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CORSO DI DOTTORATO IN IPERTENSIONE E PREVENZIONE DEL RISCHIO CARDIOVASCOLARE

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A successful experimental model for intimal hyperplasia prevention using a resveratrol delivering balloon.

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“May the Force be with you”

Obi-Wan Kenobi. Star Wars

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Introduction

Restenosis due to intimal hyperplasia is a major clinical problem that compromises the success of angioplasty and endovascular surgery [1,2]. The pathogenesis of restenosis is multifactorial, involving such events as endothelial injury, inflammation, platelet activation, and hyperplasia of the intima, primarily due to vascular smooth muscle cell replication (vSMC).[3] The incidence of intimal hyperplasia varies in different risk populations, e.g., diabetic patients, up to 35% of whom require bare metal stent implantation; clinical evidence has shown that this value is reduced but continues to cause problems after the implantation of drug-eluting stents (DES)[4]. Overall, however, despite many years of clinical experience with drug-eluting balloons (DEB), the number of large, high-quality, randomised clinical trials is low, and further data are urgently needed across the spectrum of clinical indications. Taxol and other cytostatic drugs destroy a cell's ability to use its cytoskeleton in a flexible manner, and, considering the clinical results, further research on a more physiologic mechanism of action should be pursued. Antioxidants are currently under

investigation due to their protective activity within the vessels. Rosenbaum et al.[5] showed that the endothelialisation of prosthetic grafts was significantly reduced, and anastomotic hyperplasia, significantly increased in rabbits on a high cholesterol diet. Treatment with an antioxidant improves endothelial cell coverage, decreases intimal hyperplasia and reduces oxidative stress, promoting the patency of prosthetic grafts. Resveratrol is a polyphenolic phytoalexinic antioxidant that is produced by grapes and other plants in response to injurious infections. There are several pioneering reports on RSV, including studies on the inhibition of the arachidonate metabolism via interactions with the 5-lipoxygenase and cyclooxygenase pathways in leukocytes[6-12]. However, RSV attracted little interest until 1979, when the "French Paradox"[13] reported the positive benefit of an RSV-containing diet, in particular the moderate consumption of red wine, for coronary heart disease. The molecular structure of RSV, unfortunately, reduces its immediate clinical application for three main reasons: 1) its status as a highly lipophilic molecule; 2) its fast drifting from the TRANS- to CIS-phase, representing an oxidised and inactive state, respectively; and

3) its low circadian bioavailability for rapid hepatic metabolism. As consequence of these features, the oral bioavailability of RSV is negligible since it is rapidly conjugated to improve the solubility of the compound. The disposition of ^{14}C-labeled RSV, as orally and intravenously administered in normal, healthy volunteers, was evaluated to estimate the extent of the oral dose absorbed, the bioavailability of the unchanged drug, and the drug's metabolic phase. RSV demonstrated a high oral absorption but a rapid and extensive metabolism, resulting in only trace amounts of unchanged RSV remaining until systemic circulation[14]. Five major metabolites were detected in the urine samples14, plasma and colo-rectal cancer tissues[15], although all were only measured qualitatively due to a lack of available reference materials. Metabolite 1 (M1) was a RSV monoglucuronide, and metabolite 2, an isomeric RSV monoglucuronide (M2), was found in greater abundance. M3 was a dihydroresveratrol monoglucuronide, whereas M4 and M5 were two poorly resolved RSV sulphates, i.e., a resveratrol monosulphate (M4) and a dihydroresveratrol sulphate (M5). Despite the controversial results on the efficacy of RSV reported in

the literature, very recent data obtained in colon cancer cells have supported the notion that RSV, in spite of its low bioavailability, is able to act through its metabolites, mainly the sulpho-conjugate but also the combination of sulphate/glucuronide derivatives[15,16]. Despite the wide literature on RSV, only few preclinical studies have demonstrated the efficacy of RSV in an animal model or investigated the possibility of locally administering this antioxidant by eluting stents[6,7]. Based on our experience, we decided to set up a sterile, injectable, and hydrophilic RSV-containing compound (RSV-c). This solution was locally administered in the common iliac artery of adult male New Zeeland white rabbits using a dedicated device.

Methods

In vitro study

Cell seeding. Human Coronary Artery Smooth Muscle Cells (HSMC) (C-017-5C, GIBCO-Invitrogen) were seeded, according to a kit's instruction (2.5 x 10^3 cells/cm^2 to reach 80% confluence in 7-9 days) and maintained in culture at a lower density in 6-well plates to determine the proliferation rate. Experiments were conducted on cells between passages 4 and 6.

Cell proliferation study. HSMC were divided in three experimental groups:

1- CONTROL: medium (GIBCO-Invitrogen Medium 231 with Supplement), n=8.

2- RESVERATROL (RSV, Biotivia Italia, Verona. Italy): (25 mM dilution in ethanol), final concentration 100 µM in medium, n=12.

3- VEHICLE (VEH): ethanol 0.4 µL/mL in medium, n=12.

All groups were incubated for 48 h at 37°C. The medium and treatments were changed every 24 h to prevent RSV degradation. On day 2, the cells were washed twice with phosphate-buffered saline (PBS), harvested by trypsinisation, and counted

with a coulter counter (Beckman Coulter Z2).

Preparation of the compound (RSV-c)

To obtain a sterile, injectable solution, we optimised the solubility of RSV and its viscosity. RSV was dissolved in a 0.40% w/v tamarind seed polysaccharide (TSP) and 2.50% w/v KOLLIPHOR^{TM} HS15 (BASF, KHS) solution that had been previously sterilised by vapour steam under pressure. Afterwards, the solution was filtered by disposable, sterile and pyrogen-free PES filters at the nominal porosity of 0.22 µm into a sterile amber Type I glass container at a laminar airflow work bench. A media fill program assured the validation of the aseptic process. The containers were stored at room temperature until use. A placebo formulation was prepared according to the procedure reported above. To avoid the fast removal of RSV by the blood stream after intimal administration, a high viscosity solution was prepared. TSP, a well-known biocompatible polymer, was used to confer a suitable cinematic

viscosity to the vehicle for administration by drug-eluting balloon. According to our previous experience[12], the viscosity of the preparation was based on the non-ionic contrast medium iomeprol (Iomerol®, Bracco, Italy; kinematic viscosity at 37°C: 5.617±0.034 $mm^2 \cdot s^{-1}$). The final kinematic viscosity of the RSV-c was very close to that of the reference solution (6.097±0.0379 $mm^2\ s^{-1}$). KHS is a surfactant admitted for parenteral preparations and was added to the formulation in order to dissolve 140 µg/mL of RVS.

In vivo study

Animals.

Thirty-six male New Zealand white rabbits, weighting from 2.8 to 3.6 kg, were assigned randomly and in equal numbers to different study subgroups (Table 1). The animals were housed in a dedicated facility and fed with standard diet with free access to water.. All experiments were conducted in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals, as well as in accordance

with the ethical committee guidelines from the Università degli Studi di Milano. The research protocol was approved by the Italian Ministero della Salute.

Table. In vivo study design[a]

	Group			
Design	*PK*	*Sham*	*Carrier*	*RSV-c*
Subgroup[b]	2, 6, 24	3, 30	3, 30	3, 30
Time	2, 6, and 24 h	3 and 30 d	3 and 30 d	3 and 30 d
Compound	RSV-c	None	Carrier	RSV-c
Procedure	DDC	Simple PTA	PTA+DDC	PTA+DDC

DDC, Drug-delivery catheter; *PK*, pharmacokinetic; *PTA*, percutaneous transluminal angioplasty; *RSV-c*, resveratrol compound.
[a]Administration of 20 mL carrier or RSV-c by DDC.
[b]n = 4 for each subgroup.

Angioplasty and delivery procedures.

Using a pre-operative colour-Doppler ultrasound (Titan™, Sonosite USA), all animals were scanned to measure their mean arterial size and femoral artery velocimetry [peak systolic velocity (PSV), end-diastolic velocity (EDV)] to obtain preoperative morphologic and velocimetric data. The mean right iliac artery diameter was 3±0.6 mm. The right femoral artery PSV and EDV were, respectively, 90 and 60 cm/s.

The animals were treated with an anaesthetic protocol to ensure the full

unconsciousness of the subjects during the surgical procedures and an excellent level of perioperative analgesia. Rabbits were premedicated with a subcutaneous injection of dexmedetomidina (80 μg/kg; Dexdomitor, Orion Corporation, Italy), ketamine (25 mg/kg; Ketavet, Intervet Production, Italy) and buprenorphine (20 μg/kg; Temgesic, Schering Plough Spa, Italy). After the induction, a steady depth of anaesthesia was maintained during the experimental protocol by the continuous infusion of a dilute solution of propofol (1-3 mg/kg/h; Fresenius Kabi, Italy), into the auricular vein. All animals were heparinized (80 UI/Kg of heparin sulfate; Phararepa, PharmaTex, Italy) 2 minutes before inserting the introducer sheath. After the surgical incision, the superficial fascia and muscles were separated bluntly, layer by layer, until the right common femoral artery was exposed. Proximal and distal vascular controls were assured with two 2-mm silicon ligatures to minimise the bleeding. Using the modified Seldinger technique, we directly inserted a 4 French *Cook Micropuncture (William Cook Europe ApS)* sheath. To induce and establish intimal hyperplasia in the rabbit's iliac artery, we performed a traumatic angioplasty with a balloon catheter (BANTAM

ALFA Clearstream, Ø: 3.0 mm, length: 2 cm) with Doppler ultrasound monitoring. Injury was created by inflating the balloon to 8 atm with a manometer syringe for 3 min. Afterwards, the catheter was removed, and the drug delivery catheter (Genie™, Acrostak. Geneve, CH) was introduced into the aortic-iliac bifurcation by colour-Doppler ultrasound monitoring, in order to deliver the 20 mL of carrier or RSV-c (Table 1). This balloon catheter is designed to dilate and treat arteries via the local delivery of the proposed solution (RSV-c) and reference compounds (vehicle), ensuring a fully controlled release to the vessel wall. Clinical experiences with Genie™ suggested us to deliver RSV in 2 minutes maintaining a mean inflating pressure of 6 atm. At the end of the procedure, the right common femoral artery was sutured with 7/0 polypropylene interrupted stitches. The blood supply of the leg was not affected by this surgical procedure. Pain control and antibiotic coverage were achieved through the subcutaneous administration of buprenorphine (15 mg/Kg Temgesic, Schering Plough Spa, Italy) + meloxicam (0.2 mg/Kg; Mobic, Boehringer Inghelheim, Italy) + Enrofloxacine (10 mg/Kg; Baytril, Bayer SpA, Italy). At the

time points indicated from the experimental protocol (Table 1), the balloon-treated aorto-iliac bifurcation was surgically explanted, and the animals were sacrificed with an anaesthetic overdose.

Tissue and serum measurements of RSV-c

Before, immediately after the delivery procedure and then at predetermined times ranging from 15 min and 90 min, blood samples (2 mL) were collected from the auricular vein. Samples were immediately centrifuged (3500 rpm for 15 min at 4°C) and serum was then frozen and stored at -80 °C until further processing. 250 µL de-frozen serum were added to 1 mL methanol, vortexed 1 min and centrifuged (5000 rpm for 15 min at 15°C); then the surnatant was analyzed by the HPLC method reported below.

The animals were sacrificed at 2, 6 and 24 h after administration (Table 1). Segments of the iliac artery were removed and washed with a physiologic solution, and any visible blood coagula or residual fat tissues were carefully removed. The tissue was cut into a small specimen, placed into a vial containing 0.2 mL methanol and

sonicated by a ultrasound probe (Microson™ ultrasonic cell disruptor) in an ice bath for 30 min.

Afterwards, the concentration of RSV was assayed using the HPLC method reported below.

RSV was analysed by HPLC (ChemStation HP 1100, Agilent, G), as previously described[14]. Briefly, an ODS Hypersil analytical column was used as the stationary phase (4.6 x 100 mm - particle size: 3 μm, Thermo Scientific, I), and a combination of MilliQ™ water/methanol/trifluoroacetic acid (65/35/0.3 %, v/v/v) was used as the mobile phase. The flow rate was controlled at 0.9 mL/min. The effluent was monitored at 304 and 286 nm for the determination of *trans*-RSV and *cis*-RSV (or isomeric RSV), respectively. The injection volume was 10 μL, and the analysis was performed at 30°C. As authentic RSV metabolites were not available as reference materials, the amounts of the metabolites were calculated as "RSV equivalents," using the assumption that the recovered characteristics and relationship between peak area ratio and concentration were the same as for the parent RSV.

Histological measurements

The harvested iliac arteries of sham, carrier and RSV-c groups were fixed in 10% buffered formalin; then, cross-sections were cut and embedded in paraffin. A morphological evaluation of the vessel wall thickness and intimal hyperplasia was performed on each tissue block, cutting 4-μm sections. Haematoxylin and eosin staining clearly show the internal elastic lamina, the external elastic lamina, the intimal thickness and the cells in the vessel wall. Each histological section was scanned, the intimal and external and internal elastic lamina were manually identified. Intimal layer thickness (i.e., distance between the lumen and internal elastic lamina), medial layer thickness (i.e., distance between internal and external elastic lamina), lumen area, intimal layer area and medial layer area were measured using a software for image analysis (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014). All thickness were measured at four points (i.e., cardinal points - N, E, S, W), and the mean value was taken. Then, in order to compare different treatment groups the following ratios were

used for statistical analysis: ratio intimal/medial thickness; ratio intimal/medial area and intimal proliferation index (IPI) (i.e., intimal area [intimal+medial] area). Moreover, for group RSV-c30 and sham30, proliferating cells in the intimal layer were identified by immunohistochemistry, using antibodies against Ki-67 protein (Clone MIB-1, DAKO, Glostrup, Denmark). Results of Ki-67 positive cells count were normalised for square .micron (Ki67/μm^2)

All histological analysis were performed in a blinded fashion.

Statistical analysis

Data were reported as the means ± standard deviation. The data from the proliferation studies were analysed by taking the means of three counts for each well and then considering each of the independent wells as a separate data point. The comparisons between groups were performed by analysis of variance (ANOVA) with Bonferroni's correction. $P<0.05$ was considered significant.

Results

In vitro evaluation

The number of cells was expressed as cells/mL. The cell proliferation in the presence of vehicle was similar to that in the CONTROL group (25±5 cells/mL in VEH group respect to 25±4 cells/mL in CONTROL, P=.8, N.S.). RVS significantly inhibited HSMC proliferation (Figure 1), compared to the VEH and CONTROL group (20±3 cells/mL in RSV, compared to 25±5 and 25 ±4 cells/mL in VEH and CONTROL, respectively, $P<0.01$).

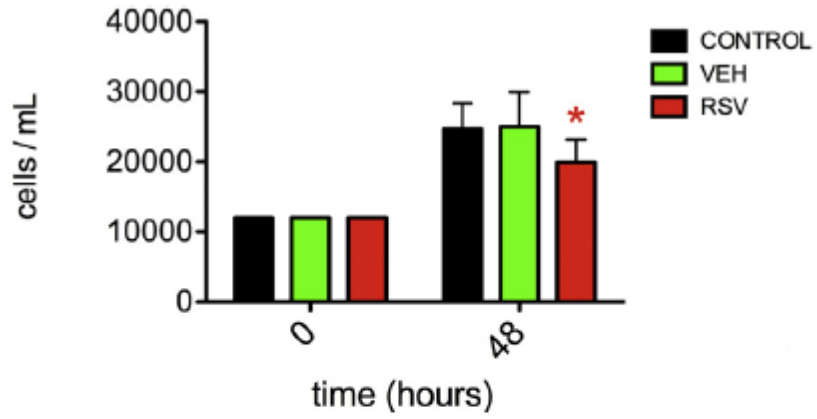

Fig 1. In the human coronary artery smooth muscle cells (HSMCs) proliferation study, resveratrol (*RSV*) slowed cell growth in the absence of pathologic stimuli after 48 hours of treatment. Vehicle (*VEH*) did not alter proliferation compared with the control. **P* < .01 RSV vs VEH and control.

Tissue measurements of RSV-c

The RSV was detectable and quantifiable in serum only immediately after administration (less than 0.2 µg/mL) and no one of its metabolites was found.

As far as the recovery from the iliac vessel is concerned, only traces of unchanged RSV were detectable in the arterial samples until 6 h after the ballooning procedure (Figure 2). However, RSV monoglucoronide (M1) and RSV monosulphate (M4) were qualitatively identified within 2 h and persisted over the considered time period. After prolonged periods of time, the concentrations of such two metabolites

decreased and the dihydroresveratrol conjugates (M2 and M3) became detectable. Based on these data, we posit that the RSV infused in a solution at the site of the artery by a drug delivery catheter was retained in the tissue and then underwent an extensive and rapid metabolism.

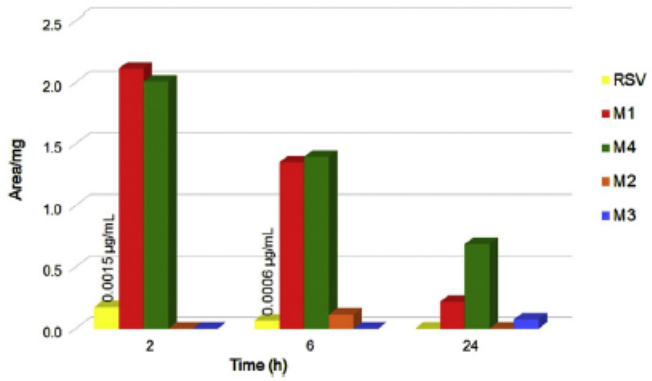

Fig 2. Recovery of resveratrol (*RSV*) and its metabolites in artery tissue after the administration of RVS solution (10 mL) by a drug-eluting balloon. Four major metabolites were identified as RSV monoglucuronide (*M1*), isomeric RSV monoglucuronide (*M2*), dihydroresveratrol monoglucuronide (*M3*), RSV monosulfate (*M4*). Because standards are not commercially available, RSV metabolites are expressed as the peak area at 286 nm corrected for the conversion factor 1.5 normalized for the artery sample weight. The correction factor was calculated as the ratio of peak area of RSV and photodegraded RSV at 304 and 286 nm.

In vivo evaluation and histological measurements

No procedure-related deaths were documented during the housing period. Before their sacrifice, all rabbits were submitted to color-Doppler ultrasound to test for

patency and velocimetric patterns. The RSV-c30 group always showed the patency of their iliac vessels, which was associated with an average value of PSV and EDV of 70±7 and 40±5 cm/s, respectively. In the sham30 and carrier30 groups, we found an average value of PSV and EDV of 30±20 and 20±15 cm/s, respectively. When we analysed the data together, we found a significant difference ($P<0.05$) between the RSV-c30 and the sham30 or carrier30 groups. The review of the literature showed a lack of comparisons of the data on velocimetry and vessel diameters in the rabbit; nevertheless, we considered the flow reduction (PSV from 90 to 30 cm/s) in the femoral artery in the sham30 and carrier30 groups to be a result of a tight stenosis. Autoptic specimens showed no macroscopic differences among the groups. Those animals sacrificed at the early time point (sham3, carrier3, and RSV-c3) showed ialin degeneration as a result of PTA, irrespectively of the treatment. However, we noted some peculiar differences in the disposition of ialin degeneration in RSV-c3. Complete circumferential lesions were present in groups sham3 and carrier3, but not in RSV-c3. Despite the presence of ialin lesions in RSV-c3, we did not observe the

same homogenous ialin intimal layer as was found in sham3 and carrier3. Micrographs were able to spot some cellularised areas in RSV-c3 (Figure 3 - panel A). Regardless of whether an empirical evaluation of this early result could be interpreted as a positive effect of RSV-c after PTA, we considered this phenomenon only as a part of the modulatory effects of RSV-c on the local inflammatory response after PTA.

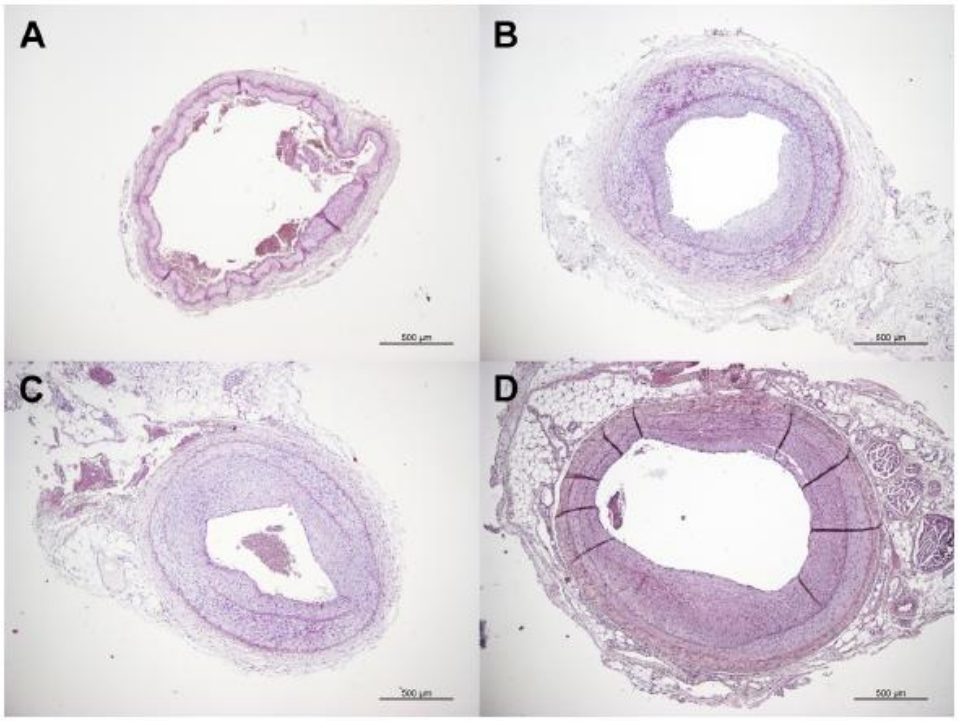

Fig 3. Hematoxylin and eosin-stained histologic cross-sections of rabbit iliac artery after angioplasty (original magnification ×40). **A,** Group RSV-c3 (resveratrol compound at 3 days)—intimal layer denudation with patchy cellularized medial layer and diffuse ialin degeneration of vessel wall. **B,** Group RSV-c30 (resveratrol compound at 30 days)—moderate reduction in lumen size due to slight intimal hyperplasia. **C,** Group carrier30 (group that received carrier at 30 days)—marked reduction in lumen size and asymmetrical thickening of the wall, mainly due to intimal hyperplasia. **D,** Group sham30 (group that received sham treatment at 30 days)—marked intimal hyperplasia, resulting in high intima/media ratio.

The morphological data at day 30 are summarized in Figure 4. The intimal hyperplasia resulted significantly greater in the case of sham30 group with respect to the other treated groups (one way ANOVA with Bonferroni's correction $p<0.05$).The comparison between the carrier 30 and RSV-c30 evidenced the beneficial effect of RSV. Indeed, the mean IPI value obtained with the RSV-c resulted about 25% lower than that obtained with the carrier. Nevertheless, this difference did not result significant according to our statistical evaluations.

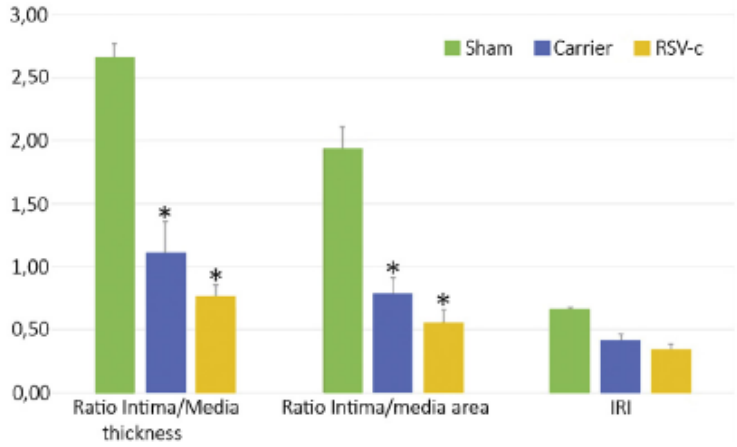

Fig 4. Parameters calculated from histologic measurements: comparison of sham30 (sham group at 30 days), carrier30 (carrier group at 30 days), and RSV-c30 (resveratrol compound at 30 days). Data are shown as mean ± standard error. *IRI*, Intimal proliferation index (ratio of intimal area to [intimal + medial] area). $*P < .05$ compared with sham30 group.

Differences between RSV-c30 and sham30 were statistically significant ($p<0,05$)

when counting Ki-67 positive cells in the whole vessel wall (2,42±0,73 $x10^{-6}$ Ki-67/µm^2 *vs.* 8,65±1,48 $x10^{-6}$ Ki-67/µm^2). When considering only the intimal layer, divergence was still noteworthy (9,20±3,20 $x10^{-6}$ Ki-67/µm^2 *vs.* 11,78±2,20 $x10^{-6}$ Ki-67/µm^2), but it was not significant according to our statistical evaluations (Figure 5).

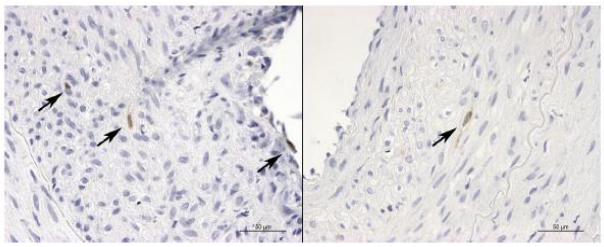

Fig 5. High magnification photomicrograph of rabbit iliac arteries without any treatment after angioplasty (group sham30). Immunohistochemistry cross-sections show nuclear positivityfor Ki-67 protein (*arrows*) in proliferating cells of the intimal layer (immunohistochemistry,original magnification ×400).

Discussion

Many papers have reported the good results of RSV for treating IH. However, despite the positive experiences, we have observed only a fair expectation of clinical use due to the limitations of its molecular structure and biodynamics. To overcome this boundary, we worked on an experimental model in the hope of establishing an intra-vessel, elutable RSV-containing compound. The delivery procedure assured a minimal lack of RSV in the blood. Indeed, less than 5% of the administered RSV was

detected in serum and, in agreement with literature data[16], it was completely eliminated within 15 min.

The rabbit model used in our study allowed us to test the vessels with a mean diameter similar to that of a human coronary artery[8]. Thus, the data obtained could be relevant to intimal vSMC proliferation and hyperplasia after balloon dilation in humans. Furthermore, the lesions generated in the present model encouraged the participation of vSMC proliferation, which, together with vSMC migration and the abnormal production of the extracellular matrix, are considered to be the primary features of the restenotic process in humans. In rabbits, as in humans, ultrasound data offer the possibility of evaluating velocimetric patterns pre- and post-operatively in order to highlight not only the local morphologic effects of resveratrol but also the relevance of these effects in terms of flow modulation below the lesion. It is notable that the segments treated with RSV-c had better velocimetries in groups sham and carrier.

We decided to use the Genie™ catheter[13] because, rather than being restricted to stent

struts, catheter-based local antiproliferative therapies offer the advantage of a homogenous drug transfer to the whole vessel wall, thereby allowing for intravessel pharmacotherapies without adding additional layers of other molecules[17]. Moreover, the delivery system of the Genie™ catheter allowed us to use the compound immediately. In this experimental model, we aimed to solve the problem of restenosis induced by angioplasty and, at the same time, to control the host reactions induced by a carrier layer.

RSV acted as an anti-oxidant, inducing a local anti-inflammatory response, and the carrier showed no particular activities on intimal hyperplasia, as observed in sham and carrier groups (Figure 3 and 4), which showed no significant differences in intimal hyperplasia. The Genie™ catheter was applied in the coronary vessels[4], and the local delivery resulted in an effective reduction in the rate of restenosis after both POBA and stenting. As a matter of fact, RSV-c forced into the artery wall by balloon expansion might accumulate in the interstitials and/or within cells, thereby avoiding the washing-out of solutions. We did not use salicylate to provide complementary

data, because we considered that the antiplatelet activity of salicylate might act as a bias that could weaken or strengthen the role of RSV-c, either way confounding the final evaluations. Despite the lack of antiplatelet activities, the good results observed in RSV-c group could encourage further investigations on RSV-c with respect to other treatments against intimal hyperplasia that might necessitate a mandatory double antiplatelet therapy.

Beside the main goal, the project also provided information of general interest in the fields of optimising compound production; localising compounds after their administration within the arteries' walls by ballooning; and assessing peri-vascular tissue responses to local, long-acting solutions. To successfully implement the local administration of RSV, many challenges would have to be considered. The localisation of RSV after infusion, i.e., intracellular, intercellular, or both, has not yet been investigated. Moreover, the drug residence time at the injured artery should probably be prolonged.

Conclusions

Control of intimal hyperplasia and restenosis remain the main goals of post-angioplasty therapy. Early and late results have shown that almost 40%[4,17] of the endovascular procedures were associated with recurrences. Even if taxol and similar treatments helped in controlling such events, the final solution would remain unclear. In our experience, magnification micrographs have shown a significant inhibition of intimal proliferation when RSV-c is applied. Moreover, no adverse events have been documented in either in vitro or in vivo studies. We believe that the know-how acquired with this experimental work towards the development of a sterile injectable compound with an effective anti-oxidant properties can be considered a positive scouting experience with future clinical relevance, as well as a strong urging for further investigations.

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