Tocopherol induced angiogenesis in placental vascular network in late pregnant ewes

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Abstract

Background: Tocopherols have biphasic, proangiogenic and antiangiogenic therapeutic effects. The objective of this clinical trial was to clarify tocopherol’s placental angiogenic potential in late pregnant ewes following oral supplementation.

Methods: Eighteen pregnant ewes during late gestation were selected for this study. Ewes were given oral supplementation of 500 mg of alpha-tocopherol (aT; N = 6) or 1000 mg of gamma-tocopherol (gT; N = 7) or placebo (CON; N = 5) once daily from 107 to 137 days post breeding. Serum was obtained at weekly intervals and tissue samples were obtained at the end of supplementation to: 1) evaluate tocopherol concentrations in serum, uterus and placentome; 2) evaluate relative mRNA expressions of Vascular Endothelial Growth Factor (VEGF), Placental Growth Factor (PIGF), endothelial Nitric Oxide Synthase (eNOS) and Hypoxia Inducible Factors (HIF) in uterus, caruncle and cotyledon; 3) analyze the morphometry of the placental vascular network.

Results: Supplementation of aT or gT resulted in increased concentrations in serum, placentome and uterus compared to control (P < 0.05). In aT group, mRNA expressions of PIGF, eNOS and HIF-1α in cotyledon were greater than the CON group. In gT group, mRNA expressions of VEGF, eNOS, HIF-1 alpha and HIF-2 alpha in caruncle and uterus, and HIF-1α in cotyledon, were greater than the CON group. Morphometry analysis revealed increased angiogenesis in the supplemented groups.

Conclusion: Daily oral supplementation of aT or gT increased angiogenesis in the placental vascular network in pregnant ewes during late gestation. Increase in placental angiogenesis may provide nutrients required for the development and growth of fetus during late pregnancy.

Background

Angiogenesis is the process of development of new blood vessels from an existing vascular network [1]. Hypoxia constitutes a basic regulatory mechanism in vascular development during embryonic and fetal growth in utero [2]. In endothelial cells, hypoxic conditions drive the transcription of multiple genes which control vascular function, expansion and remodeling. Although tissue hypoxia is the main driving force for angiogenesis, a growing body of evidence has demonstrated that oxidative stress can also be a potent trigger for the development of new vessels [3-6].

Two families of radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are very crucial to growth, differentiation, apoptosis, and aging. The transition from growth to degeneration of cells is finely tuned by the relative concentration of oxidants. For instance, in ambient conditions, H2O2 in nano-micromolar concentrations are necessary for angiogenesis [7], while its excessive accumulation at levels more than 150-200 μM prompts endothelial damage [8]. An imbalance of free radicals attacks and alters cell constituents, resulting in lipid peroxidation, protein peroxidation, oxidative inactivation, mutation of DNA, and destruction of vitamins and other functions that protect cell components [9]. Excessive accumulation of free radicals affects placental development and function, and may subsequently impact both the fetus and dam [10-13]. The dietary antioxidants are important in attenuating the oxidative damage.
damage produced by radicals [13,14]. Tocopherols are micronutrients which act as free radical scavengers. The major tocopherols found in mammalian tissue are alpha-tocopherol (aT) and gamma-tocopherol (gT). Studies on tocopherol in humans have shown that aT constitutes 80 to 90% of vitamin E in plasma, which is due to the preferential incorporation of aT into very low-density lipoprotein via tocopherol-binding protein, and a rapid catabolism of gT within the liver [14]. Consequently, aT has received the most attention and is the primary form of vitamin E in supplements. However a recent work has suggested that under specific circumstances gT might scavenge reactive species more effectively than aT thereby, being more beneficial [15,16]. Gamma tocopherol affords higher protection against lipid peroxidation as compared to aT [17]. It has been demonstrated that high levels of aT intake can decrease gT [17]. Also gT has synergistic effect with other tocopherols and suggested as most promising agent to use. Hence we decided to use both tocopherols in this study.

Optimal placental development is critical for fetal development and growth. Normal fetal growth is genetically pre-programmed and regulated by fetal, placental, maternal and environmental factors. A range of pathophysiological factors including maternal stress due to poor nutrition, hyperthermia or metabolic diseases such as pregnancy toxemia and eclampsia may affect important metabolic, transport and hemodynamic functions of placentas. This may lead to fetal stress due to poor supply of nutrition and thus impairment in fetal growth. A recent study demonstrated that glucocorticoid induced restriction of fetal and placental growth is mediated through reduced placental expression of VEGF and associated reduction in placental vascualrization during late pregnancy [24]. Supplementation of antioxidants may negate oxidative stress, promote angiogenesis and increase nutrient supply to the fetus. The differential expression of VEGF and placental growth factors (PIGF) throughout gestation indicates that they both play essential roles in placental angiogenesis. Hypoxia inducible factor (HIF)-1α or HIF-2α combine with HIF-1β to form the functional basic helix-loop-helix transcription factor HIF-1 to stimulate transcription of the VEGF gene via interaction with the hypoxia response element (HRE) [25]. Several genes encoding growth factors and tyrosine kinase receptors essential for endothelial cell growth and function such as erythropoietin (EPO), VEGF, endothelial Nitric Oxide Synthase (eNOS) and Flk-1 were shown to contain HIF-binding sites in their promoter/enhancer sequences.

We hypothesize that oral supplementation of tocopherols during late gestation in ewes will increase tocopherol levels in the circulation, resulting in increased concentrations in the uterus and placenta and thereby increasing the expression of angiogenic genes and exerting an angiogenic effect. The objectives of the study were to: 1) compare aT and gT concentrations in serum, uterine and placental tissues among supplemented and CON groups; 2) compare angiogenic gene (VEGF, PIGF, eNOS, and HIFs) expressions in the uterus, caruncle and cotyledon among supplemented and CON groups; and 3) compare angiogenic morphometric parameters of the uteroplacental vascular network of late pregnant ewes supplemented with aT, gT, or placebo.

Methods
Eighteen pregnant ewes (2 to 6 years of age and weighing approximately 68 kg; impregnated by different sires by natural service), with similar breeding dates were selected for the current study and were maintained under normal pasture conditions. One week prior to the trial, the selected ewes were moved to the research facility and were penned by treatment groups. The ewes had access to 35 sq ft/ewe paddock lots. In addition they were fed 250 g of concentrate/ewe/day and free choice hay. The composition of hay is given in Additional file 1, Table S1. This study was approved by Virginia Tech institutional animal care and use committee (IACUC; 04-068-CVM). Tissue Use Protocol was approved by IACUC at Washington State University (ASAF #03922-001).

The ewes were randomly assigned to three groups: 1) aT group (n = 6) - received 500 mg of aT (Nature’s Bounty, Bohemia, NY 11716), 2) gT group (n = 7) - received 1000 mg of gT (Kemin Industries Inc., Des Moines, Iowa 50317), 3) Control (CON) group (n = 5) - received a placebo. The average tocopherols concentrations in the supplement is given in Additional file 2, Table S2. Animals were supplemented orally, once daily, from approximately 100 to 137 days post breeding (dpb) (Figure 1). Blood samples were collected one week prior to onset of supplementation and then weekly until the end of the supplementation period [94 dpb (presupplement level), 107 dpb, 114 dpb, 121 dpb, 128 dpb, 136 ± 1 dpb] for serum aT and gT evaluation (Figure 1). At the end of the supplementation period (136 ± 1), all ewes were euthanized and tissue samples were collected from the gravid uterus and placentomes to evaluate aT and gT concentrations. Tissue samples were snap frozen and stored at -70°C until analysis. Caruncle, cotyledon and intercaruncular uterus samples were also collected.
and stored in RNAlater (Qiagen Inc., Valencia, CA 91355) and frozen at -70°C to evaluate mRNA expression. Cotyledones were separated from caruncles by applying strong pressure. Placentomal and uterine tissues were collected close to the umbilical cord for consistency.

Serum tocopherol estimation
The High Performance Liquid Chromatography (HPLC) method described by Panfili et al (2003) was employed for the quantification of tocopherols [26]. For each sample, dose-dependent (10, 50, 100, 500 and 1000 mg/L) tocopherols (Sigma-Aldrich, St. Louis, MO 63103) positive control were prepared. In a glass tube, 1 mL of serum sample and 20 μL of aT acetate (1000 mg/L) in ethanol (internal standard to estimate yield of extraction) was added and vortexed. To this 1 mL of ethanol containing 0.01% of butylated hydroxytoluene (BHT) was added. The tocopherols were extracted 3 times with 1 mL of cyclohexane containing 0.01% BHT. After evaporation of the pooled cyclohexane fractions under a stream of nitrogen, the residues were reconstituted in 200 μL of ethanol. The samples were filtered through a Pall Gellman Acrodisc® (0.2 μm/13 mm) into HPLC vial and subjected to a HPLC analysis to quantify the tocopherol’s concentration.

Uterine and placental tissue tocopherol estimation
The HPLC method described by Katsanidis and Addis (1999) was employed for quantification of tocopherol concentrations in uterus and placental samples [27]. Each uterine and placental tissue sample was homogenized separately in water (1:2) containing ascorbic acid (62.5 g of ascorbic acid in 250 mL of double distilled water) to protect tocopherol from air oxidation during saponification process [27]. The tissue homogenates were aliquoted and frozen and stored at -70°C until analysis. One point five grams of uterus or placental homogenate was placed in a screw cap tube and 0.125 g of ascorbic acid and 3.5 mL of the saponification solution [55% ethanol in distilled water and 11% KOH (w/v)] were added to the tissue. The tube with sample was heated to 80°C for 15 min. After saponification, the tubes were cooled under tap water for 1 min, and then 1 mL of distilled water was added. The solutions were extracted 3 times with 1 mL of cyclohexane containing 0.01% of BHT. The 3 extracts were combined and the solvent was evaporated under reduced pressure by a rotovap. The residue was reconstituted in 500 mL of ethanol containing 0.01% BHT.

An Agilent Technologies 1100 series liquid chromatography system (Agilent Technologies Inc. Santa Clara, CA 95051, USA) was used to perform HPLC analysis to quantify tocopherols in supplement, serum, and tissue samples. The analysis was conducted in normal phase, with a HPLC column Agilent Technologies Zorbax Rx-Si 150 × 4.6 mm, 5 μ with an elution solvent constituted of hexane-isopropanol (99.7:0.3) with a flow rate of 1 mL/min and an injection volume of 10 μL. The tocopherols were analyzed by fluorescence with an excitation wavelength of 295 nm and emission wavelength of 330 nm.

Real Time Polymerase Chain Reaction
Total RNA was extracted with RNeasy Mini Kit (QIAGEN Inc. Valencia, CA, USA) according to the manufacturer’s protocol from uterine and placental tissues. Total RNA (1 μg) was reverse transcribed into cDNA with a SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA 92008, USA) using random hexamers as a primer. Primers for the genes examined are listed in Table 1. To ensure the amplification of a single amplicon of the expected size, the product was viewed on an ethidium bromide stained electrophoresis gel. The RT-PCR analyses for VEGF, PGF, eNOS, HIFs and 18 S rRNA were performed using the ABI 7300 Sequence Detection System instrument and software (PE Applied Biosystems Inc., Foster City, CA, USA). To compare mRNA expression levels among samples, mRNA for each gene of interest was normalized to the expression of a housekeeping gene, 18 s RNA, and differences in gene expression were calculated as a fold change to the mean of the vehicle group using the 2-ΔΔCt method [28,29].

Morphometry analysis
The placenta samples collected for histological evaluation were fixed in 10% neutral buffered formalin, sectioned at 5 microns, and stained with hematoxylin and eosin. They were evaluated on a Nikon E400 Eclipse microscope, and photomicrographs (100×) were taken with a Nikon camera with a 3 chip. Images were processed with Nikon Act 1 software. Image processing and morphometry analysis were performed using ImageJ 1.42 q (NIH, USA) to evaluate the fractal dimension and
lacunarity [30,31]. The main processing steps applied to the images are shown in Figure 2. Briefly, after downloading the original image in the software, they were converted to 8 bit black and white images, and thresholding was performed by selecting mean value as threshold value. The images were made binary and were skeletonized.

Fractal analysis is a contemporary method of applying nontraditional mathematics to patterns that defy understanding with traditional Euclidean concepts. Fractal analysis measures complexity using the fractal dimension. A fractal dimension is, in essence, a scaling rule comparing how a pattern’s detail changes with the scale at which it is considered. The fractal dimension is a

| Gene   | Primer | Sequence 5’ to 3’       | GenBank accession number |
|--------|--------|-------------------------|--------------------------|
| VEGF   | Forward| TGTAATGACGGAAAGTCTGGAG   | AF071015                 |
|        | Reverse| TCACCCGCTCGCTTGTACCA     |                          |
| PI GF  | Forward| TGCCGGTCATGAGCCTGT       | NM_002632*               |
|        | Reverse| GCAGTCATGAGAAGTGTGAC     |                          |
| eNOS   | Forward| TGGGCCGCATCAGTCG         | NM_001129901             |
|        | Reverse| GAACATCTCTGTGCTAGCTG     |                          |
| HIF-1α | Forward| TCAGCTATTTCGTCAGG        | EU340260                 |
|        | Reverse| TCCAAATTCAGCACAACG        |                          |
| HIF-1β | Forward| AGATGCAGGAACTGGG          | EU340261                 |
|        | Reverse| CCTGCTTCTAAGAACC          |                          |
| HIF-2α | Forward| AGATGCAGCCTGGAAGG         | EU340264                 |
|        | Reverse| TCACACACATCAGTGC          |                          |
| HIF-2β | Forward| AGATGCAGGTTGGAATGC        | EU340265                 |
|        | Reverse| CCTCAGAGTGGCAGAACC       |                          |

*Homology to human

Figure 2 Steps involved in image processing of histological sections of placentome using ImageJ 1.42 q. a. histological image of placental vascular network (Bar = 50 μm; 100x magnification); b. 8 bit image; c. Image smoothened; d. Image with edges; e. Binary conversion; f. Skeletonization.
valuable parameter to describe the complexity. FarclLac 2.5 release 1b5 m (NIH, USA) was used to perform fractal dimension. The FracLac scan images using a shifting grid algorithm that can do multiple scans from different locations on each image. The fractal dimension of the binary skeleton was estimated using the box counting method at multiple origins. The basic procedure is to systematically lay a series of grids of decreasing calibre (the boxes) over an image and record data (the counting) for each successive calibre. To minimize grid location effects, the algorithm started from number of locations, generating a set of variables for fractal dimension. The average value over all locations was considered as the final estimate of fractal dimension. During the same analytical process ‘Lacunarity’ was also calculated. This parameter is a measure of homogeneity of structure or the degree of structural variance within an object. It was estimated as the average of the coefficient of variation for pixel density over all grid sizes and locations. The field demarked as a square in Figure 3 is one location. A total of 30 locations were evaluated for each sample.

**Statistical analyses**

The MIXED procedure of the SAS System (SAS version 9.12, SAS Institute Inc., Cary, NC 27513) was used to perform a repeated measures analysis of covariance to test for effects of treatments (aT, gT and CON), days post breeding [94 dpb (pre-supplement level), 107 dpb, 114 dpb, 121 dpb, 128 dpb], 136 ± 1 dpb] and their interactions on the serum concentration of tocopherols. Covariation of repeated measurements was modeled as compound symmetric and model adequacy was assessed using plots of standardized residuals. The differences in the serum tocopherol concentrations between pre-supplement stage and post supplement stages of gestation were tested by creating contrast statements. Analysis of variance was used to determine the within treatment between days post breeding differences and within days post breeding between treatment differences. Since the data were not normally distributed, a non-parametric procedure was used to perform the Kruskal-Wallis method test to compare the median tocopherol concentrations in serum, placenta and uterus among the treatment groups (Table 2). For responses with significant differences, pair-wise comparisons were performed using Minitab 12.1 (Minitab Inc., State College, PA, USA).

The RT-PCR data were subjected to ANOVA using 2 ΔΔCt values to ascertain statistical significance of any differences in VEGF, PIGF, eNOS and HIFs expression in placebo vs. tocopherol-treated groups [29]. The differences in fractal dimension and lacunarity among treatment groups were tested by non-parametric Kruskal-Wallis method. There were 3 ewes (2 in CON and 1 in

**Figure 3 Morphometric analysis of placental vascular network of late pregnant ewes supplemented with alpha tocopherol, gamma tocopherol or placebo**

a. Morphometric parameter of placental network of ewe supplemented with γ-tocopherol. Field demarked as red-square had Fractal dimension 1.6204 and lacunarity 0.4967. A total of 30 fields were evaluated for each sample; b. Morphometric parameter of placental network of ewe supplemented with α-tocopherol. Field demarked as red-square had Fractal dimension 1.7030 and lacunarity 0.5884. A total of 30 fields were evaluated for each sample. c. Morphometric parameter of placental network of ewe treated with placebo. Field demarked as red-square had Fractal dimension 1.6263 and lacunarity 0.6033. A total of 30 locations were evaluated for each sample.
Table 2 Median (25th and 75th percentile) tissue concentrations of alpha- and gamma-tocopherols between the treatment groups in ewes (N = 18)

| Tissue   | Treatment Groups | aT (mg/kg) | gT (μg/kg) |
|----------|------------------|------------|------------|
| Placenta | aT               | 9.19 (6.77, 12.1) * | 45.5 (10.0, 83.3) * |
|          | gT               | 2.74 (2.26, 6.22) * | 607 (477, 1057) * |
|          | Control          | 2.88 (1.85, 4.16) * | 924 (574, 1530) * |
| Uterus   | aT               | 5.23 (4.38, 6.50) * | 0 (0, 189) * |
|          | gT               | 3.28 (2.34, 3.54) * | 737.7 (607.1, 979.6) |
|          | Control          | 2.34 (1.91, 3.87) * | 156.5 (110.3, 237.1) |

* Different superscripts between treatment groups within tissue are significantly different (P < 0.05); aT - alpha tocopherol; gT - gamma tocopherol; CON - Control

Pair-wise comparison of median tocopherol levels between stages within treatment groups was different for the gT group (P < 0.05) but not in aT and CON groups (P > 0.1).

Uterine and placental tocopherol concentrations
The median aT tocopherol concentration in placenta and uterus was greater in ewes supplemented with aT compared to ewes in gT and CON groups (P < 0.05; Table 2). The median gT concentrations in placenta and uterus were greater in ewes supplemented with gT compared to ewes in the aT and CON groups (P < 0.05; Table 2). The median gT in the uterus of CON ewes was greater than aT supplemented ewes (P < 0.05; Table 2).

Vascular Endothelial Growth Factor, Placental Growth Factor, endothelial Nitrous Oxide Synthase and Hypoxia Inducible Factors expression

Figure 5a shows expression of VEGF, PI GF and eNOS in placenta and uterus in tocopherol supplemented groups relative to CON, where control is 1. In cotyledon, the PI GF and eNOS expression in ewes supplemented with aT was significantly higher compared to the CON group. The VEGF expression was not different between aT and the CON groups. The VEGF, PI GF and eNOS expressions in ewes supplemented with gT were not different from the CON group. In caruncle and uterus, the VEGF and eNOS expressions in ewes supplemented with gT were not different from the CON group.

The hypoxia inducible factors gene expression in tocopherol supplemented ewes relative to CON (1.0 fold) are given in Figure 5b. Ewes that received aT showed an increase in HIF-1α mRNA expression in cotyledon compared to control (P < 0.05). In gT supplemented ewes, the mRNA expressions of HIF-1α in cotyledon, and HIF-1α and HIF-2α in caruncle and uterus were higher compared to controls (P < 0.05). There was no HIF-1β expression in cotyledon, caruncle and uterus.

Morphometry analysis
The results showed that aT or gT supplemented ewes had increased fractal dimension and decreased lacunar-ity in their placental vascular network compared to CON ewes (Figure 6; P < 0.05).

Discussion
The goal of this study was to investigate the differences in angiogenic potential of alpha- and gamma-tocopherol. We evaluated biweekly serum, and uterine...
and placental concentrations of tocopherols, angiogenesis gene (VEGF, PIGF, eNOS and HIFs) mRNA expressions, and morphometry of placental vascular network following daily oral supplementation in late pregnant ewes. The findings of this study indicated that oral supplementation of tocopherols to pregnant ewes during late gestation increased their serum concentrations; increased their concentrations in uterine and placental tissue; alpha tocopherol increased mRNA expression of PIGF, eNOS and HIF-1α in cotyledon compared to placebo; gamma tocopherol increased VEGF and eNOS mRNA expressions in caruncle and uterus, and HIF-1α in cotyledon, HIF-1α and HIF-2α in caruncle and uterus compared to placebo; morphometry analysis of angiogenic parameters favored increased angiogenesis.
In this study, the serum and tissue level of αT (mg/kg) was higher than gT (μg/kg) even with increased gT daily dose. Gamma-tocopherol is initially absorbed in the same manner as alpha-tocopherol following administration, however, only small amounts of gamma-tocopherol are detectable in blood and tissue [32]. The bioavailability of αT is 5 to 10 times more than gT, could explain the reason for lower serum and tissue gT [33,34]. Other factors that could be responsible include high fat intake [35], oxidative stress [36,37], dose and half-life [38], species and cell type in question, and the chemical vitamer. The d2-gamma-tocopherol half-life was 13 ± 4 h compared with 57 ± 19 h for d6-alpha-tocopherol [38]. Considering the half-life, the daily oral supplementation and biweekly serum sample collection in this study occurred in the mornings, between 0800 and 0900 h, and could also be the possible explanation for the lower gamma tocopherol levels in the serum. Also, it has been demonstrated that αT transfer protein is present in early and late term placentae in other species [39]. Hence it is possible that αT was better able to transfer across to the cotyledon and thereby increased tissue αT concentration.

Figure 5 Relative mRNA of target genes expressed in uterus and placenta of late pregnant ewes following oral supplementation of alpha tocopherol, gamma tocopherol or placebo. a. Vascular endothelial growth factor, endothelial nitric oxide synthase, and placental growth factor mRNA expression in placenta and uterus following alpha or gamma tocopherol oral supplementation (× expression relative to control = 1; * P < 0.05); b. Hypoxia inducible factors (HIF-1α, HIF-2α, HIF-2β) expression in placenta and uterus following alpha or gamma tocopherol oral supplementation (× expression relative to control = 1; * P < 0.05).
Tocopherols exhibit complex biological effects reflecting their diverse nutritional, antioxidant and signaling properties [26]. The biologically active range of tocopherols is relatively narrow (~3-4-fold variation). Because of this relatively narrow "window of efficacy," benefits from tocopherols might require continuous consumption to be effective. Once ingested, orally administered vitamin E plateaus in the plasma by 24 h but does not fully distribute to all biological compartments until at least ~1 week after ingestion [27]. It is consistent in this study that the serum concentrations of both aT and gT spiked during the first week and maintained at increased levels during rest of the study.

Several factors including VEGF, PIGF, basic Fibroblast Growth Factor (bFGF), epidermal growth factor, eNOS and angiopoietins 1 and 2 have been recognized as important regulators of placental development [2,40,41]. In the present study, alpha tocopherol increased mRNA expression of PI GF in cotyledon compared to placebo, and gamma tocopherol increased VEGF in caruncle and uterus. In ewes, maternal placental vascularization is dependent upon VEGF and angiopoietins expression. In contrast, fetal placental vascularization depends on numerous angiogenic factors [42,43]. It has been demonstrated that both VEGF and PIGF driven angiogenesis acts via an eNOS activated, nitric oxide mediated pathway [44]. In this study, aT increased the eNOS in cotyledon and gT increased the eNOS in caruncle and uterus. In pioneering studies, Cooney et al [14,45] showed that tocopherol reduces nitrogen dioxide to the less harmful nitric oxide. Because endothelium derived nitric oxide is a key regulator of vascular homeostasis, up-regulation of endothelial nitric oxide synthase and nitric oxide formation by tocopherol could be important in promoting angiogenesis [46].

In this study aT, increased mRNA expression of HIF-1α in cotyledon compared to placebo. The gT increased mRNA expressions of HIF-1α in cotyledon, and HIF-1α and HIF-2α in caruncle and uterus compared to placebo. Hypoxia-inducible factor is the main regulator of the cellular response to low oxygen levels in mammalian species, playing a key role in regulating many physiological functions. The 2 principal HIF-α subunits, HIF-1α, which is ubiquitously expressed and HIF-2α, which shows a spatially restricted expression, both play important roles in vascular development and endothelial function [47-49]. Hypoxia inducible factor-1α or HIF-2α combine with HIF-1β to form the functional basic helix-loop-helix transcription factor HIF-1 to stimulate transcription of the VEGF gene via interaction with the hypoxia response element (HRE) [25]. This occurs under hypoxic conditions, but is down regulated under normoxic conditions due to targeted proteolysis of HIF-1α and HIF-2α, mediated by the von Hippel-Lindau tumor suppressor gene product (pVHL). Additionally, VEGF is regulated post-transcriptionally through stabilization of its mRNA by interaction with heterogeneous nuclear ribonucleoprotein L (hnRNP L). The basic helix-loop-helix transcription factor HIF-2α is also capable of heterodimerizing with HIF-1α to form functional HIF-1. Several genes encoding growth factors and tyrosine kinase receptors essential for endothelial cell growth and function such as EPO, VEGF, eNOS and Flk-1 were shown to contain HIF-binding sites in their promoter/enhancer sequences.

Although placental and fetal growth and development are driven by the program encoded in its genome, the genetic regulation is influenced by the intra-uterine environment in which the fetus grows. The size and nutrient transfer capacity of the placenta plays a central role in determining the prenatal growth trajectory of the fetus. Angiogenesis is the process of formation of new vessels from preexisting vessels. Although tissue hypoxia is the main drive for angiogenesis, a growing body of evidence has demonstrated that oxidative stress can also be a potent trigger for the development of new vessels. Hence it seems reasonable that the presence of no or high oxidative stress may result in formation of new vessels. Angiogenic sprouting is controlled by the balance between pro-angiogenic signals (VEGF) and factors that promote quiescence (pericyte) or VEGF inhibitors [50]. In conditions that favor angiogenesis, some endothelial cells can sprout, whereas others fail to respond. In the present study, morphometric analysis revealed ewes supplemented with alpha- or gamma-tocopherol had increased fractal dimension and decreased lacunarity compared to placebo treated ewes indicated increased angiogenesis in utero-placental vascular network. It should be noted that even though not significant reduced mRNA expressions for VEGF, PI GF and eNOS in uterus and placenta were observed in this study.
Conclusions
This study evaluated cellular responses to supplementation in order to understand the mechanisms behind the biological effects of tocopherols on placental angiogenesis during late gestation in pregnant ewes. The tocopherol concentrations of uterus and placenta were increased following oral supplementation. In αT supplemented ewes, mRNA expression of PlGF, eNOS and HIF-1α in cotyledon were greater than the CON group. In γT supplemented ewes, VEGF and eNOS mRNA expressions in caruncle and uterus, and HIF-1α in cotyledon, HIF-1α and HIF-2α in caruncle and uterus were greater than the CON group. Morphometry analysis revealed angiogenic parameters favoring increased angiogenesis. Given the findings, it is plausible that the angiogenesis observed in this study might be a prooxidant and proangiogenesis pathway. Based on the results observed in this study we confer that the tocopherol supplementation can be employed to promote placental angiogenesis during late gestation. The oral tocopherol supplementation may be considered as an option to overcome pregnancy complications due to placental ischemia during late gestation.

Additional material

Additional file 1: Supplemental Table S1: Composition of hay fed choice to the pregnant ewes during the trial period
Additional file 2: Supplemental Table S2: Average tocopherols content in supplements

Additional file 3: Supplemental Table S3: P values for the effect of treatment and stage of gestation on serum concentration of alpha and gamma tocopherol in ewes (N = 18) supplemented with tocopherols

Additional file 4: Supplemental Table S4: P values for the comparison of differences in the serum alpha and gamma tocopherol concentrations during different stages of gestation in ewes (N = 18) supplemented with tocopherols

Acknowledgements
This study was funded by College of Veterinary Medicine, Washington State University, Pullman WA 99164 and by Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA 24061. The authors acknowledge Geraldine Magnin-Bissel and Jonathan Dohanich, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA 24061 for the technical support.

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Authors’ contributions
RK did the work of acquisition of funding, conception, design and collection of data, morphometry analysis, data analysis and interpretation, and drafting of the manuscript, tables and figures. VK contributed the design and conception, PCR analysis, collection of data and drafted the manuscript. JR contributed to the work of morphometry analyses. KD did the work of feed supplementation, euthanasia, and collection of samples. PDS contributed to the work of postmortem tissue sample collection and histology. CDT contributed to the work of HPLC, and revised the manuscript for content and language. All authors read and approved final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 23 November 2009 Accepted: 12 July 2010
Published: 12 July 2010

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