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Serenoa Repens and N-acetyl Glucosamine/milk proteins complex differentially affect the paracrine communication between endothelial and follicle dermal papilla cells.

Running Title: *Serenoa repens* and NAG/ milk proteins complex on *in vitro* cocultures.

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Abstract

Current treatments for hair follicle (HF) disruption are based on 5-α reductase inhibitors and prostaglandin modulators. Botanicals and nutraceutical compounds interfere with hair loss or stimulate its partial regrowth. Here we used *in vitro* cocultures to investigate the activity of *Serenoa repens* (*SR*) and N-Acetyl Glucosamine+milk proteins (NAG/Lac) on the paracrine interactions between human microvascular endothelial cells (HMVEC) and HF dermal papilla cells (FDPC). Both *SR* and NAG/Lac-induced endothelial tubulogenesis were enhanced by FDPC. *SR* promoted proliferation of both the cell types, while NAG/Lac was effective on endothelium. VEGF production, enhanced by *SR*, was further augmented by FDPC. In FDPC 5-α reductase-II and β-catenin expressions were modified by *SR* and less by NAG/Lac, with no additional effect by HMVEC. *SR* and NAG/Lac prevented lipid

peroxidation, whereas *NAG/Lac* was effective on IL-1β production. Finally, SR and NAG/Lac differentially affected HMVEC permeability and tight junction proteins content. These data provide a mechanistic background for the potential use of these compounds as promoters of HF vascularization.

INTRODUCTION

The dermal papilla is a cluster of mesenchymal cells located at the base of the hair follicle (HF) which have a number of important roles in the regulation of hair growth (Topouzi et al., 2017). HF dermal papilla controls hair growth and is characterized in the anagen phase by a highly developed vascular network with enhanced expression of angiogenic growth factors and cytokines (Yano et al., 2001). Nutritional deficiency may impact both hair structure and hair growth. Effects on hair growth include acute telogen effluvium (TE), a well-known effect of sudden weight loss or decreased protein intake (Mubki 2014). Other studies reported a potential association between nutritional deficiency and chronic telogen effluvium, Androgenetic alopecia (AGA), female pattern hair loss (FPHL), and Alopecia areata (Guo, Katta 2017). Clinical observations, as well as animal studies and *in vitro* techniques have been developed to investigate and promote hair reconstitution (Ohyama, Veraitch 2013). Recently, new therapeutical options with topical 5-α reductase inhibitors, prostaglandin modulators, and treatments with stem cells have been proposed in order to overcome the limitations of oral finasteride and topical minoxidil (MXD), the current gold standard for AGA treatment (Valente Duarte de Sousa, Tosti 2013).

A number of botanicals or dietary supplements are available that can effectively slow or reduce hair loss and inflammation observed in AGA or stimulate partial hair regrowth (Rondanelli et al., 2016; Prager et al., 2006). *Serenoa repens (SR)* is a low-growing palm tree that is endemic to all counties of Florida, (USA). Permixon, a pharmaceutical extract from fruit *SR*, is anti-androgenic and has been used to relieve symptoms of benign prostate hyperplasia (Ooi Pak 2017). *SR* is one among the many naturally occurring 5-α-reductase inhibitors which has gained popularity as a remedy for AGA (Murugusundram 2009). Liposterolic extract of *SR* (LSESr) and its glycoside, *β*-sitosterol, were tested in subjects with AGA which showed a highly positive response to treatment. The blockade of inflammation

using a composition containing LSESr as well as two anti-inflammatory agents (carnitine and thioctic acid) alters the expression of molecular markers of inflammation in *in vitro* system. These findings suggest that 5-α reductase inhibitors combined with blockade of inflammatory processes could represent a novel two-pronged approach in the treatment of AGA with improved efficacy over current modalities (Chittur et al., 2011). Notably, *in vitro* assays revealed the ability of LSESr to inhibit 5-alpha-reductase expression 3-fold better than Finasteride (Chittur et al., 2011). Recently, *SR* has been proposed as a 'natural' alternative to conventional treatments for male AGA as well as for other hair disorders (Wessagowit et al., 2016).

In this work we compare the effects of *SR* to a mixture of N-acetyl-Glucosamine (NAG) and milk proteins (β-Lactoglobulin + α-Lactalbumin) (Lac). The precursor for Hyaluronic Acid, NAG, is a hexosamine sugar that is metabolized by the hexosamine biosynthesis pathway and stimulates O-linked N-acetyl-glucosamine (O-GlcNAc) modification (O-GlcNAcylation) of cytosolic and nuclear proteins (DeAngelis et al., 2013). Clinical tests of formulations containing NAG have shown that it can also reduce the appearance of facial hyperpigmentation, especially when used beside niacinamide (Sarkar et al., 2013). Due to the versatile functions of Glucosamine and its derivative NAG, they are considered valuable ingredients for accelerating wound healing, improving skin hydration, and decreasing wrinkles and colors (Bisset et al., 2007; Vournakis et al., 2008). Interestingly, poly-N-acetyl glucosamine nanofibers have been recently reported to regulate endothelial cell migration leading to angiogenesis (Vournakis et al., 2008). The hair apparatus contains considerable amounts of complex carbohydrates with different saccharide residues (alpha-D-mannose, beta-D-glucose, alpha-L-fucose and NAG). Moreover, the presence of glycogen in outer root sheath cells might enable these cells to provide other hair cells with energy when necessary (Ishii et al., 2001).

Milk is considered one of the most nutritionally complete foods available, it is mainly composed of water, lipids, lactose and proteins, and their relative shares widely vary among species (Kuligowski et al., 2017). The two most prominent and abundant proteins of whey protein fraction are β-lactoglobulin and α-lactalbumin (Li et al., 2017). β-Lactoglobulin is the major whey protein in ruminant species; for its amino-acid sequence and 3D structure it belongs to lipocalins, a widely diverse family of proteins, most of which bind small hydrophobic ligands and thus may act as specific transporters, similar to serum retinol binding protein (Brownlow et al., 1997). In addition, β-Lactoglobulin from buffalo colostrum inhibits cell proliferation, micro-vessel sprouting, cell migration and tube formation of human umbilical vein endothelial cells (Chougule et al., 2013). A recent study reported the ability of Gly-Leu-Phe (GLF), an immune-stimulating peptide derived from α-lactalbumin, to prevent alopecia induced by the anticancer agent etoposide in a neonatal rat model after intraperitoneal injection (Tsuruki et al., 2005).

We previously established transwell-based cocultures to show that FDPC promote viability, proliferation and tubulogenesis of HMVEC, as well as to test the effects of biomimetic peptide Sh-Polypeptide 9 (CG-VEGF) (Bassino et al., 2015, 2016). Here we took advantage of this approach to evaluate the bioactivity of both *SR* and NAG/Lac.

MATERIALS AND METHODS

Compounds

Serenoa repens (SR) (Euromed S.A. BARCELONA (SPAIN), N-Acetyl Glucosamine (NAG) (Polichimica Srl Bologna Italia), milk proteins (β-Lactoglobulins + α-Lactalbumin: Lac) (DAVISKO FOODS INTERNATIONAL Inc. USA) (milk proteins: 75% β-Lactoglobulins and 22.03% α-Lactalbumin, NAG/milk proteins ratio: 1:1) were used at different concentrations (2.5 mg/ml, 25 μg/ml and 2.5 μg/ml).

Cell Culture

Human hair follicle dermal papilla cells (FDPC) were obtained from PromoCell (Germany; lot 2040301.35). FDPC were grown in Follicle Dermal Papilla Cell Medium (PromoCell) with 1% antibiotic/antimycotic (Invitrogen, Grand Island, NY, USA). In order to avoid phenotypical changes occurring during cell culture, only FDPC at 2-7 passages were used for all experiments. Human microvascular endothelial cell line (HMVEC) was purchased from Lonza and grown in EGM 2-MV medium (Lonza, Basel, Switzerland). All cell types were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cocultures

HMVEC were thawed from frozen stock and seeded in 24 well plates at a density of 2.5 x 10^3 cells/well, medium was discarded after 18 hrs, then complete medium was replaced with DMEM 2% FCS to reduce cell proliferation for 24 hrs and finally replaced with DMEM 0% + compounds (NAG/Lac or *SR,* used at different concentrations) alone or in the presence of the insert with FDPC. For cell proliferation in coculture, FDPC were thawed, centrifuged, recounted and seeded at a density of 2.5 x 10^3 cells/well for 2 days in Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Calf Serum (FCS) until 100% confluence on Transwell

clear polyester membrane inserts with 0.4-um pore size and a 0.3-cm^2 area (Corning). The proliferation of HMVEC (alone or in the presence of FDPC on the insert) was analyzed after 24 hrs of coculture. As described above for FDPC, HMVEC were seeded on the Transwell clear polyester membrane inserts and exposed to the same experimental protocol.

Cell viability

For proliferation assays in cocultures, HMVEC or FDPC (2.5 x 10^3 cells/well) were seeded in 24-well plates following the protocols described in the previous chapter. Cell number was evaluated by the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS). MTS conversion into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. Formazan product was measured with a F5 FilterMax microplate reader (Molecular Devices, Sunnyvale, CA, US) at 490 nm, as absorbance is directly proportional to the number of viable cells.

Tubulogenesis

Matrigel (Collaborative Biomedical Products, USA) was used to make up a basement membrane matrix gel solution for cell suspension. Sub-confluent HMVEC were trypsinized and 5.0×10^4 cells were added to each well of a chilled 24-well plate and allowed to gel for 30 min at 37°C in a humidified 5% CO_2 atmosphere. HMVEC, with or without insert containing FDPC at the different experimental conditions, were maintained in a stable humidified 5% CO_2 atmosphere for all the experimental time course (18 hrs). Image acquisition and statistical analysis were performed with Metafluor and ImageJ softwares, respectively. Tubulogenic indexes were obtained with Angiotool software and include total vessel length

and total number of junctions.

ELISA Assays

β-catenin, VEGF, Occludin, Claudin-5 and IL-1β (in cell lysates or medium) were assessed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Sigma ELISA Kit). Briefly, 100µL of medium (for VEGF and IL-1β) or cell lysate (for occludin, and nuclear cell lysate for β-catenin; See below '*Nuclear protein extraction for β-catenin quantification'*) were incubated into an antibody-coated 96-well plate at room temperature for 2.5 hrs. The wells were washed four times with wash buffer solution. Then 100 µL anti-human β-catenin or IL-1α antibody was added and the samples were again incubated for 1h at room temperature. The plate was washed four times, 100µL of streptavidin-peroxidase conjugated was applied for 1h at room temperature. After a final washing, 100 µL tetramethylbenzidine substrate was added and allowed to develop for 30 min in the dark at room temperature. After stopping the reaction with 50 µL stop solution containing citric acid 2.0 $mmolL^{-1}$, absorbance was read at 450 nm using a F5 FilterMax microplate reader (Molecular Devices, Sunnyvale, CA, US). Sample concentration was calculated from the standard curve.

Nuclear protein extraction for β-catenin quantification

FDPC were grown to 70-80% confluence. Afterwards, cells were scraped using fresh PBS, collected into an appropriate conical tube and centrifuged (5 min at 450 x *g)*. Then the supernatant was discarded and 1 mL Lysis Buffer (10 mM Tris HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.3 M Sucrose, including DTT and protease inhibitors) added to 200 µL of packed cell volume (PCV) for 15 min. Suspended cells were centrifuged for 5 minutes at 420 x *g*. Pellet of packed cells was resuspended in 400 µL (2X PCV) of Lysis Buffer and

fragmented using a syringe with a narrow-gauge. The disrupted cells in suspension were centrifuged for 20 min at 10,000 x *g*. The supernatant was transferred into a fresh tube and this fraction corresponds to the cytoplasmic fraction. Subsequently the pellet was resuspended in 140 µl of extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) Glycerol added with 1.5 µL of the 0.1 M DTT solution and 1.5 µL of the Protease Inhibitor Cocktail) and centrifuged for 5 min at 20,000 x *g*. The resulting supernatant is the nuclear protein extract. It is finally collected to a clean tube and analyzed with β-catenin ELISA assay.

Fluorescence microscopy

5-α reductase II expression was detected using immunofluorescence assay. Briefly, after the treatments cells were fixed in 4% paraformaldehyde for 25 min at room temperature. Then, the fixed cells were washed three times with ice cold PBS solution, incubated 20′ with 0.3% Triton, and 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in PBS, and stained with the primary antibody 24 hrs at 4°C. Cover slides were washed twice with PBS and incubated 1h at room temperature with the secondary antibody. After two washes in PBS, cover slides were mounted on standard slides with DABCO (Sigma) and observed after 24 hrs with by fluorescence microscopy (Nikon T-E microscope, 10×objective). For each experiment, we randomly acquired three fields/sample evaluating the mean of fluorescence intensity/cell/field (about 30 cells/field).

Lipid peroxidation

Lipid peroxidation (LP) was analyzed using Click-iT® LAA reaction kit (LifeTechologies). Briefly, Linoleic acid is the most abundant polyunsaturated fatty acid found in mammals and its LP products likely account for the majority of lipid-derived protein carbonyls. When

incubated with cells, LAA (Linoleamide alkyne) incorporates into cellular membranes. Upon LP, LAA is oxidized and produces 9- and 13-hydroperoxy-octadecadienoic acid (HPODE). These hydroperoxides decompose to multiple α, β-unsaturated aldehydes, which readily modify proteins at nucleophilic side chains. These alkyne-containing modified proteins can be subsequently detected using Click-iT® chemistry and multiplexed with other probes appropriate for fixed cells. Briefly, HMVEC were grown and recovered overnight at 37°C alone or with FDPC and treated with H_2O_2 used alone or with NAG/Lac or *SR*, respectively. Then we added Click-iT® LAA stock solution to the cells in complete growth medium at a final concentration of 50 μM and we treated the cells with the compound of interest for the desired time. Cells were washed three times with PBS to remove free Click-iT® LAA from the cells, and immediately fixed and permeabilized. Finally we added 0.5 mL of Click-iT® reaction cocktail to each well containing a coverslip. Cells were incubated for 30' at room temperature, protected from light. Fluorescence (Alexa Fluor® 488) was acquired using a F5 FilterMax microplate reader (excitation emission maxima: 495/519nm) (Molecular Devices, Sunnyvale, CA, US).

Transendothelial Resistance (TEER) measurement

The transendothelial resistance (inverse of permeability) of HMVEC monolayer treated with the different compounds was evaluated with TEER measurements, using an Endohm 12 electrode chamber and an endothelial volt/ohm meter EVOM^2 (World precision Instruments, USA). TEER values were obtained from three independent experiments.

Statistical analysis

Statistical significance in all experiments was evaluated by Kaleidagraph software (Synergy Software, USA) using nonparametric Wilcoxon test. The Wilcoxon test was chosen for the

survival assays analysis because for each condition in each experiment five biological replicates were done and they were not normally distributed. The Wilcoxon test was also used for cell tubulogenesis analysis because the percentages of tubulogenesis were not normally distributed. All values are presented as the mean ± standard error (SEM). For each experimental condition five technical replicates were performed; three biological replicates were done for each experiment: N=3. Results with *p*-values < 0.05 were considered statistically significant: *p<0.05; **p<0.01; ***p<0.001.

RESULTS

Effects of SR and NAG/Lac on tubulogenesis and VEGF production by HMVEC grown alone or cocultured with FDPC.

SR enhanced HMVEC tubulogenic potential measured by endothelial tube formation assay both in terms of total tubule length and number of junctions (Fig. 1B-F, see Fig. 1A for set-up configurations); FDPC significantly increased both tubulogenic indexes only in presence of the highest dose of *SR* (Fig. 1, see Fig. 1A for set-up configurations). All concentrations of NAG/Lac enhanced HMVEC tubulogenic indexes (Fig. 1B-F, see Fig. 1A for set-up configurations), although the effect was more prominent for the highest dose of NAG/Lac (2.5mg/ml).

The presence of FDPC further increased tubulogenic indexes, the effect was statistically significant only for the lowest dose (Fig. 1B-F). Accordingly, *SR* and NAG/Lac were both able to significantly promote VEGF production from endothelial cells; coculture with FDPC produced a significant additional enhancement only upon *SR* treatment (Fig. 1G).

SR and NAG/Lac affect proliferation of HMVEC and FDPC grown separately or in coculture.

NAG/Lac (25μg/ml) promoted proliferation of FDPC grown alone, without any additional effect of the coculture with HMVEC (Fig. 2B, see Fig. 2A for set-up configurations). All concentrations of *SR* significantly increased FDPC number (Fig. 2B). The presence of HMVEC, in coculture configuration, significantly enhanced the response of 25μg/ml *SR* (Fig. 2B). NAG/Lac significantly increased cell number of HMVEC grown alone, and the coculture with FDPC further enhanced the response in presence of 2.5μg/ml NAG/Lac (Fig. 2C). All concentrations of *SR* promoted HMVEC proliferation (Fig. 2C). An additional gain of the response to *SR* (2.5 μg/ml) was observed in the presence of FDPC (Fig. 2C).

Effects of SR and NAG/Lac on the expression of 5-α reductase II and β-catenin by FDPC.

The 5-α reductase II enzyme catalyzes the conversion of testosterone to dihydrotestosterone (DHT), the most potent male hormone that causes AGA (Jang et al., 2007). Here, we tested the role of both compounds in the modulation of 5-α reductase II expression in testosterone treated cells. As indicated by the graph, in coculture configuration, HMVEC drastically reduced 5-α reductase II expression in FDPC treated with testosterone. *SR* significantly prevented 5-α reductase II expression triggered by 100 nM testosterone in FDPC (Fig. 3A, B $_{(i, ii)}$) (24 hrs). NAG/Lac completely failed to counteract the response to testosterone.

Moreover, *SR* increased the nuclear expression of β-catenin in FDPC with no further variations in coculture with the endothelium. Conversely, NAG/Lac was ineffective on β-catenin expression in FDPC; the presence of HMVEC raised this parameter only in the presence of NAG/Lac (2.5mg/ml) (Fig. 3C).

Effects of SR and NAG/Lac on IL-1β production and lipid peroxidation by HMVEC alone or in coculture with FDPC.

We asked whether *SR* and NAG/Lac could protect endothelium against oxidative stress typically occurring during aging, skin wounding and inflammation. HMVEC were treated with H_2O_2 (400μM, 24 hrs) to induce a strong oxidative stress. 400μM H_2O_2 strongly induced endothelial lipid peroxidation (LP) (Fig. 3D). The FDPC-derived CM significantly prevented LP (Fig. 3D). NAG/Lac and *SR* drastically reduced oxidative stress, but the presence of FDPC-derived CM abolished *SR* effect (Fig. 3D). *NAG/Lac* also prevented IL-1β production by HMVEC treated with H_2O_2 and in presence of FDPC. No changes were observed when endothelial cells were treated with *SR* (2.5mg/ml) (Fig. 3E).

SR and NAG/Lac modify endothelial permeability: evaluation of TEER and tight junction protein content.

HMVEC were grown at confluence and TEER measurements were performed for 7 days (Fig. 4A). As indicated by the graph, the highest concentration (2.5mg/ml) of *SR* and NAG/Lac reduced endothelial resistance upon 24 hrs of treatment. The TEER values did not recover up to 96 hrs of treatment. Accordingly, NAG/Lac decreased the content of Occludin, a well-known component of the tight junctions (24 hrs), while no variations were induced by *SR* treatment (Fig. 4B). Finally, both compounds reduced the cytosolic levels of Claudin-5, another protein included in tight junctions (24 hrs), although the effect was statistically significant for the only *SR* (2.5mg/ml) (Fig. 4C).

DISCUSSION

We recently reported that human follicle dermal cells strongly support endothelial survival, proliferation and tubulogenesis in transwell cocultures (Bassino et al., 2015). Here the same approach was employed to investigate the activity of nutritional supplements. Recently, has been reported that the diet supplementation with omega -3 and -6 and antioxidants acts efficiently against female hair loss in improving hair density and reducing the telogen percentage and the proportion of miniaturized anagen hair (Le Floc'h et al., 2014). In this work we tested the role of *SR* and NAG/Lac compounds in the regulation of the paracrine cross-talk between endothelium and follicle dermal papilla cells. Here, we show that *SR* and NAG/Lac exert differential activities. In particular, the results reveal that *SR* is more powerful than NAG/Lac in promoting endothelial production of VEGF and tubulogenesis; notably, the activity of *SR* is more effective than previously measured for the widely used topical drug minoxidil (see Bassino et al., 2016). *SR* enhances β-catenin expression by FDPC, protects vascular endothelium from oxidative stress and prevents 5-α reductase II expression induced by testosterone. Recently, the topical application of *SR* extract was suggested to be an alternative treatment in male patients who cannot tolerate the side-effects of standard medications (Wessagowit et al., 2016). Specifically, the lipido- sterolic extract of SR, LSESr, highly enriched with fatty acids and phytosterols, was reported to block both isoforms of 5-α reductase (types I and II) in contrast to finasteride (Chittur et al., 2011). Moreover, LSESr promotes hair regeneration and repair in mouse models by activating TGF-β signaling and mitochondrial pathway (Zhu et al., 2018). Finally, we observed that both compounds affect endothelial permeability ad alter the expression of occludin and claudin-5, two critical components of the tight junction complexes. These results unveil a novel functional role for *SR* and NAG/Lac that could efficiently lower vascular endothelium resistance. In conclusion, our *in vitro* study points to *SR* an NAG/Lac as effective compounds; nonetheless, further

mechanistic studies, strengthened by *in vivo* and clinical trials, are required to shed more light on their applicative potentiality.

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L.M. designed the research study, wrote the paper and contributed essential reagents.
Competing interests: Conflicts of interest none declared

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FIGURE LEGENDS

Figure 1. *SR and NAG/Lac promote tubulogenesis and VEGF production by HMVEC.*
A) Scheme of the experimental setup and relative contrast phase images of tubule formation at 18hrs. Arabic numbers (1-5) are referred to the mono-culture and coculture configurations. 1) HMVEC in DMEM 10% or HMVEC in DMEM 10% + FDPC; 2) HMVEC in DMEM 10% + NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml); 3) HMVEC in DMEM 10% + *SR* (2.5mg/ml, 25μg/ml and 2.5μg/ml); 4) HMVEC in DMEM 10% + NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml) and in presence of FDPC; 5) HMVEC in DMEM 10% + *SR* (2.5mg/ml, 25μg/ml and 2.5μg/ml) and in presence of FDPC.
B) Total number of junctions were evaluated in HMVEC (grown alone or cocultured with FDPC) treated with *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).
C) Total tubule length was analyzed on HMVEC (grown alone or in coculture with FDPC) treated with *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).
D) VEGF quantification in HMVEC (grown alone or cocultured with FDPC) treated with *SR* and NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).

Figure 2. *SR and NAG/Lac promotes proliferation.*
A) Scheme of the experimental setup. Arabic numbers (1-4) are referred to the mono-culture and coculture configurations. 1) FDPC or HMVEC in DMEM 10%; 2) FDPC or HMVEC in DMEM 0% + *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml); 3) HMVEC in coculture with FDPC + *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml); 4) FDPC in coculture with HMVEC + *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).
B) Cell proliferation (24 hrs) of FDPC grown alone or in coculture with HMVEC and in presence of *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).
C) Cell proliferation (24 hrs) of HMVEC grown alone or in coculture with FDPC and in presence of *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).

Figure 3. *SR and NAG/Lac differently affect 5-α reductase II, β-catenin expression and pro-inflammatory processes.*
A) Scheme of the experimental setup. Arabic numbers (1-4) are referred to the monoculture and coculture configurations. 1) FDPC + testosterone (100nM) or H_2O_2 (400μM) and treated with NAG/Lac (2.5mg/ml); 2) FDPC were grown in presence of CM of HMVEC + testosterone (100nM) or H_2O_2 (400μM) and treated with the NAG/Lac (2.5mg/ml); 3) FDPC + testosterone (100nM) or H_2O_2 (400μM) and treated with *SR* (2.5mg/ml); 4) FDPC were grown in presence of CM of HMVEC + testosterone (100nM) or H_2O_2 (400μM) and treated with *SR* (2.5mg/ml).
B) Representative images of FDPC grown alone or in coculture with HMVEC + 100nM testosterone and NAG/Lac or *SR* (2.5mg/ml). Images of 5-α reductase-II fluorescence intensity were taken with Olympus fluorescence microscope after 24hrs of treatment (i). Statistical analysis of 5-α reductase-II fluorescence intensity (F.I.) (ii).
C) β-catenin quantification in FDPC grown alone or in coculture with HMVEC + *SR* or NAG/Lac (2.5mg/ml, 25μg/ml and 2.5μg/ml).

D) Lipid peroxidation in HMVEC (treated with H_2O_2) grown alone or in presence of FDPC + *SR* or NAG/Lac (2.5mg/ml).
E) IL-1β production in HMVEC (treated with H_2O_2) grown alone or in presence of CM of FDPC + *SR* or NAG/Lac (2.5mg/ml).

Figure 4. *SR and NAG/Lac affect endothelial permeability of HMVEC monolayer and tight junctions content.*

A) TEER measurement in HMVEC treated for 7 days with NAG/Lac (2.5mg/ml) and SR (2.5mg/ml).
B) Occludin quantification in HMVEC lysates. HMVEC were treated for 24 hrs with NAG/Lac (2.5mg/ml) or SR (2.5mg/ml).
C) Claudin-5 quantification in HMVEC lysates. The cells were treated for 24 hrs with NAG/Lac (2.5 mg/ml) or *SR* (2.5mg/ml).