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António Augusto Ferreira Pinto de Figueiredo

Real-time PCR study of Ang1, Ang2, Tie-2, VEGF and KDR expression in human erectile tissue during aging

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Trabalho efectuado sob a Orientação de:

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Real-time PCR study of Ang1, Ang2, Tie-2, VEGF and KDR expression in human erectile tissue during aging

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Running title:
Real-time PCR of Ang1, Ang2 and VEGF in human penis in aging

Abstract

Introduction: Aging is a recognized risk factor for erectile dysfunction (ED), contributing independently to vascular damage of penile tissue. Vascular maintenance depends on angiogenic factors balance in tissues. Vascular endothelial growth factor (VEGF) is a potent mitogen and survival factor for endothelial cells, whose functions are mediated by specific receptor kinase domain region (KDR). Other factors, such as angiopoietins, crosstalk with VEGF. Angiopoietin-1 (Ang1) and Angiopoietin-2 (Ang2) compete for binding to Tie-2 and, while Ang1 promotes vascular stabilization, Ang2 acts as a partial agonist or antagonist of Ang1 signalling, depending on VEGF bioavailability.

Aims: The aim of this study is to quantify the expression of Ang1, Ang2, Tie-2, VEGF and KDR by real-time polymerase chain reaction (PCR) in human corpus cavernosum (CC) from young and aged healthy individuals.

Methods: Human CC fragments were obtained from organ donors without known risk factors to ED which were further divided in two groups according to age: young (16-28 years) and aged (59-74 years). RNA was extracted and converted to cDNA. Real-time PCR reactions employed appropriate primers.

Main Outcome Measures: The quantification of mRNAs of Ang1, Ang2, Tie2, VEGF and KDR in human CC during aging. The ratio of Ang1/Tie2 and Ang1/Ang2 values was also calculated for each individual.

Results: Real-time PCR results showed an almost four-fold significant reduction in the Ang1/Tie-2 ratio during aging (p=0,01). Ang2 expression was also lower in CC of older individuals, although without statistical significance. Ang1/Ang2 ratio, VEGF and KDR expression results were highly variable. Nevertheless, it was evident a reduction in the Ang1/Ang2 ratio and KDR expression with aging.

Conclusions: The obtained results suggest that aging is associated with a lower angiogenic potential, particularly in those mechanisms that depend on angiopoietins/Tie-2 system. This may compromise the vascularization of the CC in the elderly, thus contributing to aging-associated ED.

Keywords: Aging, erectile dysfunction, human penile tissue, angiogenesis, angiopoietin-1, angiopoietin-2, Tie-2, VEGF, KDR

Introduction

Aging is a recognized risk factor for erectile dysfunction (ED) contributing independently to vascular damage of penile tissue [1-2]. Hence, CC emerges as an appropriate tissue to study vascular aging. In fact, corpus cavernosum (CC) of aged individuals present structural modifications, such as loss of smooth muscle fibres coupled to fibrosis [3-4], even in the absence of concomitant pathologies. Moreover, it has also been demonstrated that patients with cardiovascular disease (CVD), the main cause of mortality in the elderly, present increased prevalence of ED, when compared with healthy individuals [5-6]. Taking into account this evidence, ED is actually considered equivalent to endothelial dysfunction and the first marker of systemic CVD [6-7]. However, the molecular mechanisms involved in vascular aging are not yet fully clarified. It is known that this process is associated to decreased angiogenic potential [8], which is supported by the previous demonstration of down-regulation of vascular endothelial growth factor (VEGF) protein expression in the CC of aged rat [9] and human [4]. VEGF is expressed in every vascularized tissue acting as a potent mitogen and a survival factor for endothelial cells [10]. It exerts these effects by binding to two specific membrane receptors: VEGFR-1 or Flt-1 (*fms*-like tyrosine kinase) and VEGFR-2 or KDR (kinase domain region) but most of the effects currently atributed to VEGF result from KDR activation. Although VEGF is the most important angiogenic mediator [10], other factors, such as angiopoietins, crosstalk *in vivo* with it, modulating angiogenesis and vascular remodeling. Angiopoietin-1 (Ang1) binds specifically to receptor Tie-2, promoting anti-inflammatory and anti-apoptotic effects on endothelial cells [11]. Thus, Ang1 strongly contributes to vessel stabilization and maturation. In contrast, the role of angiopoietin-2 (Ang2) in angiogenesis is not fully understood. It is a pro-inflammatory cytokine, activating the endothelium and inducing vascular permeability [12]. It competes with Ang1 for binding to Tie-2, however with less affinity [13], and may act as a partial agonist or antagonist of Tie-2, depending on VEGF bioavailability [14]. When enough VEGF is available, it induces overexpression of Ang2, promoting angiogenesis. In fact, Ang2 is highly expressed only at sites of vascular remodeling in the adult [15]. However, if VEGF levels are low, vascular destabilization and regression will occur [14]. So, Ang2 and VEGF contribute together to the proliferation and organization of the endothelium, whereas Ang1 is responsible for the maintenance of blood vessels [14-15]. As expression of both Ang1 and Tie-2 is primarily constitutive, changes in Ang2 expression, or in the ratio of Ang1/Ang2, seem to be an essential point of regulation of angiogenesis and vessel stability [14].

Aims

The aim of this study is to quantify the expression of Ang1, Ang2, Tie-2, VEGF and KDR by real-time polymerase chain reaction (PCR) in human CC fragments, removed from young and aged healthy individuals, which as far as we know, was never reported before. We intent to elucidate the molecular mechanisms associated with vascular remodeling and stability during aging. Clarifying these mechanisms will disclose novel therapeutic perspectives for the age related vasculogenic erectile dysfunction.

Methods

Penile tissue processing

Human CC fragments were obtained from organ donors (protocol approved by Faculty of Medicine of Universidade do Porto and Hospital S. João Ethics' Committee). None of the patients presented risk factors for ED. The CC fragments were immediately frozen at -80 °C and maintained until processed. The samples were divided in two groups according to the age of the individuals, young (16-28 years) and aged (59-74 years).

Real-time PCR

Extraction of total RNA was performed using TRIZOL reagent (Invitrogen Corporation, CA, USA) according to the manufacturer's protocol. Total RNA was quantified by the measured absorvance at 260 nm in a spectrophotometer Beckman DU640 (Beckman Fullerton, USA). BioScript™ reverse transcriptase was used to generate cDNA using five micrograms of RNA and oligo(dT) primers, according to manufacturer's protocol (Bioline, MA, USA). Real-time PCR was performed in 96-well 0.2 ml thinwall PCR plates using the iQ5 real-time PCR detection system (Bio-Rad Laboratories, CA, USA) and carried out with iQ SYBR Green Supermix (Bio-Rad). The primers' sequences and amplification programs are summarized in table 1. Amplification reactions were performed in duplicate and the amount of cDNA in the reactions was normalized with an internal control, the constitutively expressed gene GAPDH. The specificity of the amplification of the expected DNA fragments was confirmed by 2% agarose gel electrophoresis and by analysis of the melting curves. An amplification reaction control with no reverse transcriptase enzyme (termed RT-) was performed in order to assess the interference of potential genomic DNA in the RNA solution. Relative gene expression was calculated from the formula: $2^{\Delta CT}$; ($\Delta CT = CT_{GAPDH} - CT_{target}$).

Statistical analysis

Statistical analysis was done employing Microsoft Office Excel version 2007 for Windows (Microsoft, USA) and difference of mean values between groups was assessed by one-tail *t* test. A value of $P < 0.05$ was considered significant.

Main outcome measures

The expression of Ang1, Ang2, Tie-2, VEGF and KDR was quantified in the CC of young and aged men, employing real-time PCR. The Ang1/Tie-2 and Ang1/Ang2 ratios were also calculated. It is expected a better understanding of the angiogenic processes in the CC during aging.

Results

The real-time PCR amplified products were analysed by agarose gel electrophoresis and, as shown in Figure 1, only one band, of the expected molecular length, was detected for each experiment.

The mRNA expression of Ang1, Ang2, Tie-2, VEGF and KDR was quantified in all CC samples. Both Ang1 and Tie-2 expression levels showed a high intra-group variability. However, Ang1/Tie-2 ratio values were similar within each group. It was evident an almost four-fold significant reduction in the aged group 31,4±12,1 when compared with the young one 113,1±43,3 ($p=0,01$) (Figure 2). Regarding Ang2, it was observed a lower expression in the aged comparing to the young CC tissues, although without statistic significance (Figure 3). The Ang2/Tie-2 ratio revealed to be very variable within both groups (data not shown). The Ang1/Ang2 ratio (Figure 4), VEGF (Figure 5) and KDR (Figure 6) levels also presented high intra-group variability, nevertheless, Ang1/Ang2 ratio and KDR evidenced a decreasing tendency in the aged individuals (Figures 4 and 6, respectively).

Discussion

ED is a highly prevalent disease [1,16] particularly in the developed countries where aged population tends to progressively increase. In effect, ED was classified for a very long time as a normal consequence of the aging process. Nonetheless, nowadays it is receiving much more attention from physicians since it is considered the first symptom of cardiovascular diseases [6] that are the leading cause of mortality in the elderly. Aging is a complex and gradual process, genetically determined and environmentally modulated, that results in loss of function of the organs and systems. Vascular system is particularly prone to manifest aging-related dysfunction, which is characterized by increased oxidative stress, impaired nitric oxide (NO) bioavailability and also modification of the gene expression pattern [17]. The NO dependence for erectile mechanism coupled to specific structural organization of the CC, justify the increase of ED prevalence affecting man in the elderly [18]. In addition, aging provokes down-regulation of angiogenic capacity that interferes with vascular quiescence and remodeling in the CC. Therefore, the quantitative study of the expression of angiogenic factors in human CC of healthy individuals here reported, strongly contributes to the understanding of the pathophysiology of ED that occurs during physiologic aging. In the present study we used CC fragments obtained from healthy individuals that had no ED or risk factors for ED. This selective procedure significantly reduced the number of samples. However, previous studies carried out in our laboratory showed that CC tissues obtained from prosthesis insertion surgeries due to ED caused by denervation or male-to-female sex change surgeries express higher quantities of angiogenic growth factors, which led us to reject these samples for the present study. Hence, the differences observed in the expression of the angiogenic factors in our work are primarily due to aging.

The Angiopoietin/Tie-2 system acts as a vascular specific ligand/receptor system to control endothelial cell survival and vascular maintenance [14]. Ang1 and Ang2 are specific

ligands for Tie-2 and both depend on VEGF bioavailability in tissues for exerting their functions. Ang1 is an anti-inflammatory and a pro-survival factor that binds agonistically to the receptor Tie-2, playing a major role in the promotion of vessel stability and maturation [11]. It exerts a fundamental role in the stabilization of preformed vessels, being able to restore vascular architecture even in the absence of mural cells [19]. In our study we verified a considerable intra-group variability both in Ang1 and in Tie-2 expression levels. Besides this lack of age-related correlation for Ang1 and Tie2 when measured alone, an almost four-fold reduction in the Ang1/Tie-2 ratio was observed in the CC samples of aged individuals when compared with young specimens (p=0,01). This finding strongly suggests that the available relative amount of Ang1 to activate Tie-2 diminishes during aging, so does the stimulus to stabilize the vascular net. This effect may contribute to increased susceptibility of CC to pro-apoptotic and pro-inflamatory factors in the elderly. No such relation between Ang2 and Tie-2 was observed. In fact no significant difference was observed between young and old CC samples. All the same, Ang2 expression was lower in the aged. Although the results are not statistically significant, all the quantification values of the young are higher than those of the aged tissues, evidencing a strong tendency of Ang2 to decrease expression during aging. This result was unexpected taking in account previous Western-blotting data demonstrating that the CC of aged individuals present higher Ang2 levels than those of the young [4]. This apparent disagreement could be justified considering that Ang2 is stored in the Weibel-Palade bodies in endothelial cells before secretion in response to specific stimulus [20]. We hypothesize that the increased protein levels of Ang2 observed in CC of aged individuals could be due to a less intense angiogenic stimulus which leads to a lower release from Weibel-Palade bodies. Thus, greater levels of stored peptide in Weibel-Palade bodies in the elderly could negatively regulate the expression of Ang2 mRNA. Nevertheless, as far as we know, no demonstration of aging influence on endothelial Weibel-Palade bodies number and composition was reported, so that further studies will be necessary to clarify this assumption.

Ang2 is an important angiogenic factor that cooperates with VEGF, both taking part in vascular remodeling [14]. Its decrease will compromise the capacity of the tissue to stimulate proliferation of endothelial cells and their organization in new blood vessels. The Ang1/Ang2 ratio was also calculated and, besides not statistically significant, a decreasing tendency was observed in the samples of CC obtained from older patients that present an average value ten-fold lower. The concomitant reduction observed in the expression of Ang2, and in the Ang1/Tie-2 and Ang1/Ang2 ratios observed in the CC samples of aged individuals, may impair both angiogenesis and vascular stabilization. These modifications could also contribute to aging-related erectile dysfunction, since a well-developed and stable vascular bed is necessary to a normal erection. Additionally, this data suggests the existence of a correlation between regulation of expression of Ang1 and Tie-2, but not between Ang2 and Tie-2. This finding is supported by previous demonstration that Ang1 is the main ligand of Tie-2 while Ang2 competes for the binding to Tie-2 with less affinity [11]. Moreover, classifying Tie-2 as the main receptor of Ang2 is not a peaceful statement. In fact, Ang2 signaling is not fully understood and some of its effects may be due to interaction with other proteins [14]. Our results seem to support this view as no relationship between the independent expressions of Ang2 and Tie-2 was observed.

We also quantified the expression of VEGF and its receptor KDR. VEGF is considered the main angiogenic growth factor and is the best studied so far [21]. It promotes, after interaction with its receptor KDR, proliferation, sprouting and tube formation of endothelial cells. In fact, most of the effects normally attributed to VEGF result from KDR activation. Although a significant variation in the expression of these factors was not found, the analysis of KDR expression evidenced an average eight-fold decrease in the aged group, which agrees with previous studies carried out in the rat [9]. Further studies are being performed to clarify VEGF-KDR physiology during aging in human CC.

It is well established that the effects of Ang2 depend on VEGF availability and are tissue and context-specific [14]. It has a pro-angiogenic role when VEGF is highly expressed but when VEGF levels are decreased, Ang2 contributes to vessels regression and destabilization. Our data suggests that no evident relationship exists between the expression of Ang2 and VEGF in the healthy human CC during aging, however further studies in a larger sample will be necessary to confirm this assumption.

Conclusions

In brief, the obtained results suggest a lower angiogenic potential of the CC of the aged individuals, particularly in those mechanisms that depend on angiopoietins/Tie-2 system. That goes in agreement with what we expected, since an overall reduction of the angiogenic capacity with aging was also described for other tissues. In fact, aging appears to independently cause molecular alterations in the CC that may contribute to ED. However, in ED patients, additional risk factors other than age, may also contribute to impair angiogenesis. In effect, Ryu *et al* reported a decrease in angiopoietins and VEGF expression [22] as well as in erectile function [23] of hypercholesterolemic rats that was restored after combined Ang1 and VEGF intracavernous delivery. Clinical trials revealed angiogenesis induction by local administration of angiogenic factors as a promising treatment for ischemic pathologies [24-25]. This strategy could also be used as a powerful weapon in the treatment of ED, considering that downregulation of cavernous vascularization mechanisms is a common ethiology and no curative treatment for ED is available. Thus, clarification of the molecular mechanisms that accompany CC aging and aging-associated ED will open new perspectives to develop novel mechanism-based therapeutic approaches.

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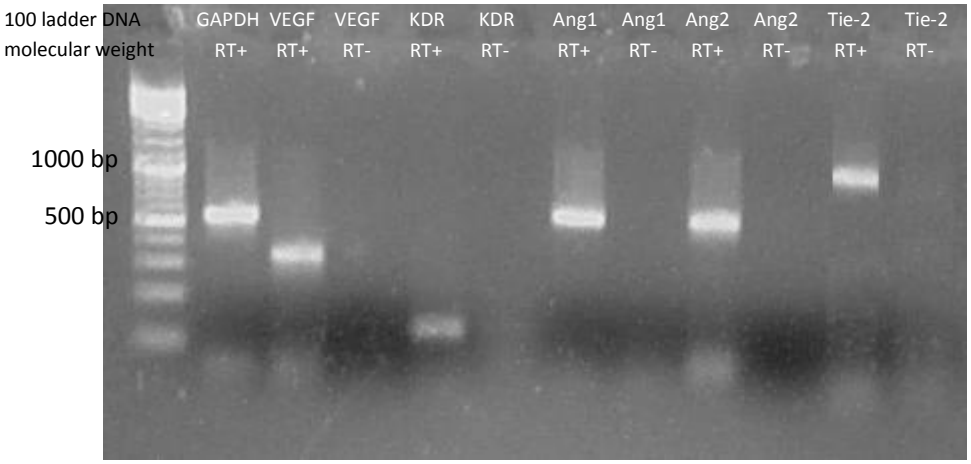

Figure 1: Analysis by agarose gel electrophoresis of GAPDH, VEGF, KDR, Ang1, Ang2 and Tie-2 real-time PCR amplification (RT+). RT- lanes indicate amplification reaction in the absence of reverse transcriptase enzyme.

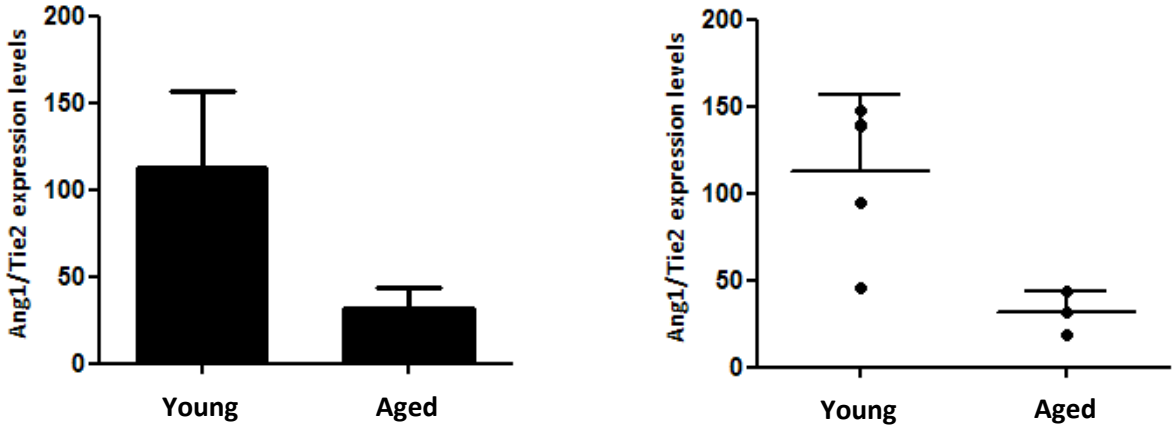

Figure 2: Ang1/Tie-2 expression in the CC of Young (n=5) and Aged (n=3) donors. There is a statistically significant reduction in its value during aging (p=0,01).

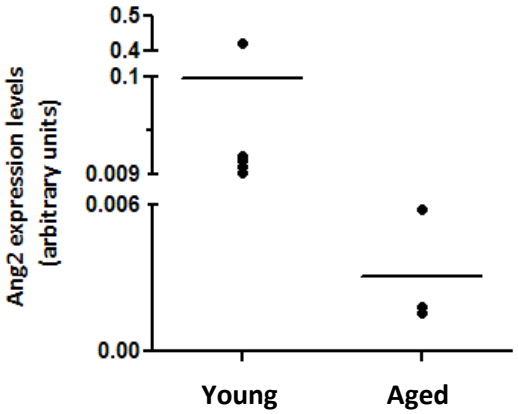

Figure 3: Ang2 expression values in the CC of Young (n=5) and Aged (n=3) groups. Mean values in the Aged (0,003±0,002) are lower than those in the Young (0,09±0,2) groups.

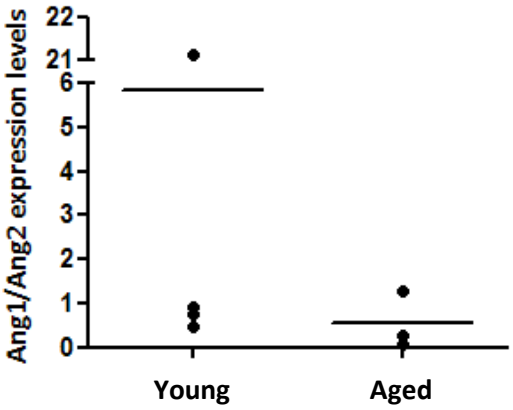

Figure 4: Ang1/Ang2 expression in CC of Young (n=4) and Aged (n=3) groups. Mean values in the Aged (0,53±0,76) are lower than those in the Young (5,8±10,2).

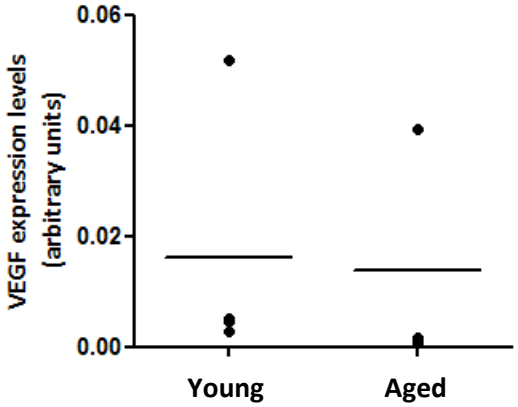

Figure 5: VEGF expression values in the groups Young (n=4) and Aged (n=3). The results are highly variable and no difference is evident between the Aged (0,014±0,022) and the Young (0,016±0,02).

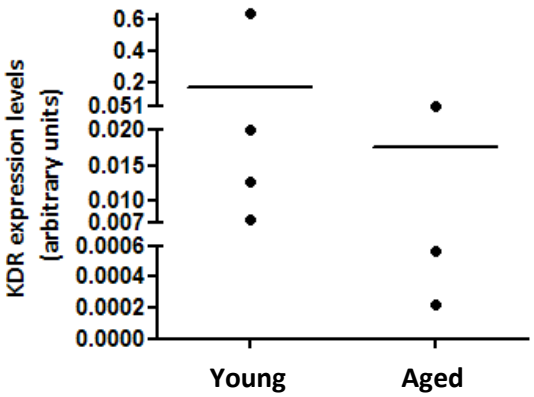

Figure 6: KDR expression values in the groups Young (n=4) and Aged (n=3). Mean values in the Aged (0,02±0,03) are lower than those in the Young (0,2±0,3).

Table 1: Description of the primers' sequences and real-time PCR programs.

mRNA	Accession number	Sequence (5'→3')	Length of the amplified product (bp)	Real-time PCR program
GAPDH	NM_002046	Upper – GGTGAAGGTCGGAGTCAACG Lower – CAAAGTTGTCATGGATGACC	496	The same programs used for the other mRNAs
Ang1	NM_001146	Upper – GCCTACACTTTCATTCTTCCAGA Lower – TCTTCCTTGTGTTTTCCTTCCAT	500	5' 94 °C, 40x (1' 94 °C, 1' 58 °C, 2' 72 °C)
Ang2	NM_001147	Upper – GGCAGCGTTGATTTTCAGAGGACT Lower – TTTAATGCCGTTGAACTTATTTGT	429	10' 95 °C, 40x (15'' 95 °C, 1' 60 °C, 1' 72 °C)
Tie-2	NM_000459	Upper - TACTAATGAAGAAATGACCCTGG Lower - GGAGTGTGTAATGTTGGAAATCT	826	5' 94 °C, 40x (1' 94 °C, 1' 56 °C, 2' 72 °C)
VEGF	NM_001025366	Upper – ATGAACTTTCTGCTGTCTTGGGT Lower – TGGCCTTGGTGAGGTTTGATCC	323	5' 94 °C, 40x (30'' 94 °C, 30'' 62 °C, 30'' 72 °C)
KDR	NM_002253	Upper – TGCCTACCTCACCTGTTTC Lower – GGCTCTTTCGCTTACTGTTC	114	5' 94 °C, 40x (30'' 94 °C, 30'' 62 °C, 30'' 72 °C)

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