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Capsaicin inhibits the adipogenic differentiation of bone marrow mesenchymal stem cells by regulating cell proliferation, apoptosis, oxidative and nitrosative stress

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Abstract

Obesity is a global health problem that needs utmost attention. Apart from the other factors the trans-differentiation of mesenchymal stem cell (MSC) differentiation into adipocyte is an added detrimental cause for the intensification of obesity. The main objective of this present study is to analyses whether capsaicin can capable of inhibiting the differentiation of BMSCs towards adipocytes. Bone marrow mesenchymal stem cells (BMSCs) were obtained and exposed to different concentrations of capsaicin for the period of 6 days following 2 days of adipogenic induction. The capsaicin exposed cells were collected at three different time points (2, 4 and 6 days) and subjected to various analyses. BMSCs after exposure to capsaicin showed dose and time dependent reduction in cell viability and proliferation. Interestingly, capsaicin induced cell cycle arrest at G_0-$G_1$and increased apoptosis by increasing reactive oxygen species (ROS) and reactive nitrogen species (RNS) production. Capsaicin significantly inhibited the early adipogenic differentiation, lipogenesis and maturation of adipocytes with concomitant repression of PPARγ, C/EBPα, FABP4 and SCD-1. Taken together the present study has clearly emphasized that capsaicin potentially inhibits the adipogenic differentiation of mesenchymal stem cell multifaceted mode (anti-proliferative, apoptotic and cell cycle arrest) via stimulating of ROS and RNS production. Thus, the capsaicin not only suppress the maturation of pre-adipocyte into adipocyte but also inhibit the diffrentiation of mesenchymal stem cells in to adipocytes.

Key words: Human Bone Marrow Mesenchymal stem Cell, Capsaicin, Adipogenesis, Oxidative Stress and nitrosative stress.

Introduction

Obesity is the global health problem that is gradually posing a devastating threat to life of mankind. The sedentary lifestyle of man coupled with unhealthy hygiene and modern cultural adaptation has increased the prevalence of obesity in both developed and developing countries reducing the quality of human life. Obesity is considered to be one of the most significant risk factor for metabolic disorders such as coronary heart disease, hypertension, type 2 diabetes, cancer, respiratory complications and osteoarthritis [1]. Thouth number of factors involved in obesity, the differentiation of mesenchymal stem cell (MSC) into adipocyte is an added detrimental cause for the intensification of obesity in addition to the pre-adipocyte maturation into adipocyte [2]. Therefore, scientific studies on MSC differentiation and pre-adipocyte maturation into adipocyte have become the current focus to fight against obesity.

Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide) is one of the main constituent present in red pepper that is extensively used as spice [3]. Its therapeutic value was known since ancient time with its wide usage in the Indian and Chinese system of medicine however with lack of proper scientific validation. Studies have reported that it serves as a potent analgesic and anti-inflammatory agent [4], afferent nerve stimulant [5] and as a regulator of food intake, appetite, subsequent protein and fat intakes [6] and enhance energy metabolism [7]. Furthermore, capsaicin is shown to inhibit the growth of various immortalized and malignant cells [8] and induce apoptosis in transformed cells [9]. The experimental animal studies have demonstrated its potency in the attenuation of body weight and total body fat [10] and improvisation of the glucose tolerance [11]. In experimental mice models, oral administration of capsaicin with diet has enhanced energy metabolism and also has suppressed body fat

accumulation [12]. It has been reported to enhance the fat oxidation and fecal lipid excretion [13]. Additionally, it was found to regulate lipid catabolism through acetyl-CoA carboxylase and fatty acid synthesis [7]. A clinical study has revealed a positive association between the consumption of foods containing capsaicin and a lower prevalence of obesity [14-17]. It also reported that Capsaicin along with other supplements capsaicine significantly reduced the body weight, hip and waist girth and increased the secretion of adipokines and energy expenditure in overweight men and women [18, 19]. The findings from Lee *et al.* (2013) [20] indicate that even tropical application of the capsaicin might limit fat accumulation in adipose tissues and also reduce inflammation and increase insulin sensitivity. However, whether the capsaicin has the potency to inhibit the adipogenic differentiation of MSCs has yet to be understood. The main objective of this present study is to analyses whether capsaicin can capable of inhibiting the differentiation of BMSCs towards adipocytes.

Materials and Methods

Isolation of BMSCs; adipogenic induction and capsaicin exposure

To test out objective the bone marrow mesenchymal stem cell (BMSC) line was established according to the methods described elsewhere [21].The established cells were seeded onto 12-well plates and grown until 80% confluence was attained. Adipogenic differentiation in BMSC was induced by using an adipogenic cocktail containing 10 μg/mL insulin, 5 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) for a period of 2 days. Henceforth, the cells were treated with various concentrations (10, 50, 100, and 200 μM) of capsaicin (Sigma Aldrich, USA) for a period of 6 days. The treated cells were harvested at different time points (2, 4 and 6

days) for various analyses. The concentration of capsaicin used in the present study was adapted from the previous report [22, 26].

Measurement of cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the effect of capsaicin on the cell viability of differentiating BMSCs. The protocol was carried out as per the manufacture's instruction (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells (1×10^5/well) were seeded onto 12-well plates and upon attaining 80% confluence, cells were treated with capsaicin (10, 50, 100and 200 μM). At 2, 4 and 6 days of post-treatment, cells were used for MTT assay. Finally, the absorbance was measured at 570 nm using a Countess® automated cell counter (Invitrogen, Carlsbad, CA, UAS).

Cell cycle analysis

The effect of capsaicin on the cell cycle was analyzed by using the propidium iodide (PI) staining technique according to the method described previously [22]. Briefly, cells (1×10^5/well) were seeded onto 12-well plates and upon attaining 80% confluence, cells were treated with capsaicin (10, 50, 100 and 200 μM). At 2, 4 and 6 days of post-treatment, cells were used to determine the cell cycle distribution using FACS Caliber flow cytometry system (BD Biosciences, USA).

Oil red O staining and quantification

The effect of capsaicin on adipogenic differentiation was assessed by Oil Red O staining technique. Briefly, cells were rinsed twice with phosphate-buffered saline

(PBS, pH 7.4) and fixed in 10% (v/v) formalin in PBS for 20 min, washed with 60% (v/v) isopropyl alcohol, and stained in 2% (w/v) Oil Red O (Sigma-Aldrich) for 30 min at room temperature (RT). Cells were washed in isopropyl alcohol followed by repeated washes in distilled water. Staining was also quantified by extraction of Oil Red O into 100% isopropyl alcohol. Absorbance was measured at 510 nm.

Estimation of intracellular ROS and RNS production by fluorescent microscopy

The production of intracellular ROS (H_2O_2, •HO) was measured by peroxide sensitive fluorophore 2', 7'-dichloro-dihydro-fluorescein diacetate (DCF-DA) (Sigma-Aldrich, USA) method described by Lee *et al.,* [23]. The intracellular superoxide ($O_2^{\bullet -}$) was measured by hydroethidine (HE) (Invitrogen, USA) method [24]. The intracellular NO was measured by DAF-2DA (Invitrogen, USA) [25]. Analysis of fluorescent intensity was done analyzed under fluorescent microscopy with appropriate filters.

Quantification of apoptotic cells by Annexin-V and DAPI staining

The effect of capsaicin on programmed cell death in differentiating BMSC was evaluated by Annexin V staining kit (Sigma-Aldrich, USA). Briefly, cells (1×10^5/well) were seeded onto 12-well plates and upon attaining 80% confluence, cells were treated with capsaicin (10, 50, 100 and 200 μM). At 2, 4 and 6 days post-treatment, they were assayed for cell death by Annexin-V staining according to the manufacturer's instruction and analyzed under FACS Caliber flow cytometry system (BD Biosciences, USA).

Nuclear fragmentation (late apoptosis) was studied by chromatin staining with DAPI, as previously described [26]. At the end of the experiment, the supernatant was

discarded and cells were fixed with 3.5% formaldehyde in PBS for 30 min at RT, then washed four times with PBS, and exposed to DAPI (2 μg/ml) for 30 min at room temperature. Excess stain was removed by washing with PBS twice for 5 min each and examined under fluorescence microscope (Olympus Optical, Japan) with ultraviolet illumination. The number of apoptotic cells were quantified by manual count of 200 cells/field and totally 5 field/sample was counted.

Immunofluorescent study

BMSCs (1×10^5) were cultured on the coverslip for immunocytochemistry according to the method described by Glynn and McAllister, [27]. After the capsaicin exposure, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at RT, permeabilized with 0.2% Triton-X in PBS for 10 min at RT. The non-specific site was blocked with blocking buffer (2% bovine serum albumin) for 30 min at RT. The cells were incubated with rabbit polyclonal C/EBPα, PPARγ (1:100) (Abcam, USA), FABP4, and SCD (1:200) (Santa Cruz, USA) antibodies at 4°C for overnight. After a wash with PBS, the cells were incubated with secondary antibodies (goat anti-rabbit IgG conjugated with FITC and TR). The cells were mounted with anti-fade solution containing the DAPI (Invitrogen) and analyzed under fluorescent microscopy with appropriate filters.

RNA extraction and real-time RT-PCR analysis

BMSCs were harvested after 2, 4 and 6 days of adipogenic differentiation. Total RNA was extracted using the trizol reagent (Molecular Research Center, Cincinnati, OH). Total RNA levels were quantified by absorbance at 260 nm, and RNA integrity

was checked by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining of the 28S and 18S bands.

Total RNA (1 μg) was reverse-transcribed into cDNA using an iScriptc DNA Synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Real-time RT-PCR was performed using Quantitect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA) and a 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, PCR was done in a final reaction volume of 25 μl containing 200 ng cDNA, 12.5 μl SYBR Green RT-PCR Master Mix, and 1.25 μl of each of two primer solutions (10 μM). The thermal cycling parameters were as follows: 50°C for 2 min and 95°C for 15 min followed by 40 cycles at 94°C for 15 s, 62°C for 30 s, and 72°C for 30 s. All primers were designed with reference to sequences published by the National Center for Biotechnology Information (Table 1). The 2–ΔΔCt method was used to determine relative changes in mRNA levels (Livak and Schmittgen, 2001). All data were normalized to the expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

The data were subjected to the "One-way ANOVA" [28]. The significance was determined using "Tukey's post-hoc Test" with p value <0.05 considered as statistically significant.

Results

Effect of capsaicin on cell viability of differentiating BMSCs

The effect of capsaicin on cell viability in differentiating BMSCs was assessed by measuring the mitochondrial activity by MTT assay. Fig 1 illustrates the dose dependent inhibitory effect of capsaicin on cell viability, in all the time points. The significant inhibition was observed in capsaicin levels as low as 10µM within 48 h (p<0.001). The IC_{50} was observed in the concentration of 200µM for the period of 2 days. The highest inhibition of 83.8% was observed at the dose of 200µM capsaicin exposure for the period of 6 days.

Effect of capsaicin on lipid deposition in differentiating BMSCs

The quantitative analysis of Oil red O is staining showed that capsaicin significantly (p<0.001) reduced the lipid accumulation in differentiating BMSCs at dose and time dependent manner when compared to control (Fig. 2 A). Significant reduction (p<0.001) was observed even at low dose (10µM) exposed for 2 days and the reduction has significantly increased with respective increase in dose of capsaicin. However, the 50% inhibition was observed at 75.88 µM for 2 days and 89.9% inhibition at 200 µM for 6 days exposure. Associated with the quantitative analysis, the qualitative analysis also showed dose and time dependent reduction in Oil red O positive cell and also decreased the size of lipid drops in all capsaicin exposed cells when compared to unexposed cells (Fig. 2 B).

Effect of capsaicin on apoptotic cell death in differentiating BMSCs

Apoptosis was evaluated by the Annexin-V and DAPI staining by flow cytometer and fluorescent microscopy. Correlation with reduced cell viability (MTT

assay), the Annexin-V staining also revealed that the capsaicin significantly increased the externalization of Phosphatidyl-serine at both dose and time dependent manner. The positive cells were detected in the minimum dose of 10 μM (15.8%) at 2 days post capsaicin exposure and the number was increased to 41.6% in 200 μM exposure for 6 days. In correlation with Annexin-V study, DAPI staining has also showed significant increase in cells with apoptotic nuclear morphology at dose and time dependent manner. The 50% of apoptotic cells was observed at the dose of 100 μM for 4 days and 68% of cell death was observed in 200 μM capsaicin treatment for 6 days. Both Annexin-V and DAPI staining clearly indicates that capsaicin induces the externalization of Phosphatidyl-serine (early apoptosis) (Fig. 3A& 3B) and DNA damage (late apoptosis by DAPI stain) (Fig.4A and4B).

Effect of capsaicin on Cell cycle in differentiating BMSCs

The cell cycle analysis was done by PI staining using flow cytometer. In fig. 5, the data depicts that capsaicin significantly decrease the synthesis phase of the cell division with concomitant increase of G_0-G_1in dose and dime dependent manner. This data clearly emphasize that the capsaicin exposure induces the cell cycle arrest at G_0-G_1 phase when compared with control at dose and time dependent manner (Fig. 5).

Effect of capsaicin on ROS and RNS production in differentiating BMSCs

The effect of capsaicin on intracellular ROS production during adipogenic differentiation was estimated. The levels of ROS were found to be markedly increased with increasing concentration of capsaicin (Fig. 6A & 6B). Interestingly, the capsaicin induced ROS production, was observed even at low dose of 10 μM at 48 h. The

production was found to be markedly increased corresponding to the dose and time compared to control. The Fig. 7 depicts that capsaicin enhances the production of RNS in the differentiating adipocytes. Interestingly, the induction was observed as early as 48 h and at the concentration of 10 μM and this was increased markedly with respect to dose and time. However, the most notable alteration was observed in 100 and 200 μM capsaicin treated cells for all the time when compared with control (Fig. 7).

Effect of capsaicin on adipogenic and transcription factor expression in differentiating BMSCs

Immunohistochemistry data revealed that capsaicin exposure markedly reduced the expression of adipogenic transcription factor (C/EBPα and PPARγ) and adipogenic proteins (FABP4 and SCD) at dose and time dependent manner when compared to control. The suppression was markedly observed even at short duration and low concentration (2 days and 10 μM).The reduction was increased with respect to the concentration and duration of the capsaicin exposure (Fig. 8 and 9).

Effect of capsaicin on mRNA expression of adipogenic genes during differentiation of BMSCs

PPARγ is known as a key station protein that is expressed early in the adipogenic differentiation prior to C/EBPα. In the present study, capsaicin exposure significantly (p<0.001) suppresses the mRNA expression of PPARγ and C/EBPα (Fig. 10 A, B, C and D) with concomitant stimulate the mRNA expression of FOXO1 and IRF1 (Fig.11 A and B) at dose and time dependent manner. The fold changes were observed in all the doses, but it was more significant at 50, 100 and 200 μM in all the

experimental periods. However, the stimulatory effect of capsaicin on anti-adipogenic factors was observed even at low concentration in all the time point. Additionally, capsaicin also significantly (p<0.001) repressed the expression of maturation or lipogenic genes (FABP4 and SCD) at dose and time dependent manner (Fig. 10 A, B, C and D).

Discussion

Currently, Obesity and its associated secondary complications are the fastest growing health problems, which reduce the quality of human life. The present findings support the earlier studies that capsaicin potentially inhibits the adipogenesis via ameliorating the ROS and RNS mediated inhibition of cell viability, inducing cell cycle arrest at G_0/G_1 stage and repression of adipogenic genes (C/EBPα, PPARγ, FABP4 and SCD) with concomitant stimulating the FOXO1 and IRF1.

The free radical estimation has revealed that capsaicin enhances the production of ROS ($O_2{\bullet}^-$, $HO{\bullet}^-$ and H_2O_2) at dose and time dependent manner. This increased $O_2{\bullet}^-$level may be due to the activation of the NADPH oxidase system in differentiating adipocytes. Earlier report by Lee *et al.* (2004) [29] has demonstrated that the capsaicin potentially activates the NADPH oxidase in HepG2 cells. Additionally, the capsaicin also alters the mitochondrial electron transport chain via repressing the activity of mitochondrial complex-I and complex-III enzymes [30] and thereby, induces the production of ROS (H_2O_2, •HO and $O_2{\bullet}^-$).

Normally ROS (at physiological level) attenuates the adipogenesis via activating the mitotic clonal expansion (MCE) [31], which is the first phase of

differentiation and is characterized by the production of pre-adipocytes via the subsequent cycle of cell divisions [32]. In the present study, the inhibitory effect on viability (MTT assay) and induction of cell cycle arrest at G_0/G_1 stage might be due to activation of super-physiological level of ROS production by capsaicin. Furtheremore, the levels of NO also was found to be significantly increased at dose and time dependent manner, which clearly emphasizes that capsaicin, not only stimulate the production of ROS but also activates the production of NO in both early and late differentiation. This indicates that capsaicin has the potency to activate the various nitric oxide synthase (NOS) system [33, 34] thereby, it induces nitrosative stress. Activation of both ROS ($O_2{\bullet}^{-}$) and NO could lead to the formation of $ONOO^{-}$ [35] which is more toxic to the dividing and differentiating cells. This could be the crucial factor in the inhibition of adipogenesis.

Interestingly, when the dividing cells are exposed to ROS and RNS, they might undergo cell cycle arrest to either repair the DNA or initiate apoptosis [36]. In the present study, capsaicin induced both cell cycle arrest and apoptosis, which could be due to the ROS and RNS mediated activation of AMP-activated protein kinase (AMPK) [26] and repression of the cyclin dependent kinase pathway [37, 38] thereby, it arrests the cell cycle and inhibits cell proliferation. Furthermore, the increase in the externalization of Phosphatidylserine as determined by annexin-V staining (early apoptosis) and the DNA fragmentation DAPI staining (late apoptosis) suggest that capsaicin induces apoptosis. This may be due to the direct interaction of ROS and RNS to DNA and mitochondria that could have resulted in the loss of mitochondrial membrane potential leading to release of cytochrome-C and activation of caspase-3 and BAX [39]. Consistent with our present study, the earlier study has shown that capsaicin

induces apoptosis in rat hepatoma Fao cells and 3T3-L1 cells via activation of caspase 3 and BAX [22, 26]. However, they used 100 μM of capsaicin to induce apoptosis in 3T3-L1 cell lines but in the present study, we observed apoptosis even at 10 μM for 48 hour exposure and the effect was increased in relation to the dose and time. This variation might be attributed to the nature of cell type used. BMSCs may be more sensitive to even low concentration at the time of differentiation.

Furthermore, the earlier study by McBeath *et al.* [40] has demonstrated that BMSCs culture in high density have a propensity to become adipocytes than that of low density. Interestingly, in the present study, capsaicin induced cell cycle arrest, inhibited cell proliferation and induced apoptosis which ultimately led to reduced cell population/density that in turn might repress the adipogenic differentiation. In the present study inhibition of adipogenic differentiation of BMSCs is more than that of induction of apoptosis. Which clearly demonstrate that capsaicin has great potency suppress the adipogenic differentiation independently rather through apoptosis.

The qualitative and quantitative analysis of Oil red O staining showed that capsaicin reduces the number of adipogenic cells at dose and time dependent manner with association of reduced expression of adipogenic transcription factors and other genes that are involved in adipogenesis. These data clearly indicate that capsaicin inhibits the adipogenesis. Supporting with our present study some of clinical study showed that capsaicin potentially reduce the adipogenesis via increasing metabolism and energy expender in overnighted subjects [18, 19]. The expression of PPARγ, a transcription factor is the master regulator of adipogenesis [41]. Our results have shown that capsaicin significantly suppresses the expression of PPARγ, which could be due to

TRPV1 channels mediated activation of induced calcium influx [42]. This increased calcium influx might activate various cascade via simulating oxidative stress and nitrosative stress thereby it induces the ROS and RNS mediated activation of AMPK [26], inhibits the binding of C/EBP to its promoter region [43]. It also suppress the expression of FOXO1 and IRF1 which could leads to direct repression of PPARγ finally leading to adipogenic inhibition. However, repression of PPARγ and C/EBPα and stimulation of FOXO1 and IRF1 clearly demonstrate that capsaicin has the inhibitory effects on both early and late adipogenesis.

C/EBPα is a member of the C/EBP family basic-leucine zipper class of transcription factors, and is known to form a positive feedback loop with PPARγ to reinforce the expression of adipocyte-specific genes [43]. In particular, during normal adipogenesis, the activated PPARγ protein binds to the promoter regions of adipocyte-expressing genes, including FABP4 and SCD-1 to activate lipogenesis or maturation of adipocyte [44, 45]. In the present study, capsaicin repressed the expression of adipocyte specific gene and proteins such as SCD-1 and FABP4. These results clearly demonstrate that capsaicin not only suppress the early differentiation via repression of PPARγ and C/EBPα but also inhibit lipogenesis and/or maturation of adipocyte via repression of FABP4 and SCD expressions. Thereby it suppress both adipogenic differentiation of and maturation of adipocytes from BMSCs. Further, the capsaicin also stimulate the mRNA expression of anti-adipogenic factors (FOXO1 and IRF1) which clearly indicates that the capsaicin inhibit the adipogenesis not only by repressing the adipogenic factor but also via enhancing anti-adipogenic factors.

Taken together, we conclude that capsaicin inhibits adipocyte differentiation by down-regulating the expression of adipogenic related factors (PPARγ, C/EBPα, FABP-4

and SCD), reduces cell viability, cell cycle arrest and apoptosis via stimulating ROS and RNS production. Target molecule in which the capsaicin inhibits the adipogenic differentiation of BMSC is still unclear. However, activation of ROS and RNS production by calcium influx via TRPV1 channel and its associated cascade might be major role in anti-adipogenic differentiation of capsaicin. Therefore, habitual consumption of capsaicin not only inhibit the pre-adipocyte in to adipocytes but also act as a potential inhibitor of differentiation of mesenchymal stem cell in to adipocytes. Since, the mesenchymal stem cell (MSC) differentiation into adipocyte plays a detrimental cause for the intensification of obesity the capsaicin could act as good remedy for anti-obesity therapy. Further, *in-vivo* and pharmacokinetic studies are warranted to establish the therapeutic potential of capsaicin in the treatment of obesity.

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Abbreviations

MSC, mesenchymal stem cell; BMSCs, Bone marrow mesenchymal stem cells; ROS, reactive oxygen species; RNS, reactive nitrogen species; Annexin V-FITC, annexin V-fluorescein isothiocyanate; C/EBPR, CCAAT enhancer binding protein alpha; FABP4, Fatty acid binding protein; SCD, Stearoyl-CoA desaturase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GPDH, glycerol-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; IC_{50}, 50% growth inhibitory concentrations; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PPARγ, peroxisome proliferator-activated receptor-gamma; FOXO1, Forkhead box O1, IRF1, interferon regulatory factor 1; DAPI, 4',6-Diamidino-2-Phenylindole; FITC, Fluorescein isothiocyanate;

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Legends

Figure 1. Graph showing the effects of capsaicin on MTT activity in the differentiating bone marrow mesenchymal stem cells. Each point indicates the mean ± SEM of % of inhibition. * - p<0.05, ** - p<0.01 and 0.001.

Figure 2. Photomicrograph and graph showing the effects of the capsaicin on lipid accumulation in differentiating the bone marrow mesenchymal stem cells. *Details:* Figure 2A shows the qualitative analysis of adipogenesis in differentiating BMSCs by oil red O staining (20 X magnification). The 2B indicates the quantitative analysis of oil red O substance (OROS) by spectroscopic method. Each point indicates the mean ± SEM (n=3) of OROS. # - p<0.05, @ - p<0.01 and $ - p<0.001.

Figure 3. Photomicrograph showing apoptotic cells in capsaicin exposed differentiating

BMSCs by Annexin – V (early) staining. *Details:* Figure 3A indicates the flowcytometric image of apoptotic cell distribution. Figure 3B indicates the quantitative representation of the apoptotic cells with respect to dose and time of capsaicin exposure. Each point indicates the mean ± SEM (n=3) of Annexin-V positive cells. @ - $p<0.01$ and $ - $p<0.001$.

Figure 4. Photomicrograph showing the apoptotic analysis (Late) in the differentiating BMSCs by using DAPI staining. *Details:* Figure 4A indicates the DAPI staining with apoptotic nuclear morphology (Arrow head) in various capsaicin exposed groups (20 X magnification). Figure 4B indicates that quantification of cells with apoptotic nuclear morphology. Each point indicates the mean ± SEM (n=3) of nucleus showed apoptotic morphology. # - $p<0.05$, @-$p<0.01$ and $ - $p<0.001$.

Figure 5. Photomicrograph showing the effects of capsaicin on cell cycle of differentiating BMSCs. *Details:* Figure 5A indicates the flowcytometric analysis of various stage of cell cycle in dose and time dependent exposure of capsaicin. Figure 5B indicates the quantitative analysis of each stage of cell cycle in time and doe dependent exposure of capsaicin. Each point indicates the mean ± SEM (n=3) of various stage of cell cycle. # - $p<0.05$, @-$p<0.01$ and $ - $p<0.001$.

Figure 6. Photomicrograph showing the effects of capsaicin on Free radical production in differentiating BMSCs induced using 2', 7'-dichloro-dihydro-fluorescein diacetate (DCF-DA) and hydroethidine (HE). *Details:* Figure 6A indicates *in-situ* localization of hydrogen peroxide (H_2O_2) and hydroxyl ($HO\cdot^-$) radicals production (Green) the intensity of fluorescent directly propositional to amount of radicals produced (20 X magnification). Figure 6B indicates the *in-situ* localization of $O_2^{\cdot-}$ production (Red). The intensity of fluorescent directly propositional to amount of $O_2^{\cdot-}$ radical produced.

Figure 7. Photomicrograph showing the effects of capsaicin on *in-situ* $\cdot^-NO$ radical production in differentiating BMSCs by using DAF-2DA. The intensity of fluorescent (green) directly propositional to amount of ^-NO radicals produced (20 X magnification).

Figure 8. Photomicrograph showing immunolocalization of the SCD and C/EBPα in capsaicin exposed and non-exposed differentiating BMSC cells. The cytoplasmic (green) expression of SCD and nuclear (Red) expression of C/EBPα showed in adipocyte. The nucleus was localised with DAPI staining (40 X magnification).

Figure 9. Photomicrograph showing immunolocalization of the FABP4 and PPARγ in capsaicin exposed and non-exposed differentiating BMSC cells. The cytoplasmic (green) expression of FABP4 and nuclear (Red) expression of PPARγ showed in adipocyte. The nucleus was localised with DAPI staining (40 X magnification).

Figure 10. Graphs to show the effect of capsaicin on mRNA expression of adipogenic (FABP4 and SCD) and transcription factors (PPARγ and C/EBPα) in differentiating BMSCs. Each point indicates the mean ± SEM (n=3) of % of mRNA expression. . # - $p<0.05$, @-$p<0.01$ and $ - $p<0.001$.

Figure 11. Graphs to show the effect of capsaicin on mRNA expression of anti-

adipogenic factors (FOXO1 and IRF1) in differentiating BMSCs. Each point indicates the mean ± SEM (n=3) of % of mRNA expression. #- $p<0.05$, @ - $p<0.01$ and $ - $p<0.001$.

Table 1. Oligo nucleotide primer sequence of the adipogenic genes 1) CCAAT/enhancer binding protein, 2) Fatty acid binding protein 4, 3) Peroxisome proliferator activated receptor γ, 4) Stearoyl-CoA desaturase, 5) Glyceraldehyde-3-phosphate dehydrogenase, 6) Forkhead box O1 and 7) interferon regulatory factor 1.