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Characterization of the expression of Ang1, Ang2 and Tie2 in the corpus cavernosum of the Rat during aging

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Characterization of the expression of Ang1, Ang2 and Tie2 in the corpus cavernosum of the Rat during aging

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Abstract

Aging is the single most significant risk factor for erectile dysfunction (ED), leading to structural modification of cavernous tissue and altering expression of vascular growth factors. Angiopoietin/Tie2 system has been recently considered as a potential target for therapy of vascular disorders, including ED. Hence, the aim of this study was to analyze expression of angiopoietin1 (Ang1), angiopoietin2 (Ang2), and their receptor Tie2 in corpus cavernosum (CC) of rat during aging (6, 12, 18 and 24 months).

The expression of Ang1, Ang2 and Tie2 was studied by immunohistochemistry and immunofluorescence, followed by semi-quantification after Western blotting.

Both Ang1 and Ang2 were localized mainly in perivascular smooth muscle and endothelial cells, still Tie2 was strictly detected at vascular endothelium. A significant decrease in Ang2's expression was observed at 12 months when compared with 6 months aged rats, a tendency that reverses in older animals. No significant differences were demonstrated for Ang1 or Tie2, which is consistent with their constitutive expression in CC. The ratios Ang1/Tie2 and Ang2/Tie2 were also calculated and both decrease during aging, while no marked variation was observed for Ang1/Ang2.

Our results suggest that angiopoietin/Tie2 system participate in the vascular maintenance and remodeling of the CC during aging.

Key words: Erectile dysfunction; aging; angiogenesis; rat; corpus cavernosum; angiopoietins; Tie2.

Running title: Ang1, Ang2 and Tie2 in aged rat´s erectile tissue

Introduction

Erectile dysfunction (ED), defined as the consistent inability to achieve and maintain an erection sufficient for satisfactory sexual intercourse, is a highly prevalent disease worldwide. According to a recent European survey, aging, diabetes mellitus, and hypertension are the most important risk factors for the development of ED (Ponholzer et al., 2005). However, aging was recently considered as the single most significant risk factor (Ghalayini et al., 2010).

Despite all epidemiological studies carried out, consensus has not yet been reached on ED's exact magnitude. According to *The Massachusetts Male Aging Study*, 34.8% of men aged 40 to 70 years old have moderate-to-complete ED, and 15% of men aged 70 years old have complete ED. This study also concluded that the risk of manifesting ED increases markedly with age (Feldman et al., 1994). Moreover, it was found that the prevalence of severe ED increases from 2.7% in men in their twenties to 38,6% in their sixties and 46% in those aged 70 years and above (Ghalayini et al., 2010).

The corpus cavernosum (CC) of the penis is the main anatomic structure involved in erection and consists of a spongelike tissue, where numerous vascular spaces, lined by endothelial cells (EC) (Gumus et al., 2004), are supported by smooth muscle layers and connective tissue trabeculae. Not surprisingly, ED has a predominantly vasculogenic origin (Ryu et al., 2005).

The erection of penis starts with an excitatory neuronal stimulus that induces nitric oxide (NO) and prostaglandin production by ECs which are critical for relaxation of the smooth muscle components of CC (Burnett et al., 1992; Burnett, 1995). This mechanism is followed by a coordinated vascular response that directs blood perfusion to dilated sinusoidal spaces of the CC and, after veno-oclusion, generates rigidity of the penis sufficient for coitus (Aboseif & Lue, 1988). The loss of endothelium integrity leads to decrease in NO bioavailability in perivascular smooth muscle which results in a clinical condition named endothelium

dysfunction, and seriously compromises the erectile capacity of CC. This evidence leads several authors to consider ED equivalent to endothelium dysfunction (Goldstein, 2003; Guay, 2007). Therefore, ED is now considered a barometer for vascular health and incipient cardiovascular disease (Cheitlin, 2004).

Aging induces structural modification of the CC, increasing the percentage of connective tissue constituents, such as fibroblasts and collagen fibers comparatively to smooth muscle cells (Cordeiro et al., 2008; Tomada et al., 2008; Tomada et al., 2010*a*). Augmentation of the vascular spaces was also observed in aged individuals (Costa & Vendeira, 2008; Tomada et al., 2010*a*). Moreover, a decrease in penile endothelial and neuronal activity of NO synthase (NOS, responsible for nitric oxide synthesis) has been observed during aging (Garban et al., 1997), a condition that is often associated with damage of intracavernous vascular structures, as described for both rodent and human (Burchardt et al., 2000; Pinheiro et al., 2000).

The highly ordered process of formation of new capillaries from the preexisting blood vessels is named angiogenesis (Risau, 1997; Yancopoulos et al., 1998; Carmeliet & Jain, 2000). It was reported that angiogenesis is especially downregulated in the aged individuals (Carmeliet, 2003; Fiedler & Augustin, 2006). Even so, vascular integrity and remodeling characteristics of each adult tissue are determined by the balance of local pro- and anti-angiogenic factors, and strictly depend on their regulated interaction with specific membrane receptors expressed mainly by endothelial and mural cells. Vascular endothelial growth factor (VEGF) is the most important angiogenic factor expressed in every vascularized tissue. It induces proliferation, sprouting and tube formation of ECs *in vitro* (Ferrara et al., 2003) usually after a hypoxic stimuli (Otrock et al, 2007). Angiopoietin 1 (Ang1) and Angiopoietin 2 (Ang2), originally identified in tissue culture (Peters, 1998), belong to the family of Angiopoietins and both crosstalk *in vivo* with VEGF (Eklund, 2005). Ang1 and Ang2 are mainly produced by pericytes and ECs, respectively (Bach et al., 2006; Fiedler et al., 2004) and bind with the

same affinity to the tyrosine kinase with immunoglobulin and EGF homology domain-2 (Tie2) receptor, expressed primarily on ECs, that mediate their biological effects (Schnurch & Risau, 1993). Ang1 was shown to act as an obligatory agonist inducing Tie2 phosphorylation, that increments microvascular endothelium maturation and integrity (Suri et al., 1996), and promotes cell-cell and cell-extracellular matrix interactions, cooperatively with VEGF (Eklund & Olsen, 2005). It also presents anti-permeability and anti-inflammatory properties that exert a protective effect on the endothelium. Ang1 is widely expressed in normal human CC where it features a paracrine signal of stabilization or survival through a low-level constitutive expression (Tomada et al., 2010*a*; Tomada et al., 2010*b*).

Conversely, Ang2 expression is restricted to sites of vascular remodeling, where it promotes vessel destabilization and functions as an autocrine negative regulator of the quiescent resting endothelium. Despite being considered a natural antagonist of Ang1, Ang2 is able to context-dependently induce Tie2 phosphorylation, which also provides it agonistic functions (Yuan et al., 2009). In fact, Ang2 either promote vessel growth or regression depending on the cell type and on the levels of bioavailability of other growth factors, such as VEGF. Indeed, in the presence of endogenous VEGF, Ang2 efficiently induces vessel sprouting and growth, facilitating angiogenesis. If endogenous VEGF activity is inhibited, Ang2 effectively promotes vessel regression with cell death (Lobov et al., 2002; Eklund & Olsen, 2005; Bach et al., 2006; Fiedler & Augustin, 2006). In fact, Ang2 can induce a highly specific destabilization response in microvascular ECs independently of Ang1 in pathological situations (Lobov et al., 2002), while it may prevent excessive sprouting and branching of blood vessels in normal tissues (Maisonpierre et al., 1997).

For all this, angiopoietin/Tie2 system emerges as a putative target for therapy of vascular disorders, including ED. This evidence prompted us to study Ang1, Ang2, and Tie2 expression in CC of rat during aging, which as far as we know has never been reported. It is

our conviction that successful understanding of molecular mechanisms involved in vascular maintenance and remodeling of CC will improve prevention and treatment of vasculogenic ED in the aged individuals.

Materials and Methods

Animals

Twenty male Wistar rats maintained under a 12-hour light-dark cycle and standard temperature (20-22ºC) with free access to food and water, were randomly divided in 4 groups, and sacrificed by decapitation when reach the age of endpoint (6, 12, 18 and 24 months). The trunk blood was collected to heparinized tubes, and the plasma fractions were frozen at -80ºC until analysis. Penises were dissected up to the crura and excised after rejection of the distal foreskin. Each penis was divided in two portions, one was immediately fixed in 10% buffered formaldehyde, and the other frozen at -80ºC for further molecular analysis. Animal procedures were undertaken according to the European Community guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals.

Immunohistochemistry

Formalin fixed penis fragments were embedded in paraffin, oriented along its transversal axis; 5 μm thick sections, were cut with a Leica RM2145 microtome (Leica Microsystems GmbH, Germany) and placed onto 0.1% poly-L-lysine coated microscopy slides for immunostaining. Sections were deparaffinized, hydrated in a graded series of ethanol solutions of decreasing concentrations until water, and treated for 30 min with 3% hydrogen peroxide solution in methanol to block endogenous peroxidase activity. Epitope retrieval was performed by 30 min incubation with HCl 0.1M, followed by neutralization with sodium tetraborate 0.1M for 5

min. Sections were incubated in a humid chamber at room temperature for 1 h with 10% goat normal serum and 2% bovine serum albumin (BSA) phosphate buffer saline (PBS) solution. Afterwards, they were incubated overnight at 4°C, with specific primary antibodies to Ang1 (1:25 diluted), Ang2 (1:25 diluted) and Tie2 (1:200 diluted) (Santa Cruz Biotechnology, CA, USA). Sections were further incubated at room temperature in a humid chamber with the biotinylated secondary antibody, anti-goat (1:500 diluted) (Sigma-Aldrich, Gillingham, UK) to Ang1 and Ang2, or anti-rabbit (1:500 diluted) (Santa Cruz Biotechnology, CA, USA) to Tie2, for 30 min at room temperature followed by 30 min incubation with streptavidin-horseradish peroxidase complex diluted 1:200 (Vectastain,Vector, Burlingame, CA). Sections were then washed in PBS, reacted for 5 min with 3,3´-diaminobenzidine (DAB) tetrahydrochloride solution containing 0.1% (v/v) hydrogen peroxide (H_2O_2) and counterstained with hematoxylin. The negative control was done without the primary antibody, in order to confirm the specificity of the antibody. All slides were observed, and images were captured in an optical microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), connected to an Axiocam MRm camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Immunofluorescence

Simultaneous detection of Ang1/Tie2 and Ang2/Tie2 was performed in sections processed as described before for immunohistochemistry, incubated overnight at 4°C, with the mixture of the primary antibodies in the concentrations previously used. Following washing, sections were incubated at room temperature for 1h in a humid chamber with appropriated secondary antibodies solution, anti-goat conjugated with Alexa Fluor 568® (red) / anti-rabbit conjugated with Alexa Fluor 488® (green) both 1:500 diluted (Molecular Probes, Leiden, Netherlands). Sections were then mounted with a glycerol solution in phosphate buffer (3:1) after a 30 s

incubation with 4',6-diamidino-2-phenylindole (DAPI), for nuclear DNA staining (blue). The negative control intended to exclude autofluorescence or nonspecific reactivity was also performed without the primary antibodies. Sections were visualized in an ApoTome microscope (Carl *Zeiss System*) and digital images were acquired with AxionVision 3.0 program (Carl *Zeiss System).*

Western Blotting

Penis fragments were homogenized in 0.1M NaCl, 5mM EDTA, 0.5% v/v Triton X-100, 50mM Tris pH7.2, supplemented with 1:200 (v/v) of protease inhibitor (protease Inhibitor Cocktail P8340, Sigma-Aldrich Co, UK) and 20% (v/v) of glycerol. Quantification of total proteins of each sample was done by the method of Bradford (Bradford, 1976) in a spectrophotometer Beckman DU640 (Beckman, CA, USA) and 20 μg of total protein per lane were loaded on a 10% sodium dodecilsulfate polyacrylamide gel (SDS-PAGE) (Laemmli, 1970), and allowed to run for 1 h in an electrophoresis apparatus (Bio-Rad®, CA, USA), 20mA were applied per gel.

Electrophoresis peptides were transferred to a nitrocellulose membrane (Bio-Rad®) with 0.45 μm pore, for 2 hours (Towbin et al., 1979). Membranes were stained with Ponceau S, washed and incubated for 1 h with a blocking solution (nonfat dried milk 5% in 0.1% Tween-20 Tris-buffered saline), at room temperature and with gentle agitation on a platform shaker. Membranes were then probed with the antibodies anti-Ang1, anti-Ang2, and anti-Tie2 (previously used in immunohistochemistry) and β-actin (1:200 diluted) (Molecular Probes, Leiden, Netherlands) overnight, at 4°C, with gentle agitation. After being washed, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1:5000 diluted), anti-goat to Ang1 and Ang2 (Santa Cruz Biotechnology, CA, USA), anti-rabbit to Tie2 (Molecular Probes), or anti-IgM to β-actin (Molecular Probes) for 1 h. β-actin

was used as an internal control in order to quantify the proteins in study. Labelled bands were evidenced using chemiluminescent substrate (Kit SuperSignal, Pierce Biotechnology, IL, USA) and quantified using specific software (Scion Image® for Windows). Results represent the semi-quantification of each protein in each experimental group (6, 12, 18 and 24 months).

Statistics

Values are expressed as mean ± standard error for the mean (SEM) throughout the text. All statistical analysis was performed employing absolute values using the Statistical Package for the Social Sciences (SPSS), version 14.0 for Windows (SPSS Inc., Chicago, IL, USA); the difference of mean values between groups was assessed using a two-tail t test. A value of $P<0.05$ was considered significant.

Results

In the present report, the detection of Ang1, Ang2 and Tie2 was performed by immunohistochemistry in penile tissue obtained from rats aged 6, 12, 18 and 24 months. No marked histological differences were observed among cavernous tissue isolated from the experimental groups. Under light microscope examination, tissue presented spongelike texture in every studied age (Fig. 1). It exhibited a mesh of interconnected cavernous spaces that were lined by endothelium, surrounded by a smooth muscle layer, and separated by trabecula mainly composed by connective tissue (collagen fibres and fibroblasts), as previously reported (Neves et al., 2008).

In what concerns Ang1 immunolocalization (Fig. 1), a scattered staining both in endothelium and in smooth muscle layer was observed in CC of all studied ages. An apparent decrease was observed in the CC of rats aged 12 months (Fig. 1). Equivalent distribution of stained

structures was observed after immunohistochemical detection of Ang2 (Fig. 2), which also presented an apparent reduced intensity in the CC of rats aged 12 months. As expected, the receptor Tie2 was detected mainly in the endothelium (Fig. 3) in all studied groups. These results corroborate those obtained by dual immunolabelling of angiopoietins and their receptor Tie2. In fact, simultaneous immunofluorescent detection of Ang1 (red) and Tie2 (green) demonstrated low intensity co-localization, restricted to the endothelium (Fig. 4), and, as previously observed by immunohistochemistry (Fig. 1), a slight reduction in Ang1 staining in the CC of 12 months aged rats. Simultaneous detection of Ang2 (red) and Tie2 (green) evidenced co-expression restricted to the endothelium (Fig. 5), which apparently was also decreased in CC of rats aged 12 months. Interestingly, a slight increase in endothelial expression of Tie2 was observed in aged animals, 18 and 24 months, when compared with younger rats (6 and 12 months) (Fig. 4 and 5).

The semi-quantification of Ang1, Ang2 and Tie2 in CC was carried out in Western blots following chemiluminescent detection of bands. As shown in figure 6, the studied proteins were detected with an apparent molecular mass of 60kDa for Ang1, 66kDa for Ang2 and 140kDa for Tie2. The expression of each molecule was analyzed, for each studied age. Although having no statistical significance, it was observed a slight increase in Ang1's expression in older rats (18 and 24 months) when compared with younger ones (6 and 12 months) (Fig. 7). On the other hand, it was found a significant decrease in the expression of Ang2 in animals with 12 months when compared with the group of 6 months aged rats, a tendency that reverses in the two groups of older animals (Fig. 7). The expression of Tie2 does not present significant difference among the studied groups, besides the apparent tendency to increase during aging (Fig. 7).

The ratios Ang1/Tie2 and Ang2/Tie2 for each studied animal were also calculated, and as shown in graphs of figures 9 and 10 respectively, both ratios decrease during aging. Despite

this variation, only the ratio Ang2/Tie2 observed at 24 months aged animals was significantly lower than that presented by 6 months rats. Additionally, Ang1/Ang2 was calculated for all studied animals, however it does not vary consistently during aging (Fig. 8).

Discussion

In the present report, we studied the expression of Ang1, Ang2 and their receptor Tie2 in erectile tissue of rat during aging. Despite the differences observed in the structure of rat's and human's CC, the rat is still an attractive model to study ED (Pinheiro et al., 2000; Cordeiro et al., 2008). This interest has been attributed to the small size of the animal and to the availability of correlative data on the physiology of erectile function between the two species (Fernandez et al., 1991).

Aging is an independent risk factor for ED which, in part, is motivated by structural modifications that occur in cavernous tissue. In fact, it was demonstrated that smooth muscle cells proportionally diminishes during aging (Cordeiro et al., 2008; Tomada et al., 2008; Tomada et al., 2010*a*), which is accompanied by increase in collagen deposition and decrease in elastic fibers' concentration, both in rat and human CC (Pinheiro et al., 2000). Aging is also associated with vascular remodeling which leads to increase in cavernous vascular spaces (Costa & Vendeira, 2008; Tomada et al., 2010*a*). Moreover, postmortem studies revealed increased atherosclerotic vascular alterations in the arterial bed of the penis of aged individuals (Bossart et al., 1980). Taking into account that dysfunction of endothelium antedates atherosclerosis structural derangements, it is conceivable that factors which regulate vascular functioning are a key to the understanding of such changes.

Vascular remodeling is a complex and context sensitive process that results from combined modification in the cellular composition of the tissue and in the expression of angiogenic

factors, of which VEGF is the principal. Other angiogenic factors: Ang1, Ang2 and their receptor Tie2, are not involved in the primary vasculature formation, since the process is not affected when any of these genes is knocked out (Bach et al., 2006). However, their role in angiogenesis is remarkable, beginning in early embryonic life. Either Ang1 or Tie2 deficiency results in embryonic lethality (day 12,5 and 10,5 respectively), due to important cardiovascular defects such as poorly organized vessels, fewer branches and reduced pericyte coverage (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996; Patan, 1998). In contrast, in the adult, overexpression of Ang1 or its receptor has little effect on vessel structure and heart development (Thomas & Augustin, 2009). In what concerns Ang2, although being weakly expressed in the quiescent vasculature and dispensable for early development, it seems to be dramatically up-regulated during vascular remodeling (Eklund & Olsen, 2005) and particularly in tumor growth (Stratmann et al., 1998; Scott et al., 2005; Thomas & Augustin, 2009). Overexpression of Ang2 apparently occurs in necrotic and hypoxic regions (Koga et al., 2001), provoking vascular defects such as the disruption of vessel integrity (Maisonpierre et al., 1997), destabilization of endothelium and the triggering of an inflammatory response (Fiedler et al., 2006). However, Ang2 deficient mice only show minor vascular defects, which reflect the main role of Ang2 as a regulator of vessel remodeling and vascular regression (Gale et al., 2002; Hackett et al., 2002), in which case it associates with a down regulated VEGF (Lobov et al., 2002).

Taking into account the contribution of Ang1 and Ang2 to vascular maintenance and destabilization respectively, it was hypothesized that angiopoietin/Tie2 system is a regulator of vascular remodeling of the CC of the rat during aging, thus contributing to age-related ED's progression.

Immunohistochemical detection of Ang1 showed its expression in smooth muscle cells, mainly from the vessel wall, and also in endothelium, similarly to previous observations in

human tissue (Tomada et al., 2010*a*; Tomada et al., 2010*b*). Ang2 presented an equivalent distribution in rat's cavernous tissue, despite no previous results for immunohistochemical detection in human CC were reported. Moreover, Tie2 seems to be endothelial specific, which again agrees with the localization observed in human CC (Tomada et al., 2010*a*; Tomada et al., 2010*b*).

The semi-quantification of Ang1, Ang2 and Tie2's expression during aging was made on the Western blots. In what concerns Ang2 expression, it was observed a significant decrease in the CC of 12 month-old animals, after which the tendency reverted; interestingly, VEGF level in rat CC presented an inverse variation: there was a gradual increase until 9 months, followed by a decline thereafter (Neves et al., 2006). As to Ang1 and Tie2 expression, an age-related pattern similar to Ang2 was found: a decrement in expression at 12 months, although not statistically significant, and an increase at 18 and 24 months, when compared to the youngest, 6 months old animals.

The inverse relation of Ang2 and VEGF expressions and the Ang1 and Tie2 trend provide further support to this rat model because parallel findings were described previously in human CC (Tomada et al., 2010*a*). In addition, they indicate that VEGF dependent endothelial growth in CC is forestalled in aged animals, thus suggesting that a new regulatory mechanism is taking place, combining all three factors: Ang1, Ang2 and Tie2.

Ang1 and Ang2 are well known competitors for the receptor Tie2 and their opposing activity likely results in the fine regulation of vascular remodeling. How this occurs is largely unknown and may be context sensitive. Moreover, recent evidence strongly suggests agonistic function for Ang1 and Ang2 in Tie2 activation (Bogdanovic et al., 2006; Daly et al., 2006;Yuan et al., 2009).

In the CC, the lack of statistically significant differences between the different ages is suggestive of a constitutive synthesis of all three factors; however, the relative variation in

protein level exhibited by any of them, notably Tie2 receptor level, is evidence for an age-related compensatory up-regulation that favors Tie2 receptor signaling in aging, probably resulting from growing demands of vascular remodeling due to VEGF down-regulation.

The individual variation of each angiogenic factor is difficult to discuss, nevertheless the comparison between the relative levels along aging may provide some insights.

The age-related Ang1 increase is higher than Ang2, which favors Ang1 action and provides another compensatory effect due to vascular stabilization contribution. This might be of importance as it was previously reported that the functional status of the vasculature could be inferred from the Ang1/Ang2 ratio (Fiedler et al., 2006; Pfaff et al., 2006). Accordingly, vascular quiescence maintained by constitutive Ang1-Tie2 signaling prevails over Ang2/Tie2 destabilization and pro-inflammatory effect. This is especially important taking into account previous structural studies either in rat or human, providing evidence for an age related reduction of smooth muscle cells of the perivascular spaces, and their partial replacement by fibroblasts and collagen (Cordeiro, et al., 2008; Tomada et al., 2008).

One final remark concerns the changes observed in the 12 month old animals. It is apparent from the data that at this age, CC exhibits a peculiar angiogenic pattern, which appears to disagree with the main age related tendencies. In fact Ang1, Ang2 and Tie2 decrease at 12 months, in contrast with other ages. This likely reflects specific maturational properties of unknown significance and are evident because the evaluation was made at various time-points. Since long this approach has been recommended in aging studies (Coleman et al., 1990).

As a whole, these data show a fine variation in angiogenic regulation in CC along aging. They are likely to antedate ED, as they arise before structural abnormalities are observed. Thus, the importance of studying ED goes beyond specific men's wellbeing. Indeed, it can also alert for a potential asymptomatic cardiovascular disease.

Conclusions

Aging contributes to ED's progression by causing a structural disorganization of cavernosum tissue, coupled to modification in angiogenic factors expression. In the present report, we demonstrated, for the first time, that Ang1, Ang2 and their receptor Tie2 have enhanced expression in the CC of the aged rat. Taken together, the results strongly indicate that the angiopoietins/Tie2 system participate in the vascular maintenance and remodeling of the CC. Interestingly, the combined increase of Ang1 and Ang2 expression in the aged CC suggest a cooperative function between them, which corroborate previous findings in human. They apparently compensate the loss of other angiogenic factors, such as VEGF, that occurs in the CC during aging (Bogdanovic et al., 2006; Daly et al., 2006; Yuan et al., 2009). Nonetheless, downstream mechanisms of Tie2 activation and functional evaluation of erectile capacity will be needed to elucidate the importance of Ang1 and Ang2 cavernous bioavailability in age-related ED progression.

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Figure captions

Figure 1- Immunohistochemical detection of Ang1 expression in the CC of 6, 12, 18 and 24 months animals. In all groups there was a scattered staining both in endothelium and in smooth muscle layer (arrows). An apparent decrease of Ang1 immunolocalization was observed in the smooth muscle cells of rats aged 12 months.

Figure 2- Immunohistochemical detection of Ang2 expression in the CC of 6, 12, 18 and 24 months animals. In all groups there was a scattered staining both in endothelium and in smooth muscle layer (arrows). An apparent reduced intensity was observed in the CC of rats aged 12 months.

Figure 3- Immunohistochemical detection of the receptor Tie2 expression in the CC of 6, 12, 18 and 24 months animals. Tie2 was detected mainly in the endothelium in all studied groups (arrows).

Figure 4- Dual-immunolabelling of Ang1 (red) and Tie2 (green) in the CC of rats aged 6, 12, 18 and 24 months. Simultaneous detection of Ang1 and Tie2 showed poor co-localization, restricted to the endothelium. The nucleus is stained in blue.

Figure 5- Dual-immunolabelling of Ang2 (red) and Tie2 (green) in the CC of rats aged 6, 12, 18 and 24 months. Simultaneous detection of Ang2 and Tie2 evidenced co-expression restricted to the endothelium. The nucleus is stained in blue.

Figure 6- Representative bands obtained by Western blotting analysis of Tie2 (140kDa), Ang1 (60kDa) and Ang2 (66kDa) at the CC of rats of 6, 12, 18 and 24 months. Normalization of total Ang1, Ang2 and Tie2 was performed using β-actin as an internal control.

Figure 7- Semi-quantitative analysis of Ang1, Ang2 and Tie2 by Western blotting according to the different ages (n = 3 for each group) calculated by the fraction of pixels presented by each band relative to the β-actin band used as internal control.

* Significant differences between groups ($P < 0.05$).

Figure 8- Ratio of Ang1/Ang2 analysed for each animal. The Ang1/Ang2 does not vary consistently during aging.

Figure 9- Ratio of Ang1/Tie2 analysed for each animal. A decreasing tendency was observed during aging.

Figure 10- Ratio of Ang2/Tie2 analysed for each animal. A decreasing tendency was observed during aging.

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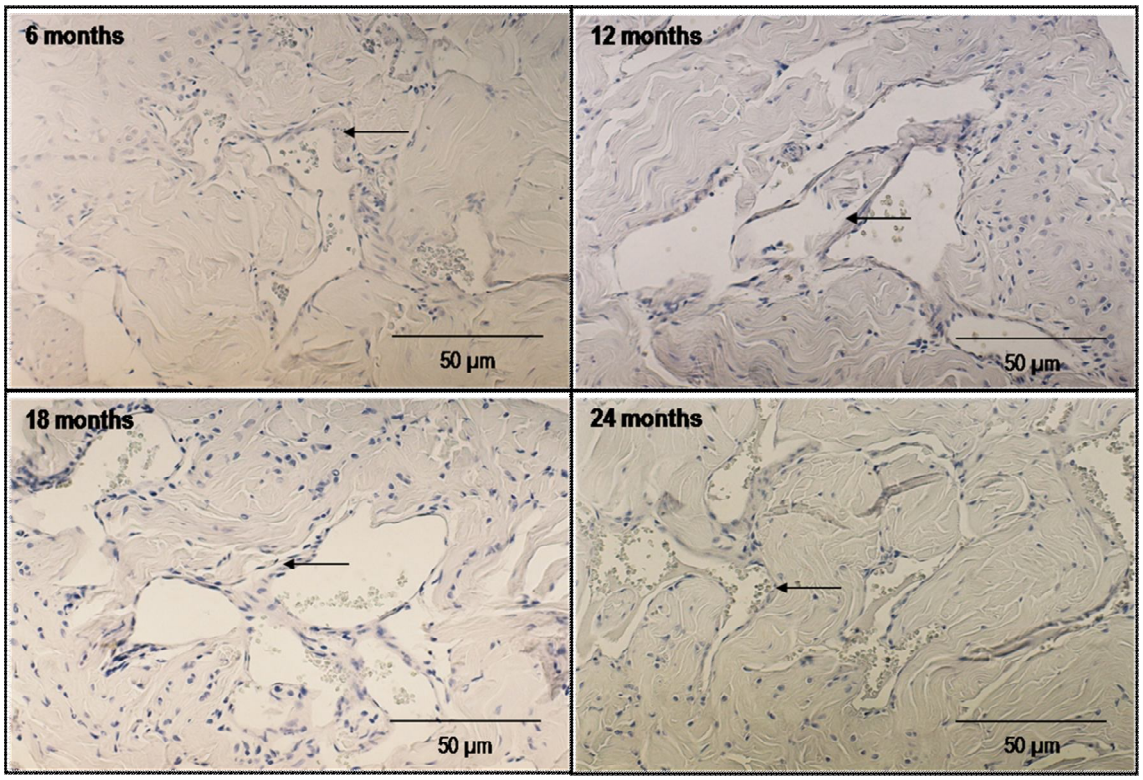

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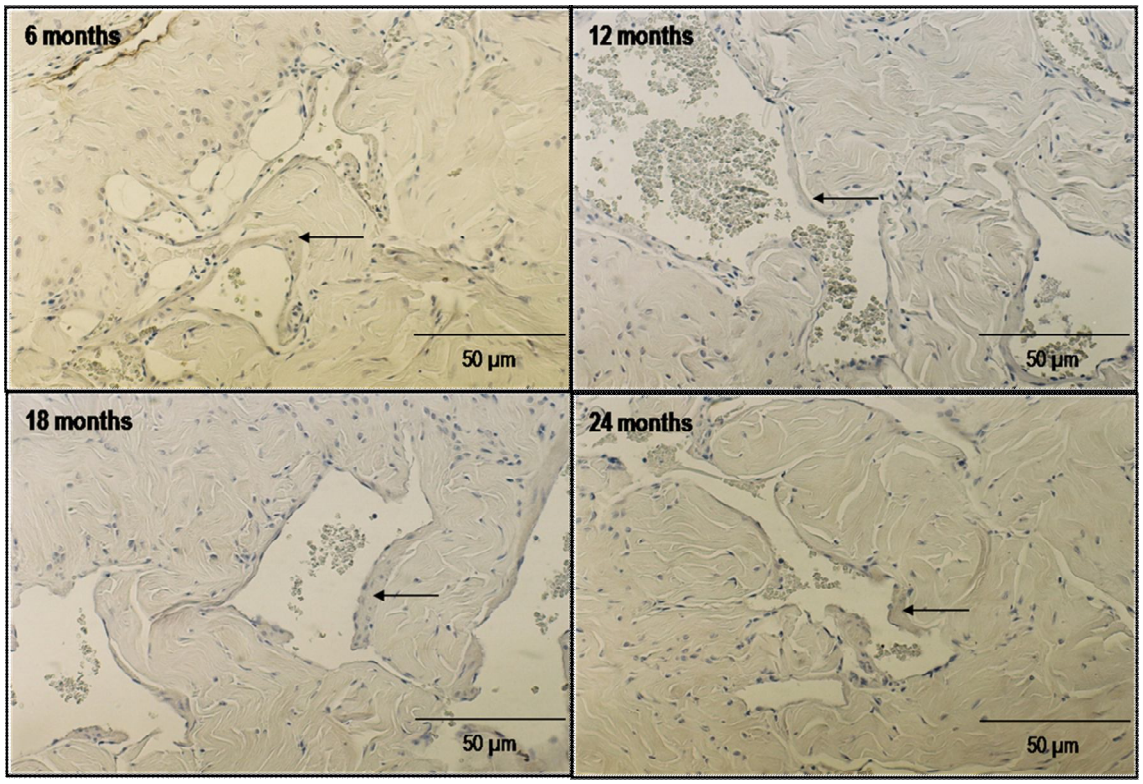

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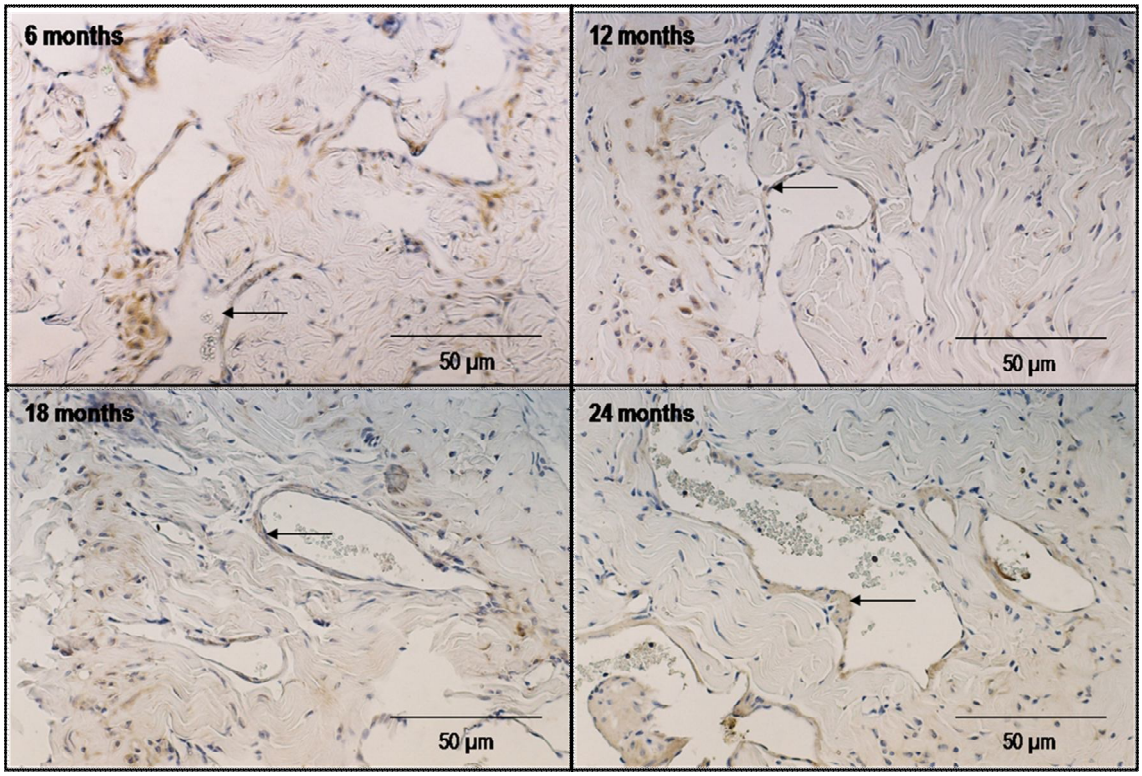

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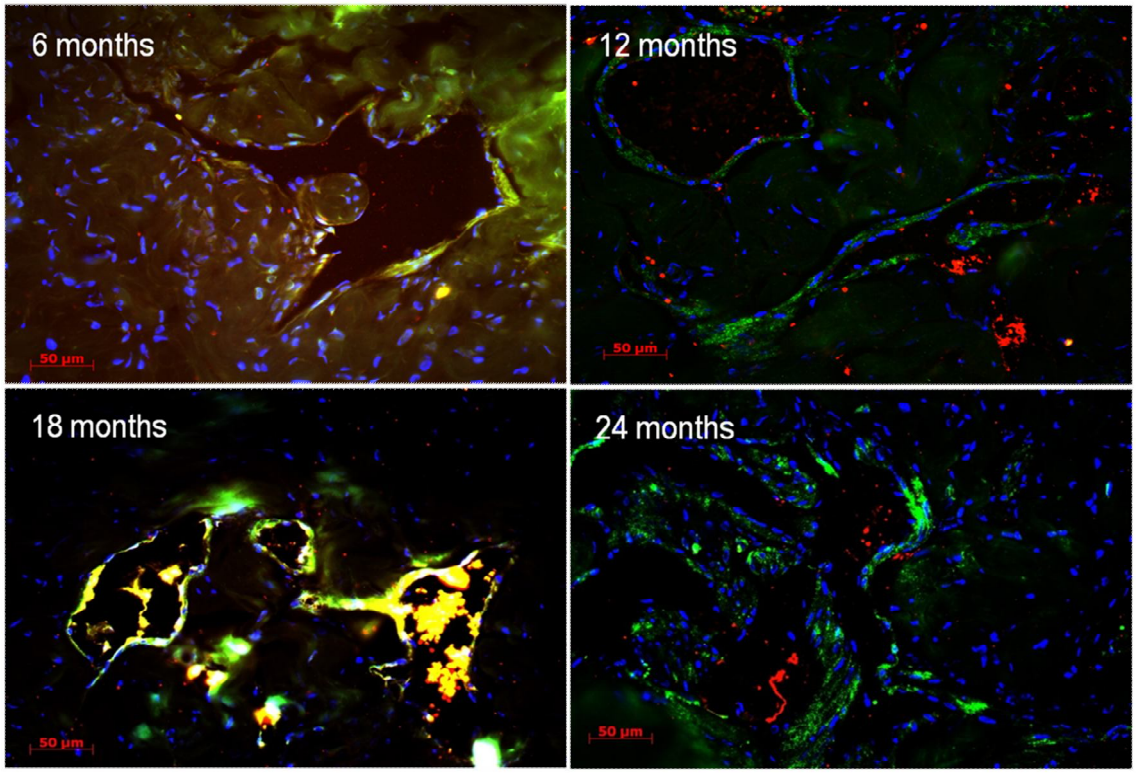

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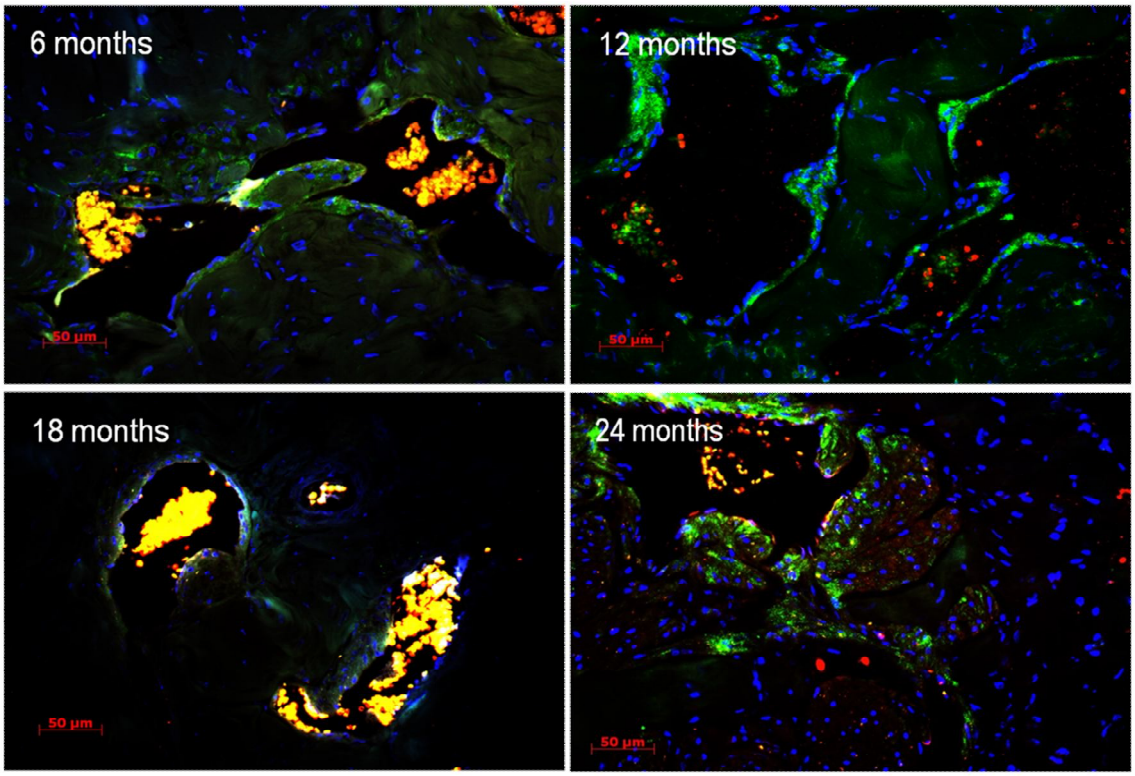

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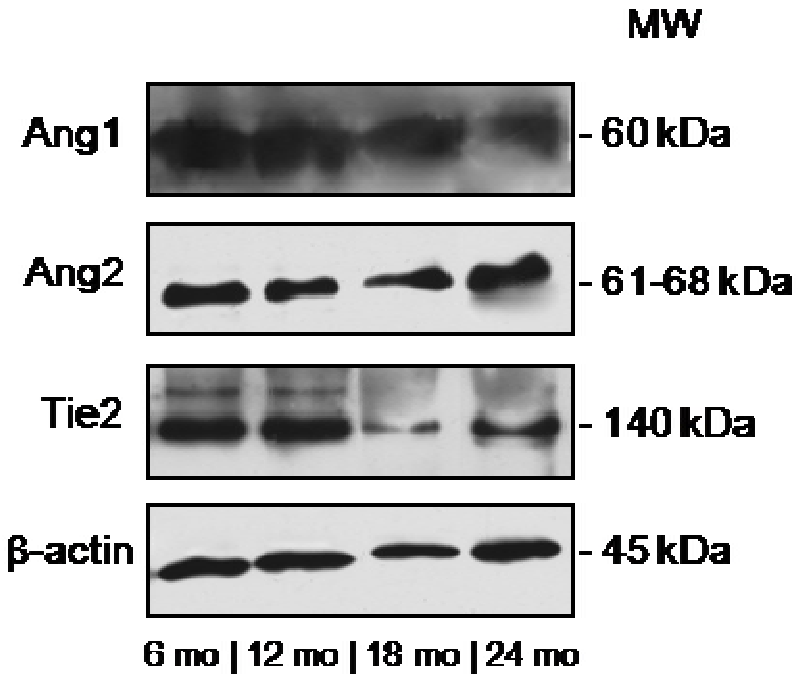

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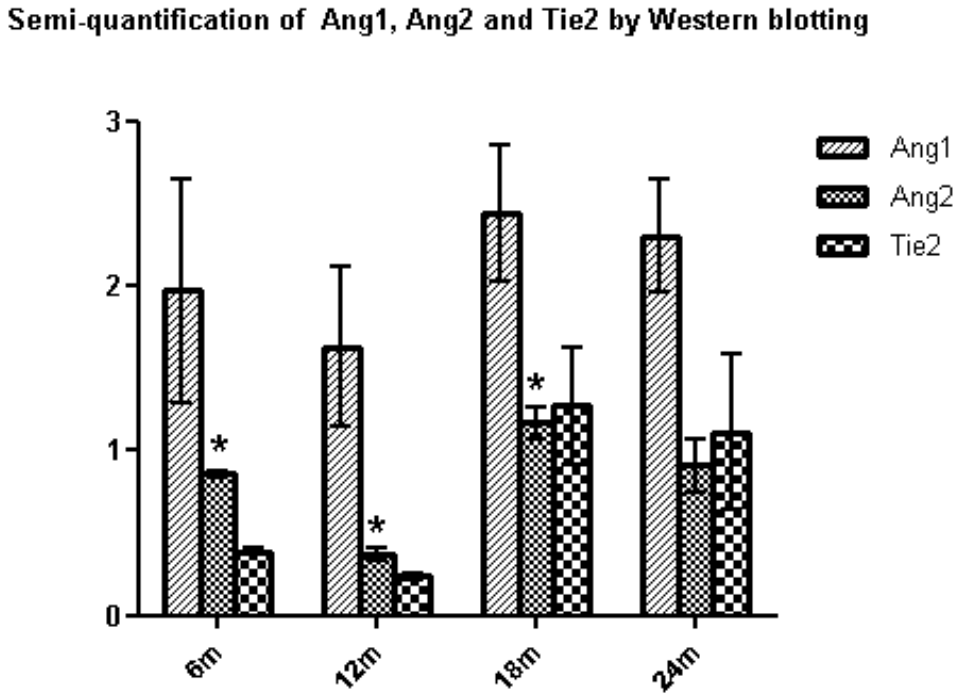

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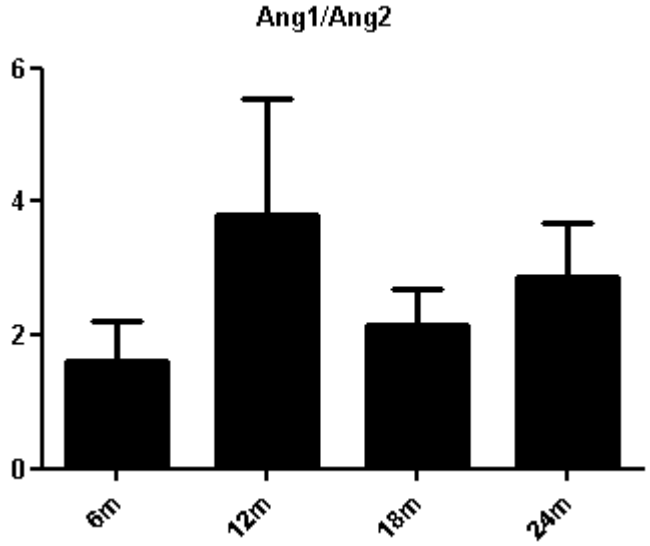

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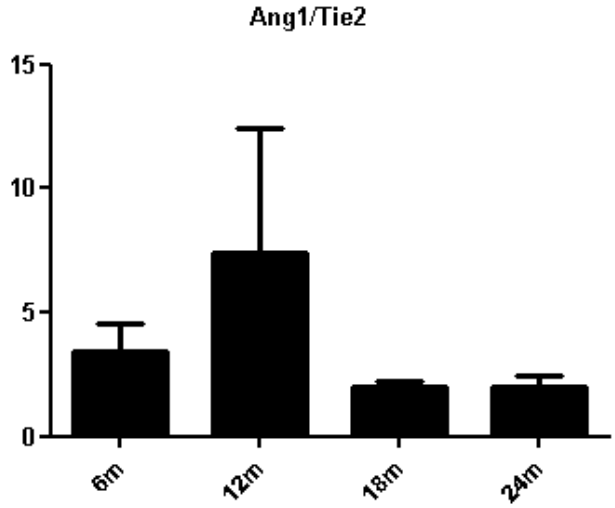

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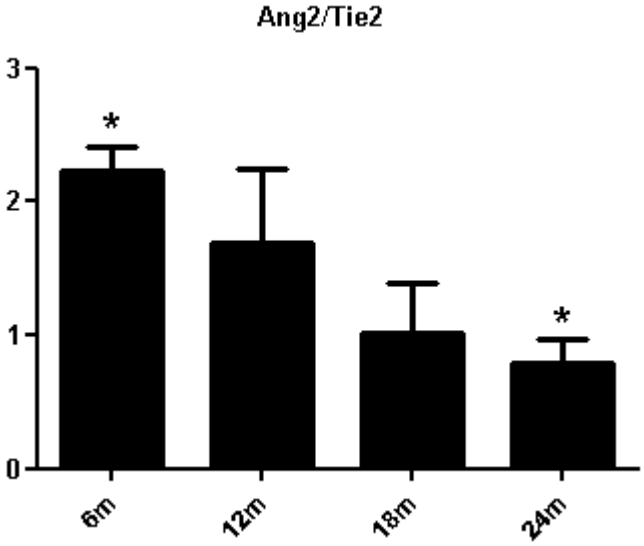

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