

Bioavailable menthol (Transient Receptor Potential Melastatin-8 agonist) induces energy expending phenotype in differentiating adipocytes

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Abstract

Recent evidences support a role of menthol, a TRPM8 agonist, in enhanced energy expenditure, thermogenesis and BAT-like activity in classical WAT depots in TRPM8 dependent and independent manner. The present study was designed to analyze whether oral and topical administration of menthol is bioavailable at subcutaneous adipose tissue and is sufficient to induce desired energy expenditure effects directly. GC-FID was performed to study menthol bioavailability in serum and subcutaneous white adipose tissue following oral and topical administration. Further, 3T3L1 adipocytes were treated with bioavailable menthol doses and different parameters (lipid accumulation, “*browning/brite*” and energy expenditure gene expression, metal analysis, mitochondrial complex’s gene expression) were studied. No difference was observed in serum levels but significant difference was seen in the menthol concentration on subcutaneous adipose tissues after oral and topical application. Menthol administration at bioavailable doses significantly increased “*browning/brite*” and energy expenditure phenotype, enhanced mitochondrial activity related gene expression, increased metal concentration but didn’t alter the lipid accumulation. Further, we used pharmacological antagonism based approach to study the TRPM8 involvement in menthol effect. In conclusion, the present study provides an evidence that bioavailable menthol after single oral and topical administration is sufficient to induce “*brite*” phenotype in subcutaneous adipose tissue.

Key words: Adipose tissue, Bioavailable, Menthol, Topical, TRPM8.

1. Introduction

The imbalance between energy intake and energy expenditure is a well-known aspect of the escalating prevalence of obesity throughout the world as a major nutritional challenge [1,2]. Multiple factors like physical activity, dietary energy intake, basal metabolic rate, variation in environmental and body temperature are reported to influence energy expenditure [3,4]. Enhancement of energy expenditure is one of the cardinal approach toward prevention of body weight gain and obesity [5]. Numerous studies have reported the direct relation of cold exposure with non-shivering thermogenesis, followed by reduction in body fat [6-8]. Interestingly, clinical studies have also reported a vital link between cold-exposure and elevated energy expenditure through adaptive thermogenesis [9]. Studies on adipocytes have also reported a relationship between cold receptor, TRPM8 and energy expenditure showing upregulated expression of UCP-1 and PGC-1 α owing to TRPM8 activation [10].

Complex physiology of the body makes it difficult to understand the exact mechanisms involved in energy expenditure. Transient receptor potential cation channel subfamily Melastatin member 8 (TRPM8) is a thermoreceptor present on the sensory nerve endings innervating the gut and skin [11-13] that senses non-noxious cold stimuli ranging from 18-25°C, hence making it attractive target for cold mimicking. Involvement of TRPM8 dependent and independent pathways in mediating cold-induced energy expenditure is well reported. Recently we have published that anti-obesity effect of menthol, a TRPM8 agonist is mediated through glucagon dependent mechanisms particularly in adipose tissues [14]. Activation of TRPM8 receptors in the gut and skin by oral and topical administration of menthol leads to increase in serum glucagon levels, thus activating several downstream catabolic processes like glycogenolysis, gluconeogenesis, browning of white adipose tissue (WAT), and activation of energy expenditure markers in WAT and brown adipose tissue (BAT) [14]. This is supposedly an indirect action of menthol on adipose tissue wherein presence of TRPM8 is not essential. On the other hand, studies from our laboratory provide a strong evidence of significant expression of TRPM8 in undifferentiated (pre-adipocytes) and differentiated (adipocytes) 3T3-L1 cells suggesting its significance in adipogenesis [15]. Also, TRPM8 is functionally expressed in rodent WAT [16] and BAT [17] as well as human WAT [18]. Alternatively, Ma et al. showed that the mouse BAT expresses TRPM8 receptor which upon activation by menthol, significantly increased the levels of p-PKA that further phosphorylates the transcription factor cyclic AMP-responsive element-binding protein (CREB), ultimately leading to enhanced UCP1 expression [17,19]. Thus, chronic dietary menthol increased thermogenesis, core body temperature, prevented diet-induced obesity and the abnormal glucose homeostasis in wild-type mice but not in UCP1^(-/-) and TRPM8^(-/-) mice [17], suggesting TRPM8 receptor presence on adipose tissue is essential. Other studies also demonstrated the induction of white-

to-brown-like phenotype through TRPM8 activation on human WAT [18, 20], summarizing the importance of the presence of TRPM8 on adipose tissue.

We hypothesize that different TRPM8 dependent and independent actions are contributing to the anti-obesity effect of menthol. The present study is an attempt to reveal whether topical (10 % w/v) or oral (200 mg/kg, p.o.) administration of menthol used during acute studies in our previous work is bioavailable at subcutaneous adipose tissue (direct or through serum). Further, using in-vitro 3T3L1 cell line model, we attempted to study that whether bioavailable menthol at subcutaneous adipose tissue is sufficient to induce direct effect on *browning* and mitochondrial energy metabolism followed by enhanced energy expenditure effects. Furthermore, this manuscript endeavours to elucidate the presence of metal ions and their role in mitochondrial electron transport chain for significant elevation in energy expenditure owing to the menthol treatment.

2. Materials and Methods

2.1 Chemicals

Mouse 3T3-L1 preadipocytes were obtained from National Centre for Cell Science (NCCS), Pune, India). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin and Phosphate-Buffered Saline (PBS) was procured from Lonza Inc. (Walkersville MD, USA). Menthol, AMTB hydrate, Isopropyl alcohol (IPA), 3-isobutyl-1-methylxanthine (IBMX), Dexamethasone (DMS), Insulin, Oil-Red-O (ORO) powder, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Dimethyl Sulfoxide (DMSO) were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). GeneZol RNA Extraction Reagent was obtained from Genetix Biotech Asia Pvt. Ltd. (New Delhi, India). Absolute Ethanol (Biotechnology grade) was procured from Amresco Inc. (Cochran Road, Solon, Ohio). Nitric acid (Trace Metal Grade) was obtained from Fisher Scientific UK Limited (Bishop Meadow Road, Loughborough, Leicestershire, UK) and Dichloromethane (DCM) was procured from Central Drug House (New Delhi, India). All other reagents used were of analytical grade and were procured from local supplier.

2.2 Animal Treatment

21 Swiss Male Albino mice (25-30g) bred in Central Animal Facility of National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India were used for the experiment. 3 mice per cage were housed on a 12 h light/dark cycle with ad libitum food and water supply. The experiment was approved by the Institutional Animal Ethical Committee, NIPER, Mohali, Punjab, India (Study approval no: IAEC/15/13). All the experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision in Experiments on Animals (CPCSEA)

guidelines on the use and care of experimental animals. Mice were randomly divided into 3 different groups consisting 3 animals for control group, 9 animals for oral administration of menthol and 9 animals for topical administration of menthol. Oral administration of menthol was given at the dose of 200 mg/Kg in 0.1% tween-80, while topical administration of menthol was given by 1mL topical application of 10% menthol in 0.1% tween 80. Animals were euthanized after 30, 60 and 120 minutes of menthol administration by cervical dislocation, blood was collected by cardiac puncture for serum isolation and subcutaneous white adipose tissues were collected for pharmacokinetic profiling of menthol ¹⁴.

2.3 Gas Chromatography-Flame Ionization Detector (GC-FID) for menthol bioavailability in serum and subcutaneous WAT

2.3.1 Extraction Procedure

Both serum and adipose tissue samples were extracted in DCM containing 200 ng/mL of thymol as internal standard. The samples were continuously vortexed for 3 minutes and centrifuged at 6000 rpm for next 3 minutes. After centrifugation, the supernatant was isolated and again extracted in DCM as discussed above. The supernatant finally obtained was used for pharmacokinetic evaluation of menthol both in serum and adipose tissue.

2.3.2 GC-FID Protocol

Separation and detection was done by injecting 1 μ L of target analyte in gas chromatograph (Agilent 7890B) equipped with split less injector system and flame ionization detector. Ultra-pure nitrogen gas was passed through molecular sieve and oxygen trap and was used as carrier gas with flow rate of 1 mL/min. The injection port was held at 240°C operated at split less mode. Separation was done using DB5 capillary column (Agilent Technologies) with dimension 30m \times 320 μ m \times 1 μ m with oven temperature kept at 40°C and then increased to 250°C at the rate of 10°C/min, hold time for 2 min. and sample run time for 23 min. the FID temperature was maintained at 280° C for detection. Flow of hydrogen gas used for FID was at the rate of 30 mL/min. The flow of air for FID was 300 mL/min along with makeup flow at the rate of 25 mL/min. Calculations were done using EZ Chrome Software.

2.4 3T3-L1 cell culture

Mouse 3T3-L1 pre-adipocytes were allowed to culture in basal medium comprising DMEM supplemented with 10%v/v FBS and 1% penicillin-streptomycin. Whole cell culture was maintained at 37° C in a humidified incubator along with continuous supply of 95% O₂ and 5% CO₂. At 60% confluency, cellular differentiation from 3T3-L1 pre-adipocytes to adipocytes was induced using

differentiation medium containing basal medium supplemented with 0.1mM IBMX, 0.1 μ M DMS and 1 μ g/ml insulin for 48 hours. Stock solutions of 0.1 μ M DMS and 0.1 mM IBMX were prepared in absolute ethanol and DMSO respectively and were directly supplemented to DMEM culture medium. Further, the cells were shifted to maintenance medium (basal medium with 1 μ g/ml insulin) for 10 days with medium replacement on every alternate day. Working solutions of 1, 50, 100, 200 and 300 μ M menthol and 10 μ M AMTB hydrate for the treatment were also prepared in absolute ethanol and DMSO respectively and directly supplemented to culture (differentiation/maintenance) medium during adipogenesis. Dose selection of menthol for *in vitro* study was done on the basis of *in vivo* pharmacokinetic profile of menthol in serum and adipose tissues. The entire cell culturing experiments were carried out as per American Type Culture Collection (ATCC) guidelines.

2.5 Cell viability assay

Cell cytotoxicity was assessed using MTT assay. Undifferentiated 3T3-L1 preadipocyte cells were seeded in a 96 well plate at density of 1×10^4 cells/well and incubated with varying concentrations (50, 100 and 300 μ M) of menthol dissolved in <0.001% ethanol at 37° C for 72 h. For control, undifferentiated pre-adipocytes treated with ethanol (<0.001%) were taken. After 72 h, media was replaced with MTT as per the instructions by manufacturer (Sigma Aldrich). Formazan crystals formed by live cells are solubilized in isopropanol and absorbance was measured at 570 nm using microplate reader (Spectra max M5e, Molecular Devices) [21] .

2.6 Oil-Red-O staining

0.5%w/v of stock solution of ORO was prepared IPA and was stored at room temperature. Working solution of ORO stain was prepared by diluting the stock solution with distilled water in the ratio 3:2. For staining, the supernatant in the culture plates after treatment with varying concentrations of menthol (1, 50 and 200 μ M) was removed and cells were washed using PBS. After washing, mature adipocytes were fixed with 10% v/v formaldehyde in PBS and kept for 30 min at room temperature followed by three subsequent washing with PBS. Then, the PBS was aspirated and 60% of IPA was added followed by incubation for 5 minutes. Further, the cells were stained in ORO working solution filled in culture plates to completely cover the plate bottom (1.25 mL per well for 6-well culture plate) and kept for incubation for 10 min. The cells were then washed with 60% IPA to rinse away excess ORO dye followed by final elution of dye in 100% IPA (2.5 mL per well for 6-well culture plate). The plates were again kept for incubation at room temperature for 10 minutes. Now, 200 μ L of each eluate was added to a 96 well microtiter plate. Also, 200 μ L of 100 % IPA was filled on the same microtiter plate as blank. Finally, intracellular lipid content was quantified after extracting ORO bound cells with 100%

IPA and absorbance was taken at 570nm using NanoQuant – Infinite M200 Pro (Tecan, Switzerland) [22,23].

2.7 ICP-MS for metal analysis

Cells from control and test samples (1, 50 and 200 μ M administration of menthol during differentiation) were pellet down and digested with nitric acid using Microwave Reaction System (Mars 6, CEM Corporation, USA). The concentration of different elements was estimated in the digested samples using standard protocol for inductively coupled plasma mass spectrometry (ICP-MS:77006, Agilent Technologies, Santa Clara, CA) [14].

2.8 qRT-PCR for Browning/Energy Expenditure genes

Total RNA from both control and treated adipocytes was extracted after 10 days of treatment using Acid guanidinium thiocyanate-phenol-chloroform extraction method with GeneZol RNA Extraction Reagent, IPA, Chloroform and was reversly transcribed to cDNA from 1 μ g of RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., CA, US). Quantitative real time-polymerase chain reaction (qRT-PCR) was performed with the cDNA as templates in 40 cycles using Custom RT²PCR Array Kit (CLAM 30615C, Qiagen Lifesciences, Maryland, USA) and RT² SYBR Green ROX qPCR Mastermix (Qiagen Lifesciences, Maryland, USA) keeping GAPDH as internal control and 7 gene specific primers for adipogenesis including *TNFRSF9*, *PPARGC1A*, *PRDM16*, *CIDEA*, *TLE3*, *HOXC10* and *NRIP1*. The qRT-PCR process was performed by programming thermocycler [CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, US)] to apply initial cycle for denaturation at 95°C for 2 min, followed by 40 cycles of annealing and elongation at 60°C for 30 s, and denaturation at 95°C for 5 s. The complete experiment was performed using three biological replicates [14]. Analysis of relative gene expression was done using $2^{-\Delta\Delta Ct}$ method [24] and values were expressed in the terms of fold change with reference to control[14].

2.9 Gene expression analysis of mitochondrial energy metabolism

Total RNA from both control and treated adipocytes was extracted after 10 days of treatment using Acid guanidinium thiocyanate-phenol-chloroform extraction method with GeneZol RNA Extraction Reagent, IPA, Chloroform and was reversly transcribed to cDNA from 5 μ g of RNA using RT² first strand kit (Qiagen Lifesciences, Maryland, USA). qRT-PCR was performed using RT² Profiler PCR Array Kit for Mouse Mitochondrial Energy Metabolism (PAMM 008ZC-12, Qiagen Lifesciences, Maryland, USA) and RT² SYBR Green ROX qPCR Mastermix (Qiagen Lifesciences, Maryland, USA). The thermocycler [Applied Biosystem 7500F Real Time PCR System (Applied Biosystems, Fosters City, CA, USA)] was programmed to apply initial cycle for denaturation at 95°C for 10 min, followed by 40

cycles of annealing and elongation at 60°C for 1 min, and denaturation at 95°C for 15 s. Three biological replicates were used for this experiment [14]. Analysis of relative gene expression was done using $2^{-\Delta\Delta ct}$ method [24] and values were expressed in the terms of fold change with reference to control [14].

2.10 Statistical Analysis

All the values are expressed as mean \pm SEM. Group comparison in *in vivo* study was done using unpaired student t-test and two-way ANOVA followed by Tukey's post-hoc test. Group comparison *in vitro* study was done using unpaired student t-test and one-way ANOVA followed by Tukey's post-hoc test. Pearson correlation was performed and representative matrix was made using Gitoools 2.3.1. (San Fernando Rd, Burbank, CA, USA). $P \leq 0.05$ was considered statistically significant. Correlation matrix and clustering was done by K means ++ Manhattan distance clusters 6.

3. Results

3.1 Pharmacokinetic Profile of Acute Oral Administration and Topical Application of TRPM8 Agonist (Menthol) in mice.

Serum menthol concentration after oral (200 mg/kg) and topical administration (10% w/v) was determined at different time intervals of 0, 30, 60 and 120 min. The peak plasma concentration after oral and topical administration was attained at 30 and 60 min respectively. Bioavailable concentration of menthol in serum after oral administration at 30, 60 and 120 min was found to be 3.28 $\mu\text{g/mL}$ (20.4 μM), 1.51 $\mu\text{g/mL}$ (9.66 μM) and 0.76 $\mu\text{g/mL}$ (4.86 μM) respectively. Serum concentration after topical administration at 30, 60 and 120 min was found to be 1.60 $\mu\text{g/mL}$ (10.23 μM), 2.71 $\mu\text{g/mL}$ (17.28 μM) and 0.82 $\mu\text{g/mL}$ (5.2 μM) respectively (Figure 1A). Area under the curve of concentration and time graph was also determined and no significant difference was observed in serum menthol concentration between oral and topical administration (Figure 1B).

Similarly, menthol concentration in the adipose tissue was also determined at the same time intervals. The peak concentration after oral and topical administration was attained at 30 min. Concentration of menthol after oral administration at 30, 60 and 120 min was found to be 28.33 $\mu\text{g/mL}$ (181.09 μM), 17.38 $\mu\text{g/mL}$ (111.2 μM) and 12.51 $\mu\text{g/mL}$ (79.98 μM) respectively. Serum concentration after topical administration at 30, 60 and 120 min was found to be 53.09 $\mu\text{g/mL}$ (339.74 μM), 28.94 $\mu\text{g/mL}$ (185.19 μM) and 20.11 $\mu\text{g/mL}$ (128.69 μM) respectively (Figure 1C). Area under the curve of concentration and time graph for topical administration was significantly higher than the oral administration which is signifying that higher amount of menthol was available in the adipose tissue after topical administration (10% w/v) (Figure 1D).

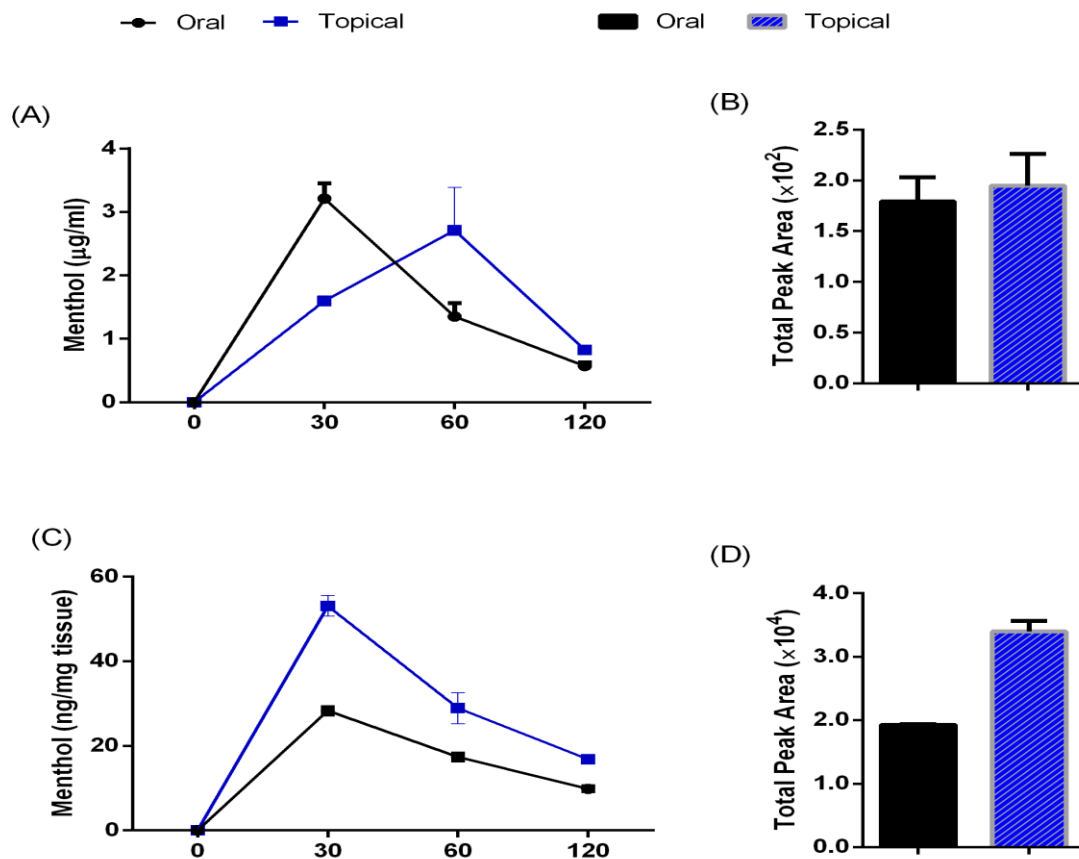


Figure 1: Pharmacokinetic of menthol administration upon oral and topical administration (A) Time dependent changes in serum concentration of menthol; (B) Area under the time-concentration curve in serum of menthol treated mouse; (C) Time dependent changes in concentration of menthol in subcutaneous adipose tissue; (B) Area under the time-concentration curve in subcutaneous adipose tissue of menthol treated mice. Oral- Single dose of oral menthol administration at 200 mg/kg. Topical- Single dose of topical menthol application at 10 % concentration (300 µL/ animal). n=3. All the values are expressed as mean \pm S.E.M; * p < 0.05

3.2 Menthol Treatment Did Not Affect Cellular Differentiation, Lipid Accumulation and Cell Viability in 3T3-L1 Cells In- Vitro.

No change in cell viability was observed with menthol treatment in comparison to vehicle treated group up to 300 µM dose (Figure 2A). Oil red O staining is used as a quantitative method to analyse the difference in the degree of differentiation and lipid accumulation in adipocytes in cell culture. The effect of varying concentrations of menthol (1 µM, 50 µM, 100 µM and 200 µM) was evaluated during adipogenesis. Slight decrease in lipid accumulation was observed with menthol 1 µM dose as compared to vehicle treated group and no change was observed with 50 and 200 µM doses (Figure 2B). No significant difference in cellular differentiation of 3T3-L1 cells was observed between the vehicle and menthol treated cells (Figure 2C).

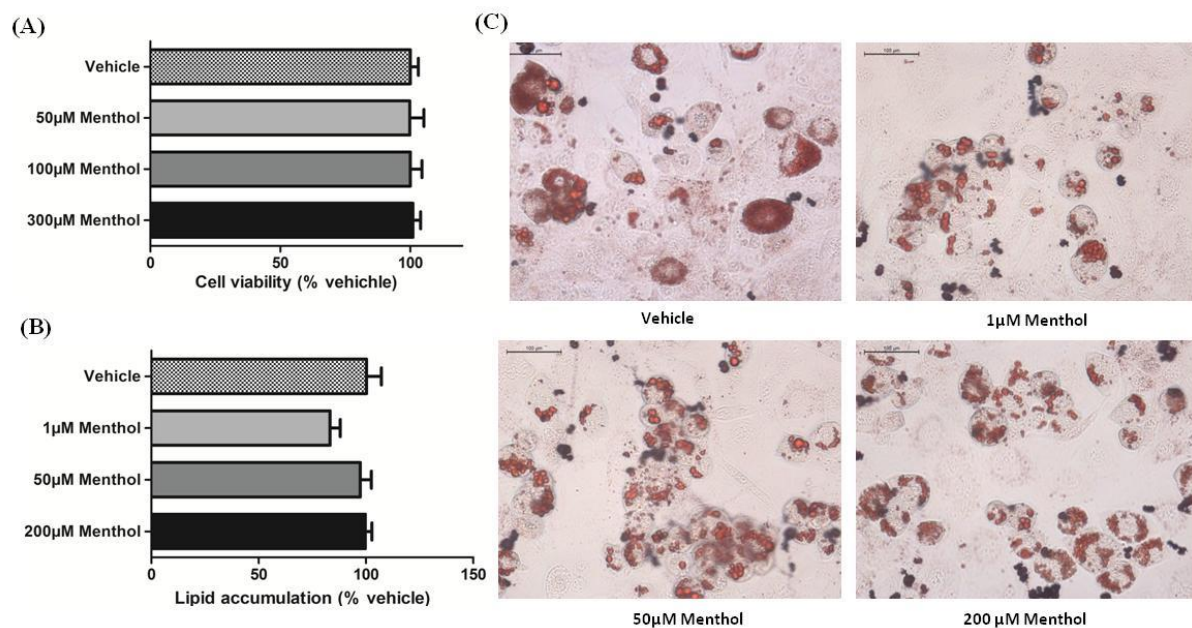


Figure 2: Effect of menthol on adipogenesis in 3T3-L1 cells. (A) Cell viability in pre-adipocytes treated with menthol for 24 hrs (B) Effect of menthol on lipid accumulation in 3T3-L1 cells. (C) Effect of various concentration of menthol on differentiation of 3T3-L1 cells. Red spots in images represents area stained by Oil Red O dye. All values are expressed as mean \pm S.E.M. (n=3). One-way ANOVA followed by Tukey's multiple comparison post hoc test was applied. *each vs vehicle.

3.3 Menthol Treatment Altered Metal Concentration and Browning Gene Markers in 3T3-L1 Pre-adipocytes In Vitro

The effect of 1, 50 and 200 (data not shown) μ M concentrations of menthol treatment during the adipogenesis process on different metals concentration in matured adipocytes was evaluated by ICP-MS. Significant increase in the concentrations of iron and copper was observed after 1 μ M menthol treatment as compared to their respective controls. Similar pattern was also observed with 50 μ M menthol treatment, showing marked elevation in levels of iron, copper and zinc as compared to their corresponding controls. However, significant reduction in cobalt metal levels was observed with both doses of menthol (1 and 50 μ M) as compared to vehicle treated group. No significant change in the level of metals like calcium and magnesium was observed with both doses of menthol (Figure 3A & 3B). There was no significant change in the metal concentration levels at 50 and 200 μ M, hence we used 1 and 50 μ M for further studies.

Treatment with 1 and 50 μ M concentrations of menthol significantly increased the mRNA expression of browning markers i.e. PR domain containing 16 (*PRDM16*), Homeobox C10 (*HOXC10*), Tumor necrosis factor receptor superfamily member 9 (*TNFRSF9*) as compared to vehicle treated group. 1 μ M menthol increased the mRNA expression of Cell death-inducing DFFA-like effector A (*CIDEA*), however treatment with 50 μ M menthol reduced the expression of *CIDEA* in insignificant manner. A

marked reduction in the mRNA expression of Peroxisome proliferator-activated receptor gamma (*PPARGC1A*), Transducin like enhancer of split 3 (*TLE3*), Nuclear receptor-interacting protein 1 (*NRIP1*) was observed with 1 μ M menthol supplementation. Although, 50 μ M menthol treatment also reduced the mRNA expression of *PPARGC1A*, *TLE3* and *NRIP1* but significant difference was only observed in expression of *TLE3* as compared to control (Figure 3C & 3D). Furthermore, same browning gene expressions were also observed with TRPM8 antagonist (AMTB) and its combinations with both doses of menthol. In 1 μ M menthol group, results depict marked increase in expression of *PPARGC1A* and *TNFRSF9* on treatment with 10 μ M AMTB (Figure 3C). However, expression of *PRDM16* and *HOXC10* were also elevated, yet the change was insignificant in comparison to vehicle treated group. Marked reduction in expressions of *TLE3*, *NRIP1* and insignificant decrease in *CIDEA* gene expression was observed with 10 μ M AMTB supplementation in comparison to vehicle treated group. Although same pattern of gene expression was found with 10 μ M AMTB treatment in 50 μ M menthol group, but significance in gene expressions was found to be changed showing marked elevation and reduction only in *TNFRSF9* and *TLE3* respectively in comparison to vehicle treated group (Figure 3D). Additionally, combination treatment of 10 μ M AMTB + μ M menthol significantly elevated expression of *PRDM16*. However, marked reduction in expression of *TLE3*, *PPARGC1A* and *NRIP1* was observed in comparison to vehicle treated group. Nevertheless, 10 μ M AMTB + 1 μ M menthol also increased the expressions of *HOXC10* and reduced the expressions of *CIDEA* and *TNFRSF9*, but change was insignificant in comparison to vehicle treated group (Figure 3C). Interestingly, combination treatment of 10 μ M AMTB + 50 μ M menthol displayed an altered behavior in comparison to combination with 1 μ M menthol showing significantly elevated expressions of *PRDM16*, *CIDEA*, *HOXC10*, *PPARGC1A* and *TNFRSF9*. Increase in expression of *NRIP1* was also observed, but change was insignificant. However, expressions of *TLE3* were markedly reduced with combination of 10 μ M AMTB + 50 μ M menthol in comparison to vehicle treated groups (Figure 3D).

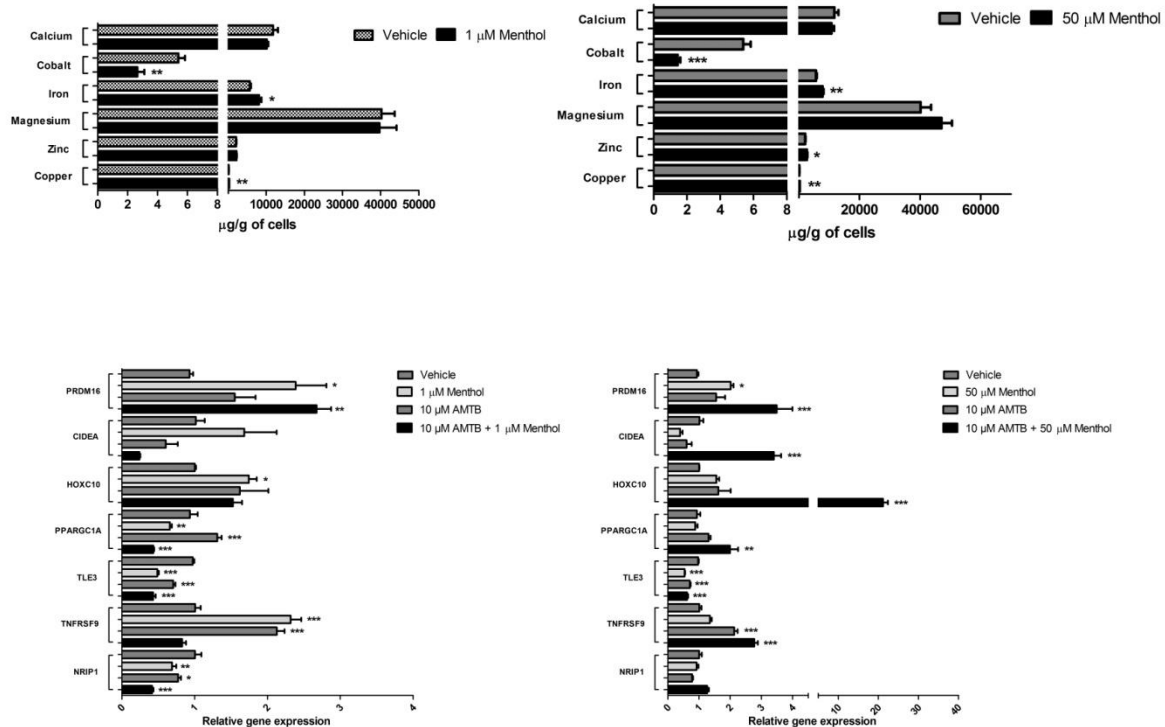


Figure 3: Effect of menthol treatment on (A) Metal concentration (Ca, Co, Fe, Mg, Zn, Cu) in 3T3-L1 cells at 1 µM dose; (B) Metal concentration (Ca, Co, Fe, Mg, Zn, Cu) in 3T3-L1 cells at 50 µM dose; (C) Relative expression of energy expenditure genes in 3T3-L1 cells at 1 µM menthol, 10 µM AMTB and 10 µM AMTB + 1 µM menthol treatment; (D) Relative expression of energy expenditure genes in 3T3-L1 cells at 50 µM menthol, 10 µM AMTB and 10 µM AMTB + 50 µM menthol treatment. n = 3-5; All the values are expressed as mean ± S.E.M; Analysis was carried out by student unpaired t-test and one-way ANOVA followed by Tukey's multiple comparison; *, **, *p<0.05, 0.01 & 0.001 1 µM menthol, 50 µM menthol, 10 µM AMTB, 10 µM AMTB + 1 µM menthol and 10 µM AMTB + 50 µM menthol treated groups vs vehicle treated group.**

3.4 Menthol Treatment Modulated Mitochondrial Activity Gene Markers For Energy Metabolism In 3T3-L1 Pre-Adipocytes In Vitro.

The effect of 1 µM menthol treatment on different mitochondrial genes was studied. In the NADH dehydrogenase (Complex I), significant increase in the gene expression of *Ndufa1*, *Ndufb2*, *Ndufc1*, *Ndufc2*, *Ndufa6*, *Ndufb3*, *Ndufb5*, *Ndufs6* was observed as compared to vehicle treated group. Significant increase in expression of *Uqcrcq* gene in cytochrome c reductase (complex III) was observed as compared to vehicle treated group. In the cytochrome c oxidase (complex IV) significant increase in expression of *Cox6a1*, *Cox6c* genes were also observed. In the F-Type ATP synthase (complex V) significant elevation in the *Atp5j* gene expression was also observed as compared to vehicle treated group (Figure 4A & 4B). Although, 50 µM menthol supplementation also displayed a modulatory effect over gene expressions

for mitochondrial energy metabolism, however, the change observed was insignificant in comparison to vehicle treated group (Figure 4A).

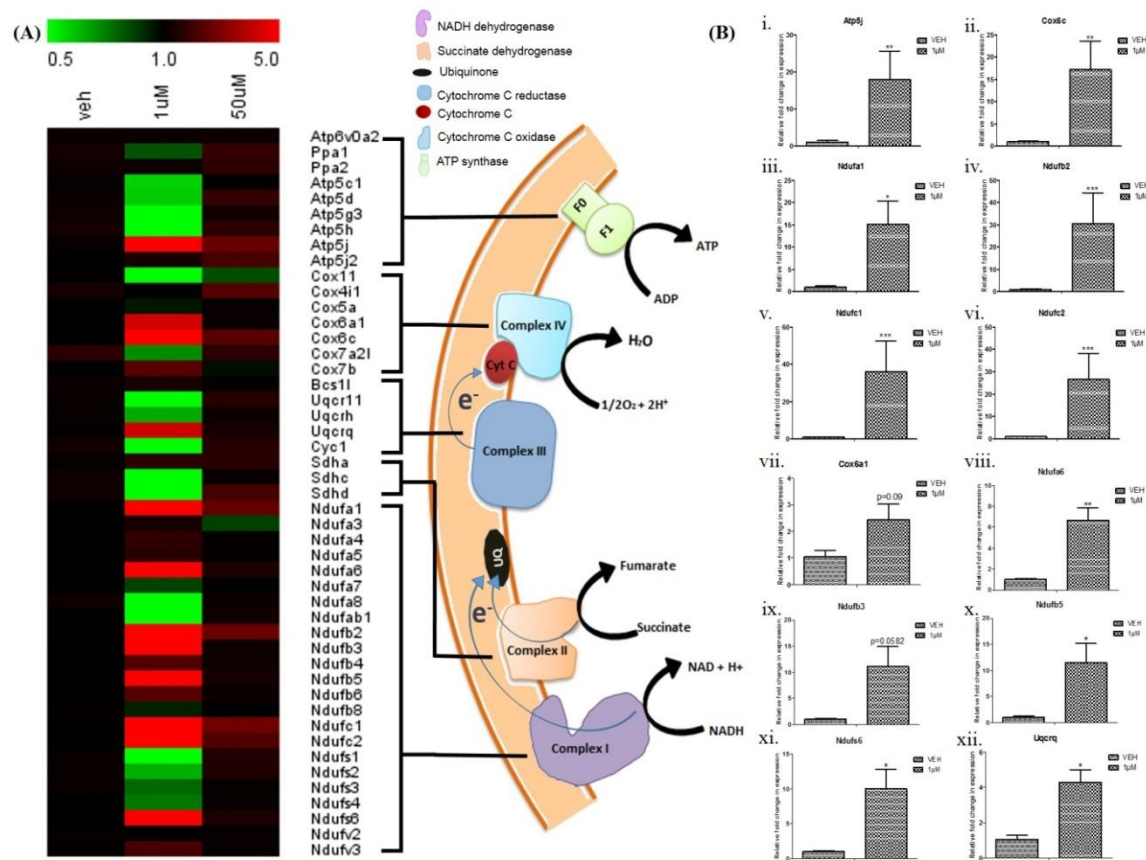


Figure 4. Heat map analysis of gene expression; and genes with statistically significant differential expression in 1µM menthol treated group. (A) Heat map shows gene expression in vehicle, 1µM and 50µM menthol treated 3T3-L1 cells (n=3 each). Color from red to green indicates high to low expression. (B) Comparative analysis of selected genes in 1µM menthol treated group. Normalization was done with reference gene B2M (β-2-microglobulin). The relative fold change in gene expression for mitochondrial biogenesis genes was compared in the three groups. Statistical analysis was done for genes i-vi using two-way ANOVA and for genes vii-xii using two-tailed unpaired t-test. *p<0.05, **p<0.01, p<0.001, each vs vehicle treated group.

4. Discussion

In our recently published manuscript, we have shown that oral and topical administration of menthol, a TRPM8 agonist, has anti-obesity potential through TRPM8 mediated glucagon dependent mechanism [14]. We provided evidence that TRPM8 mediated increase in serum glucagon and resultant increase in “glucagon machinery” in liver and adipose tissue is the signature of global shift from “fat storing state” to “fat burning state” in response to menthol administration [14]. During this work, we could suggest that the presence of TRPM8 receptor on adipose tissue is not required and the effect is selective

to the glucagon receptor present on adipose tissue, hence we may say that it is an indirect action of menthol on adipose tissue. Also, recent paper by Clemmensen and colleagues suggested that icilin, a TRPM8 agonist, effect on BAT energy expenditure cannot be explained by direct effects of icilin on adipocytes suggesting indirect actions to increase thermogenesis, likely through induction of sympathetic tone [25]. However, literature provide evidence for the presence of functional TRPM8 receptors on mouse and human adipose tissue, both white and brown [16-18, 26] and adipocyte cell lines [15]. These studies have mentioned that menthol induced increase in calcium influx in adipose tissue, mitochondrial activation and enhanced gene expression is mediated by TRPM8 receptors present on adipose tissue. The doses used in these studies are not based on the bioavailability profile of menthol, hence to establish a link between bioavailable doses of menthol and its functional effects on adipocytes is warranted. In this work, we tend to answer the question that at the acute doses showing anti-obesity effect [14], how much of menthol was bioavailable in serum and subcutaneous WAT. Also, whether this bioavailable menthol has any direct action on adipose tissue mediated through TRPM8 or others to induce energy expenditure.

Menthol was bioavailable to adipose tissues *via* both routes, oral and topical through serum and direct absorption respectively. Serum concentration of menthol reached maximum at 30 min and 60 min respectively in case of oral and topical administration, however the total area under the curve/peak concentration reached was similar over the period of 2 hours. Concentration of menthol in subcutaneous WAT reached maximum at 30 min in case of both oral and topical administration, with maximum peak concentration significantly higher in case of topical administration. We can easily argue that due to proximity of subcutaneous WAT to the site of application (topical), we see significantly higher concentration. Menthol is lipophilic in nature and its partitioning in adipose tissue through topical application is significantly higher as compared to oral administration. We understand that partitioning in rodent adipose tissue is positively predictive of partitioning in human adipose tissue [27], hence we suggest that this data has clinical utility through the direct effect of menthol on adipose tissue [14]. Previous literature also supports that L-menthol administration increases metabolic rate and thermogenesis in humans [27]. In the same study, authors have concluded that these effects are minor in oral administration as compared to topical administration due to faster metabolism of menthol (glucuronidation) and higher values of menthol glucuronides levels in blood [27]. Summarizing, route of administration is important for the action of menthol on subcutaneous adipose tissue whereas in serum both oral and topical are similarly bioavailable.

Now, the question is whether bioavailable menthol after topical administration is enough to show desired effects in adipose tissue, may be directly. We used pharmacokinetic based mathematical calculations to convert the bioavailable concentration of menthol in μM concentrations. We calculated

that there is a range of concentration *i.e.* 1 μ M to 200 μ M which will sufficiently cover the bioavailable amount of menthol on adipose tissues after topical administration. At these concentrations of menthol, 3T3L1 cells were viable as assessed by MTT assay. Also, at these doses there was no significant change in the accumulation of fat in differentiating adipose cells as shown by ORO staining. There was minor observation that some of cells were of smaller sizes.

There is lot of recent literature linking adipose tissue metal concentration with adipose tissue health, differentiation and “*browning*” of white adipose tissue. Specifically, in this regard iron and copper has major significance [28,29]. Iron and copper are essential components of the mitochondrial inner membrane complexes constituting the electron transport chain, therefore, the involvement of copper and iron in energy metabolism *via* their involvement in mitochondrial function (brown adipose tissue activation) is not surprising [29]. We performed metal analysis in differentiated adipocytes after treating cells with 1, 50 and 200 μ M of menthol during differentiation. We selected few important one based on their importance to adipose tissue health (calcium and cobalt), adipose tissue inflammation (zinc, calcium and iron), mitochondrial activation (iron and copper), cell toxicity (cobalt), glucose utilization and transport (zinc and magnesium), initiation of “*brite*” phenotype in white adipose tissue (iron and copper) for analysis [28,30,31,32,33,34,35]. Menthol administration during differentiation significantly increased the levels of iron and copper at 1 μ M whereas there was no significant change in the levels of magnesium, calcium and zinc. Cobalt concentration was significantly decreased in menthol treated differentiated adipocytes. However, at higher doses, 50 and 200 μ M (data not shown) there was significant increase in the levels of iron, copper, zinc and magnesium whereas significant decrease in cobalt concentration with no change in calcium concentration. Looking in to the metal concentration profile after menthol administration during differentiation of pre-adipocyte to adipocytes, we could suggest that menthol caused mitochondrial activation and increase in “*brite*” phenotype. This data further supports our previous finding where we linked HFD, metal concentration in adipose tissue and menthol administration in an *in-vivo* model of obesity [14].

Based on metal concentration changes, we selected 1 and 50 μ M (both 50 and 200 μ M had similar metal concentration profile) for further gene expression studies. We studied the change in expression of energy expenditure and “*browning*” related genes in differentiating adipose tissue. By critically looking into the gene expression data we could understand that (a) both 1 and 50 μ M menthol administration significantly modulated these genes which is very well corroborated with the existing literature [16], (b) these changes are not dose dependent, as at 50 μ M some of the effect is not there. Although we have not done these experiments but we may speculate that it may be due to desensitization of TRPM8 at this dose due to chronic presence of menthol, (c) AMTB, a TRPM8 antagonist, has its own effect on these genes, which may be due to its chemical nature or else. However, the effect is limited to few genes

suggesting that we cannot conclude that AMTB has “browning” effect. This may be selective effect of AMTB on these genes. (d) most striking observation was the effect of menthol in the presence of AMTB. We performed this experiment to understand whether this action of menthol is selective to TRPM8 or not. At 1 μ M, the effect of menthol was reversed in the presence of AMTB, suggesting TRPM8 dependent action. However, at 50 μ M effect of menthol was not reversed, actually it was potentiated further significantly. We suggest that in the absence of obvious target TRPM8, menthol at this higher concentration is now acting through some other mechanisms which are independent of TRPM8. There are numerous other actions of menthol which has link with energy expenditure phenomenon like its action of other excitatory or inhibitory channels, its role in TRPM8 independent increase in intracellular calcium, its role in oxidative effects [36]. Importantly at higher doses menthol acts on TRPV3 [37] and TRPA1 channels [38] which are closely related to adipogenesis, glucose utilization, hormone release and “browning”. This should be further explored to understand numerous actions of menthol. Based on these effects, we studied the effect of menthol on the mitochondrial activity complexes genes using PCR arrays (84 gene array). The effect on mitochondrial activation genes (approx. 40/84 genes) was positively correlated with energy expenditure genes, significantly higher at 1 μ M and at 50 μ M, it was decreased, which can be attributed to possible desensitization. In our previous study with TRPV1 agonist, capsaicin, we did see the same phenomenon increase in “browning” at 1 μ M and decrease at 50 μ M, which was correlated to the combination of desensitization and increase in the levels of PPAR- γ at higher doses [23]. We cannot rule out this kind of mechanisms with menthol given that both these channels are calcium permeable and in homogenous matrix their agonists can show similar effects. At 1 μ M, the changes in metal concentration, especially iron and copper, energy expenditure genes and mitochondrial complex genes are positively correlated (Fig 5). We may say that at 1 μ M menthol induces energy expenditure and mitochondrial activation, hence “brite” phenotype in differentiating adipocytes. Linking our bioavailable menthol with “brite” phenotype induction in differentiating adipocytes, we can suggest that even the topical dose less than 10 %, may be 2 % or 5 % will be sufficient to induce this effect.

There are some limitations of this study. We could have included number of doses between 1 and 50 μ M to have clear picture of desensitization or any other mechanisms responsible for decrease in effect at 50 μ M. TRPM8 independent targets of menthol should have been explored more to understand the increase in effect in our combination studies. We are planning extensive studies on these lines to establish the role of other TRP channels (TRPV3 or TRPA1) or non-TRP channels (calcium channels,

GABA/glycine receptors) in menthol's action. Also, topical

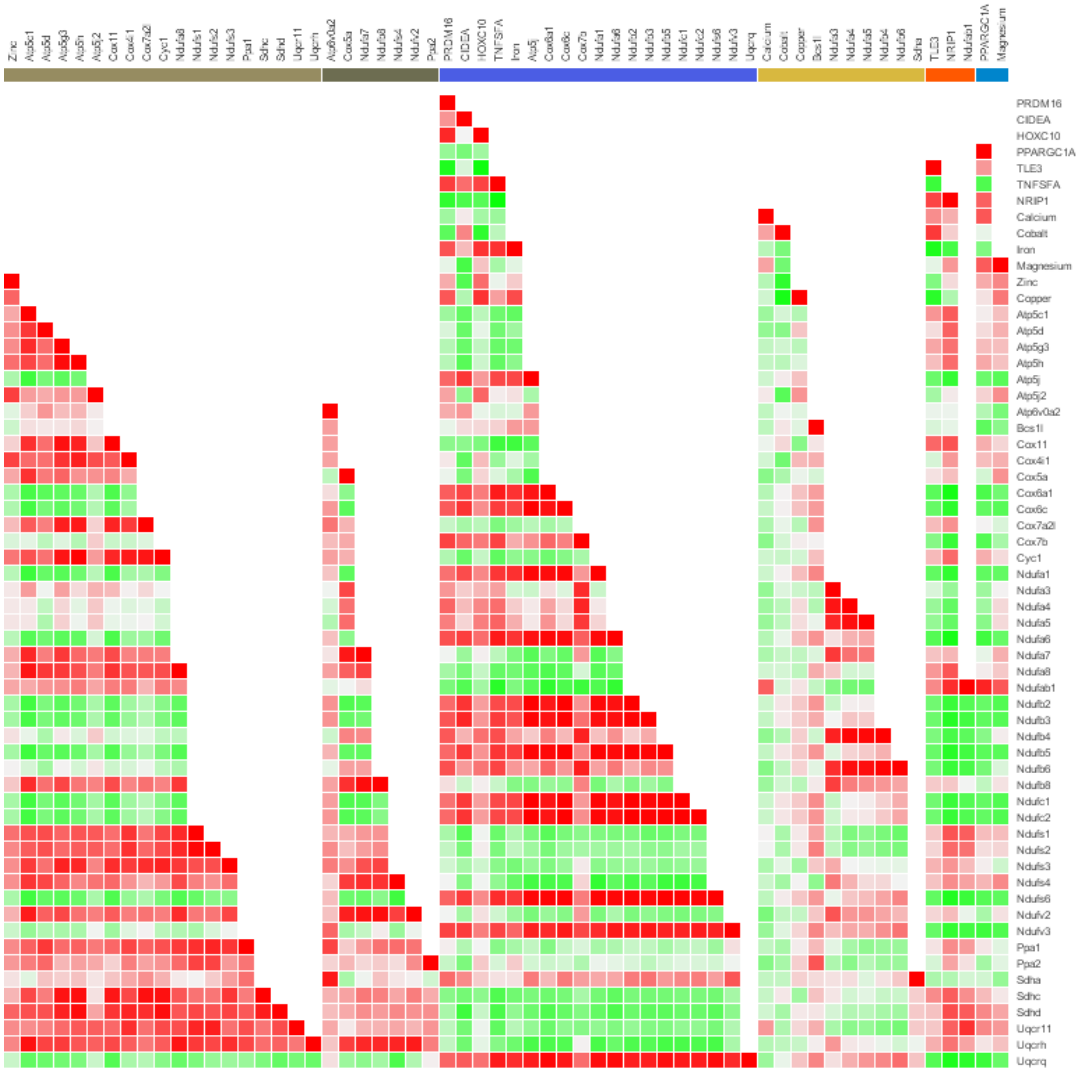


Figure 5. Correlation matrix and clustering of all the data generated through the experiments.

administration at multiple doses should be done to clearly establish cause and effect relationship. These studies will help us to develop topical menthol application as therapeutic/preventive strategy against obesity and related co-morbidities.

5. Conclusion

Over all, summarizing this study, we have provided evidence that single dose of menthol is sufficiently bioavailable to induce energy expanding “brite” phenotype in differentiating adipocytes which is dependent on TRPM8 at lower concentration (Fig. 6). These finding can be further exploited to devise

dietary (mint based nutraceuticals) or non-dietary (cold mimicking) approaches to combat obesity and type-2 diabetes.

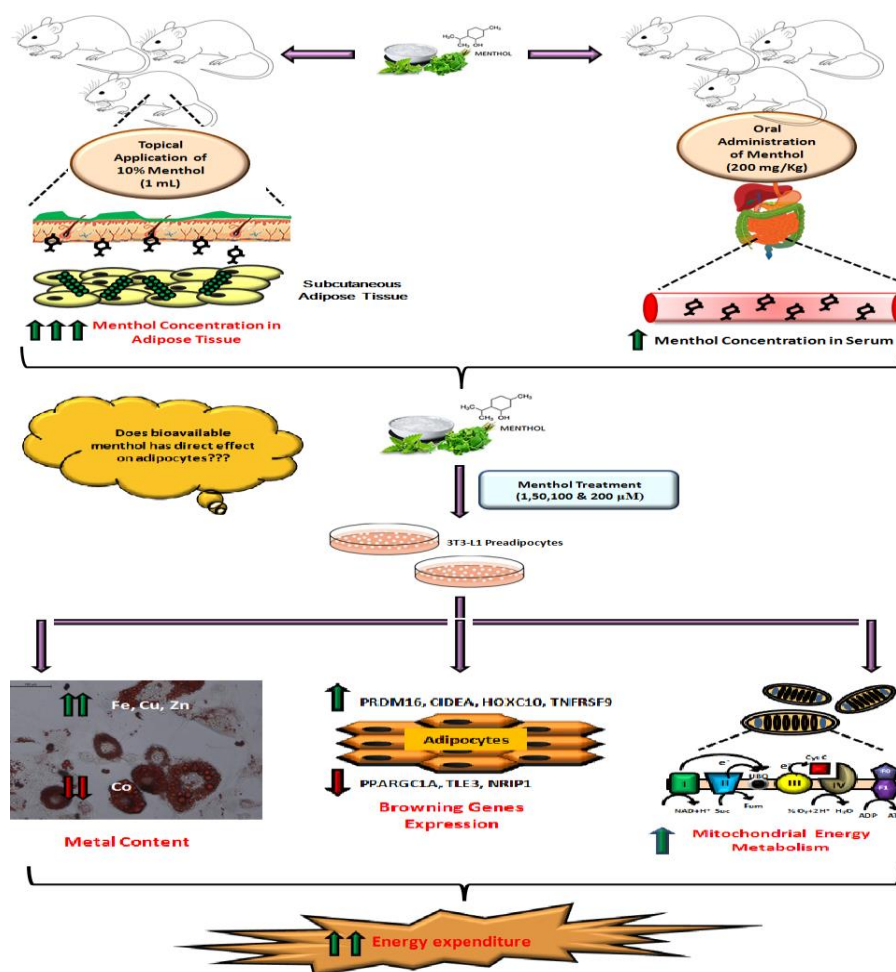


Figure 6. Summary of effect of bioavailable menthol on adipose tissue

Author Contributions: MB, A.G. and PK generated the hypothesis. MB, KC, PK, KKK designed the experiments and wrote the manuscript. P.K., A.C., V.K., N.M., V.K. performed the experiments and data calculation. J.K., V.K. and V.K. helped in data presentation and correlation analysis.

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