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EFFECTS OF CAFFEINE, PARACETAMOL AND β -NAPHTHOFLAVONE ON THE LIPID CONTENT AND DIFFERENTIATION OF HUMAN ADIPOSE DERIVED MESENCHYMAL STEM CELLS *IN VITRO*

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ABSTRACT

Obesity is a complex multifactorial disorder involving the differentiation of pre-adipocytes to mature adipocytes which is achieved through the process of adipogenesis via interaction with different adipogenic transcription factors and mediators. Many drugs and their interactions has been implicated with the common feature of adipose tissue enlargement through hyperplasia and hypertrophy. Caffeine and Paracetamol are few of the most common used drugs which have been theorized by previous researches to have some influence on the adipogenic process. In our study, we investigated the effects of Caffeine, Paracetamol and β -Naphthoflavone on the differentiation of Human Adipose Derived Mesenchymal Stem Cells (HAD-MSC). Cells were cultured in vitro using differentiation inducing media with and without the presence of different combinations of the drugs. Biochemical markers of adipogenesis were evaluated using biochemical assays for triglyceride and glycerol quantification. Our results show that there is an increase of glycerol and triglyceride concentration in cells treated with caffeine suggesting its anti-lipogenesis characteristics through the enhancement of the lipolytic process. Paracetamol also appears to have anti-adipogenic effects due to its apparent role in suppressing the accumulation of triglycerides. In addition, only the use of β -Naphthoflavone in combination with caffeine and Paracetamol, resulted in lower triglyceride concentrations than the latter two drugs alone which may indicate its possible enhancing anti-adipogenic properties is through its interaction with the other two drugs.

Key Words: Adipogenesis, Lipogenesis, Adipocyte Differentiation, Caffeine, Paracetamol.

INTRODUCTION

Obesity is a disorder in which excessive accumulation of lipids in adipose tissue is characteristic. White Adipose Tissue (WAT) contains adipocyte precursors which under adipogenic conditions undergo adipogenesis to become mature adipocytes. Subsequent excessive proliferation of these adipocytes and their

enlargement- hyperplasia and hypertrophy respectively- are characteristic of obesity. Adipose tissue not only acts as fat storage, but also as an endocrinal organ- it secretes various important adipocytokines such as adiponectin (Desai *et al.*, 2013; Meena *et al.*, 2014). Obesity is also characterized by chronic low-grade inflammation due to over nutrition that stresses and overloads signaling networks as well as inflammatory cytokines which are released by WAT - this affects immune and metabolic systems leading to inflammation (Iyer *et al.*, 2010).

Obesity has been linked with higher risks of

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cardiovascular and musculo-skeletal disorders and its rise is now witnessed globally. Approximately 65% of the world's population now resides in geographic areas where being overweight and obese causes more fatalities than being underweight (World Health Organization, WHO). It has been suggested that individuals that suffer from obesity (BMI > 30) may be more prone to develop metabolic abnormalities. Due to the widespread effect of obesity, the search for compounds that can effectively be employed in the production of anti-obesity drugs and treatments is becoming more desirable.

Much research has been conducted to study the effects of different chemicals and substances on adipogenesis, lipolysis and their subsequent effects on obesity and fat accumulation. One of the widely studied drugs is caffeine – with it being the most used pharmacologically active agent (Tofovic *et al.*, 2002). Caffeine is available in many over the counter cosmetics and a large variety of foods and drinks that are consumed by people every day- this makes the prospect of finding therapeutic effects for it more desirable. When used in combination with other drugs (such as ephedrine) or other herbal supplements and concoctions; it has been shown to help in the treatment of obesity and promotion of weight loss- this is due to the metabolic effects that are exhibited by caffeine (Bracale *et al.*, 2014). Caffeine is a compound that acts as an antagonistic inhibitor of adenosine in the sympathetic nervous system- however; caffeine actually causes an increase in cell activity as opposed to adenosine binding which slows it down (Bracale *et al.*, 2014). Due to this, it prevents the breakdown of cyclic adenosine monophosphate (cAMP). Higher concentrations of cAMP causes an increase in protein kinase A (PKA) that subsequently increases the production of ATPase – an enzyme that is involved in energy synthesis- and this, in turn, causes an overall increase in the body's metabolic activity (Sassone-Corsi, 2012).

A key element of the adipogenic pathway related to cAMP is the cAMP-response element binding protein (CREB)- whereby its activation has been shown to be necessary for the up-regulation of several other adipogenic regulators including CCAAT/ enhancer-binding protein (C/EBP) whose isoform C/EBP β is an initiator of adipogenesis through the expression of C/EBP α and more importantly peroxisome proliferator activated receptor γ (PPAR γ) transcription factors which work together to allow adipocyte differentiation (Fox *et al.*, 2006; Darlington *et al.*, 1998).

PPAR γ is involved heavily in lipid metabolism and is greatly expressed in both white and brown adipose tissue. The deletion of PPAR γ in mice sustained with a high fat diet revealed significant differences in the pattern and amount of lipid accumulation- they exhibited a loss of white adipose tissue mass, lower overall body fat percentage and an increase in adiponectin levels (Jones *et al.*, 2005). This indicates the importance of PPAR γ in the

adipogenic pathway and its effect on expression of adiponectin which is inversely proportional to fat mass and plays a role in inflammation and energy homeostasis.

Adiponectin is an adipocytokine that causes an increase in fatty acid oxidation (Yamauchi *et al.*, 2002). Transgenic murine models designed to overexpress adiponectin in adipose tissues were found to have a decrease in both adipose mass and adipocyte size due to a higher level of energy expenditure and glucose utilization caused by higher levels of expressed adiponectin (Bauche *et al.*, 2007). The relationship between adiponectin levels and lipid accumulation has also been demonstrated in various murine obesity models as well as human obesity studies (Bauche *et al.*, 2007; Piñero *et al.*, 2005).

These findings possibly provide a therapeutic possibility to battle obesity through the use of adiponectin enhancing agents – such as caffeine-. Studies on mice models that were placed on a high fat diet and then treated with caffeine rich oolong tea have demonstrated an increase in lipolysis on fat droplets (Han *et al.*, 1999). Other studies have linked higher adiponectin serum levels and decreased lipoprotein cholesterol with increased intake of caffeine rich products such as coffee (Yamashita *et al.*, 2012). The metabolic pathways and mechanics of caffeine in the human body are also important to be considered when attempting to understand its effects.

In addition to the use of caffeine to sustain high cAMP levels, the use of Paracetamol (acetaminophen) due to its cyclooxygenase inhibiting mechanism can also affect adipocyte differentiation by effectively attenuating the expression and activity of PPAR γ . Paracetamol's mechanism of action is most likely through the inhibition of prostaglandin (PG) synthesis through the inhibition of cyclooxygenase-2 enzymes (COX-2) (Anderson, 2008). The role of PG's and subsequent hormones and enzyme synthesized is essential in adipocyte differentiation. PG's are lipid mediators that undergo synthesis through the action of different enzymes. Arachidonic acid is first released by phospholipases (PLA₂s) and undergoes conversion to PGH₂ by one of the two COX isozymes (COX-1 or COX-2). The PGH₂ that is produced is metabolized into different PG's that are involved in adipocyte differentiation which causes the activation of PPAR γ (Ueno *et al.*, 2011; Ghoshal *et al.*, 2011). Therefore, bearing this expression mechanism in mind- the inhibition of COX-2 would prevent or decrease the conversion of arachidonic acid to PGH₂ which will have the ultimate effect of reducing and/or inhibiting adipocyte differentiation. COX-2 activation has also been found to be significantly involved in development of an inflammatory response in adipose tissue (Hsieh *et al.*, 2010) and has been linked to contributing in brown adipocyte differentiation in mice (Ghoshal *et al.*, 2011).

Despite this proposed mechanism, no conclusive research has yet to provide a definitive answer to the role of COX-2 inhibition or overexpression in adipocyte

expression. Studies conducted on 3T3-L1 cell lines showed that different adipocyte specific markers which included PPAR γ and adiponectin were found to be decreased after the use of COX inhibitors (Mazid *et al.*, 2006). COX-2 deficient murine models which were supplied with a high fat diet showed lower body mass than their wild type counterparts were also found to have higher metabolic activity, lower PPAR γ , lower inflammatory markers and significantly higher levels of adiponectin (Ghoshal *et al.*, 2011). These variables coincide with other findings linking them to increased lipolysis and lower body fat and BMI thus suggesting that the use of a COX-2 inhibitor (such as paracetamol) can possibly provide a protective treatment opportunity against obesity and can be employed for future anti-obesity medication. However a minor percentage of opposing research showed an activation of PPAR γ in adipocyte cell lines when treated with non-steroidal anti-inflammatory (NSAID) drugs such as Indomethacin (Lehmann *et al.*, 1997).

Aryl Hydrocarbon Receptor (ahR) is a ligand activated transcription factor that has been shown to regulate adipogenic factors and is involved in the induction of detoxifying enzymes such as cytochrome P450 (Yoshinari *et al.*, 2006). Synthetic flavonoid α -Naphthoflavone is classified as an ahR antagonist and has been shown to promote adipogenesis and lipid accumulation in 3T3-L1 cells *in vitro* (Wang *et al.*, 2015). Inversely, utilizing ahR agonists such as β -Naphthoflavone (BNF) are hypothesized to have an opposite effect and effectively inhibit or minimize lipid accumulation. BNF has been shown to be an effective inducer of important detoxifying enzymes such as CYP1A1 through the translocation of ahR receptor bound to BNF (Yoshinari *et al.*, 2006).

METHODOLOGY

Adipose Derived Mesenchymal Stem Cells Growth and Differentiation

Adipose derived mesenchymal stem cells (HAD-MSC) purchased from HiMedia™ (code: CL007) were differentiated according to protocol using the kit purchased from HiMedia™ (code: CCK011). Day 0 involved maintaining the cells in DMEM high glucose supplemented with 10% FBS. The cells are then subcultured at approximately 70% confluency and incubated in 5% CO₂. Cell density was adjusted and 220 μ l of the cell suspension was pipetted into six well plates in accordance to the schematic diagram provided (Figure 1). A total of two controls and seven experimental wells were used. The concentrations of caffeine and Paracetamol used were 20mM while BNF was used at a concentration of 0.2mM. These drugs were added to the wells in various combinations on day 4 along with 200 μ l of differentiation media 1. After 48 hours, the spent media was aspirated and the drugs were replenished along with the addition of differentiation media 2. This protocol was repeated again

after 48 hours using complete media in place of differentiation media till complete differentiation was achieved at day 10.

Triglyceride Quantification Assay

Media samples collected from all the wells on Day 10 and Triglyceride levels in all samples were quantified following the protocol provided with the Triglyceride Quantification Assay Kit purchased from abcam™ (code: ab65336). Briefly, reaction wells were set up with five standards being set as duplicates along with samples collected from the wells on Day 10 of differentiation. 20 μ l of Lipase enzyme was added to each well and incubated at room temperature for a period of 20 minutes. This was followed by adding 50 μ l of the prepared reaction mix into each well and incubated in the dark for one hour. After the incubation period, the assay was read at an OD of 570nm to get absorbance readings for each sample. Using the readings, a standard curve was plotted and corresponding concentrations of samples were calculated.

Free Glycerol Quantification Assay

Using the Free Glycerol Assay Kit purchased from abcam™ (code: 65337); an indication of lipolysis levels was obtained. This is because as triglycerides undergo hydrolysis via the lipolysis process they release glycerol and free fatty acids.

Standards were prepared using a 1mM glycerol standard and 50 μ l was added to a 96 well plate in duplicates. Media samples collected from the wells on day 10 were also added in the same amount followed by the addition of 50 μ l of the prepared reaction mix. This is then incubated without light at 37°C for a period of 30 minutes and is then read at an OD of 570nm to obtain absorbance readings. Using the data, a standard curve was generated and corresponding concentration of samples was calculated.

MTT Assay

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay was conducted to assess cell viability of the cells using the MTT kit purchased from HiMedia™ (code: CCK003). MTT reagent was prepared by dissolving the provided MTT powder in buffer. 100 μ l cell suspension samples from each of the control wells and sample wells were pipetted onto a 96-well plate along with 10 μ l of the prepared MTT reagent. The plate was covered with foil and incubated at 37°C with 5% CO₂ for a period of four hours. The plate was then read at an OD of 570nm and 650nm. Using the resulted data, specific absorbance was calculated and plotted.

RESULTS

Cell Growth and Differentiation

Upon arrival the cells observed were healthy and had a stretched out, irregular shape which is characteristic

of preadipocytes (Figure 2A). 48 hours after differentiation media 1 was added, the cells began to take on a more regular spherical appearance and clumps had begun to form indicating the initiation of the differentiation process (Figure 2B). 48 hours following the addition of differentiation media 2, the cells began to differentiate further to form adipocytes (Figure 2C). The formation of fat droplets in the cells and increased cell clumping was observed indicating successful differentiation.

Triglyceride Concentrations

The cells that were treated solely with caffeine had higher triglyceride concentration when compared to the control (Figure 3A) as well as in comparison to all other independent and combinatory drug treatments. In contrast, significantly notable differences in triglyceride concentrations were noted in the well treated with Paracetamol (Figure 3B) whereby it had lower triglyceride content than the control. Interestingly, when both Paracetamol and caffeine are used together, their individual effects seemingly balance and an overall lower concentration of triglyceride is expressed- when compared to independent caffeine treatment (Figure 3C).

The treatment of cells using BNF independently displayed a dramatically lower triglyceride content compared to the control (Figure 4A) as well as in comparison to the cells treated with BNF + caffeine (Figure 4B) and those treated with BNF + Paracetamol and BNF + Caffeine + Paracetamol (data not shown)

Free Glycerol Concentrations

Free glycerol content is used as an indicator of lipolytic activity within the cells as it is released when triglycerides are hydrolyzed and broken down. Bearing the above in mind, the wells treated with Paracetamol, BNF and BNF + Caffeine had almost equal glycerol levels to those seen in the control with differentiated cells (data not shown). Alternatively, the well treated with BNF + caffeine + Paracetamol (Figures 5A), had significantly lower glycerol concentrations than the control. Alternatively, the well which contained caffeine + Paracetamol alone showed the opposite with higher glycerol levels (Figure 5B). Most notably out of all the samples taken, the well containing caffeine had the highest glycerol concentration overall with it being approximately 2.5 times more glycerol than the differentiated control (Figure 5C).

MTT Assay

MTT assay was used to help determine cell viability after the addition of drugs. Figure 6 illustrates the results of this test compared to the control. As observed, the wells treated with drugs had overall higher absorbance readings than the control- indicating that these drugs improved viability or most probably resulted in increase in number of cells due to an induced halt in the differentiation and adipogenesis process that kept some cells in pre-adipocyte status and preserving their dividing capability

Fig 1. Schematic outline of well plates

Fig 2A. Cells upon arrival

Fig 2B. Cells 48 hours after differentiation media 1 addition

Fig 2C. Cells 48 hours after differentiation media 2 addition

Fig 3. A comparison of different effects exhibited by caffeine and Paracetamol independently and in combination on the triglyceride concentration after differentiation of HAD-MSC's. (A) Elevated triglyceride concentration seen when cells are treated with caffeine independently. (B) Reduced triglyceride levels shown when cells are exposed to Paracetamol. (C) The effect of combining both caffeine and Paracetamol appears to null the different effects exhibited by each drug independently giving lower triglyceride content than the control

Fig 4. Cells treated with BNF independently show a significant lower concentration of triglycerides than the control with no drugs. (B) When cells are treated with both BNF and caffeine, the triglyceride concentration is raised when compared with the use of BNF alone. This provides further indication that caffeine causes an increase in triglyceride content as it seemingly attenuated the suppressive effect of BNF on triglyceride concentration of cells

Fig 5. (A) Illustrates the glycerol concentration when BNF, caffeine and Paracetamol are used in combination. The levels of glycerol are lower than the control under these conditions and therefore can suggest an inhibited or minimized rate of lipolysis. **(B)** Glycerol concentration is significantly raised when BNF is excluded and only caffeine and Paracetamol are used in conjunction with each other. The glycerol content is higher than the preceding condition (5A) and the control. **(C)** An approximate 2.5 times higher glycerol concentration observed in the well treated with caffeine alone when compared to the control. The use of caffeine independently showed the highest glycerol concentration overall indicating higher lipolysis levels

Fig 6. MTT assay absorbance readings of all wells compared to the control. All samples show absorbance readings higher than the control. The last four wells in particular – BNF + Caffeine + Paracetamol, Caffeine, Paracetamol and Caffeine + Paracetamol- show the most significant difference when compared to the control. This can indicate that all the cells were viable and that drug interactions may have contributed to this enhance viability

DISCUSSION

Triglycerides are biological molecules composed of a glycerol backbone and fatty acids which are stored in adipocytes (Prattes *et al.*, 2000). Their content within cells can be used as a direct indicator of adipogenesis- the higher the content, the more adipogenesis and adipocyte differentiation occurred. When triglycerides are hydrolyzed-process of lipolysis- glycerol is released

(Ahmadian *et al.*, 2007). Therefore, glycerol concentrations can be used as direct indicators of lipolytic activity in cells. Our results demonstrate that caffeine, Paracetamol, BNF and their varying combinations may all have effects on the processes of lipogenesis and adipogenesis.

The triglyceride and glycerol concentrations of cells treated solely with caffeine reflect lipolytic properties.

The use of caffeine resulted in the highest glycerol concentration among all tested drugs and combinations- the concentration was approximately 2.5 times higher than the differentiated control cells without addition of the drugs. As shown, triglyceride contents of caffeine were lower than glycerol- however; the triglyceride content of cells with caffeine was higher than all other samples. These traits could be an indicator that caffeine contains potent lipolytic effects but no significant effect on the process of adipogenesis itself. Perhaps caffeine can have an effect on existing triglycerides but cannot inhibit their formation. Conversely, Paracetamol appears to have no significant effect on the process of lipolysis but reduced triglyceride concentrations indicates that the independent use of Paracetamol may contain anti-adipogenic effects and prevent triglyceride formation. The suggested lipolytic and anti-adipogenic effects of caffeine and Paracetamol are further emphasized when examining their combined effect on preadipocytes differentiation. The triglyceride and glycerol concentrations under this condition are very similar to one another indicating a sort of homeostasis where the enhanced lipolytic effects expressed by caffeine are compensated by increased adipogenesis directed by Paracetamol.

The use of BNF to treat cells has varying effects based on whether it is used alone or in combination with other drugs. When BNF is used independently, triglyceride concentrations are reduced in comparison with differentiated control cells. However, glycerol concentrations under treatment with BNF are equal to the differentiated control. To further examine the consequences of utilizing BNF, it was used as an additional supplement along with caffeine, Paracetamol and their combination. When BNF is used with caffeine, the pronounced lipolytic effects of caffeine appear to become diminished and glycerol concentration levels become lower than the differentiated control. This effect on glycerol is also seen when BNF is used to supplement Paracetamol

and the combination of caffeine and Paracetamol. This may suggest that BNF has inhibitory effects on lipolysis and attenuates any potent lipolytic effects expressed by the use of caffeine and Paracetamol. In contrast to the observed inhibitory effect of BNF on lipolysis, its effects on adipogenesis favor anti-adipogenic properties. When used to treat cells independently, the triglyceride concentration of cells is lower than the differentiated control. This implied anti adipogenic effect is also observed when BNF is used to supplement caffeine and the combination of caffeine and Paracetamol. The triglyceride concentrations under these two conditions, is lower than those expressed in the same conditions without BNF supplementation. This provides further indication that BNF can be used to significantly enhance anti-lipogenic properties of caffeine and paracetamol through the attenuation of triglyceride synthesis. MTT analysis showed most probably that these drugs induce halt in the differentiation and adipogenesis process that kept some cells in pre-adipocyte status and preserving their dividing capability, especially as more cell numbers seen samples containing drugs compared with differentiated control lacking drugs. In addition we may suggest that these drugs at such concentrations were not harmful or toxic to cells

CONCLUDING REMARKS

Our results provide suggestive evidence and indication towards the anti adipogenic and lipolytic properties of Caffeine, Paracetamol and BNF. The presence of underlying mechanisms that can potentially express these properties merits further research and exploration. BNF is known to induce detoxifying CYP450 enzymes which may be a contributing factor to the anti adipogenic effects expressed. Future research to study the gene expression of CYP450 could provide further evidence of the adipogenic process that occurs during the differentiation of preadipocytes under the presence of different drugs.

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