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Concomitant proliferation and caspase-3 mediated apoptosis in response to low shear stress and balloon injury

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Abstract

Background—Arterial remodeling occurs as a response to hemodynamic change and direct vessel wall injury through the process of neointimal hyperplasia (NH). A concomitant response of vascular smooth muscle cell (VSMC) proliferation and apoptosis exists. The purpose of this study is to assess the cellular response of vessels following exposure to low shear stress (τ) and balloon injury in order to further elucidate the mechanisms underlying vascular injury. Our hypothesis is that the combination of low τ and balloon injury results in NH approximating that seen in clinical arterial restenosis, and that quantitative analysis of VSMC proliferation and apoptosis correlates with the associated increase in arterial remodeling.

Methods and Results—New Zealand White rabbits underwent surgery on the carotid artery creating low τ (n=11), balloon injury (n=11), combined low τ and balloon injury (n=11), and sham (n=13) groups. Experiments were terminated at 1, 3, and 28 days. Day 1 and 3 arteries were analyzed with immunohistochemistry for apoptotic markers, terminal transferase dUTP nick end labeling (TUNEL) and activated caspase-3, and a cellular proliferation marker, accumulated Proliferating Cell Nuclear Antigen (PCNA), as well as immunoblot analysis for activated caspase-3 and PCNA at day 3. There was significantly greater apoptosis in the combined group as compared to the other groups as assessed by quantitative TUNEL and activated caspase-3 levels at both days 1 and 3. Similarly, an increase in cellular proliferation as assessed by PCNA expression, was significantly greater in the combined group as compared to other groups. At 28 days there was no difference in NH observed in the low τ ($26 \pm 3 \mu\text{m}$) and balloon injury ($51 \pm 17 \mu\text{m}$) groups. However, significantly more NH was observed in the combined group ($151 \pm 35 \mu\text{m}$) as compared to the other groups.

Conclusions—An increase in VSMC apoptosis via a caspase-3 dependent pathway is up-regulated by 24 hrs in the face of combined low shear stress and balloon-induced vessel wall injury. Paradoxically, this increase in VSMC apoptosis is associated with a significant increase in neointimal thickening at 28 days. The concomitant increase of both apoptosis and proliferation are indicative of a robust arterial remodeling response.

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Keywords

neointimal hyperplasia; arterial remodeling; vascular injury; vascular smooth muscle cells; shear stress; balloon injury; apoptosis; hemodynamics

INTRODUCTION

Neointimal hyperplasia (NH) is an adaptive response of blood vessels to both injury^{1,2} as well as low flow hemodynamic states^{3,4}. The consequent arterial remodeling is multifaceted resulting in compensatory luminal narrowing⁵, through vascular smooth muscle cell (VSMC) proliferation and migration^{1,2}, in addition to VSMC apoptosis^{6,7}. It is the pathologic remodeling of NH that is considered to be a predominant cause of arterial restenosis following percutaneous interventions. The role of injury and hemodynamic factors, such as decreased shear stress, increased wall tension, or both on vascular remodeling have been well studied⁸⁻¹⁰. These stimuli lead to VSMC “phenotypic modulation,” migration and proliferation, in addition to extracellular matrix degradation¹¹⁻¹³, all of which are thought to be stimulated by locally released cytokines derived from activated platelets, macrophages, white blood cells and the VSMCs themselves¹⁴⁻¹⁸. As more percutaneous interventions are being performed clinically, it is important to explore the role of decreased wall shear stress and balloon injury as it relates to arterial remodeling.

Apoptosis is a non-inflammatory mechanism of cell death that has been shown to play an important role in both atherosclerosis as well as NH^{19, 20}. Isner *et al.* demonstrated that up to 93% of human arterial restenotic specimens contained foci of apoptosis, as well as 43% of primary atherosclerotic lesions¹⁹. In experimental models, apoptosis has been shown to occur early after balloon injury, and is thought to be a result of the vessel’s homeostatic response to proliferative signals when normal cell-matrix interactions are disrupted²¹. Although some studies demonstrate an increase in apoptosis following balloon angioplasty, other studies focus on the lack of apoptosis up-regulation in comparison to cell proliferation, indicating a nidus for net intimal thickening²².

Hemodynamics are of critical importance to clinical success²³. Wall shear stress (τ), a well-studied flow parameter, is due to the frictional force of the flowing blood on the endothelial cells and is known to be a major regulator of arterial remodeling. Decreasing the wall τ by reducing the blood flow is shown to produce a predictable neointimal response^{9, 10, 24}. Similarly, endothelial denudation as occurs during percutaneous transluminal angioplasty augments the development of neointimal response²⁵⁻²⁷. Moreover, it is not uncommon to find low τ following balloon injury due to technical failure, as seen with embolization of downstream vessels, presence of persistent collateral circulation, and constrictive remodeling in arteries^{28, 29}. Although several animal models of NH using either balloon injury or low τ have been studied^{7, 30, 31}, the effect of low τ following balloon injury as can occur clinically following angioplasty has yet to be fully elucidated.

The purpose of this study was to examine the *in vivo* effects of concomitant balloon injury and low τ on the arterial remodeling response, in particular to focus on smooth muscle cell viability as assessed by apoptosis and cellular proliferation. In a New Zealand White rabbit carotid model, the apoptotic and proliferative response of vascular smooth muscle cells exposed to extremely low τ , balloon injury or both were examined. Apoptosis was assessed via terminal transferase dUTP nick end labeling (TUNEL) and activated caspase-3 immunostaining and immunoblotting. Cellular proliferation was assessed via immunostaining and immunoblotting of accumulated Proliferating Cell Nuclear Antigen (PCNA), a cell proliferation marker. Resultant neointimal thickening was assessed via histomorphometry.

METHODS

Animal operations

Animals were cared for in accordance with the University of Chicago Institutional Animal Care and Use Committee (IACUC). Male New Zealand White rabbits (3 kg) were anesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg) and xylazine (5mg/kg) augmented with isoflurane (1%-3%) titrated for effect via endotracheal intubation. Antibiotic prophylaxis was provided with enrofloxacin 10 mg/kg intramuscularly daily for 3 days. Aspirin (1 mg/kg) and clopidogrel (25 mg/kg) dissolved in water was given as a loading dose 24 hours prior to surgery and a maintenance dose (aspirin –1 mg/kg and clopidogrel –10 mg/kg) was continued until sacrifice at either 1, 3 or 28 days. Intra-arterial pressure was monitored through a 22-gauge cannula placed in the contralateral ear artery.

Animal Models

For all of the animal groups, a 4 cm length of left common carotid artery (CCA) was exposed, after surgical preparation, through an incision overlying the anterior border of sternocleidomastoid muscle. Systemic anticoagulation was achieved with 200 U/kg of heparin prior to proximal and distal arterial control. For the low τ group (n=11), the left CCA was exposed as previously described and an arteriotomy was made just distal to the cranial thyroid branch. Arterial outflow was restricted by ligating the distal CCA, distal to the patent cranial thyroid branch, over an 0.014 guide wire, limiting arterial outflow through a narrowed distal CCA and cranial thyroid branch. For the balloon injury group (n=11), the left CCA was exposed as described above and an arteriotomy made just distal to the cranial thyroid branch. A 3F Fogarty balloon catheter (Edwards Life Sciences, Irvine, CA) was then inserted retrograde into the CCA. The balloon catheter was inflated with 0.1 ml isotonic saline solution to a ratio of 1.1:1 (balloon to CCA diameter) and the inflated catheter was withdrawn in a stepwise fashion to the entry point with constant rotation. The previous procedure was repeated a total of three times to create a 3-4 cm area of balloon injury in the proximal CCA. For the combined low τ and balloon injury group (n=11), the left CCA was balloon injured first as previously described and then restricted by ligating distal to the cranial thyroid branch over an 0.014 guidewire as previously described with the low τ group. The arteriotomies of all injury models were repaired using interrupted 8-0 nonabsorbable prolene surgical suture (*TE-145*; Davis-Geck, Manatee, PR) under 4.5x loupe magnification. For the sham group (n=13), the left CCA was temporally exposed as for the other groups and the incision was closed in a two layer fashion with 3-0 vicryl deep fascial and 4-0 maxon running subcuticular suture similar to the other groups. Anticoagulation was not reversed for any of the four groups.

After either 1, 3 or 28 days, the operated left CCA was re-exposed as previously described for harvest. The proximal CCA was cannulated, while the distal CCA and the cranial thyroid branch were clamped and the artery perfusion fixed with 1.25% glutaraldehyde at the last recorded mean arterial pressure for 10 minutes, followed by CCA excision. The proximal half of the harvested CCA was snap frozen in liquid nitrogen for later protein analysis, and the distal half was paraffin-embedded for later Weigert van Gieson staining, H&E staining, and immunostaining.

Flow and pressure monitoring

Blood flow and mean arterial pressures were each measured once before and after intervention and arteriotomy repair, as well as prior to artery harvest, using an Transonic flow probe (2SB) and an ultrasonic transit-time flow meter during both the initial operation and during the harvest to ensure consistency and reproducibility of our model. (HT207; Transonics Systems, Ithaca, NY) The readings were recorded with a digital data acquisition system (*Lab Master DMA*;

Scientific Solutions, Solon, Ohio). The length of the balloon-injured CCA and the external diameter of the CCA were measured with digital calipers (CD-6, Mitutoyo Corp, Osaka, Japan).

Hemodynamic Assessment

Measured hemodynamic variables included mean arterial pressure (P) and blood flow (Q) while the wall shear stress (τ_w) was calculated using the Poiseuille formula: $\tau_w = 4\rho Q / \pi r_i^3$ (r_i = internal radius; ρ = viscosity of blood, assumed to be 0.03 poise). The internal radius measurement was calculated from immunohistochemical sections at day 1 and day 28.

Immunohistochemistry

For immunohistochemistry, 5- μ m sections of the paraffin-embedded CCAs were longitudinally cut at day 1 and day 3 from the sham, low τ , balloon injury, combined low τ and balloon injury groups. All paraffin sections were deparaffinized and rehydrated in a descending alcohol series.

Detection of apoptosis was performed through TUNEL (Terminal transferase dUTP Nick End Labeling) assay using the VasoTACS *in situ* apoptosis detection kit (Trevigen, Inc. Gaithersburg, MD). The sections were incubated with proteinase K (Trevigen, Inc. Gaithersburg, MD), washed with deionized water, incubated with 3% H₂O₂ for 5 min, and washed with deionized water again. TUNEL staining was performed as per the manufacturer's instructions.

Detection of activated caspase-3, an end mediator of apoptosis, was performed using SignalStain IHC detection kit (Cell Signaling, Danvers, MA). Antigen unmasking was performed by immersing slides in 0.01 M sodium citrate buffer followed by peroxidase quenching to block endogenous peroxidase activity. To prevent non-specific binding, the sections were immersed in a 3% BSA blocking solution for 1 hour at room temperature. For antibody staining, sections were first incubated with a prediluted primary antibody at 4°C overnight, rinsed for 15 minutes with PBS, incubated with biotinylated secondary antibody for 1 hour at room temperature and rinsed for a further 15 minutes with PBS. The sections were then stained as per the manufacturer's protocol and counterstained with hematoxylin.

Cell proliferation was assessed by staining for PCNA to look for the actively cycling cells within the media and intima. After antigen unmasking and peroxidase quenching as described above, the sections were incubated in a 3% BSA blocking solution for an hour at room temperature to prevent non-specific binding. Sections were then incubated with anti-PCNA mouse monoclonal IgG primary antibody (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, then rinsed with PBS and incubated with a pre-diluted biotinylated anti-mouse secondary antibody (Dako, Carpinteria, CA) for 1 hour at room temperature followed by washing with PBS. The sections were then incubated with a DAB+Chromagen substrate buffer (Dako, Carpinteria, CA). The reaction was analyzed under a microscope and the slides were rinsed when brown precipitate developed indicating positive staining. Counterstain with hematoxylin was applied before coverslipping.

Western blot

At day 3, after perfusion fixation with 1.25% glutaraldehyde, a portion of the left CCA was excised, snap frozen in liquid nitrogen, and morselized via mortar and pestle with subsequent homogenization on ice in lysis buffer containing 20 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM DTT, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 2 μ M leupeptin, 400 μ M PMSF and 10 U/ml aprotinin (Sigma Chemical, St. Louis, MO). The homogenate was incubated for 30 minutes at 4°C and then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was removed and protein concentration determined using the Bradford method. Equal amounts of

each protein extract (50 µg/lane) were heated at 95°C for 10 minutes in sample buffer (94 mM phosphate buffer, pH 7.0, 1% SDS, 2 M urea and 3% β-mercaptoethanol) and then separated onto 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane (Millipore, Bedford, MA) in a Novex Western transfer apparatus (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. The blots were washed and incubated with anti-caspase-3 rabbit polyclonal primary antibody (Calbiochem, San Diego, CA) and anti-rabbit secondary antibody (Pierce Biotechnology, Rockford, IL) for detection of caspase-3. For detection of PCNA, HRP conjugated mouse monoclonal antibody specific for PCNA (Dako, Carpinteria, CA) was used. GAPDH monoclonal antibody (Chemicon, Temecula, CA) was used as a loading control. The blots were developed using an ECL Western blotting system (Amersham Biosciences; Piscataway, NJ) and densitometry analysis was performed using a KODAK Image Station 440cf system (Rochester, NY).

Image analysis

For evaluating regions with positive TUNEL, caspase-3 and PCNA staining, ImageJ, a processing and analysis software was used³². In brief, digital images were acquired with a microscope equipped with a 3.0 mega pixel digital camera (Olympus; Melville, NY). The regions of interest were photographed in 8-bit grayscale at a magnification of x4, the background normalized and the density thresholds set to 130 (minimum) and 255 (maximum). The image was then inverted to give the positive staining as red on a black background. A region of 0.04 mm² was selected to measure the positive area in eight equidistant regions on the section in both media and intima and analysis performed by using the ImageJ particle analysis algorithm in a blinded fashion. The percent positive area was calculated by dividing the positive staining area by the total area (0.04 mm²) of the region selected. For each region of interest, a spot check was performed by visually counting the cells under the microscope, and the consistency of results obtained with both methods was found to exceed 95%.

Assessment of morphology

For histomorphometry, 5-µm sections of the paraffin-embedded CCA were longitudinally cut at day 28 from the sham, low τ, balloon injury, and combined low τ and balloon injury groups, and stained using the Weigert van Gieson stain. Sections of each specimen were analyzed histomorphometrically in a blinded fashion. Digital images were acquired with a microscope equipped with a 3.0 mega pixel digital camera (Olympus; Melville, NY). Neointimal thickness, medial thickness, and luminal radius were digitally measured using ImageJ software, while the intima:media ratio and neointimal area were calculated using the previously measured values.

Data Analysis

Data analysis was performed using standard software (*Microsoft Excel Data Analysis; Redmond, WA*). Data is presented as mean ± standard error of the mean (SEM). Comparison of means was achieved using One-way analysis of variance (ANOVA) with Tukey's Honestly Significant Differences test, as well as the Student's t-test, with a significance assigned at $P < 0.05$.

RESULTS

Hemodynamic assessment

Hemodynamic data are given in Table 1 and Table 2. As expected, restriction of the CCA by ligating the distal CCA over a 0.014 guide wire in the low τ group resulted in very low flow both at day 1 (2.90 ± 0.72 ml/min) and day 28 (3.65 ± 0.78 ml/min), in addition to shear stress at days 1 (0.64 ± 0.16 dyne/cm²) and 28 (0.73 ± 0.14 dyne/cm²), as compared to the sham (27.25 ± 1.36 ml/min, 25.2 ± 2.92 ml/min; 4.59 ± 0.53 dyne/cm², 6.65 ± 2.07 dyne/cm²). The

measurements in the combined low τ and balloon injury group at day 1 and day 28 (3.58 ± 0.89 ml/min, 5.75 ± 2.62 ml/min; 0.50 ± 0.11 dyne/cm², 0.54 ± 0.26 dyne/cm²) were statistically similar to the low τ group, and significantly lower when compared to both the sham group and the balloon injury group alone (17.00 ± 1.26 ml/min, 32.58 ± 2.72 ml/min; 2.57 ± 0.41 dyne/cm², 6.49 ± 1.47 dyne/cm²).

Assessment of apoptosis

In situ detection of cell death using TUNEL assay revealed a significant increase in the percent positive area at day 1 and day 3 for the combined low τ and balloon injury group as compared to the other three groups. Although there was a significant difference in the percent positive area of apoptosis between the low τ and balloon injury groups at day 1, there was no significant difference at day 3 (Fig.1 & 2 a,b).

A key mediator of apoptosis, caspase-3, was significantly greater in the combined low τ and balloon injury group as compared to the other three groups at day 1 and day 3 on immunostaining. Unlike the TUNEL assay apoptosis data at day 1, there was no difference in the percent positive areas of apoptosis between the low τ and balloon injury groups at day 1. (Fig. 1 & 2 a,b) These findings may result from potential areas of cell necrosis, that may be stained with TUNEL assay. Although, at day 3, similar to the TUNEL assay apoptosis data, there was no difference in caspase-3 percent positive area between low τ and balloon injury groups, and both continued to be significantly greater than the sham group (Fig.1 & 2 a,b). The western blot densitometry further supports these results for caspase-3, demonstrating a 3-4 fold increase in caspase-3 in the combined low τ and balloon injury group as compared to the other three groups at day 3 (Fig. 3 a,b).

Assessment of proliferation

Cell proliferation as assessed by percent positive area of PCNA, was also significantly greater in the combined low τ and balloon injury group as compared to the other three groups at day 1 and day 3. (Fig.1 & 2 a,b). Although at day 1, the PCNA percent positive area of the sham group as compared to the low τ and balloon injury groups is higher, this lacked statistical significance. (Fig. 1b) At day 3, these results differed demonstrating an increase in cell proliferation with all of the injury groups as compared to the sham group, with the combined low τ and balloon injury group creating statistically the greatest cell proliferation than all the other three groups. (Fig. 2b) Supportively, our western blot analysis at day 3 from the combined low τ and balloon injury group revealed more than a 2.5 fold increase in the PCNA expression at day 3 as compared to either the low τ or the balloon injury group alone. There was no PCNA expression in the sham group (Fig.4 a,b).

Carotid artery morphology at 28 Days

Representative samples from all of the different cohorts; sham (n=6)(28 day carotid artery morphology data from 4 of the 6 sham animals was previously published ³³), low τ (n=4), balloon injury (n=4), and combined low τ and balloon injury (n=4), are shown in Figure 5, and numeric data given in Table 3. All arteries remained patent at sacrifice at 28 days. All three injury models, low τ (26 ± 3 μ m), balloon injury (51 ± 17 μ m), and combined low τ and balloon injury (151 ± 35 μ m), resulted in a significant increase in the neointimal thickness as compared to the sham group (0 μ m). Although when comparing the NH observed in the low τ group with the balloon injury group, there was no significant difference. However, a significantly greater NH was observed with the combination of low τ and balloon injury as compared to all of the other three groups. Similarly, a significantly greater intima:media ratio was observed for all injury models when compared to the sham group, with statistically the greatest ratio observed with the combined low τ and balloon injury group. The resultant neointimal area was also significantly greater in all of the injury models, as compared to the sham. Although there was

a dramatic trend towards increased neointimal area for the combined low τ and balloon injury as compared to both the low τ and balloon injury groups alone, there was no statistical significance other than when compared to the sham group.

DISCUSSION

Balloon-induced direct vessel wall injury and low flow hemodynamic states lead to neointimal hyperplasia (NH) as a result of induced arterial remodeling. Resultant NH plagues the success of catheter-based therapies for arterial occlusive disease, with intermediate-term patency rates varying between 50-90%^{34, 35}. Although studies demonstrate the effect of balloon injury as well as low flow hemodynamic states alone on NH⁶, our study evaluates the effect of low flow hemodynamic states, as assessed via shear stress, and balloon injury on NH, as well as the effect of combined low shear stress and balloon injury on NH – a phenomenon that frequents the clinical realm.

In this study we evaluated the effect of low shear stress and balloon injury on vascular smooth muscle cell proliferation and apoptosis underlying arterial remodeling following injury. Evidence suggests that there is an integral role of programmed cell death in vessel remodeling and neointimal lesion development¹⁹. Animal models have shown resultant smooth muscle cell apoptosis following low flow hemodynamic states⁶, as well as balloon injury²¹. In the present study, we studied both the apoptotic and cellular proliferative response in a combined low shear stress and balloon injury animal model. Our model focused not only on the occurrence of both cell proliferation and apoptosis, but also the onset of cellular response to injury.

Our results revealed an early onset of both cellular proliferation and apoptosis by 24 hours which continued for at least 72 hours after injury. The resultant cellular proliferation and apoptosis was statistically greater following combined low shear stress and balloon injury, than either low shear stress, balloon injury, or uninjured arteries alone - concluding that arterial exposure to the combination of low shear stress and balloon injury creates a synergistic response to both smooth muscle cell proliferation and apoptosis. This early onset of apoptosis is supported by other investigators evaluating apoptosis following balloon injury alone in rat carotid arteries²¹. However in our study, we not only demonstrated an early onset of apoptosis following arteries exposed to balloon injury alone as well as low shear stress alone, but the largest apoptotic response to vessels following exposure to concurrent balloon injury and low shear stress states.

To further evaluate the up-regulated apoptotic response, we selectively isolated caspase-3, a critical member of the caspase/interleukin-1 β -converting enzyme protease family, which is an end mediator of programmed cell death^{36, 37}. Through immunostaining and western blot analysis, we showed that the apoptotic response to vessel injury is caspase-3 mediated, with concomitant low shear stress and balloon injury producing the greatest response.

Coexistent to the apoptotic response was a robust proliferative response as shown by early PCNA activation by 24 hours. This proliferative response is a well known response in NH and has been well studied¹¹. Kamenz *et al.* investigated both the proliferative and apoptotic response following balloon angioplasty in rabbit carotid arteries, showing an overall greater incidence in cellular proliferation in comparison to cellular apoptosis, leading to overall net NH at 28 days²². They eluded the existence of a delicate balance between cell proliferation and apoptosis within the smooth muscle cells of the vessel wall, and that small shifts lead to either net cell accumulation or cell death²².

NH is a complex physiologic response with multiple physiologic variables³⁸ and the balloon injury-low flow model has helped elucidate many of its mediators^{31, 39}. With this in mind, we

limited the study to the assessment of concomitant proliferation and apoptosis. In our study, we demonstrated an up-regulation of both cellular proliferation and apoptosis with almost every injury model as compared to our control rabbits, with the combination of low shear stress and balloon injury leading to the greatest up-regulation. We believe this up-regulation of both apoptosis and cellular proliferation indicates not necessarily a balance, but a well coordinated response of cellular demise and cell survival underlying arterial remodeling, since the greatest NH in our study resulted from the combined low shear stress and balloon injury, which also produced both the greatest cellular proliferation and apoptosis. One putative explanation for the considerable increase in NH despite increased apoptosis in the combined low shear stress and balloon injury model may be that the apoptotic cells induce a release of growth factors and cytokines that by a paracrine effect, induce ERK1/2 pathways leading to increased smooth muscle cell proliferation, thereby leading to increased NH. Certainly, the NH response has been shown to be mediated through mitogen activated protein kinase signaling pathway⁴⁰, modulation by the cytoskeleton due to changes in the physical flow parameters⁴¹ or by inflammatory mediators and platelets⁴². Although it is difficult to ascertain whether the cellular events of apoptosis lead to cellular proliferation, or vice versa, it is reasonable to conclude that the combination of both low shear stress and balloon injury lead to a significant increase in both cell proliferation and cell apoptosis, which in turn results in a synergistic increase NH approximating that seen in clinical restenosis.

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Figure 1.

Panel a) *In situ* detection of apoptosis using TUNEL assay and Caspase-3 immunostaining, as well as cell proliferation using PCNA immunostaining. Sham, Low τ , Balloon Injury, and combined Low τ and Balloon Injury at day 1. The arrow indicates positively stained cells. (Magnification x40)

Panel b) The percent positive area by TUNEL assay, Caspase-3, and PCNA immunostaining for the different injury models at day 1.

Figure 2.

Panel a) *In situ* detection of apoptosis using TUNEL assay and Caspase-3 immunostaining, as well as cell proliferation using PCNA immunostaining. Sham, Low τ , Balloon Injury, and combined Low τ and Balloon Injury at day 3. The arrow indicates positively stained cells. (Magnification x40)

Panel b) The percent positive area by TUNEL assay, Caspase-3, and PCNA immunostaining for the different injury models at day 3.

Figure 3.

Panel a) Western blot analysis for Caspase-3 expression for the different injury models at day 3. Lane 1 represents the positive control, lane 2 – Sham group, lane 3 – Low τ group, lane 4 – Balloon Injury group, and lane 5 – combined Low τ and Balloon Injury group.

Panel b) The densitometry data for Caspase-3 expression at day 3 for the different injury models.

Expressed as mean $\pm$ SEM

* $P < 0.05$ compared to Sham Model.

† $P < 0.05$ compared to Balloon Injury Model.

‡ $P < 0.05$ compared to Combined Low τ and Balloon Injury Model.

Figure 4.

Panel a) Western blot analysis for PCNA expression at day 3. Lane 1 represents the positive control, lane 2 – Sham group, lane 3 – Low τ group, lane 4 – Balloon Injury group, and lane 5 – combined Low τ and Balloon Injury group.

Panel b) The densitometry data for PCNA expression at day 3 for the different injury models.

Figure 5. Rabbit CCA exposed to Sham (A,a), Low τ (B,b), Balloon Injury (C,c), and combined Low τ and Balloon Injury (D,d) harvested after 4 weeks and stained with Weigert-van Gieson stain. $\rightarrow$ represents Internal Elastic Lamina while $\leftrightarrow$ indicates NH. Note profound NH in the combined Low τ and Balloon Injury group (A, B, C, D – x4; a, b, c, d – x40 magnification).

Table 1

Carotid artery hemodynamics at harvest (Day 1)

Model	Blood Flow Day 0 (ml/min)	Blood Flow at harvest Day 1 (ml/min)	Shear stress at harvest Day 1 (dyne/cm ²)
Sham (n=4)	31.50 ± 2.87	27.25 ± 1.36	4.59 ± 0.53
Low τ (n=4)	3.30 ± 0.45 * [†]	2.90 ± 0.72 * [†]	0.64 ± 0.16 * [†]
Balloon Injury (n=4)	29.87 ± 3.18 [‡]	17.00 ± 1.26 * [‡]	2.57 ± 0.41 [‡]
Low τ and Balloon Injury (n=4)	2.91 ± 0.69 *	3.58 ± 0.89 *	0.50 ± 0.11 *

Expressed as mean ± SEM

* P < 0.05 compared to Sham Model

[†] P < 0.05 compared to Balloon Injury Model[‡] P < 0.05 compared to Combined Low τ and Balloon Injury Model

Table 2

Carotid artery hemodynamics at harvest (Day 28)

Model	Blood Flow Day 0 (ml/min)	Blood Flow at harvest Day 28 (ml/min)	Shear stress at harvest Day 28 (dyne/cm ²)
Sham (n=4)	31.3 ± 3.06	25.2 ± 2.92	6.65 ± 2.07
Low τ (n=4)	3.98 ± 0.85 * [†]	3.65 ± 0.78 * [†]	0.73 ± 0.14 * [†]
Balloon Injury (n=4)	23.75 ± 1.11 [‡]	32.58 ± 2.72 [‡]	6.49 ± 1.47 [‡]
Low τ and Balloon Injury (n=4)	3.59 ± 0.25 *	5.75 ± 2.62 *	0.54 ± 0.26 *

Expressed as mean ± SEM

* P < 0.05 compared to Sham Model

[†] P < 0.05 compared to Balloon Injury Model[‡] P < 0.05 compared to Combined Low τ and Balloon Injury Model

Table 3

Carotid artery morphometry at 28 Days

Model	Neointimal thickness (μm)	Medial thickness (μm)	I:M	Neointimal area (mm ²)
Sham (n=6)	0	149 ± 13	0	0
Low τ (n=4)	26 ± 3 * [‡]	165 ± 14 [‡]	0.16 ± 0.02 * [‡]	0.12 ± 0.01 * [‡]
Balloon Injury (n=4)	51 ± 17 * [‡]	120 ± 17	0.45 ± 0.17 * [‡]	0.67 ± 0.18 *
Low τ and Balloon Injury (n=4)	151 ± 35 *	107 ± 15 *	1.41 ± 0.19 *	2.05 ± 0.81 *

Expressed as mean ± SEM

* P < .05 compared to Sham Model

[‡] P < .05 compared to Balloon Injury Model[‡] P < 0.05 compared to Combined Low τ and Balloon Injury Model