescence microscopy, 2.5 x objective, focused on the superficial vascular plexus. A. Some radial vessels are enlarged and branching in the retinal periphery; capillary net is rarefied in several small regions. Some spots of leaked FITC are present. B. Some radial vessels show short hypoperfused segments; capillary net is rarefied in some regions. No FITC leakage. C. Radial vessels show normal size for the whole length; capillary net is very thick and regular; no FITC leakage can be seen. *: optic nerve head. Bar: 1 mm

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affect the activity of endothelial nitric oxide synthase (eNOS) and several other proteins, as well as the expression of metalloproteases, which play key roles in endothelial cell migration. To our knowledge, the present paper constitutes the first attempt to investigate Topotecan antiangiogenic ability in a physiological condition and during the maturation of a hierarchically organized vascular network.

The results described through observation, by fluorescence and confocal microscopy, of retinal fluorangiographies obtained from animals treated with Topotecan 1 mg/kg of body weight can be summarised as follows:

i) the alterations did not consist of large non-vascularised regions but a broad range of shape and position alterations of retinal vessels; these results are in agreement with those we previously obtained in fluorangiographies of DEHP-treated suckling rats (Zei et al., 2009);

ii) the alterations were substantially similar after the different ways of drug administration (subcutaneous, intravenous or peribulbar injection).

In particular, one week after treatment (at 14 days of life), main alterations affected radial vessels which showed non-perfused segments with abnormal branching and enlargements, as well as capillaries of superficial plexus, which appeared coarsely arranged, absent from the periarteriolar regions and from several randomly located regions. Deep plexus also appeared less regularly organised and spots of extravascular FITC were detected. Interestingly, some segments of radial arterioles or venules appear mislocated in the deep retinal tissue, where normally capillaries belonging to the deep vascular plexus are found. Two weeks after the treatment (at 21 days of life) alterations were sensibly milder than at the previous stage.

It is well known that in retina of normal rats the capillary-free periarteriolar regions, caused by the relative hyperoxia present in close proximity to immature arterioles, disappear during the second postnatal week, probably due to maturation of the arteriolar wall and consequent periarteriolar normoxia. On such a basis, the here described persistence of capillary-free periarteriolar regions in 14 day-old Topotecan-treated pups can be interpreted as a sign of delay in arteriolar wall maturation. The presence of vessels with increased permeability, revealed by the frequent spots of leaked FITC, might be due to a delay in covering new capillaries with pericytes and mural cells. Together these two alterations allow to suggest a Topotecan-induced impairment in the establishment of vascular hierarchy. Moreover, the vertical mislocation of vessels, especially radial arterioles and venules, which should be found only in the superficial plexus, allows to suggest that the Topotecan-induced extensive remodelling of retinal vessels includes also a derangement in vertical architecture. Such a loss of alignment between vascular plexuses and neuronal and astrocytic laminae might be especially important in this developmental stage, when neuronal development is

heavily influenced by local variations in oxygen tension.

Taken as a whole, our results confirm that rodent retina, particularly during the second week of postnatal life, is a highly valuable system for studying angiogenesis modulation. In this period, in fact, superficial vascular plexus forms perpendicular branches into deeper layers, finally leading to the formation of the mature vascular system, and at the same time establishes a hierarchical architecture consisting of distinct arteries, veins, and capillaries (Uemura et al., 2006). Therefore, both delays in capillary growing and alterations in the construction of a highly organized vascular architecture can be easily detected. In particular, these alterations in vertical architecture can be important because of their consequences on neuronal development and, so, should also be considered in other experimental models of retinopathies.

Concerning the access of Topotecan, administered in different ways, to vitreous and posterior eye tissues, the similarity in vascular alterations found in subcutaneously, intravenously and peribulbar injected animals, together with the presence of comparable, although milder, alterations in the controlateral eye of locally treated pups, suggest that in suckling rats, as well as in rabbits (Carcaboso et al., 2007) and in transgenic mice (Tsui et al., 2008), the major route of drug delivery is hematogenous rather than transscleral.

In any case, the differences in severity of systemic damage (body weight decrease, relative weight increase, walking ability defect, etc.) should be kept in mind when choosing a modality of Topotecan administration. In animal retina, in order to investigate the antiangiogenic or proangiogenic properties of a drug, it appears more practical to utilise a systemic treatment, such as an easy intraperitoneal injection (although it causes systemic damage), because results are more repetitive, instead of a local administration, technically more difficult and which can cause individually different systemic damage.

By contrast, when Topotecan is used as a chemotherapeutic drug, especially for retinoblastoma, or possibly as an antiangiogenic factor in a multifactorial therapy of proliferative retinal vasculopathies, a local administration must be utilised, and many studies are currently performed in order to minimize systemic exposure and achieve selective transscleral penetration. To this purpose recently in rabbits the insertion of Topotecan-loaded biocompatible polymer implants into the episclera caused a high accumulation of the lactonic form of the drug in locally exposed ocular tissues, while its concentrations in plasma and in controlateral eye were minimal or undetectable (Carcaboso et al., 2010).

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