Apigenin Induces Browning in White Adipocytes Mediated by VEGF-PRDM16 Signaling.

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Abstract

The white adipose tissues are metabolically inert which results in deranged biological signalling disorders resulting in obesity. Lack of vascularisation in these tissues is mainly responsible to make them metabolically inert. Not much work has been done in this direction to understand the role of angiogenesis in white adipocytes metabolism. In the present study, we evaluated the effect of angiogenic modulator in modulating the metabolism in white adipocyte. Nutraceuticals apigenin (Apg) was employed as angiogenic modulator. The results indicated that promoting angiogenesis by Apg enhanced the de novo differentiation and trans-differentiation of white adipocyte into brown like phenotype by triggering vascular endothelial growth factor A. Cross talk between endothelial and adipocytes were observed in co-culture studies. The metabolic shift in white adipocytes was observed to be due to the upregulation of PRDM16 cascade. The study provides new insights for inducing metabolic shift in white adipocytes by modulation of angiogenesis in white adipocyte to trigger browning for the treatment of obesity. Further the study opens scopes for development of medical devices for obese subjects, an area that needs to be addressed specifically with reference to soft tissue engineering as commercial soft tissue engineering scaffolds does not suit the obese patients.

Introduction

Adipose tissues are the primary energy reservoir in our body and they act as the largest endocrine gland, it produces hormones, growth factors, free fatty acids and cytokines [1]. Two types of adipocytes exist: brown and white adipocyte. Brown adipocytes are multilocular adipocytes specialized for non-shivering thermogenesis and dissipation of energy. Expansion and activation of these adipocytes increase metabolic activity [2]. In contrast, unilocular white adipocytes are energy-storing cells and abnormal expansion of these cells leads to fat accumulation and obesity [3, 4]. Although numerous medications and therapies are available for obesity, its side effects limit its accessibility [5]. Enhancing metabolic status and energy dissipation in white adipocytes can be used as a promising therapeutic strategy against metabolic disorders.

In any tissue, proper vascularization and blood perfusion are compulsory for its growth, expansion, and metabolic status [6]. Angiogenesis plays significant role in adipogenesis [7]. Ironically angiogenesis which play a significant role in adipogenesis significantly reduces once white adipose tissue formation completes leaving behind a hypoxic microenvironment [8]. The hypoxic microenvironment in adipose tissue deters several biological processes in particular wound healing process [9]. Interestingly, the hypoxic microenvironment which triggers significant angiogenesis in tumor microenvironment [10] remains inert at adipose tissue to trigger any angiogenic response [11]. The tissue regeneration in obese significantly gets affect delaying the wound healing process due to lack of angiogenesis [12]. It has been reported that hypoxia inducible factor (HIF) does not play any role in triggering angiogenic response in white adipose tissue [11]. Hence, strategies to modulate angiogenesis in white adipose microenvironment may help in promoting tissue regeneration and wound healing in obese subjects. Not much work has been done to understand how modulation of angiogenesis affects white adipose microenvironment.
Understanding the molecular event and mechanism how triggering angiogenesis in white adipocyte microenvironment would pave ways in developing of medical devices for obese subjects with reference to soft tissue engineering. Further whether these angiogenic modulators would have any role directly on the adipocyte cell lineage is also not known.

Here in this study, Apigenin (4',5,7-trihydroxyflavone) which is a natural flavonoid present in vegetables, fruits and medicinal plants was used as angiogenic modulator in white adipocyte to understand its effect on the cellular behavior of white adipocyte. Owing to its biocompatibility, anti-inflammatory, anti-cancerous, free-radical scavenging, anti-hyperglycemic effects, antiosteoporosis, immune regulation, it is also being used as an alternative medicine for several health issues [13]. In addition, it also has beneficial effects on biological organ protection [13]. Apigenin exerts remarkable anti-adipogenic activity by lowering lipid content [14]. However, its action on white adipocyte has not been delineated. Hence, in this manuscript, we attempted to modulate angiogenesis in white adipocyte using nutraceutical, apigenin. The efficacy of apigenin to induce angiogenesis and its effect on the cellular biochemistry and physiology of white preadipocytes and white mature adipocytes were studied. After treatment with Apg, white pre-adipocyte subpopulation attained brown like character. The results revealed that apigenin can induces both \textit{de novo} differentiation of white preadipocytes and trans-differentiation of mature white adipocytes into brown adipocytes by modulating angiogenesis. The results indicated that Apg promoted angiogenesis independently on ECs alone and also promoted VEGF expression in white adipose cells that can act in paracrine way to activate the nearby ECs to angiogenic phenotype. The study indicated the cross talk between ECs and white adipocytes mediated via VEGF-VEGFR2-PRDM16 signaling resulting in simultaneous induction of angiogenesis in ECs and browning in white adipocytes.

\textbf{Materials And Methods}

\textbf{Materials and Reagents}

All the chemicals were purchased from Sigma-Aldrich, Missouri, United States unless if stated. Culture grade chemicals were used for all cell culture experiments. All tissue culture wares were procured from TPP, Switzerland and Greiner, Austria. The primers were custom synthesized and procured from Priority Life Science, Coimbatore, India. Antibodies were purchased from Santa Cruz Biotech, United States. Mouse 3T3-L1 cell line passage number 7 was procured from the National Centre for Cell Sciences (NCCS), Pune, India. Human endothelial cells, EA.hy926 passage number 11 was procured from the American Type Culture Collection (ATCC), United States.

\textbf{Experimental}

\textbf{Cell culture and maintenance}

Mouse white pre-adipocytic cells, 3T3-L1 and Human endothelial cells, ECs (EA.hy926) were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS) and streptomycin (100µg/ml), penicillin (100units/ml), gentamicin (30µg/ml), amphotericin B
(2.5µg/ml) and maintained in a humidified CO₂ incubator with 5% CO₂ and 37°C temperature. Once cells got 80-90% confluent, it was harvested and used for further experiments.

**Cell viability assay**

To study the cytotoxicity of apigenin (Apg) on 3T3-L1 and ECs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed [15]. When the cells got 80-90% confluent, cells were harvested using 0.05% Trypsin-EDTA and counted using a hemocytometer. Approximately, 12 000 cells/well were seeded on 48 well plates and left overnight in a humidified CO₂ incubator with 5% CO₂ and 37°C temperature. Cells were treated with different concentration of apigenin (5µM, 10µM, 15µM, 20µM, 25µM, 30µM, 35µM). After 24 hours, the spent medium was removed and 0.5 mg/ml MTT was added and incubated in dark at 37°C. After 3 hours, MTT was removed and purple formazan crystals were solubilized using dimethyl sulfoxide (DMSO). The optical density was measured at 570nm using Bio-Rad Elisa plate reader, California, USA.

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\text{Cell viability(\%)} = \frac{\text{Absorbance of samples} \times 100}{\text{Absorbance of control}}
\]

**Live/Dead Assay**

Approximately 30 000 cells/well were seeded on 24 well plates and allowed to attach. Cells (3T3-L1 and ECs) were treated with apigenin (20µM and 30µM) in DMEM with 10% FBS and antibiotics. After 24 hours of treatment, the spent medium was removed and 2µM Acridine Orange(AO) in PBS was added to the cells and incubated for 30 minutes at 37°C. Later the cells were washed with PBS to remove the unbounded AO and the cells were treated with 4µM Propidium Iodide(PI) in PBS for 15 minutes at 37°C [16]. The fluorescent images were taken using Leica DMi8 microscope, Leica microsystems, Wetzlar, Germany.

**Tube formation assay**

In order to study the angiogenic efficacy of apigenin, endothelial tube formation assay was carried out using collagen matrix [17]. Collagen matrix was prepared by reconstituting 0.3mg/ml of acid-soluble collagen pH 7.4 with(20µM and 30µM) and without apigenin. Collagen solutions were coated in 24 well cell culture plate and allowed to form gel by incubating at 37°C. ECs were trypsinized and approximately 30 000 cells/well were seeded on collagen gel coated plates. Morphology of the cells was periodically observed through microscope for tube formation. Tubular network formation was confirmed by staining the cells with 2µg/ml of calcein in PBS. The stained cells were examined through Leica fluorescence microscope.

**Aortic sprout formation assay**

Sprout formation efficacy of apigenin was studied using aortic sprout formation assay [18]. Ten days old chicken embryo was crack opened and the embryo was transferred to a sterile Petri plate for the isolation
of aortic arch tissues. The aortic arch was separated by dissecting the embryo ventrally using a sterile surgical blade. The separated aortic arch was transferred to sterile PBS and washed several times to remove the fat and other tissues. The aortic arch was rinsed with DMEM and cut into 1mm sized pieces. These pieces were placed into 24 well culture plates coated with collagen Matrigel premixed with apigenin (20µM and 30µM), VEGF (10ng/ml) and Thalidomide (200µg/ml) and DMEM medium was added. The plate was incubated in a humidified CO₂ incubator with 5% CO₂ and 37°C. Culture medium was replaced every alternative day and images were taken. Sprouting endothelial cells were stained with fluorescent dye Calcein(3µM) and photographed. The total tubule length was quantified using Angiosys 2.0 software, Cellworks, UK [19].

**Chorioallantoic membrane (CAM) assay**

CAM assay was performed to check the pro-angiogenic efficacy of apigenin [20]. Fertilized Giriraj chicken eggs (day 3) were procured from Krishi Vigyan Kendra, Potheri, Tamil Nadu, India and acclimatized by incubating the eggs at 37°C in a humidified atmosphere for 24 hours. The eggs were disinfected with ethanol and a small window was made on the blunt end of eggs without disturbing the CAM. Apigenin (20µM and 30µM) was added carefully to the blood vessels. The embryonic models treated with VEGF (10ng/ml) were used as the positive control whereas; the embryo treated with thalidomide (200µg/ml) was used as the negative control. Images were taken (0th hour) and eggs were further sealed with sterile parafilm and incubated at 37°C. After 24 hours development of blood vessels was photographed and capillary plexus were quantified.

**Co-culture**

Interaction between preadipocytes and ECs upon apigenin treatment was were analyzed using co-culture studies [21]. Separately maintained 3T3-L1 and ECs were mixed and seeded into 24 well plates. The next day cells were treated with 20µM and 30µM of apigenin. After 24 hours, the morphology of cells and interaction between cells were observed. To visualize the cytoskeletal interaction between both cells, cells were fixed, permeabilized and stained with phalloidin. Images were captured using Leica DMI8 microscope.

**De novo differentiation of preadipocytes**

White preadipocyte 3T3-L1 was used as a cell differentiation model for beige adipocyte differentiation. 3T3-L1 cells were harvested and approximately 1 lakh cell/well were seeded on 6 well plates and incubated in a humidified CO₂ incubator with 5% CO₂ and 37°C for 48 hours. Once confluent, differentiation was induced by treatment with induction medium containing Insulin(100nM), Dexamethasone(100nM), Isobutyl methyl xanthine(0.5mM), Rosiglitazone(1µM), triiodothyronine(2nM) and Indomethacin(125µM) [22, 23, 24, 25]. After 48 hours, differentiation was initiated by treating it with differentiation medium supplemented with 0.1µM Insulin and 2nM triiodothyronine with and without apigenin for succeeding days [24]. In order to evaluate the effect of apigenin on de novo differentiation of
preadipocytes, 20µM and 30µM apigenin were used. Both induction and differentiation medium was supplemented with 20µM and 30µM apigenin.

**Trans-differentiation of white adipocyte into beige by apigenin**

To examine whether apigenin can induce trans-differentiation in white adipocytes, 3T3-L1 preadipocytes were allowed to differentiate into white adipocytes by treating it with induction medium containing Insulin(1µM), Dexamethasone(20nM), Isobutyl methyl xanthine(0.5mM) and Rosiglitazone(1µM) and maintenance medium containing1µM insulin. Differentiated cells were treated with 20µM and 30µM of apigenin.

**Oil Red O(ORO) Staining**

Assessment of degree of adipocyte differentiation and browning was performed using Oil Red O staining [25]. ORO stain is a lysochrome diazo dye which predominantly binds to lipid droplets. After differentiation, the cells were fixed and stained with ORO stain for 10 minutes at room temperature and images were acquired in Leica phase contrast microscope (Leica microsystems, Wetzlar, Germany) to visualize the lipid accumulation. Later the stain was eluted and optical density was measured at 500nm to quantify the lipid accumulation in differentiated cells.

**JC1 staining**

To discriminate the energized and de-energized mitochondria, JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) staining had been carried out [26]. Cells were treated with different concentrations of apigenin (20µM and 30µM) for 24 hours and treated cells were stained with 1µg/ml of JC1 stain at 37°C for 20 minutes. Cells were washed and observed for green(481nm) and red fluorescence(550nm) under fluorescence microscope (λ_{ex}:450-490nm; λ_{em}:535-550nm).

**Mito-Red staining**

To investigate the effect of apigenin on mitochondrial mass and mito-biogenesis, Mito-red staining was performed [27]. 3T3-L1 was treated with different concentrations (20µM and 30µM) of apigenin for 24 hours. Treated cells were washed with PBS and incubated with 100nM of Mito Red stain in culture medium (Mito Red buffer) at 37°C for 30 minutes. Later the Mito Red buffer was removed and cells were washed with culture medium and images were acquired through Leica DMi8 microscope (λ_{ex}:569nm; λ_{em}:594nm). The mitochondrial mass of the control and treated cells was calculated by relative fluorescence intensity using Image J software, NIH, USA [28].

**Assessment of gene expression:**

The expression of genes specific for angiogenesis and browning was analyzed to understand the role of Apg induced angiogenesis in browning. The total RNA was extracted with TRIzol reagent (Invitrogen, California, United States) following the manufacture's instructions. Apigenin treated ECs and 3T3 were
harvested by homogenizing it with TRIzol reagent. Followed by phase separation and RNA precipitation. The RNA pellet was washed and dissolved in diethylpyrocarbonate (DEPC) water. The RNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific, USA) and normalized. Complementary DNA (cDNA) was synthesized from normalized RNA using iScript cDNA Synthesis kit (Bio-Rad). The primer sequence and optimum annealing temperature are given in Online Resource 1 (Table S1). The iProof high-fidelity PCR master mix (Bio-Rad) was used for PCR. The conditions were initial denaturation at 98°C for 2 min, denaturation at 98°C for 10s, annealing temperature was set as per specific primer for the 30s, extension at 72°C for 30s and final elongation at 72°C for 7 min. The obtained amplicons were separated by 1.8% agarose gel electrophoresis using a direct load PCR 100 base pairs ladder. Band intensity was calculated using Image lab software, Bio-Rad Laboratories, California, USA.

The gene expression was further assessed by quantitative real time PCR (Step One Plus 7500 instruments, Applied Biosystems) using SYBR green chemistry (SsoAdvanced Universal SYBR Green Supermix, Bio-rad) with the same set of primers. ROX dye was used as passive dye to normalize non-specific fluorescence. Each sample was analysed in triplicates for all the genes. The cycling conditions were: 95°C for 30s, 40 cycles of (a) 95°C for 15s, (b) 60°C for 1 min and followed by melt curve analysis. The fold change in gene expression was evaluated by \( \Delta \Delta C_T \) method by normalizing \( C_T \) of target genes with the \( C_T \) of RPL32 (housekeeping gene). The significance of differential expression between groups was tested using student’s t-test and the p-value indicated.

**Analysis of protein expression**

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA was performed to study the expression of UCP1, PLIN, PRDM16, VEGF, VEGFR2 [29]. After differentiation, cell lysates were collected and protein concentration was normalized using the bicinchoninic acid method. Normalized protein was coated in ELISA plate and probed with specific primary antibody and HRP conjugated secondary antibody. After incubation with the substrate, the concentration of proteins was calculated by the absorbance of the colored product.

**Western Blotting**

Equivalent protein concentration of all samples was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% separating gel. The separated protein samples were transferred to polyvinylidene fluoride membranes (immunoblot PVDF membrane, BioRad, USA). The membrane was blocked with 3% Bovine serum albumin (BSA) in TBST (TBS containing 0.05% tween) for 1 hour, followed by overnight incubation with primary antibodies (1:1,000): anti-gapdh, anti-vegf, anti-flk-1, anti-ucp1, anti-perilipin, anti-prdm16. (Santa Cruz Biotechnology Inc.) at 4°C. After wash with TBST, the membranes were incubated with Alkaline phosphatase conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology Inc.) for 1 hour, followed by TBST wash. Membrane was treated with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium, Sigma Aldrich) substrate to detect the alkaline phosphatase activity. The stable purple-blue colored bands obtained were visualized in ChemiDoc MP.
Imaging system (Bio-Rad, California, USA) and the band intensity was calculated using Image lab software.

**Immunocytochemistry**

To investigate the gene expression of UCP1 and PLN, Immunocytochemistry was performed [27]. Cells were fixed with formaldehyde for 15 minutes at room temperature. The fixed cells were blocked with blocking buffer for 60 minutes and subsequently washed with PBST 3 times. The cells were permeabilized with permeabilization buffer for 10 minutes and rinsed with PBST thrice. Then cells were incubated with primary antibody overnight at 4°C. After washing with PBST, corresponding secondary antibodies were added and incubated for 2 hours at room temperature. The unbounded antibody was washed off by rinsing with PBST thrice and subsequently counterstained with DAPI. The images were captured using Leica DMi8 microscope.

**Statistical analysis**

Data were analyzed using Graph pad prism software (www.graphpad.com) [27]. For cellular studies, data represented are values from at least three independent biological samples. Multiple comparisons with control were analyzed using one-way analysis of variance (ANOVA). Comparison between two groups was done using unpaired t-test. Statistical significance was accepted at p <0.05. The error bar on the graphs represent mean±SE.

**Results**

**Cytocompatibility of apigenin on pre-adipocytes and EC**

The cytotoxicity profile for apigenin on 3T3-L1 and ECs were studied by analyzing the mitochondrial dehydrogenase activity using MTT assay. The results indicated 88% cell viability for both cells up to the concentration of 35µM (Fig. 1A). Cell morphology was not compromised till 30µM in both cell types(Fig. 1B). Hence the optimum concentration 20µM (Apg20) and 30µM (Apg30) was chosen for further experiments. Cyto compatibility of apigenin in both cells was further confirmed by Acridine Orange(AO) / Propidium Iodide(PI) assay. The results indicated that the membrane integrity and cell viability has not been compromised after apigenin treatment (Fig. 1C).

**Pro-angiogenic ability of Apg in vitro model, organotypic model and in vivo chick chorioallantoic membrane.**

Angiogenic efficacy of apigenin was investigated by *in vitro* endothelial tube formation assay. The results showed concentration-dependent tube formation(Fig. 2A). At 20µM of apigenin, cell elongation and cell patterning were observed. When the concentration of apigenin was increased to 30µM, elongated cells formed cell to the cell network and capillary tube-like structures. The results indicated a dose-dependent tube formation of endothelial cells after treatment with apigenin.
The pro-angiogenic role of apigenin was further confirmed using aortic sprout formation assay. Superior sprouting was elicited on the aorta placed on matrigel premixed with apigenin (Fig. 2B). Aorta placed on matrigel with positive control also shown remarkable sprouting. No sprouting was observed in the negative control. The quantification of data confirms a concentration-dependent increase in sprouting on apigenin treatment (Fig. 2C). Compared to the positive control, Apg20 and Apg30 show a 3-fold and 6-fold increase respectively.

To examine the pro-angiogenic efficacy of apigenin on \textit{in vivo} model, chorioallantoic membrane (CAM) assay on the chick embryonic system was performed. The chick CAM was treated with 20µM and 30µM of apigenin, capillary plexus was analyzed after different time intervals. The growth of capillaries was significant in CAMs treated with apigenin compared to control (Fig. 2D). The results were consistent with the \textit{in vitro} tube formation assay. Embryos treated with the positive control also showed an increase in capillary plexus, whereas in embryos treated with negative control the blood vessel growth was negligible. The quantification of the capillary plexus implies that the development of blood vessels was dose-dependent (Fig. 2E).

**Apg induces angiogenic markers and upregulates VEGF and VEGFR2 in EC.**

The previous assays on angiogenic potential on \textit{in vitro}, \textit{in vivo} and organotypic systems suggested remarkable pro-angiogenic efficacy of the apigenin. To analyze the gene expression of \textit{vegf a}, \textit{vegfr2} on EC, total RNA was extracted after apigenin treatment and PCR was performed. The results are shown in Fig. 2F. Apg treated cells showed increase in expression of \textit{vegf a} and \textit{vegfr2}. The expression of internal controls was comparable in all the cases indicating uniform loading of samples. Real time PCR was performed to quantify specific gene expression relative to housekeeping gene (RPL32). There was increase in \textit{vegf a} and \textit{vegfr2} expression by 3.2 and 1.5fold respectively. The results indicated that Apg upregulates \textit{vegf a} and \textit{vegfr2} expression and induce angiogenesis in EC (Fig. 2G).

Protein expression of angiogenic markers on treated cells was analyzed by ELISA. There was a remarkable increase in VEGF and VEGFR2 protein in differentiated cells treated with apigenin (results are provided in Online Resource fig. S1). Quantification of protein expression of VEGF and VEGFR2 was done by western blotting (Fig. 2H). Band intensity was assessed and fold change to control was calculated. There was 1.5 and 1.6fold increase respectively for VEGF and VEGFR2 relative to control (Fig. 2I). The results substantiated previous results and confirms the pro-angiogenic efficacy of Apg.

**Apigenin augments ECs & pre-adipocytes connection**

The previous results indicated that apigenin significantly promoted angiogenesis hence to understand whether the same efficacy is maintained by apigenin in adipocyte microenvironment a co-culture study was performed. Expanded co-cultures of ECs in presence of adipocyte treated with apigenin developed cell to cell connections and capillary tube-like structures (Fig. 3A). The results confirmed the pro-angiogenic efficacy of apigenin under adipose microenvironment.
Apigenin induces de novo differentiation of pre-adipocytes and as well as trans-differentiation of white adipocytes

Angiogenic assays revealed the excellent pro-angiogenic efficiency of Apg in in vitro, organotypic and in vivo chick chorioallantoic membrane model. Co-culture studies also revealed augmentation of cross-talk between endothelial and pre-adipocyte cells. To understand the effect of Apg on white adipocytes, studies were performed in white pre-adipocytes and analyzed for morphological changes in these cells on treatment with apigenin.

White pre-adipocytes were treated with and without Apg. The preadipocytes cells treated with apigenin showed morphological changes similar to that of brown-adipocytes with smaller lipid droplets and low lipid accumulation compared to the control cells (Fig. 3B). This result indicated the ability of Apg to enhance de novo differentiation of white pre-adipocytes to a beige adipocyte. In order to study the effect of Apg on mature adipocytes, white pre-adipocytes were differentiated into mature white adipocyte and then treated with Apg. The result indicated that the mature white adipocytes (WAC) treated with apigenin resembled morphological characteristics typical of brown-like (beige) adipocytes with numerous small lipid droplets compared to their respective control cells which retained white adipocytic feature [30] with large lipid droplet (Fig. 3C). This reveals the potential of Apg to induce trans-differentiation browning in mature white adipocytes. The differentiation experiments were carried out with Apg20 and Apg30. The results indicated a concentration-dependent effect of apigenin on the differentiation. Hence, the concentration of Apg30 was used for all further studies. The results suggested that apigenin has the potential to induce browning in both white pre-adipocytes (de novo differentiation) and in mature white adipocyte (trans-differentiation).

Apigenin induces browning and decreases lipid accumulation

To investigate the degree of browning induction and to quantify the lipid content in differentiated cells, Oil Red O staining was performed. White cells showed significant staining due to high lipid content compared to brown-like cells. The results indicated that the de novo differentiated preadipocyte cells showed numerous small fat droplets compared to control brown adipocyte (Fig. 3D). During trans-differentiation, in control white adipocyte a single large fat droplet was observed with small rim of cytoplasm whereas in apigenin treated white adipocytes small droplets of lipids were observed. The results confirm apigenin induced browning in white pre-adipocytes and mature adipocytes(Fig. 3E). To quantify the lipid accumulation after differentiation, stain retained in the cells was eluted and absorbance at 500nm was measured. Quantification of lipid accumulation in both de novo (Fig. 3F) and trans-differentiation (Fig. 3G) indicated a remarkable decrease in lipid content in cells treated with apigenin compared to the control cells. The results are consistent with the preliminary differentiation studies and suggested the ability of apigenin to modify the lipid accumulation and trigger both de novo and trans-differentiation browning of white adipocytes.
Active Mitochondria and upsurges in mitochondrial mass upon treatment with apigenin

The mitochondrial membrane potential is a key indicator of mitochondrial activity [31]. To study the outcome of apigenin treatment on mitochondrial activity, mitochondrial membrane potential was measured using JC1 staining. Apigenin treated cells and control cells exhibited bright red fluorescence indicating higher membrane potential of cells (Fig. 4A). The results indicate mitochondria are active and cells are healthy even after treatment with apigenin. To investigate the effect of apigenin on mitochondria mass and biogenesis, apigenin treated cells were stained with membrane potential independent dye, Mito-red. Mito-red is the rhodamine based dye which directly accumulates into the cells and emits red fluorescence. The fluorescence intensity is directly proportional to the mitochondrial mass which indirectly indicates the mitochondrial biogenesis. The results showed that the fluorescence intensity of apigenin treated cells was significantly higher than the control cells and the increase in intensity was directly proportional to the concentration of apigenin. Results show that on treatment with apigenin, the mitochondrial mass was significantly increased. Fig. 4B represents the quantification of relative fluorescence intensity. Cells treated with apigenin exhibited 1.8 and 2.5 times more fluorescence intensity than that of the control (C) cells. The results suggested a significant increase in mitochondrial mass upon apigenin treatment.

Apigenin modulates angiogenesis and induces browning

The effect of apigenin on browning was further investigated by analyzing the gene level expression of *vegf-a*, *vegfr2*, *ucp1* and *prdm16* after differentiation. Total RNA was extracted after differentiation and PCR was performed. The results are shown in Fig. 5A. In both de novo and trans-differentiated cells treated with Apg showed significant increase in angiogenic markers (*vegf-a* and *vegfr2*) and browning markers (*ucp1* and *prdm16*). Real time PCR was preformed to quantify specific gene expression relative to housekeeping gene (RPL32). There was increase in *vegf a* and *vegfr2* expression by 3.1 and 1.4fold respectively in de novo differentiated cells with Apg (BACA). Expression of browning markers were also analyzed, 3.1 and 1.6fold increase was observed in *ucp1* and *prdm16* respectively in BACA. (Fig. 5B). In trans differentiated cell with Apg (WACA), expression of angiogenic and browning markers were similar to that of brown control (BAC). The results are shown in Fig. 5A. The results indicated that Apg modulates angiogenesis by upregulating *vegf-a* and *vegfr2* and induce browning in both white pre-adipocytes and mature white adipocyte by enhancing browning markers *ucp1* and *prdm16*.

To confirm the significant role of angiogenic modulator (Apg) in white adipocyte browning, protein level analysis was carried out. ELISA results reveal significant upregulation of UCP1, PRDM16 and downregulation of PLIN expression in BACA and WACA (results were provided in Online Resource fig. S2). In order to quantify and confirm the expression western blotting was done (Fig. 5C). Relative fold change was calculated from the band intensity (Fig. 5D). The expression of angiogenic (VEGF-A and VEGFR2) and browning (UCP1 and PRDM16) marker was increased by 1.5fold in BACA. In WACA, the expression of
both angiogenic and browning marker was similar to that of BAC. In typical white adipocytes (WAC) the levels of PLIN were high, whereas in BACA and WACA expression was decreased by 0.4fold. Beiging of mature white adipocyte by apigenin (WAC A) showed protein expression comparable to control brown adipocyte. The protein expression results are corroborated with gene expression studies. Immunostaining at the cellular level was carried out, it confirmed the upregulation of UCP1 (Fig. 5D) and downregulation of PLIN (Fig. 5E) on BACA and WACA. Upregulation of PRDM16 and UCP1 will initiate thermogenesis and downregulation of PLIN will activate the lipolysis and prevent further lipid storage. Immunostaining results are in concordance with ELISA and western blot results. The results comprehended the pro-angiogenic efficacy of Apg. This results further substantiated the prominent role of angiogenic modulator in browning of white adipocytes.

**Discussion**

The present study was performed to elucidate the role of angiogenesis in white adipocytes metabolism. It has been reported that bioactives in particular nutraceuticals exhibit differential activity and cytotoxicity depending on various cell types [32]. ECs are the principal cells involved in angiogenesis and 3T3-L1 cells represent preadipocyte, the principal cell type involved in adipogenesis. The cytotoxicity profile for apigenin in both the cell type revealed the compatible nature of apigenin and the effective safe concentration (20 µM and 30µM) was confirmed by AO/PI assay. Inorder to use as angiogenic modulator, the pro-angiogenic efficacy of apigenin was studied. Apigenin exhibited a concentration-dependent proangiogenic effect in *in vitro* model, organotypic model and *in vivo* chick chorioallantoic membrane assay. The gene expression studies on endothelial cells revealed induction of *vegf a* and *vegfr2* expression upon Apg treatment. Apg exhibited excellent pro-angiogenic features in all studied models by activating VEGF-A and VEGFR2. The association between ECs and white pre-adipocytes in presence of Apg was analyzed by co-culture studies. A significant increase in capillary network on ECs was observed in co-culture treated with Apg. Thus, the pro-angiogenic efficacy of apigenin was confirmed in co-culture and Apg was used as an angiogenic modulator to study the its role white adipocytes cell physiology.

In order to study role of angiogenic modulator in white adipocytes, pre-adipocytes and mature adipocytes were allowed to differentiate with and without Apg. Pre-adipocytes are the key cell type that differentiates into mature adipocyte either as brown adipocyte or white adipocyte depending upon the cellular signaling and cues from the microenvironment [33]. Mostly, pre-adipocytes are triggered to differentiate into white adipocytes except in myogenic factor 5 expressing cell lineage [34]. White adipocyte deposition is a major factor in obesity. Modulating angiogenesis in these hypoxic cells can be a strategy to activate metabolism and lipolysis. Morphological analysis and ORO staining of cells differentiated in the presence of Apg exhibited multilocular brown-like cells. Quantification of ORO staining confirms the decrease in lipid accumulation in both *de novo* differentiation and trans-differentiation induced by apigenin. Apigenin effectively induced browning in both white pre-adipocytes and mature white adipocytes by activating lipid metabolism. Our data showed that modulating angiogenesis enhances lipolysis and induce browning in these quiescent cells. The strategy to control obesity is converting white adipocytes to beige/ brown-like
adipocytes by utilizing the lipid [35]. This can be achieved by giving proper trigger to convert pre-adipocytes to brown adipocyte (de novo differentiation) [36] or mature white adipocyte to brown adipocyte (trans-differentiation) [37]. The differentiation of WAC to BAC requires a significant change in the metabolic state of the cells that require a high nutrient and O₂ supply chain to the BAC microenvironment. Hence a direct relationship between browning of adipocytes and blood vessel formation is required for the proper differentiation of WAC. Moreover being thermogenically active with elevated mitochondrial activity, brown adipocytes needs to be well vascularized [38]. Reports suggested that pro-angiogenic factors can induce browning of white depots [39].

Besides lipid droplet size, another major difference between white and brown adipocytes is the presence of active mitochondria in the brown adipocyte. Mitochondria are dynamic organelle that regulates adipocyte differentiation [40]. Dysfunctional mitochondria have unfavorable effects on lipid metabolism and thermogenesis [40]. JC-1 staining confirms that the mitochondrial activity is not compromised upon treatment with apigenin. Mitochondrial membrane potential was not altered after treatment with apigenin, mitochondria were active with higher mitochondrial membrane potential. Studies revealed that treatment with natural dietary compounds and mitochondria targetting antioxidants induce browning by improving mitochondrial function [41]. Mito-red staining results revealed a concentration-dependent increase in mitochondrial mass upon apigenin treatment. Recent studies proposed that activation of mitochondria and high mitochondrial biogenesis in brown adipocytes is the key reason for thermogenesis and high metabolism. An increase in mitochondrial activity on treatment with apigenin might be the reason behind the browning of white adipocyte cells. Apg promoted VEGF expression in white adipose cells that can act in paracrine way to activate the nearby ECs to angiogenic phenotype. Further Apg was also shown to promote angiogenesis independently on ECs alone in absence of white adipocytes. The study indicated the cross talk between ECs and white adipocytes mediated via VEGF-VEGFR2-PRDM16 signaling resulting in concomitant angio-induction in ECs and browning both (trans and de novo) in white adipocytes.

Browning of white adipocytes by apigenin was indicated by the characteristic multilocular cells and confirmed by ORO staining. Apigenin treated cells also exhibited higher mitochondrial mass. Protein level expression studies illustrated upregulation of UCP1, PRDM16 and downregulation of perilipin in apigenin triggered browning. Thermogenin/UCP1 is a brown specific marker responsible for non-shivering thermogenesis. This protein is responsible for energy expenditure and lipid metabolism in brown adipocytes [42, 43]. Apigenin induces browning by upregulating UCP1 protein and thereby activating thermogenesis both in white preadipocytes and white adipocytes via de novo and trans-differentiation respectively. Previous reports suggested that the expression of UCP1 in white depots indicates browning. Perilipin (PLIN) is a prominent lipid-associated protein present in adipocytes [44]. These proteins confiscate the lipid droplets from lipase action. It has been reported that the defective PLIN in mice caused low adiposity and showed small multilocular lipid droplets than unilocular [45]. Our results point towards the activation of lipid metabolism by downregulating PLIN on apigenin treatment. Brown adipocytes selectively express the PRDM16 transcription factor that regulates the thermogenic genes
Preceding reports proposed expression of PRDM16 in white preadipocytes induces brown phenotypic characters, upregulates UCP1 and activates mitochondrial biogenesis [46, 47]. UCP1, PRDM16 and PLIN expression illustrate that apigenin is inducing browning in both white preadipocytes and mature adipocytes by activating thermogenesis marker and mitochondrial biogenesis.

From differentiation studies using apigenin has proved its excellence in browning of white adipocytes by its pro-angiogenic efficacy. Gene expression studies showed apigenin increases vegf-a and vegfr2. Protein expression studies in de novo and trans-differentiated beige adipocytes treated with apigenin showed upregulated expression of VEGF-A and VEGFR2. Previous studies proposed that proangiogenic moieties like VEGF-A can induce browning in white adipocytes. In our study, the enhanced expression of VEGF-A by ECs on treatment with apigenin showed that VEGF-A may aid in paracrine signaling and further activate the adipocyte browning. Literature evidence suggests overexpression of VEGF-A prevents diet-induced obesity by activating thermogenesis in adipocytes [39]. Our study indicated significant expression of VEGF by pre-adipocytes as well as mature adipocytes during de novo and trans differentiation browning. The VEGF was observed to mediate these differentiation via PRDM16 cascade. Schematic representation of apigenin induced browning by modulating angiogenesis is illustrated in Fig. 6.

Conclusion

Abnormal deposition of metabolically inactive white adipocytes will halter normal homeostasis of body. To study the role of angiogenesis in white adipocyte and effect of modulation of angiogenesis in these cells, Apg was used as an angiogenic modulator. Apg modulated angiogenesis and induced browning in white pre-adipocytes and mature white adipocytes by activating VEGF-A/VEGFR2 which in turn activates PRDM16. PRDM16 activates UCP1 regulated thermogenesis, mitochondrial biogenesis and lipid metabolism by downregulating PLIN. Upregulation of VEGF-A by ECs upon treatment with Apg may activate paracrine signaling and further enhance angiogenesis in adipose microenvironment. The present study illustrates the strategy of angiogenic modulation of white adipocytes to activate energy metabolism and lipolysis.

Statements and Declarations

Declarations

Ethics approval and consent to participate: “This study does not involve any human or animal subjects, hence did not require ethical approval and consent”.

Consent for publication: “Manuscript does not contain any personal data in any form, hence consent for publication is not applicable in this study”.

Data Availability: “The datasets generated during current study are available from corresponding author on reasonable request. The datasets of gel and blots analyzed during current study are available in
supplementary information”.

**Competing Interest:** “The authors have no relevant financial and non-financial interests to disclose”.

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Figures
Figure 1

Biocompatibility of apigenin on pre-adipocytes and EC

(A) Histogram depicting in vitro biocompatibility.

(B) Phase-contrast images.

(C) Fluorescence images of AO/PI assay.
Figure 2

Pro-angiogenic efficacy of apigenin

(A) Angiogenic property of Apg on endothelial tube formation on collagen matrix.

(B) Sprouting of cells in chick aorta; PC-positive control and NC-Negative control.
Quantiﬁcation of chick aortic sprouting assay.
* Represents statistical signiﬁcance compared to control with p <0.05.

Chorio-allantoic membrane assay on chick embryo; PC-positive control and NC-Negative control.

Quantification of capillary plexus on chick chorioallantoic membrane.

Gene expression of angiogenic markers in EC by reverse transcription PCR studies: C- control, T-EC treated with ApG30, M- 100bp ladder.

Graphical representation of gene expression of angiogenic markers quantiﬁed by real-time PCR.
* Represents statistical signiﬁcance compared to control with p <0.05.

Western blot analysis of protein expression of VEGF-A and VEGFR2.

Graphical representation of protein expression of VEGF-A and VEGFR2 quantiﬁed by western blotting.
* Represents statistical signiﬁcance compared to control with p <0.05.
Figure 3

Apigenin induced browning

(A) Cross talk between ECs and Pre-adipocytes on coculture microenvironment stained with phalloidin.

(B) *De novo* differentiation.

(C) Trans-differentiation.

(D) ORO staining of de novo differentiation.

(E) ORO staining of trans-differentiation.

(F) Quantification of lipid accumulation in *de novo* differentiation.

(G) Quantification of lipid accumulation in trans-differentiation of white adipocyte.

* Represents statistical significance with p <0.05.

Figure 4

Apigenin activates mitochondrial biogenesis

(A) Mitochondrial activity and mitochondrial mass analysis using JC-1 and Mito red staining.

(B) Relative fluorescence intensity of cells stained with Mito Red.
* Represents statistical significance compared to control with $p < 0.05$.

Figure 5

Apigenin promotes browning by inducing angiogenic markers
(A) Gene expression analysis of angiogenic markers and browning markers on brown and white adipocytes by reverse transcription PCR studies: BAs- brown adipocytes, WAs- White adipocytes, C- control, T-treated with Apg30, M- 100bp ladder.

(B) Graphical representation of gene expression of angiogenic markers and browning markers quantified by real-time PCR.

* Represents statistical significance compared to control with p <0.05.

(C) Western blot analysis of protein expression of VEGF-A, VEGFR2, UCP1, PRDM16 and PLIN.

(D) Graphical representation of protein expression of VEGF-A, VEGFR2, UCP1, PRDM16 and PLIN quantified by western blotting.

* Represents statistical significance compared to control with p <0.05.

(E) Immunostaining of UCP1

(F) Immunostaining of PLIN.

Figure 6

Schematic representation of apigenin induced browning by modulating angiogenesis
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