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Xanthine analogues and adipose tissue metabolism

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Resumo

A obesidade tem aumentado nas últimas décadas e é um dos problemas de saúde mais preocupantes a nível mundial. As metilxantinas, como é o caso da cafeína (1,3,7-trimetilxantina), teofilina (1,3-dimetilxantina) e teobromina (3,7-dimetilxantina), demonstraram propriedades anti-obesogénicas bastante promissoras. Estes compostos naturais encontram-se bastante difundidos na dieta humana, nomeadamente no café, no chá e no chocolate. De entre estes fitoquímicos, a cafeína é descrita como moduladora do metabolismo da glucose e ácidos gordos, assim como o seu consumo aparenta ser inversamente proporcional ao aumento de peso corporal. Com base na estrutura química das metilxantinas, vários compostos análogos têm sido sintetizados. Foi colocada a hipótese de que um desses compostos, a 8-(3-fenilpropil)-1,3,7-trietilxantina, poderia apresentar um potencial anti-obesogénico bastante elevado. Deste modo, o nosso projeto visou avaliar os efeitos desta nova molécula no perfil metabólico e oxidativo de adipócitos, com o objetivo de avaliar o seu potencial como opção farmacológica para o tratamento da obesidade e suas complicações. Para este fim, foi utilizada como modelo *in vitro* a linha celular de pré-adipócitos de rato 3T3-L1 e a cafeína, de origem sintética, para fins comparativos. As células foram incubadas na presença de concentrações crescentes de cafeína e 8-(3-fenilpropil)-1,3,7-trietilxantina (0.1, 1, 10 e 100 μM) e o perfil citotóxico de ambos os compostos foi avaliado espectrofotometricamente pela redução do sal de tetrazólio (MTT) e quantificação de lactato desidrogenase (LDH) libertada. Os metabolitos presentes no meio de cultura foram identificados e quantificados recorrendo à ressonância magnética nuclear de protão (^{1}H-NMR) e as células foram recolhidas para a caracterização do perfil oxidativo. Os nossos resultados demonstram que a 8-(3-fenilpropil)-1,3,7-trietilxantina não induziu citotoxicidade em nenhuma das concentrações estudadas. Comparativamente à cafeína, este composto aumentou significativamente o consumo de glucose, piruvato e glutamina, assim como a produção de lactato, alanina e acetato. Estes resultados ilustram o elevado potencial da 8-(3-fenilpropil)-1,3,7-trietilxantina como modulador metabólico, mesmo quando comparado com a cafeína. Adicionalmente, a 8-(3-fenilpropil)-1,3,7-trietilxantina promoveu um efeito antioxidante, diminuindo os níveis de oxidação proteica e protegendo contra os danos causados pelo stress oxidativo. Em suma, a 8-(3-fenilpropil)-1,3,7-trietilxantina apresenta-se como um ótimo candidato para o *design* de fármacos anti-obesidade seguros e inovadores.

Palavras-chave

8-(3-fenilpropil)-1,3,7-trietilxantina; Cafeína; Fármacos anti-obesidade; Metabolismo de adipócitos; Obesidade; Xantina.

Resumo Alargado

O tecido adiposo é um órgão metabolicamente ativo que atua como o principal repositório de energia do corpo humano, sob o formato de gordura (triacilgliceróis). Este órgão complexo e dinâmico atua como um tampão energético, armazenando e libertando energia consoante as necessidades do organismo. Sempre que existir um excesso energético, o tecido adiposo irá armazenar esta energia sob a forma de moléculas de triacilgliceróis, dentro das gotas lipídicas da sua célula predominante, o adipócito. Este processo é denominado lipogénese. Em contrapartida, sempre que existir um défice de energia, o adipócito irá proceder à lipólise, ou seja, à hidrólise de moléculas de triacilgliceróis em glicerol e ácidos gordos livres, que são transportados e oxidados de forma a produzir grandes quantidades do nucleótido adenosina trifosfato (ATP).

A obesidade tem aumentado nas últimas décadas e é um dos problemas de saúde mais preocupantes a nível mundial. Estima-se que em 2015 mais de 2.2 biliões de indivíduos apresentavam peso excessivo (índice de massa corporal (IMC) > 25 kg/m^2), dos quais 710 milhões eram obesos (IMC > 30 kg/m^2). Esta condição é definida pela acumulação excessiva de gordura no tecido adiposo e é geralmente atribuída a maus hábitos alimentares e a sedentarismo, contudo pode ser igualmente atribuída a fatores genéticos, epigenéticos, fisiológicos e ambientais. A acumulação de massa adiposa é acompanhada pela hipertrofia e hiperplasia de adipócitos, ou seja, um crescimento em tamanho e número. Este constitui um mecanismo adaptativo ao excesso de gordura, protegendo o organismo de lipotoxicidade. No entanto, a quantidade de gordura que o tecido adiposo consegue armazenar é limitada e, quando ultrapassada, poderá levar a complicações metabólicas, como a resistência à insulina e, consequentemente, diabetes mellitus tipo 2, assim como a disfunções cardiovasculares e infertilidade. Deste modo, é crucial prevenir e tratar a obesidade. Atualmente, os pilares do tratamento da obesidade assentam na restrição calórica da dieta associada ao aumento da atividade físico. Contudo, este é um processo lento e difícil, pelo que existe uma crescente procura por opções farmacológicas seguras e eficazes que possam agilizar e simplificar o processo.

O estudo de compostos naturais com potencial anti-obesogénico tem suscitado interesse entre a comunidade científica. De entre estes, as metilxantinas apresentam um potencial anti-obesogénico particularmente elevado. As metilxantinas mais estudadas e conhecidas são a cafeína (1,3,7-trimetilxantina), a teofilina (1,3-dimetilxantina) e a teobromina (3,7-dimetilxantina), muito presentes na dieta através do café, chá e chocolate. Estes compostos têm a capacidade de modular o metabolismo da glucose e ácidos gordos, promovendo a lipólise e inibindo a diferenciação de adipócitos, ou adipogénese, assim como o seu consumo aparenta ser inversamente proporcional ao ganho de peso. Existem vários mecanismos sugeridos para explicar estes efeitos, sendo que o predominante é o antagonismo

de recetores de adenosina. Ao bloquear a cascata de sinalização da adenosina nos adipócitos, as metilxantinas induzem um sinal lipolítico, levando à hidrólise de triacilgliceróis em glicerol e ácidos gordos livres.

Devido às suas propriedades e efeitos benéficos para a saúde, a estrutura química das xantinas naturais tem sido utilizada como modelo para a síntese de compostos inovadores com novas características. Um desses compostos, a 8-(3-fenilpropil)-1,3,7-trietilxantina, foi sintetizado pela equipa de Van der Walt e Terre'Blanche (School of Pharmacy, North-West University, South Africa). Este composto apresenta uma alta afinidade por recetores de adenosina, característica esta que poderá estar associada a um potencial anti-obesogénico elevado. Deste modo, este projeto visou caracterizar a modulação conferida por esta nova molécula no perfil metabólico e oxidativo dos adipócitos, com o objetivo de avaliar o seu potencial como opção farmacológica para o tratamento da obesidade e suas complicações. Para este fim, utilizou-se como modelo *in vitro* a linha celular de pré-adipócitos de rato 3T3-L1 e a cafeína, de origem sintética, para fins comparativos. As células foram cultivadas na presença de doses crescentes de cafeína ou 8-(3-fenilpropil)-1,3,7-trietilxantina (0.1, 1, 10 e 100 μM) e o perfil citotóxico de ambos os compostos foi avaliado espectrofotometricamente pelos ensaios da redução do sal de tetrazólio (MTT) e quantificação da lactato desidrogenase (LDH) libertada. Os metabolitos presentes no meio de cultura (lactato, alanina, acetato, piruvato, glutamato e glucose) foram identificados e quantificados recorrendo à ressonância magnética nuclear de protão (^{1}H-NMR) e as células foram recolhidas para a caracterização do perfil oxidativo, através da análise da oxidação e nitração proteica, assim como da peroxidação lipídica.

Os nossos resultados demonstraram que a 8-(3-fenilpropil)-1,3,7-trietilxantina não induz citotoxicidade nas concentrações estudadas, ao passo que a cafeína induziu na concentração mais elevada (100 μM). Adicionalmente, a 8-(3-fenilpropil)-1,3,7-trietilxantina aumentou significativamente o consumo de glucose, piruvato e glutamina, assim como a produção de lactato, alanina e acetato comparativamente aos grupos expostos à mesma concentração de cafeína. Estes resultados sugerem que a 8-(3-fenilpropil)-1,3,7-trietilxantina tem uma capacidade superior à da cafeína para modular o metabolismo celular para produção de energia. Adicionalmente, a 8-(3-fenilpropil)-1,3,7-trietilxantina reduziu a oxidação proteica, revelando propriedades antioxidantes e um possível papel protetor contra o stress oxidativo. Por outro lado, a cafeína exibiu comportamentos pro-oxidantes acompanhados pelo aumento da oxidação proteica.

Em suma, o composto 8-(3-fenilpropil)-1,3,7-trietilxantina aparenta ser um ótimo candidato para o *design* de fármacos anti-obesidade seguros e inovadores.

Abstract

Obesity has been increasing in the last decades and is one of the most prolific health concern worldwide. Methylxanthines, such as caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine), have demonstrated potential anti-obesity properties. These natural compounds are widely distributed in the human diet, especially food products such as coffee, tea, and chocolate. In fact, caffeine is known to modulate glucose and fatty acid metabolism and its consumption seems to be inversely associated with body weight increase. Based on methylxanthines chemical structure, several xanthine analogues have been synthetized. We hypothesized that one of those compounds, 8-(3-phenylpropyl)-1,3,7-triethylxanthine, may have a promising anti-obesity potential. Our study aims to characterize the modulation conferred by 8-(3-phenylpropyl)-1,3,7-triethylxanthine in the metabolic and oxidative profile of adipocytes in order to evaluate its potential as a pharmacological option to address obesity and its complications. For this purpose, the anti-obesogenic potential of 8-(3-phenylpropyl)-1,3,7-triethylxanthine was evaluated in mouse preadipocyte cell line 3T3-L1, using synthetic caffeine for comparative purposes. Cells were cultured in the presence of increasing concentrations of caffeine or 8-(3-phenylpropyl)-1,3,7-triethylxanthine (0.1, 1, 10 and 100 μM) and the cytotoxic profile was accessed spectrophotometrically by reduction of tetrazolium salt (MTT) and quantification of released lactate dehydrogenase (LDH). The metabolites in culture medium were identified and quantified by proton nuclear magnetic resonance (^{1}H-NMR) and cells were collected for analysis of the oxidative profile. Our results show that 8-(3-phenylpropyl)-1,3,7-triethylxanthine presented no cytotoxicity at all studied concentrations. When compared with caffeine, 8-(3-phenylpropyl)-1,3,7-triethylxanthine significantly increased glucose, pyruvate, and glutamine consumption, and lactate, alanine, and acetate production. These findings illustrate that 8-(3-phenylpropyl)-1,3,7-triethylxanthine has a high potential to act as a metabolic modulator, even when compared with caffeine. Additionally, 8-(3-phenylpropyl)-1,3,7-triethylxanthine promoted an antioxidant environment, decreasing protein oxidation, and protecting against oxidative stress-induced damage. Thus, 8-(3-phenylpropyl)-1,3,7-triethylxanthine appears as a promising candidate for new and safe anti-obesity drug design.

Keywords

8-(3-phenylpropyl)-1,3,7-triethylxanthine; Adipocyte metabolism; Anti-obesity drug; Caffeine; Obesity; Xanthine.

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^{13}C-NMR	Carbon-13 nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
4-HNE	4-hydroxynonenal
5'AMP	5' adenosine monophosphate
AC	Adenylyl cyclase
ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotropic hormone
ADSCs	Adipose-derived stem cells
Akt	Protein kinase B
AMPK	5'AMP-activated protein kinase
aP2	Adipocyte protein 2
AR	Adenosine receptor
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	ccaat enhancer binding protein
C/EBPα	ccaat enhancer binding protein alpha
C/EBPβ	ccaat enhancer binding protein beta
C/EBPδ	ccaat enhancer binding protein delta
CAF	Caffeine
cAMP	Cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
DNP	2,4-dinitrophenyl
DNPH	2,4-dinitrophenylhydrazine
EDAC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
ERK	Extracellular signal-regulated kinase
ETX	Synthetic xanthine
FDA	Food and Drug Administration
GLUT4	Glucose transporter type 4
GPDH	Glycerolphosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
HSL	Hormone-sensitive lipase
IBMX	3-Isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor 1

IR	Insulin receptor
K_i	Inhibitory constant
LDH	Lactate dehydrogenase
MGL	Monoacylglycerol lipase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methylxanthine
NCS	Newborn calf serum
OS	Oxidative stress
PBS	Phosphate buffered saline
PDE3B	Phosphodiesterase 3B
PDE4	Phosphodiesterase 4
PFK-2	Phosphofructokinase-2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PPARγ	Peroxisome-proliferator activated receptor gamma
PVDF	Polyvinylidene difluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TAG	Triacylglycerol
TFA	Trifluoracetic acid
UV	Ultra violet
VLDL	Very low-density lipoprotein

I. Introduction

I. Introduction

1.1. The human adipose tissue

The adipose tissue is a metabolically active organ that acts as the main energy repository in the human body and as an endocrine organ able to synthesize several biologically active molecules that regulate metabolic homeostasis (Coelho et al., 2013). Different cell types compose this dynamic and complex organ (Figure 1A), including adipocytes, which are the dominant cells, preadipocytes, vascular endothelial cells, pericytes, macrophages and fibroblasts (Cedikova et al., 2016; Geloen et al., 1989).

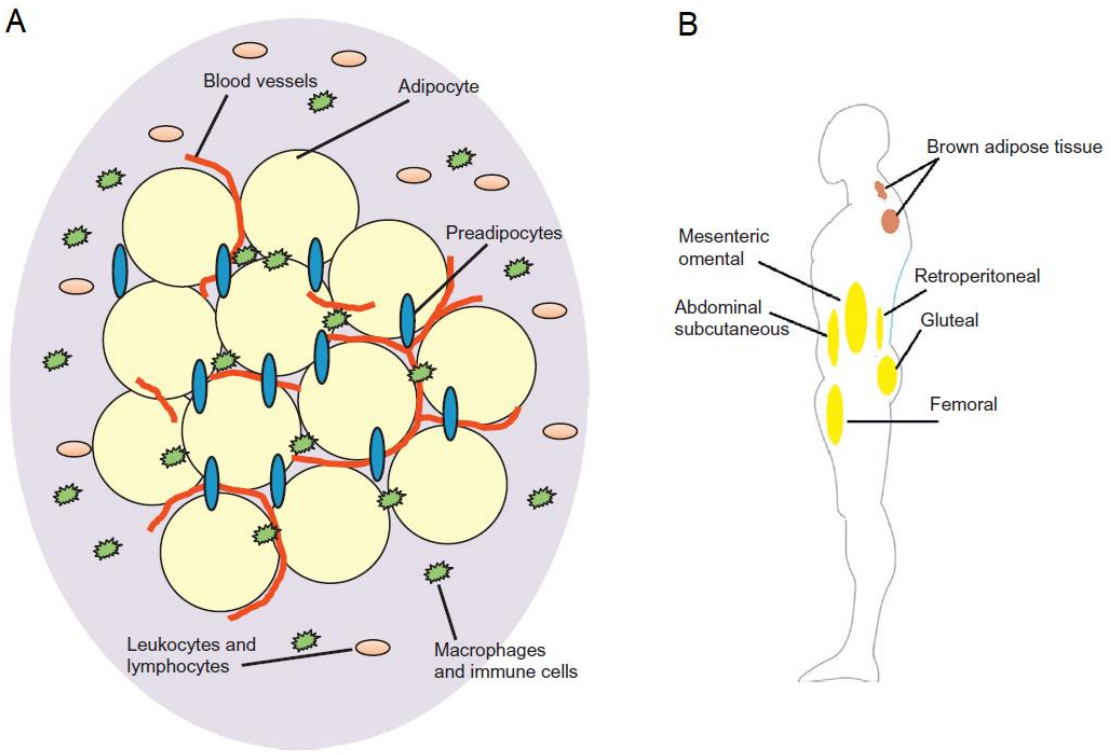

Figure 1 – Anatomy of the adipose tissue and its composition in humans. **(A)** The adipose tissue is highly vascularized and composed by different cell types, such as adipocytes, preadipocytes and immune cells. **(B)** Brown and white adipose tissue are distributed through the human body. The brown adipose tissue is mainly located in the interscapular region, while the white adipose tissue is mainly located subcutaneously. The main depots of white adipose tissue are located in the abdominal and gluteal-femoral region, or near the kidney and digestive organs, constituting the retroperitoneal or mesenteric and omental adipose tissue, respectively (adapted from Tsiloulis & Watt, 2015).

The adipose tissue fulfils several functions, which may vary among fat depots due to its size, distribution, and heterogeneity according to their molecular, morphological, and metabolic profiles (Ibrahim, 2010). In humans, there are two main types of adipose tissue, white and brown, with several differences in adipocytes' morphology and function

(Figure 1B). The brown adipose tissue is mainly located in the interscapular region and its size is reduced from birth to adulthood (Coelho et al., 2013). While this tissue also stores energy in form of fat, its main activity is heat production, or thermogenesis. Brown adipocytes are described as being smaller in comparison to white adipocytes, with relatively abundant cytoplasm, lipid droplets of different sizes and numerous mitochondria that produce heat by fatty acids oxidation (Saely et al., 2012). Although its participation in thermogenesis is nearly irrelevant, white adipose tissue presents much broader functions. White adipose tissue is widely distributed in the human body. Most of this tissue is located subcutaneously, storing 80% of total body fat, with the main depots found in the abdominal and gluteal-femoral region. The remaining 20% are located around the digestive organs, constituting the visceral adipose tissue (mesenteric and omental), and around the kidney (retroperitoneal) (Tsiloulis & Watt, 2015). By involving organs and infiltrating tissues, the white adipose tissue not only offers mechanical protection, but also plays an important role in the regulation of the body temperature, acting as a thermal insulator (Fonseca-Alaniz et al., 2007; Saely et al., 2012). Furthermore, white adipose tissue accomplishes multiple other functions, such as immune, endocrine and regenerative (Coelho et al., 2013; Thomou et al., 2010).

Although the white adipose tissue presents several functions, its main function is storing and releasing energy in response to the energetic needs, acting as a buffer for energy imbalance not only in cells, but also in whole-organisms. White adipose tissue stores energy whenever there is a surplus and releases energy for other organs whenever needed, such as in fasting conditions. This energy storage takes place in the form of triacylglycerol within adipocyte lipid droplets, which occupies most of the adipocyte volume. On the other hand, energy is released in the form of free fatty acids (or non-esterified fatty acids) and glycerol (Tsiloulis & Watt, 2015). These are highly efficient energy storage species, since they can be readily oxidized to produce large quantities of adenosine triphosphate (ATP) (Kiess et al., 2008).

Fat accumulation and the size of the white adipose tissue is determined by the balance between triacylglycerols synthesis and its breakdown into free fatty acids and glycerol (Figure 2). Both these pathways are controlled by a complex protein-protein interactions, intracellular signalling and neurohumoral regulators (Lass et al., 2011). Lipogenesis is the process that results in the production and storage of energy in the form of triacylglycerol within adipocyte lipid droplets. This process is stimulated when there is a surplus of energy, with high free fatty acids and glucose concentration in bloodstream (Kersten, 2001). With high glucose concentration, insulin's release is stimulated and insulin-dependent glucose transporter 4 (GLUT4) transports glucose into the adipocytes. Glycolysis occurs and glycerol-3-phosphate is produced. Together with fatty acids coming from the liver and intestines, both substrates are esterified to form lipid droplets of triacylglycerol (Bernlohr et al., 2002). On the other hand, in fasting state or when there is a lack of energy, lipolysis occurs. Triacylglycerol is hydrolysed to glycerol and fatty acids for energy production (Tsiloulis & Watt, 2015). Glycerol is transported out of adipocytes via aquaporin-type

molecules and is used in the liver for oxidation or gluconeogenesis. Released fatty acids are carried by albumin to the liver, muscles and other tissues for oxidation, being converted into acetyl coenzyme A, in a process designated ß-oxidation (Bernlohr et al., 2002; Laclaustra et al., 2007).

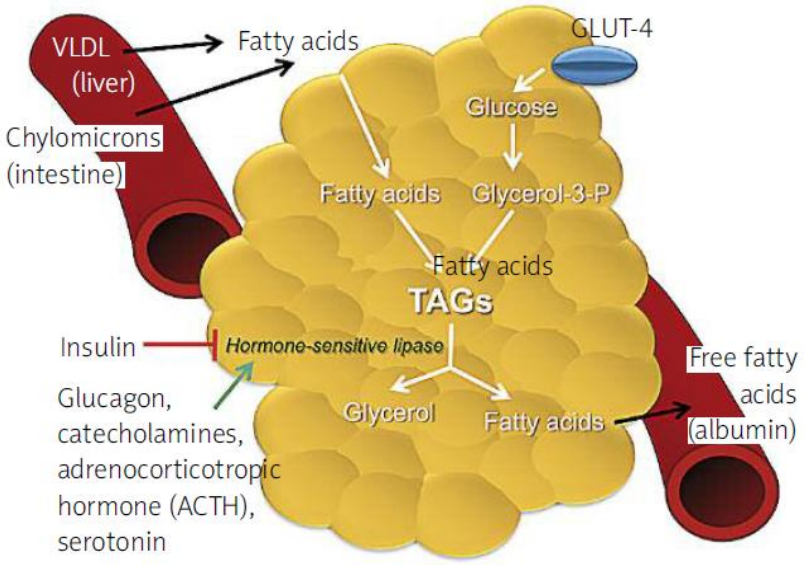

Figure 2 – White adipose tissue as an energetic buffer. When there is an energy surplus, there is an uptake of glucose by adipocytes, through GLUT4. Glycolysis occurs and glycerol-3-phosphate is produced. Glycerol-3-phosphate and fatty acids, that are carried by VLDL from the liver and chylomicrons from the intestine, are esterified to form TAGs, which is stored in lipid droplets. However, when energy is needed, TAGs are hydrolysed into glycerol and fatty acids, which are released and transported to be oxidized and produce energy. In the bloodstream, the fatty acids are bounded and transported by albumin. Both the lipogenic and lipolytic pathways are regulated by several molecules, activating or inhibiting key molecules in the process, such as one of the most important enzymes in the TAG hydrolysis, the hormone-sensitive lipase. For instance, insulin is known to inhibit its activity, stimulating the accumulation of lipids in the adipocyte. On the other hand, several other molecules may stimulate lipogenesis, such as glucagon, catecholamines, ACTH or serotonin (adapted from Coelho et al., 2013). *Abbreviations:* ACTH – Adrenocorticotropic hormone; Glycerol-3-P – glycerol-3-phosphate; GLUT4 – glucose transporter 4; TAGs – triacylglycerols; VLDL – very low-density lipoprotein.

1.2. Obesity

Obesity is clinically defined by the excessive accumulation of body fat. Obesity usually leads to an impaired health status, evidencing the deleterious impact of this chronic condition. The increasing prevalence of obesity makes it a global concern among modern societies (do Carmo et al., 2008; Ng et al., 2014). It is estimated that in 2015 more than 2.2 billion individuals were overweight (body mass index (BMI) from > 25 kg/m^2), where over 710 million of these were obese (BMI > 30 kg/m^2) (Afshin et al., 2017). This condition has multifactorial causes that include genetic, epigenetic, physiological, sociocultural, and environmental factors. Overall, obesity is a consequence of a positive energy balance that occurs when the energy intake is higher than the expenditure. This scenario is generated by

an excessive consumption of food, while having a lack of physical activity, thus leading to the accumulation of the extra energy as fat (Bray et al., 2016; Pi-Sunyer, 2002). Genetic predisposition is also an important factor to consider in the onset of obesity.

White adipose tissue mass accumulation is associated with a bigger size and a higher number of adipocytes. When the organism has an energy surplus it is accumulated as lipids in adipocytes, which then suffer hypertrophy. This white adipose tissue buffering activity is an adaptive response to energy excess, which protects other tissues from lipotoxicity (Chavey et al., 2013). The maintenance of white adipose tissue homeostasis also includes pre-adipocyte hyperplasia, where these cells proliferate and differentiate into mature adipocytes (adipogenesis) (Nishimura et al., 2007). Simultaneously, there is an excessive extracellular matrix deposition in the form of fibrosis (Figure 3), which seems to limit cell hypertrophy and thus promote adipocyte hyperplasia. In fact, it has been suggested that hyperplasia and hypertrophy may be reciprocally regulated, although the mechanisms underlying these processes are still poorly understood (Muir et al., 2016). Altogether, obesity depends on the hypertrophy of pre-existing adipocytes, but also on hyperplasia, since there is a formation of new adipocytes from precursor cells through adipogenesis. However, in severe obesity conditions, a hypertrophic threshold may be reached, exceeding adipocyte buffering capacity and leading to decreased pre-adipocyte proliferative capacity, impaired metabolic functions, ectopic lipid deposition, inflammation, and dysregulated leptin and adipokines secretion (Cotillard et al., 2014; Landgraf et al., 2015; Ryden et al., 2014).

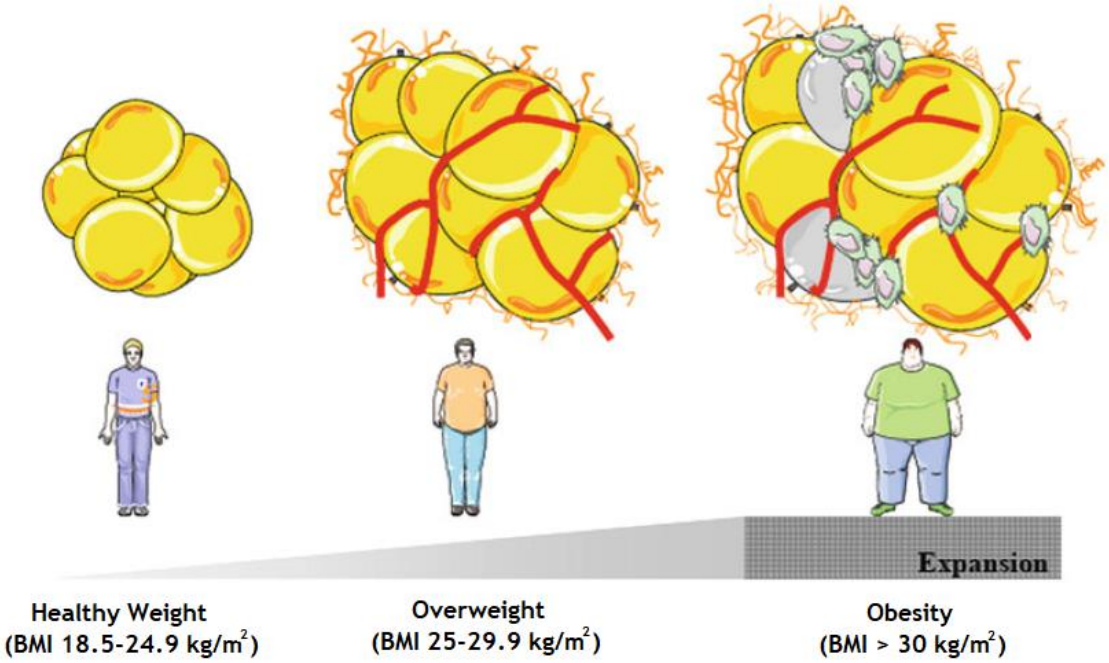

Figure 3 – White adipose tissue expansion during obesity scenarios. This mass accumulation is associated with an increase in size and number of adipocytes, fibrosis, impaired metabolic and endocrine functions, inflammation and hypoxia (adapted from Alligier et al., 2013).

A decreased capacity for lipid clearance accompanies the increased lipid storage due to the dysregulation of lipolysis, which leads to increasing lipid accumulation. The white adipose tissue expandability hypothesis supports the argument that if an individual has the capacity to store fat in this tissue, the process is harmless and there is no ectopic deposition of lipids with resulting metabolic complications (Langin, 2011; Langin & Mouisel, 2013; Virtue & Vidal-Puig, 2008). However, when this storage capacity is overridden, there is an ectopic deposition of lipids in the skeletal muscle and in the liver (Figure 4). This ectopic lipid deposition may favour several complications through lipotoxic mechanisms, including insulin resistance and ultimately diabetes mellitus (type 2) (Castro et al., 2014; Zimmet et al., 2001), cardiovascular dysfunctions (Luna-Luna et al., 2015) and infertility (Esmaeilzadeh et al., 2014; Mission et al., 2015; Palmer et al., 2012).

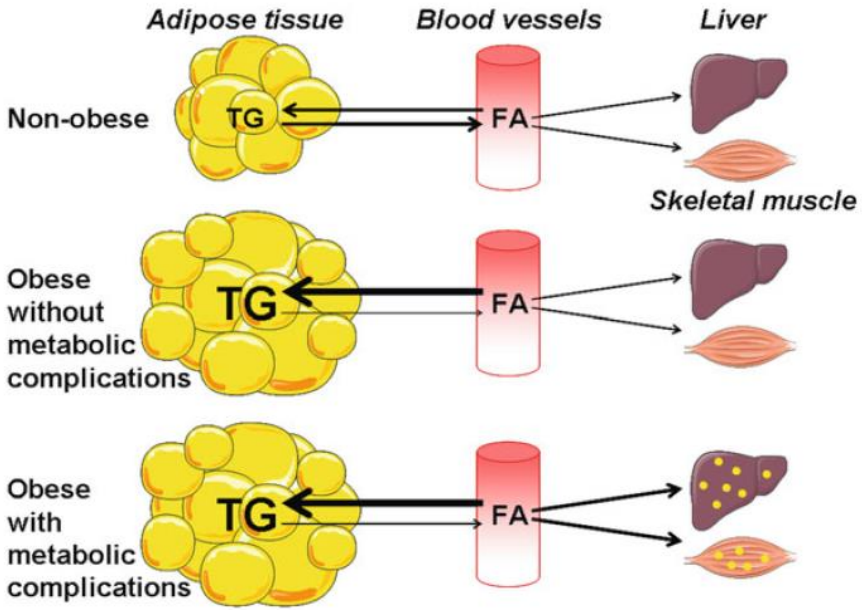

Figure 4 – Evolution from non-obese to severe obesity with ectopic lipid deposition. Increased fat storage and reduced lipolysis increases the fat accumulation in obesity. If the white adipose tissue capacity of storage is overcome, there is ectopic lipid deposition in the skeletal muscle and liver, a metabolic complication that may lead to diabetes and cardiovascular dysfunctions (adapted from Langin & Mousiel, 2013). *Abbreviations:* FA – Fatty acids; TG – triacylglycerols.

Thus, it is crucial to prevent and treat obesity. Currently, low-calorie diets and increased physical activity are the ground stones in obesity treatment. The pharmacological approaches to address obesity are scarce and the existing drugs with anti-obesity potential are prone to tolerance and only provide short-term weight loss (Bray, 2008; Snow et al., 2005). Although several possible targets for obesity treatment have been recently identified (Monteiro, 2014), there is still a need to find new, safe and cost-effective therapeutic approaches.

The study of natural products along the years has evidenced their preventive and therapeutic potential in the control of obesity. A variety of medicinal plants, fruits, and

vegetables have been used in different anti-obesity products as food supplements to promote weight loss (de Resende et al., 2015; Sun et al., 2016). Methylxanthines constitute a group of natural occurring molecules with growing interest for researches in this field. These compounds are naturally produced by both animals and plants, being caffeine, theobromine and theophylline the most well-studied (Monteiro et al., 2016). These compounds can be found in tea leaves (*Camellia sinensis* L.), cocoa (*Theobroma cacao* L.), and coffee beans (*Coffea* sp.), food products that are consumed worldwide on a daily basis. Moreover, methylxanthines have proven to have biological effects that could contribute not only for obesity management (Liu et al., 2015), but also for neurological (Fredholm et al., 1999; Nehlig et al., 1992; Roll, 1980), respiratory (Barnes, 2013; Dent et al., 1994), cardiac (Moffat, 1986; Roll, 1980) and renal disorders (Osswald & Schnermann, 2011), as well as in male reproductive function (Brokaw, 1987; Dias et al., 2015; Dias et al., 2014; Yamaguchi et al., 2009).

1.3. Methylxanthines

1.3.1. Chemical structure, biosynthesis, properties, and natural sources

Methylxanthines are methylated xanthine-derivatives that may be produced in plants and animals and can be found in most human fluids and tissues (Zrenner et al., 2006). These purine bases are heterocyclic complexes that result from the coupling of pyrimidinedione with imidazole rings (Talik et al., 2012). Among the most well-known methylxanthines (Figure 5) are 1,3,7-trimethylxanthine, 1,3-dimethylxanthine, and 3,7-dimethylxanthine, also commonly recognized as caffeine, theophylline, and theobromine respectively.

In plants, xanthosine is the primary substrate for methylxanthines' biosynthesis. Xanthosine can be produced through the degradation of adenine nucleotides, adenosine and guanosine nucleosides, and/or *de novo* purine biosynthesis (Ashihara et al., 2011). The pathway that leads to caffeine production starts with xanthosine methylation to 7-methylxanthosine, then this ribose is hydrolysed to form 7-methylxanthine and subsequent methylations yield theobromine and, finally, caffeine (Koshiishi et al., 2001; Misako & Kouichi, 2004). While theobromine may be a precursor of caffeine, theophylline can be considered as a catabolite. In almost all caffeine-producing plants, caffeine is degraded by three consecutive demethylations, with the formation of theophylline in the process (Ashihara et al., 2011). On the other hand, the major demethylated metabolite of caffeine in animals is paraxanthine (1,7-dimethylxanthine), an isomer of theobromine and theophylline, but it is not naturally produced by plants (Monteiro et al., 2016). Additionally, caffeine (Zajac et al., 2003), theobromine (McKeague et al., 2016), theophylline (Nanjundaiah et al., 2016) and paraxanthine (Müller et al., 1998) can also be obtained by chemical synthesis.

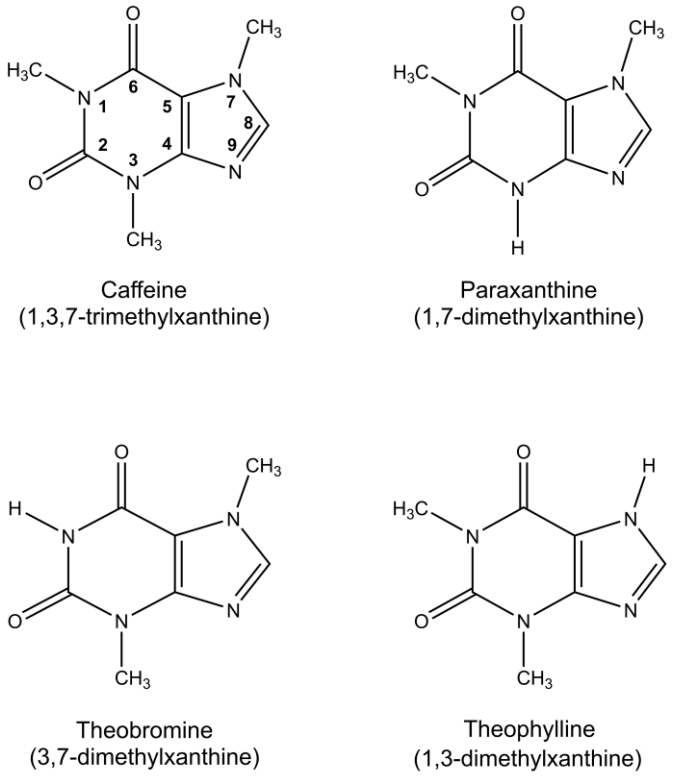

Figure 5 - Chemical structures of the three predominant methylxanthines (caffeine, theobromine and theophylline) and paraxanthine, the major dimethylated metabolite of caffeine.

Methylxanthines are considered weak Brønsted bases due to the imino nitrogen at position 9. Caffeine possesses methyl groups at position 1, 3 and 7, which confers electrophilic properties. Although theophylline shares the same electrophilic sites at position 1 and 3, it has a proton at position 7 instead of a methyl group, becoming a Brønsted acid site (Monteiro et al., 2016). For that reason, only theophylline may become a proton donor. In fact, this molecule acts as a proton donor in most pharmaceutical systems (Beale & Jr John, 2011). Theobromine differs from both compounds due to the absence of the methyl group at position 1. The presence of this methyl group was reported to be the responsible of several physicochemical properties of caffeine and its physiological effects (Pavia, 1973). Additionally, caffeine structure comprises lipophilic properties (Salihović et al., 2014), which should improve its permeability through cell membranes and cross biological barriers, such as the blood-brain barrier (McCall et al., 1982). Several synthetic modifications of these natural compounds have been produced to pursue pharmacological purposes, resulting in drugs like dyphylline, proxyphylline and enprofylline. Moreover, novel modifications are being investigated for different therapeutic applications (Baraldi et al., 2007; Moro et al., 2006), thus enhancing the interesting properties of these compounds in health promotion.

Plants containing methylxanthines are common in food products daily ingested worldwide, such as coffee, tea, cocoa, and chocolate. Coffee and tea are globally consumed and are the principal sources of caffeine intake through diet (Frary et al., 2005). Although

coffee is usually expected to contain more caffeine than tea, it can be found in considerable amounts in certain types of tea (*Camellia Sinensis* species). One study (Gilbert et al., 1976) showed that the median caffeine concentration in tea is 27 mg per cup (median size of 225 mL), while in coffee is 74 mg per cup (median size of 200 mL). Theobromine is the predominant methylxanthine found in cocoa beans, where it represents about 2% of its dry weight. Furthermore, some *Camellia* species contain more theobromine than caffeine (Ashihara et al., 2008). Despite caffeine and theobromine are prominent in dietary sources, the same does not happen with theophylline. This methylxanthine is naturally present in tea leaves and in vestigial amounts in cocoa and coffee beans (Barnes, 2013). For that reason, only few amounts of this methylxanthine are thought to be obtained through diet (Stavric, 1988). Although coffee, tea and cocoa are the most well-known plant-derived products containing methylxanthines, there are other plants reported as methylxanthines producers, such as *Paullinia* sp. (guarana), *Cola* sp. (cola beverages), *Ilex paraguariensis* A. St.-Hil. (mate), and *Citrus* sp. (Atawodi et al., 2007; Baumann et al., 1995; Kretschmar & Baumann, 1999; Weckerle et al., 2003).

Methylxanthines have been described to exert multiple physiological effects in the human body, including in the nervous (Fredholm et al., 1999; Nehlig et al., 1992; Roll, 1980), respiratory (Barnes, 2013; Dent et al., 1994) and cardiac systems (Moffat, 1986; Roll, 1980). They stimulate the skeletal muscle and promote diuresis (Tarka, 1982). Male fertility is another field in which these compounds may have positive outcomes. For instance, methylxanthines have been described to be beneficial for sperm Ca^{2+} transport (Tash & Means, 1982). Moreover, methylxanthine-rich beverages, such as tea, and caffeine were also reported to be effective additives for sperm storage and *in vitro* fertilization (Brokaw, 1987; Dias et al., 2014; Yamaguchi et al., 2009). Caffeine by itself has also improved the nutritional support of spermatogenesis by Sertoli cells (Dias et al., 2015). Some of these beneficial effects were associated with methylxanthines antioxidant properties (Azam et al., 2003; Grucka-Mamczar et al., 2009; Leon-Carmona & Galano, 2011; Ofluoglu et al., 2009). More recently, the anti-obesity potential of methylxanthines has also been reported. These compounds may interact with the adipose tissue and its effects could comprise a promising therapeutic approach for obesity management.

1.3.2. Anti-obesity potential

In the past couple of decades, the search for new pharmacological tools to address obesity has increased. Phytochemicals, such as methylxanthines, have been reported to promote weight loss in obese individuals, thus attracting the interest of several researchers. For instance, it has been reported that trained and untrained young (20-40 years) males consuming 4 mg/kg of caffeine after an overnight fast demonstrated increased resting metabolic rate values, although the magnitude of this effect was greater in trained subjects

(LeBlanc et al., 1985). Higher levels of free fatty acids were also observed in the plasma, as well as increased lipid oxidation, due to enhanced lipolysis. Furthermore, when caffeine consumption is associated with physical exercise, the effects are exacerbated (Schubert et al., 2014). A controlled trial was performed with lean individuals (18-45 years) taking 3 mg/kg of caffeine 90 min before, and 30 min after doing physical exercise. After performing physical activity (1 h of cycling, with periods of rest 1 h before and 2 h after), the participants had a test meal in *ad libitum* conditions. It was shown that the combination of physical exercise and caffeine supplementation led to an increase in energy expenditure and fat loss relative to placebo. In addition, when exposed to caffeine the participants reduced the energy and fat intake and the exercise was perceived as less difficult and more enjoyable. Another study (Gavrieli et al., 2013) showed that obese and overweight individuals consumed less food in an *ad libitum* meal after an intake of 2-4 cups of coffee than those with lower or no coffee consumption, which suggest that caffeine may have appetite suppressing effects. Besides, caffeine intake has been linked with an increased energy expenditure per day in lean subjects, but also in post-obese participants during weight loss maintenance (Dulloo et al., 1989). Caffeine also demonstrated to improve weight loss, when following a diet with caloric restriction, and prevent weight regain (Davoodi et al., 2014). Overweight females having a calorie shifting diet for 6 weeks and then a follow-up diet for 4 weeks with caffeine treatment (5 mg/kg/day) or placebo, demonstrated that after the calorie shifting diet period, the females consuming caffeine exhibited more weight and fat loss in comparison with the placebo group. Moreover, after the follow-up diet period, the weight loss on the caffeine group persisted, while the placebo group weight was regained.

Methylxanthines' consumption can be also combined with other natural compounds for superior results (Zheng et al., 2004). Female mice were fed with a standard powder diet containing several combinations of caffeine, green tea catechins and L-theanine for 16 weeks. The combination of 0.05% caffeine and 0.3% green tea's catechins was the most effective for preventing weight gain, in comparison with the normal diet group from the 4^{th} to the 16^{th} week. Furthermore, it was also shown that a diet with a theobromine-rich cocoa powder promoted weight loss in Wistar rats, compared to control (Eteng et al., 2006).

Altogether, the molecular activity of methylxanthines seems to lead to the stimulation of lipolysis and inhibition of adipogenesis, decreasing the accumulation of the adipose tissue. By this means, methylxanthines can promote weight loss in overweight and obese subjects. Several mechanisms were proposed to explain their actions, which will be addressed on the following topics.

1.3.3. Lipolytic activity

1.3.3.1. Adenosine receptors antagonism

Lipolysis is the pathway that leads to free fatty acids and glycerol release from triacylglycerols, being unique to adipocytes. One known regulator of lipolysis is the adenosine receptor (AR), which can be divided in four types: A_1, A_{2A}, A_{2B} and A_3. These are present in every cell, with the four types exhibiting a differential cell and tissue expression (Olah & Stiles, 1995). Adenosine is an endogenous nucleoside released from adipose tissue during sympathetic nerve activation. In 1972, it was shown for the first time that adenosine and its analogues could inhibit lipolysis in adipocytes (Fain et al., 1972). Later, it was reported that this lipolysis inhibition by adenosine was mainly mediated by activation of the A_1R. Moreover, it has been demonstrated that these receptors are important regulators of lipolysis, fatty acid storage and tissue partitioning of fat (Johansson et al., 2007). Adipose tissue has a high expression of A_1R, which are functionally active in differentiated adipocytes (Trost & Schwabe, 1981; Vassaux et al., 1993). However, accumulating evidence indicates that the number of receptors may differ among fat repositories. Data from animal studies reported much higher affinity and binding capacity of white adipose tissue for one specific A_1R agonist in comparison to brown adipose tissue, suggesting that white adipose tissue might have a higher expression of A_1R than brown adipose tissue (Saggerson & Jamal, 1990). The human subcutaneous adipose tissue showed a higher number of receptors than the visceral adipose tissue, supporting the hypothesis that the A_1R may exert a major role in the regulation of subcutaneous fat storage (Barakat et al., 2006). Furthermore, it has been proposed that these receptors are efficiently coupled with a G_i protein in adipocytes and cannot be affected independently (Liang et al., 2002). This feature suggests that G proteins inactivation cannot be overcome by the activation of a higher number of A_1R (Dhalla et al., 2009). Besides, functional uncoupling of A_1R from these proteins leads to the synthesis of more receptors (Jajoo et al., 2006). Each receptor appears to activate more than one G protein and signal amplification seems to be independent of the total number of receptors (Baker et al., 2000). Overall, in adipocytes, A_1R activation by its agonists (e.g. adenosine) inhibits of the adenylyl cyclase activity, which consequently reduces cyclic adenosine monophosphate (cAMP) formation, inhibits protein kinase A (PKA) and, ultimately, blocks phosphorylation of the lipases involved in lipolysis, leading to its inhibition (Dhalla et al., 2009). Additionally, adenosine and its analogues might modulate insulin action and sensitivity in adipose tissue through A_1R, by increasing insulin sensitivity and potentiating insulin-induced activation of phosphoinositide 3-kinase (PI3K), resulting in the decrease of cAMP concentration in adipocytes (Budohoski et al., 1984; Rolband et al., 1990; Takasuga et al., 1999). On the other hand, while A_1R and A_3R stimulation leads to lower concentrations of cAMP via G_i proteins, the stimulation of $A_{2A}R$ and $A_{2B}R$ increases cAMP concentration via G_s proteins, translating into lipolysis (Fredholm, AP, et al., 2001). However, the expression of these receptors during

adipocyte differentiation is low and, consequently, its role in lipolysis is reduced (Borglum et al., 1996).

A vital mechanism of methylxanthines' anti-obesity activity is the non-specific antagonism of AR, as these molecules are able to block and inhibit, in a competitive manner, the role of adenosine in the cells, thus stimulating lipolysis (Figure 6A) (Chen & Chern, 2011). Methylxanthines have the capacity to inhibit the four subtypes of AR at physiological doses (<100 μM), but most of their actions seem to be mediated through the inhibition of A_1 and A_{2A} subtypes (Sattin & Rall, 1970). The AR inhibitory effects are mostly mediated by the 1-methyl group of methylxanthines structure (Green & Stanberry, 1977). In fact, caffeine and theophylline were shown to be potent inhibitors of AR (Boulenger et al., 1982). Still, in other studies (Daly et al., 1983; Fredholm, Irenius, et al., 2001; Klotz et al., 1997) theophylline and paraxanthine were reported to have slightly higher affinities than caffeine for the A_1R (in similar concentrations). On the other hand, theobromine does not possess the 1-methyl group and was described to have lower affinity for A_1R and $A_{2A}R$ subtypes (Carney et al., 1985; Schwabe et al., 1985; Shi & Daly, 1999).

Additionally, methylxanthines are also nonselective competitive inhibitors of phosphodiesterases at pharmacological doses (> 1 mM) (Beavo et al., 1970; Butcher & Sutherland, 1962; Cardinali, 1980; Nicholson et al., 1989). Phosphodiesterases are responsible for hydrolysing cAMP and have a central role in regulating cAMP signalling, most of which is accountable to phosphodiesterase-4 (Wu & Rajagopalan, 2016). Reversible inhibition of phosphodiesterases by methylxanthines impairs the hydrolysis process, preventing the degradation of cAMP and consequently increasing its concentration (Sassone-Corsi, 2012). Therefore, phosphodiesterase-4 inhibition increases lipolysis via activation of hormone-sensitive lipase that is induced by the increased cAMP concentration (Figure 6A) (Wu & Rajagopalan, 2016). The three natural-occurring methylxanthines are described as competitive inhibitors of phosphodiesterases, where theophylline is reported to be a more potent inhibitor than caffeine (Daly, 2007; Stavric, 1988).

A possible drawback of methylxanthines use is the increased release of free fatty acids into circulation as an end product of lipolysis that is observed after A_1R antagonism. Ultimately, this scenario could potentially lead to the worsening of insulin resistance (Dhalla et al., 2009). Interestingly, A_1R antagonists were reported to improve glucose tolerance, which could be attributed to a selective increase in the adipose tissue receptors (Xu et al., 1998).

While natural occurring methylxanthines have affinity for the AR, synthetic compounds based on the xanthine chemical structure have been developed, evidencing more potent and selective antagonism activity for all four receptor subtypes (Moro et al., 2006). In general, substitutions at position 8 with aryl or cycloalkyl groups were reported as promising potential for the identification of novel adenosine A_1R and $A_{2A}R$ antagonists. Conversely, it was concluded that ethyl substitution at the positions 1, 3 and 7 may enhance adenosine A_1R affinity when compared to methyl substitutions (Baraldi et al., 2007; Van der Walt &

Terre'Blanche, 2015). Moreover, several ring-extended xanthines have been developed to increase phosphodiesterase-4 inhibition (in the nM range) (Arnold et al., 2002; Pissarnitski et al., 2004).

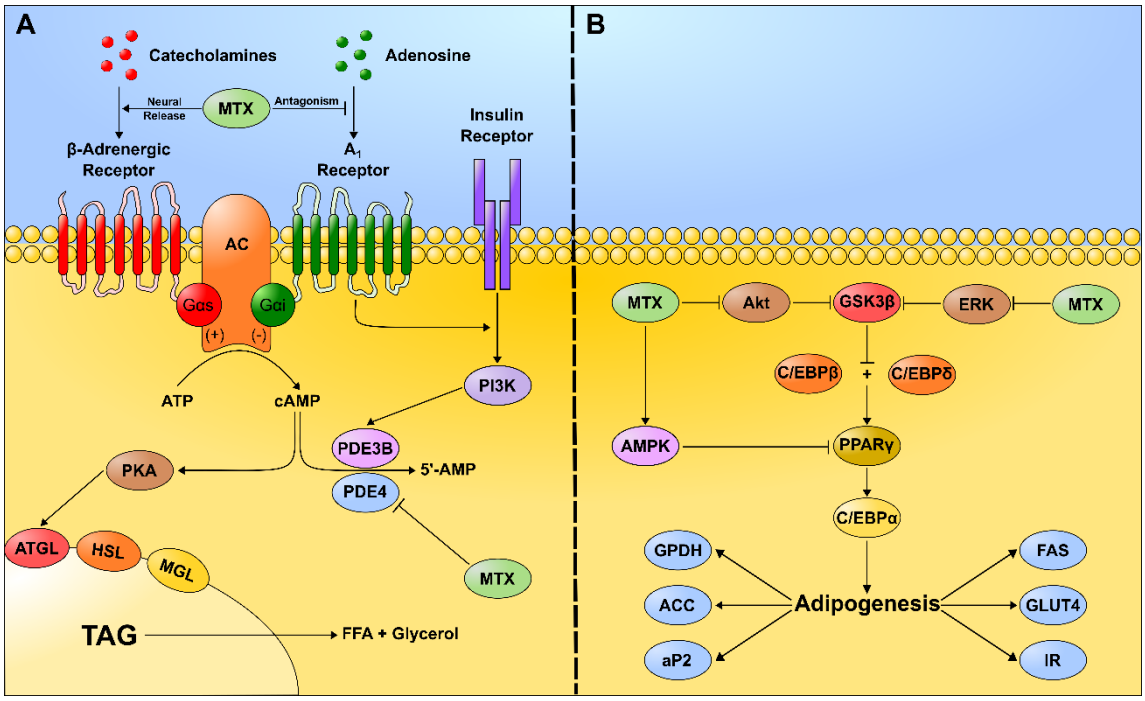

Figure 6 - Schematic illustration of methylxanthines' activity in adipocytes. Methylxanthines can stimulate lipolysis (A) due to the inhibition of A_1 receptors, increased concentration of catecholamines and inhibition of PDE4. This results in an increased cAMP concentration, which activates PKA and the lipases involved in lipolysis. Methylxanthines were also reported to inhibit adipogenesis (B), by disturbing the Akt and ERK axis and by activating AMPK. Altogether, its action leads to the decrease expression of C/EBPα and PPARγ, the two main adipogenic transcription factors. Abbreviations: 5'AMP - 5' adenosine monophosphate; AC - Adenylyl cyclase; ACC - Acetyl-coA Carboxylase; Akt - Protein kinase B; AMPK - 5'AMP-activated protein kinase; aP2 - Adipocyte protein 2; ATGL - Adipose triglyceride lipase; ATP - Adenosine triphosphate; C/EBPα - ccaat enhancer binding protein alpha; C/EBPβ - ccaat enhancer binding protein beta; C/EBPδ - ccaat enhancer binding protein delta; cAMP - Cyclic adenosine monophosphate; ERK - Extracellular signal-regulated kinase; FFA - Free fatty acids; FAS - Fatty acid synthase; GLUT4 - Glucose transporter type 4; GPDH - Glycerolphosphate dehydrogenase; GSK3β - Glycogen synthase kinase 3 beta; HSL - Hormone-sensitive lipase; IR - Insulin receptor; MGL - Monoacylglycerol lipase; MTX - Methylxanthine; PDE3B - Phosphodiesterase 3B; PDE4 - Phosphodiesterase 4; PI3K - Phosphoinositide 3-kinase; PKA - Protein kinase A; PPARγ - Peroxisome-proliferator activated receptor gamma; TAG - Triacylglycerols.

1.3.3.2. Modulation of catecholamines' release

Catecholamines, such as adrenaline and noradrenaline, are major regulators of lipolysis in humans (Morigny et al., 2016). These compounds activate or inhibit lipolysis by bounding/unbounding to adrenergic receptors. Adrenergic receptors can be divided in two major types, α and β, each with several subtypes (Langin, 2006). In general, when β-adrenergic receptors are stimulated, a lipolytic response is triggered by its action on the stimulatory G alpha (G_s) subunit of heterotrimeric G proteins. Subsequently, adenylyl cyclase is activated and the conversion of ATP into cAMP is catalysed (Mauriege et al., 1988). On the

other hand, the activation of α-adrenergic receptors induces an antilipolytic signal, since they are coupled with inhibitory G alpha (G_i) subunit of heterotrimeric G proteins, thus inhibiting adenylyl cyclase activity and cAMP production (Stich et al., 1999). Therefore, lipolysis regulation relies on the relative affinity of catecholamines for the distinct adrenergic receptors and on the expression of each receptor on the adipocytes' membrane (Morigny et al., 2016).

Methylxanthines can stimulate the sympathetic nervous system, leading to the release of noradrenaline and activating the ß-adrenergic receptors (Acheson et al., 2004). It has been observed that caffeine increases catecholamine levels (Chen et al., 1994). Moreover, catecholamines and caffeine can act synergistically, increasing cAMP concentration in a superior extent than induced by the hormones alone, thus further promoting lipolytic activity (Figure 6A) (Butcher et al., 1968). In addition, methylxanthines have been shown to potentiate the effects of ephedrine (the most active alkaloid of *Ephedra* sp.), an α- and ß-adrenergic receptor agonist (Greenway, 2001). This sympathomimetic agent, originally used as a bronchodilator, was found to stimulate lipolysis and induce weight loss in asthmatic patients. Ephedrine also stimulates the release of noradrenaline, which then binds to ß-adrenergic receptors on adipocytes (Diepvens et al., 2007). A combined administration of methylxanthine (caffeine or theophylline) and ephedrine to obese mice for 6 weeks led to a decrease in body weight and fat, as well as an increase in energy expenditure (Dulloo & Miller, 1986a). Later, administration of the same combination of compounds (22 mg of ephedrine, 30 mg of caffeine and 50 mg of theophylline) to human subjects demonstrated that ephedrine/methylxanthine combination was more effective in raising resting metabolic rate in post-obese and lean subjects than ephedrine alone (Dulloo & Miller, 1986b). There are several other studies that corroborate these findings (Liu et al., 2013). Obese subjects that received 200 mg caffeine/20 mg ephedrine for over 24 weeks, lost 6.5% of total body weight and 12.4% of whole body fat mass. However, in 2004 the Food and Drug Administration (FDA) banned from the market the ephedra-containing supplements due to the potential adverse effects, and efforts to find an optimized replacement are ongoing (Bray & Greenway, 2007; Haller & Benowitz, 2000). Ann Liu et al. (2015) studied albuterol for that purpose. Albuterol is another selective ß2-adrenergic agonist previously reported to stimulate lipolysis when used in a dose four-fold higher than the inhalatory bronchodilation dose used for asthma treatment (200 µg) (Amoroso et al., 1993; Goldberg et al., 1975). The combination of albuterol (7-17 ng/mL) and caffeine (3-10 µg/mL) resulted in a 30-40% increase in lipolysis over buffer-treated human adipocytes. Furthermore, in human subjects, the combination of caffeine (100-200 mg) with albuterol (2-4 mg) also resulted in significant increase of energy expenditure, irrespective of treatment-dose combination. Besides, an increase in lean mass along with a decrease on fat mass also occurred in caffeine/albuterol treated rats.

1.3.4. Anti-adipogenic activity

Mild obesity results mainly from adipocytes hypertrophy, whereas more severe cases of obesity also involve cell hyperplasia. To maintain the metabolic homeostasis upon energy overload, preadipocytes are recruited to differentiate into mature adipocytes, in a process called adipogenesis. This process is important for healthy adipose tissue growth, remodelling, and expansion, which may prevent the adverse metabolic dysregulation associated with obesity (Eisenstein & Ravid, 2014; Rosen & MacDougald, 2006). Certain transcription factors have been shown to be pivotal for adipogenesis, such as the ccaat enhancer binding proteins (C/EBP) family, and peroxisome-proliferator activated receptor-gamma (PPARγ) (Rosen et al., 1999; Tontonoz et al., 1994; Wu et al., 1999). The expression of two C/EBP proteins, C/EBPβ and C/EBPδ, promotes the expression of PPARγ, possibly via binding sites on its promoter (Fajas et al., 1997; Zhu et al., 1995). Then, PPARγ stimulates the expression of another C/EBP protein, C/EBPα, which induces the differentiation process (Freytag et al., 1994; Lin & Lane, 1992, 1994). For that reason, these latter transcription factors are considered the main regulators of adipogenesis. PPARγ and C/EBPα trigger differentiation through the regulation of adipocyte-specific genes that are necessary for adipocyte function, including fatty acid binding protein/adipose protein 2 (FABP/aP2), insulin receptor, GLUT4, acetyl-coA carboxylase, fatty acid synthase, and glycerolphosphate dehydrogenase (GPDH) (Kubota et al., 1999; Spiegelman et al., 1993; Tontonoz, Hu, Devine, et al., 1995; Tontonoz, Hu, & Spiegelman, 1995). On the other hand, there is evidence that C/EBPβ and C/EBPδ are not essential for adipocyte differentiation, suggesting the existence of adipogenic transcriptional cascades that do not involve these proteins (Tanaka et al., 1997).

More recently, it was shown that methylxanthines may inhibit adipogenesis. Primary rat adipose-derived stem cells (ADSCs) and mouse bone marrow stromal cell line (M2-10B4) were used to evaluate the i*n vitro* effects of caffeine on adipogenesis (Su et al., 2013). The continuous exposure of cells to caffeine (0.1-1 mM) during differentiation showed that caffeine dose-dependently reduced lipid droplet and adipocyte differentiation in both cell types, and that it also decreased the expression of C/EBPα and PPARγ, the two main adipogenic transcription factors. Similar results were obtained in 3T3-L1 cell line when cells were incubated with a 5% coffee solution (Aoyagi et al., 2014). Later, it was described that caffeine effects in adipocytes were mediated by reducing the activation of the protein kinase B (Akt) and disturbing the Akt/glycogen synthase kinase 3 beta (GSK3β) axis. This axis is known for its role in multiple cellular processes such as metabolism, proliferation, or transcription. In fact, it was shown that reduced activation of this axis leads to the inhibition of the mitotic clonal expansion and inhibition of the C/EBPβ in 3T3-L1 cells.

Theobromine was also shown to inhibit differentiation of 3T3-L1 cells (Jang et al., 2015). When preadipocytes were exposed to theobromine (50, 100 and 150 μg/mL) for 7 days during their differentiation period, a decreased accumulation of lipid droplets and a decreased expression of C/EBPα and PPARγ were reported, in a concentration-dependent

manner. The authors also reported an increased phosphorylation of the AMP-activated protein kinase (AMPK) and a decreased phosphorylation of extracellular signal-regulated kinase (ERK), also in a concentration-dependent manner. Both protein pathways intervene in the regulation of PPARγ, whose inhibition leads to the inhibition of adipogenesis (Burns & Vanden Heuvel, 2007; Farmer, 2005).

Overall, methylxanthines seem to inhibit adipogenesis by disturbing the adipocyte signalling and inhibiting the main adipogenic transcription factors (Figure 6B). However, adrenergic and adenosine receptors also appear to have a role in adipogenesis. In fact, the expression of ß-adrenergic receptors increases during the differentiation process, whereas upon stimulation by agonists, there is an inhibition of adipocyte differentiation due to PKA activation (Li et al., 2010). Moreover, it has been reported that the activation of A_1 and A_2 receptors stimulates adipocyte differentiation, by promoting lipogenesis and lipid accumulation, which are accompanied by an increased PPARγ and C/EBPα expression, respectively (Gharibi et al., 2012). However, in other studies it appears that AR coupled to G_s proteins inhibit adipocyte differentiation, while receptors coupled to G_i proteins promote differentiation (Wang et al., 1996; Wang & Malbon, 1999). Thus, although their actions and adipogenesis mechanism are not completely understood, it has been suggested that they may play a role in cAMP-independent adipogenesis regulation (Eisenstein & Ravid, 2014).

II. Objectives

II. Objectives

Methylxanthines are widely distributed in the human diet and its consumption seems to be inversely associated with body weight increase. As previously described, caffeine (the most well-known and studied natural methylxanthine) is reported to modulate glucose and fatty acid metabolism. Recently, Van der Walt and Terre'Blanche (2015) synthetized several xanthine analogues. One of those synthetic xanthine analogues, 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX), was reported to be a very potent A_1 receptor antagonist. Thus, ETX may be a promising drug candidate for obesity treatment.

This project aims to study the modulation conferred by new this molecule in the metabolic and oxidative profile of adipocytes in order to evaluate its pharmacological potential for obesity treatment. For this purpose, the anti-obesogenic potential of ETX was evaluated in mouse adipocytes (derived from differentiation of the cell line 3T3-L1), using synthetic caffeine as comparator.

Therefore, the following specific objectives were established:

1. Culture and differentiation of 3T3-L1 preadipocytes into adipocytes;
2. Characterization of the cytotoxic profile of ETX in 3T3-L1 adipocytes;
3. Evaluation of the metabolic profile in 3T3-L1 adipocytes after treatment with ETX as compared to caffeine;
4. Evaluation of the oxidative profile in 3T3-L1 adipocytes after treatment with ETX as compared to caffeine.

III. Materials and Methods

III. Materials and Methods

3.1. Xanthine analogues synthesis

The synthetic xanthine, 8-(3-phenylpropyl)-1,3,7-triethylxanthine (Figure 7), was produced at the School of Pharmacy, North-West University, Potchefstroom, South Africa, and kindly provided by our collaborators Van der Walt and Terre'Blanche. The synthesis process was previously described (Van der Walt & Terre'Blanche, 2015; Van der Walt et al., 2013). The first step was the reaction of 1,3-dimethyl- and 1,3-diethyl-5,6-diaminouracil with carboxylic acid, such as 4-phenylbutanoic acid, phenylpropanoic acid and phenoxyacetic acid, in the presence of the coupling reagent N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDAC) at room temperature. Resulting from this acylation, several 1,3-dialkyl-6-amino-5-carboxamidouracil intermediates were obtained. The cyclization of these intermediates was obtained under strong basic conditions (aqueous sodium hydroxide solution), to produce the corresponding 1,3-dialkyl-7H-xanthine derivatives. Without purification, the 1,3-dialkyl-7H-xanthine derivatives were directly treated with excess iodomethane or iodoethane in the presence of potassium carbonate, resulting in several 7-alkylated xanthine derivatives. Finally, 8-(3-phenylpropyl)-1,3,7-triethylxanthine was purified by recrystallization and the molecular structure and purity were verified by ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry analysis.

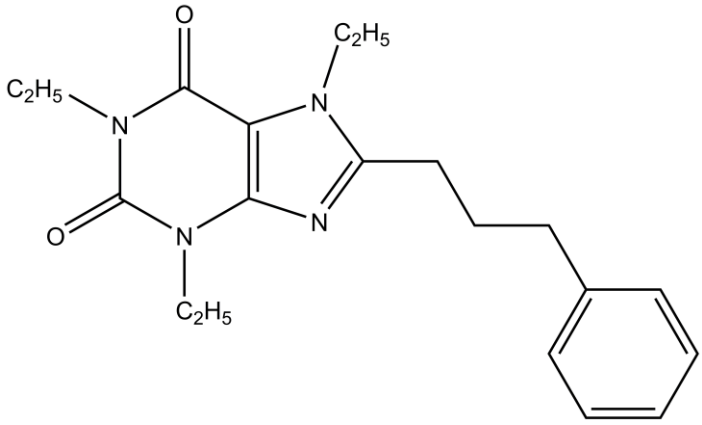

Figure 7 – Chemical structure of the new synthetic xanthine (8-(3-phenylpropyl)-1,3,7-triethylxanthine).

3.2. Mouse preadipocyte cell line 3T3-L1 culture

The 3T3-L1 is a preadipocyte cell line derived from the 3T3 cells, which were obtained from Swiss mouse embryo cells (Green & Meuth, 1974). The 3T3-L1, under

appropriate conditions, are able to differentiate into adipocytes, being widely used for *in vitro* adipose tissue studies.

Preadipocyte cell line 3T3-L1 was purchased from a commercial supplier (Zen-Bio, Durham, NC, USA). 3T3-L1 preadipocytes at passage 14-15 were cultured in DMEM High Glucose (Sigma-Aldrich, USA) supplemented with 10% newborn calf serum (NCS) (Gibco, NZ), 1% penicillin-streptomycin (Pen-Strep) (Sigma-Aldrich, USA), 2.5 µg/mL amphotericin B (Sigma-Aldrich, USA), 50 µg/mL gentamicin (Sigma-Aldrich, USA) and 50 mM HEPES buffer (Fisher BioReagents, USA). Cells were grown in 25 cm^2 and 75 cm^2 T-flasks (Thermo Scientific, Waltham, MA, USA), being re-fed every 72 h. When reaching 70% confluence, they were passaged by incubating the cells with 0.05% trypsin-EDTA solution (Sigma-Aldrich, USA).

All cell cultures were handled in a laminar flow chamber and maintained in an incubator at 37ºC with a 5% CO_2 humidified atmosphere (Heracell 150i, Thermo Scientific, Waltham, MA, USA).

3.2.1. Adipocyte differentiation

Adipocyte differentiation was induced 2 days after the cells reached full confluence and cells were maintained for 15 days until differentiation was completed (Figure 8), as described previously (Moreira et al., 2015). The culture medium was removed and cells were washed with phosphate buffered saline (PBS). Cells were incubated in a differentiation medium composed by DMEM-F12 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Germany), 1% Pen-Strep, 2.5 µg/mL amphotericin B, 50 µg/mL gentamicin, 15 mM HEPES Buffer and a differentiation cocktail containing 1 µM dexamethasone (Sigma-Aldrich, USA), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, USA) and 1 µg/mL human insulin (Actrapid, Novo Nordisk, Denmark). After 72 h, the culture medium was replaced with the last medium without the differentiation cocktail and cells were re-fed every 72 h for more 12 days.

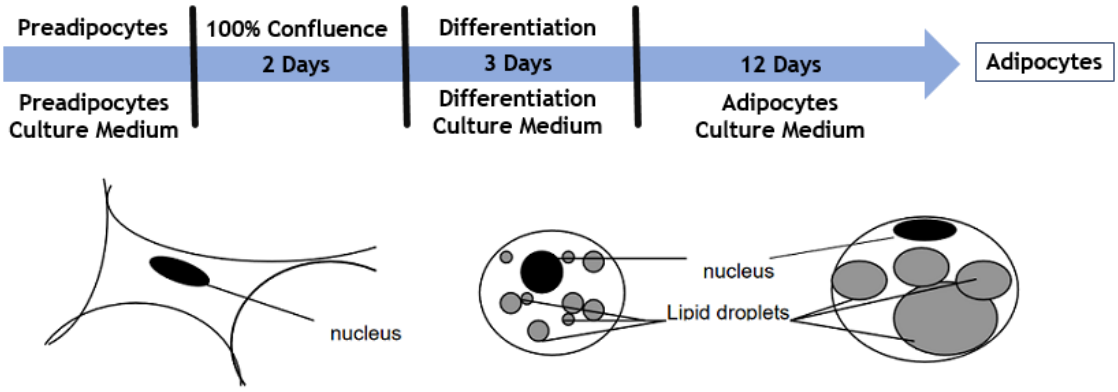

Figure 8 - Schematic illustration of the 3T3-L1 preadipocytes differentiation into adipocytes (adapted from Zen-Bio 3T3-L1 Cell Care Manual, 2015).

3.2.2. Oil Red O lipid staining

Oil Red O is a liposoluble dye used for the histological visualization of lipids. This staining technique allows the visualization of lipid droplets in tissues and cells, such as the adipocytes, by staining red these structures. When adipocytes are stained with Oil Red O, the degree of staining is proportional to lipid accumulation and cell differentiation (Ramirez-Zacarias et al., 1992). For this purpose, cells were seeded in microscope coverslips in 24-well plates (Orange Scientific, Belgium). Both coverslips and plates were sterilized by being exposed to UV for 30 min. Cell culture and differentiation were executed as described above. After the differentiation process, cells were washed with PBS and fixed for 5 min with 10% neutral buffered formalin (Sigma-Aldrich, USA). Cells were washed with distilled water and 85% propylene glycol (Merck Millipore, USA) was added, for 2 min and changed 2 times. Then, Oil Red O (0.5% in propylene glycol) (Sigma-Aldrich, USA) was spread evenly over the cells and left for 30 min at room temperature. Oil Red O was removed and 85% propylene glycol was added, for 1 min and repeated 2 times. After a wash step with distilled water, cells were stained with hematoxylin (Merk Millipore, USA) for 30 s. Hematoxylin is a dye that stains the cell nucleus. Finally, cells were washed one last time with distilled water and mounted with Aquatex® (Merck Millipore, USA).

3T3-L1 adipocytes and lipid droplets were observed and the images were recorded using an Olympus DX50 inverted light microscope (Tokyo, Japan) equipped with an Olympus DP21 digital camera.

3.3. Experimental groups

In order to evaluate the anti-obesogenic potential of synthetic caffeine (Sigma-Aldrich, USA) and ETX on 3T3-L1 adipocytes, 9 different groups were defined: a control group without caffeine or ETX and 8 other groups containing adipocyte culture medium supplemented with increasing doses of caffeine or ETX (0.1, 1, 10 and 100 µM). As caffeine was previously reported to modulate glucose and fatty acid metabolism (Santos & Lima, 2016), we decided to include caffeine exposed cells for comparative purposes. The used doses were chosen based on caffeine consumption. A dose of 1 mg/kg of caffeine is estimated to be equivalent to 1 cup of coffee, which has been reported to lead to plasma concentrations between 5 to 10 µM (Carrillo & Benitez, 2000). Moreover, since caffeine content differs significantly among caffeine-containing beverages and food products and in average the consumption of coffee is more than 1 cup (Mandel, 2002), we found pertinent to evaluate the effects of a lower concentration (1 µM) and a higher concentration (100 µM), which is estimated to be equivalent to the plasma levels attained by a high coffee consumption (more than 5-6 cups) (Magkos & Kavouras, 2005). Additionally, ETX was described as a more potent

A_1 receptor antagonist than caffeine (Van der Walt & Terre'Blanche, 2015), thus we found appropriate to include in the study an even lower concentration (0.1 µM).

Stock solutions (1 M) of caffeine and ETX were prepared. While caffeine is water soluble (Shalmashi & Golmohammad, 2010), ETX does not present this property. Thus, the stock solution was prepared in dimethyl sulfoxide (DMSO) (Labkem, Ireland) and both stock solutions were diluted in adipocyte culture medium to obtain the the intended concentrations. DMSO is known for its toxicity and the lowest concentration possible is recommended for cell culture (Galvao et al., 2014). The highest DMSO concentration resulted from the preparation of the higher concentration of ETX (100 µM), which was 0.01% (v/v) DMSO. This solvent was added to all groups at the same concentration (0.01% (v/v)).

3.4. Characterization of the cytotoxic profile

3T3-L1 preadipocytes were seeded, cultured, and differentiated in 96-well plates (Orange Scientific, Belgium), as described above. To evaluate the cytotoxic profile of caffeine and ETX, cells were treated with different concentrations of each compound (control, 0.1, 1, 10 and 100 µM of caffeine or ETX), with five repetitions for each group. Cells were kept in an incubator at 37ºC, with 5% CO_2 humidified atmosphere for 24 h. Then, two different methods were used to evaluate the cytotoxicity: MTT assay and LDH assay.

3.4.1. Characterization of the cytotoxic profile through the MTT assay

The MTT assay allows the determination of mitochondrial and, subsequently, cellular viability. This colorimetric assay is based on the capacity of metabolic viable cells to reduce the yellowish MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purplish formazan crystals. This reduction is catalyzed by mitochondrial dehydrogenases of viable cells and the proportion of crystals formed is proportional to the number of viable cells, which is quantifiable through spectrophotometry at 570 nm (Riss et al., 2004).

After the 24 h incubation, the treatment medium was removed and replaced with 150 µL of fresh adipocyte culture medium and 15 µL of MTT solution (5 mg/mL in PBS) (Amresco, USA). Then, the plates were incubated in a dark environment for 3 h 30 min at 37ºC. After incubation, the culture medium was removed and 100 µL DMSO were added, followed by agitation to solubilize the formazan crystals. The absorbance was measured at 570 nm through an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). Resulting values were divided by the mean of the control group and expressed in fold variation versus control group.

3.4.2. Characterization of the cytotoxic profile through the LDH assay

The release of the lactate dehydrogenase (LDH) into the extracellular medium was spectrophotometrically quantified using a LDH Cytotoxicity Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. This assay allows to quantitatively measure LDH, a cytosolic enzyme, released to the medium by damaged cells (Decker & Lohmann-Matthes, 1988). This colorimetric assay uses LDH as a biomarker for cellular cytotoxicity, through an enzymatic reaction that results in a red-pinkish formazan product, which is proportional to the number of dead cells and can be quantified through spectrophotometry at 490 nm. After the 24 h incubation, 50 µL of the medium were collected and transferred to a 96-well plate. As blank, fresh adipocyte culture medium was used. Then, 50 µL of Reaction Mix were added to each well and the plate was incubated in a dark environment for 30 min at room temperature. To prepare the Reaction Mix, the Substrate Mix was solubilized in 11.4 mL of distilled water and 0.6 mL of Assay Buffer, adding distilled water up to a final volume of 15 mL. After 30 min of incubation, 50 µL of a stop solution were added to end the enzymatic activity and the absorbance of the medium was measured at 490 nm using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). Resulting values were divided by the mean of the control group and expressed in fold variation versus control group.

3.5. Characterization of the metabolic profile

3T3-L1 preadipocytes were cultured and differentiated in 25 cm^2 T-flasks described above. After treatment for 24 h, culture medium was collected. Then, cells were detached from the flasks using a 0.05% trypsin-EDTA solution and collected for protein extraction. Viable cells were counted with 0.4% trypan blue (Sigma-Aldrich, USA) in a LUNA™ Automated Cell Counter (Logos Biosystems, South Korea).

To evaluate the metabolic profile of 3T3-L1 adipocytes after incubation with different concentrations of caffeine and ETX, samples of 180 µL of medium were collected and the respective ^{1}H-NMR spectra were acquired using previously described methods by Alves and collaborators (2013). ^{1}H-NMR spectrum of each sample was acquired with a magnetic field of 14.1 T at 25ºC, using a Varian NMR 600 MHz spectrometer equipped with a 3-mm Indirect Detection probe (Varian, Inc., Palo Alto, CA). Solvent-suppressed ^{1}H-NMR spectra were acquired with a sweep width of 7.2 kHz, using a delay of 4 s, a water presaturation of 3 s, a pulse angle of 30º, an acquisition time of 3 s (total interpulse delay of 10 s (4+3+3) allowed for the full relaxation of all proton nuclei required for quantitative analysis) and 32 scans. For sample analysis, 45 µL of sodium fumarate (10 mM) were mixed with 180 µL of medium and 200 µL of the mixture was transferred to a NMR tube to be analyzed. Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify the

following metabolites present in solution (multiplet, ppm): lactate (doublet, 1.33), alanine (doublet, 1.45), acetate (singlet, 1.90), pyruvate (singlet, 2.34), H1-α-glucose (doublet, 5.22) and glutamine (multiplet, 2.45). Prior to Fourier transformation, each free induction decay (FID) was processed by applying exponential multiplication (lb=0.3) and zero filling (to 128k points). Spectra were manually phased and baseline corrected. Chosen metabolite peaks were integrated using Amix-viewer (version 3.9.14, BrukerBiospin, Rheinstetten). Results are expressed as μmol/10^6 cells.

3.6. Characterization of the oxidative profile

3.6.1. Total protein extraction and quantification

Total protein was extracted from 3T3-L1 adipocytes using M-PER (Thermo Scientific, Waltham, MA, USA). This reagent contains a mild, non-denaturing detergent that dissolves cell membranes, which permits to extract total protein from most cellular compartments. For this purpose, 50 μL of M-PER were added to the cells previously collected and then the cells were homogenized by successive pippeting. Cells were left for 10 min at room temperature, with occasional agitation. Then, the lysate was centrifuged at 14000 g for 20 min at room temperature. Total protein extracted remained on the supernatant and stored at -20ºC until use.

Total protein extracted from 3T3-L1 adipocytes was quantified using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). This assay is based in the reduction of Cu^{2+} to Cu^{1+} by proteins in alkaline conditions, which is quantifiable through its reaction with bicinchoninic acid (BCA). BCA reacts with the reduced cation to form a purple-colored complex, which can be quantified through spectrophotometry. For this purpose, several standard solutions of known concentration (5, 25, 50, 125 and 250 μg/mL in PBS) of bovine serum albumin (BSA) (Sigma-Aldrich, USA) were prepared for the calibration curve. Then, 10 μL of each standard solution and 10 μL of PBS for the blank were pipetted to a 96-well plate. From the lysate previously obtained, 1 μL of the supernatant plus 9 μL of PBS were also pipetted to the plate. Subsequently, 200 μL of the kit reagent was added to each well and the plate was incubated in a dark environment for 30 min at 37ºC. Finally, the absorbance was measured at 595 nm using Anthos 2010 microplate reader (Biochrom, Berlin, Germany).

3.6.2. Protein oxidation analysis

Protein carbonyl content is commonly used as a biomarker for protein oxidation, which results from oxidative stress (Dias et al., 2015). The content of protein carbonyl groups in 3T3-L1 adipocytes from the different experimental groups was evaluated using the slot-blot

technique and a specific antibody. Protein samples were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain 2,4-dinitrophenyl (DNP) according to the method developed by Levine and collaborators (1990). Briefly, 5 µg of protein extracted from 3T3-L1 cells were diluted in filtered PBS up to a final volume of 20 µL. Then, 20 µL of 12% sodium dodecyl sulfate (SDS) were added and the suspension was homogenized. Subsequently, 40 µL of DNPH (Sigma-Aldrich, USA) (20 mM in 10% trifluoracetic acid (TFA) (Thermo Scientific, Waltham, MA, USA)) were added to the suspension. After homogenization, the samples were incubated at room temperature for 30 min in a dark environment. After the incubation, 30 µL of stop solution were added (18% ß-mercaptoetanol (Sigma-Aldrich, USA) in Tris 2 M (NZYTech, Portugal)). The derivatized samples were transferred to a polyvinylidene difluoride (PVDF) membrane using a Hybrid-slot manifold system (Biometra, Göttingen, Germany) and the resulting membranes were incubated overnight at 4ºC with a rabbit anti-DNP (1:5000, D9656, Sigma–Aldrich, St. Louis, MO, USA). Samples were visualized using a goat anti-rabbit IgG-AP (1:5000, sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany). Membranes were then reacted with ECF™ substrate (GE Healthcare, Buckinghamshire, UK) and read using a BioRad FX-Pro-plus (Bio-Rad Hemel Hempstead, UK). Densities from each band were quantified using the BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vallée, France). The density of each band was divided by the mean density of the control group and expressed in fold variation versus control group.

3.6.3. Lipid peroxidation analysis

Lipid peroxidation is one of the consequences of reactive oxygen species (ROS) damage, which may damage the cellular lipids, particularly those constituting the cell membranes. The oxidative damages of membrane lipids and the end-products of this reaction are harmful to cells and tissues (Mylonas & Kouretas, 1999). The lipid peroxidation extent can be evaluated by measuring resulting aldehydic products, such as 4-hydroxynonenal (4-HNE), as described previously by Dias and collaborators (2015). For this purpose, protein samples from each condition were diluted in PBS to a concentration of 0.001 µg/mL and used in the slot-blot technique described above. Membranes were incubated overnight at 4ºC with a goat anti-4-HNE antibody (1:5000, AB5605, Merck Millipore, Temecula, USA) and visualized using a rabbit anti-goat IgG-AP (1:5000, A4187, Sigma-Aldrich, USA). Then, membranes were incubated with ECF™ substrate (GE Healthcare, Buckinghamshire, UK) and read using a BioRad FX-Pro-plus (Bio-Rad Hemel Hempstead, UK). Densities from each band were quantified using the BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vallée, France). The density of each band was divided by the mean density of the control group and expressed in fold variation versus control group.

3.6.4. Tyrosine residues nitration analysis

The nitration of tyrosine residues to 3-nitrotyrosine is considered a biomarker for pro-oxidant processes within the cell. The incorporation of a nitro group to tyrosine can result in structural and functional changes, which contributes to the loss of cell and tissue homeostasis (Radi, 2013). To evaluate the tyrosine residues nitration, protein samples from each condition were diluted in PBS to a concentration of 0.001 µg/mL and used in the slot-blot technique described above. Membranes were incubated overnight at 4ºC with a rabbit anti nitro-tyrosine antibody (1:5000, 9691S, Cell Signaling Technology, Netherlands) and visualized using a goat anti-rabbit IgG-AP (1:5000, sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany). Membranes were then reacted with ECF™ substrate (GE Healthcare, Buckinghamshire, UK) and read using a BioRad FX-Pro-plus (Bio-Rad Hemel Hempstead, UK). Densities from each band were quantified using the BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vallée, France). The density of each band was divided by the mean density of the control group and expressed in fold variation versus control group.

3.7. Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2016 (Microsoft, USA), and GraphPad Prism 6 (GraphPad software, USA). The statistical significance of the groups was assessed by t-student test or by one-way ANOVA, followed by multiple comparisons through Fisher Least Significant Difference (LSD) method. All experimental data is presented as mean ± SEM with $p<0.05$ considered significant.

IV. Results

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4.1. 3T3-L1 preadipocytes differentiation

The cultured 3T3-L1 preadipocytes were subjected to the differentiation protocol as previously described (Moreira et al., 2015). Briefly, after being left confluent for 48 h, cells were incubated with culture medium containing a hormonal cocktail for 72 h. This cocktail is constituted by dexamethasone, IBMX and human insulin. Dexamethasone is an anti-inflammatory steroid molecule that in combination with IBMX stimulate the expression of PPARγ, promoting adipogenesis (Scott et al., 2011). Moreover, both compounds are inducers of C/EBPδ and C/EBPβ, which are essential transcription factors for adipocytes growth and differentiation (Cao et al., 1991). In addition, IBMX is a phosphodiesterase inhibitor and responsible for increasing intracellular cAMP and promoting the PKA pathway, which is required for transcriptional activation of PPARγ and adipogenic gene expression (Kim et al., 2010). On the other hand, insulin acts through the insulin-like growth factor 1 (IGF-1) receptor and stimulate the GLUT4 expression, which increases the glucose uptake and, subsequently, promotes the formation of lipid droplets through lipogenesis (Ntambi & Young-Cheul, 2000). After incubation with this cocktail, cells require nearly 12 days in order to become fully differentiated and morphological changes can be observed, such as the cytoplasm rearrangement and lipid droplets formation. To document the process and observe the morphological changes, the Oil Red staining technique was performed at day 0 (before exposition to the hormonal cocktail), 7 and 15 of the differentiation process, when several images were obtained (Figure 9).

At day 0 (Figure 9A) a fibroblast-like morphology characteristic of preadipocytes was observed. Moreover, the slight formation of small lipid droplets within the cells was also observed, even before the exposure to the differentiation medium. At day 7 (Figure 9B), cells displayed higher number of lipid droplets with slightly larger sizes. A similar situation occurred at day 15 (Figure 9C), with cells depicting a slightly increase in the number and size of lipid droplets. Still, at the end of the period that lasted the maturation protocol the cells did not show the typical morphology of fully differentiated adipocytes with a large lipid droplet covering most of the cytoplasm. The absence of the characteristic morphology of a mature adipocyte indicates that the differentiation processed was not completed at the time of cell exposure to methylxanthine stimuli. To evaluate the degree of adipocytes differentiation, we assessed the expression of differentiation protein markers by western blot technique, such as sirtuin 1 (SIRT1) and GLUT4 (data not shown). Both these proteins are highly expressed in fully differentiated adipocytes (Jin et al., 2010; Yokomori et al., 1999). Expression of these protein markers in these cells was poor, confirming the low efficiency of the differentiation protocol used.

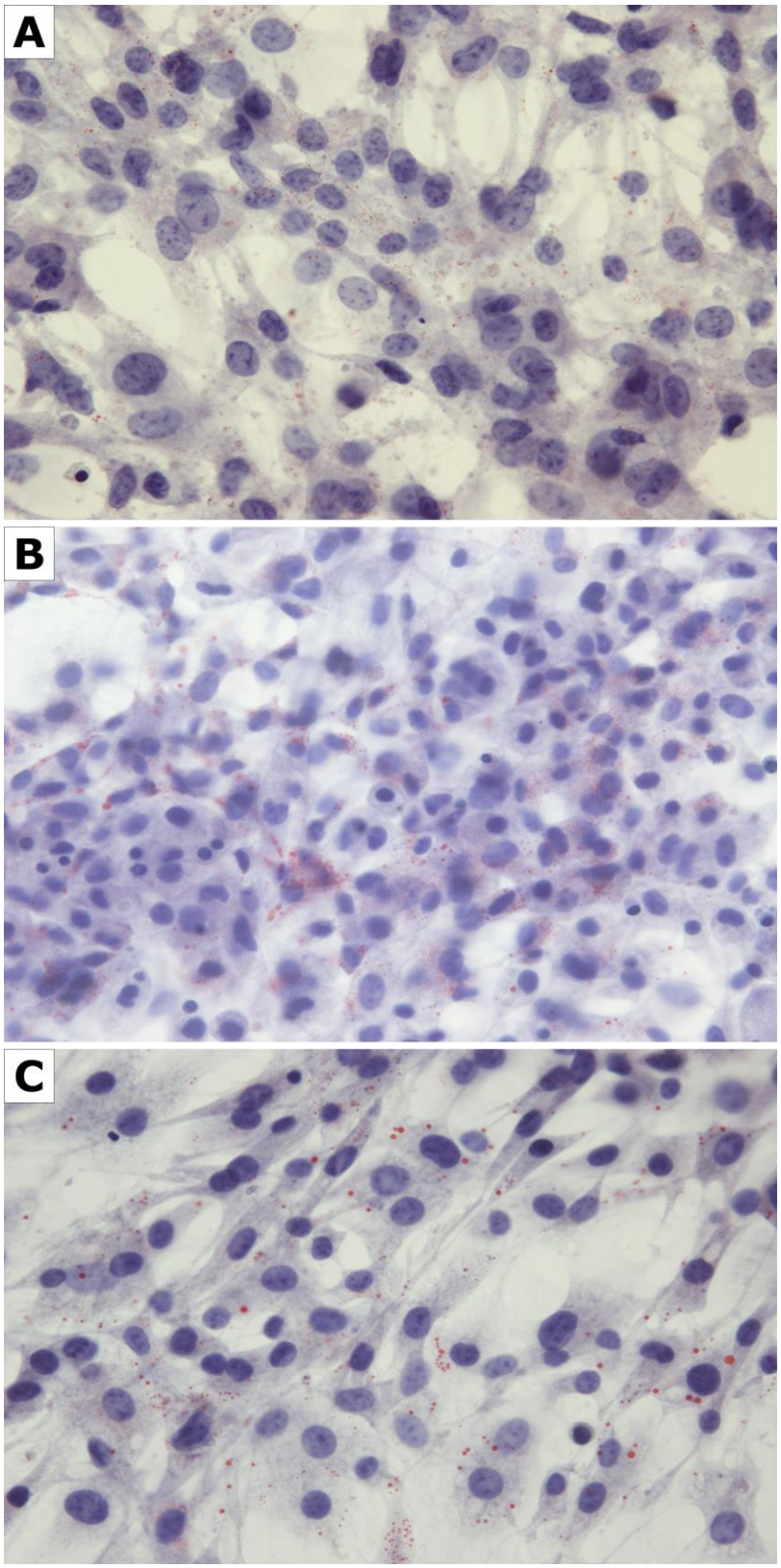

Figure 9 – 3T3-L1 preadipocytes differentiation attempt. **(A)**, **(B)** and **(C)** were obtained using an Olympus DX50 inverted light microscope equipped with an Olympus DP21 digital camera (400x magnification). **(A)** At day 0 cells displayed fibroblast-like morphology, which is characteristic of preadipocytes. Moreover, the formation of small lipid droplets within the cells was also observed, even before the exposition to the hormonal cocktail. **(B)** At day 7 lipid droplets increased in size and number. **(C)** At day 15 lipid droplets were bigger, although the cells still displayed some fibroblast-like morphology.

In order to try to overcome these results, several differentiation protocols were tested, which included changing incubation times, cocktail's reagents concentrations and even the culture flasks or plates. Unfortunately, none of the modified protocols more successful. This fact could be due to several reasons, of known or unknown origin. In fact, is well-known from the literature that 3T3 cells are difficult to handle and differentiate. For instance, after inducing differentiation, the medium became very viscous and cells started to detach, a phenomena that was previously reported (Zebisch et al., 2012). Other authors also reported low differentiation efficiency, associating this fact with certain culture plates (Mehra et al., 2007) and some associated it with high passage number of the cell culture or with previous liquid nitrogen storage (Zebisch et al., 2012).

Despite the cells were exposed to the differentiation medium for the time required to differentiate, due to the aforementioned reasons these could not be considered mature adipocytes. Therefore, the following results were obtained using 3T3-L1 preadipocytes that were previously exposed to an hormonal cocktail aimed to promote differentiation for over 12 days.

4.2. ETX presents no cytotoxic effects in 3T3-L1 preadipocytes

The cytotoxic profile of ETX and caffeine (CAF) was characterized through *in vitro* assays. Cells were incubated for 24 h with different concentrations of ETX or CAF (0, 0.1, 1, 10 and 100 µM). The MTT assay was performed to evaluate the cells' metabolic activity (Figure 10).

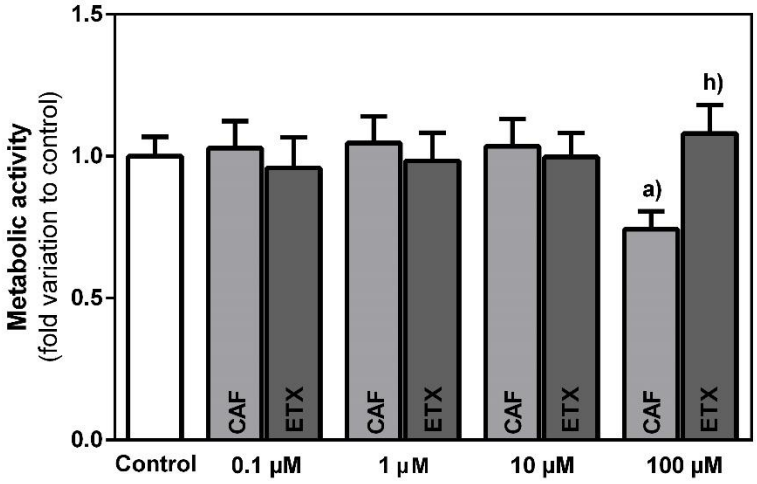

Figure 10 – Evaluation of the metabolic activity of 3T3-L1 preadipocytes after 24 h incubation with increasing concentrations of caffeine (CAF) or 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX) as determined by the MTT assay. Results are expressed as fold variation to control and mean ± SEM (n = 5 for each condition). Significantly different results ($p<0.05$) are indicated as: a) relative to control; h) relative to 100 µM CAF.

The assay showed a significant reduction in the metabolic activity (0.74 ± 0.06-fold variation to control) of CAF at the higher concentration (100 µM), while ETX presented no significant alterations. This result suggests that CAF at 100 µM may impair cells metabolism and induce some cytotoxicity, whereas the ETX has no cytotoxic effect even at this concentration.

In order to evaluate if increasing concentrations of CAF or ETX could cause cellular damage and cytolysis, the activity of the LDH released to the extracellular medium was spectrophotometrically quantified (Figure 11). This assay uses LDH as a biomarker for cellular cytotoxicity, since this cytosolic enzyme is released to the medium by damaged cells. We observed that CAF at 100 µM induced a significant LDH release (2.05 ± 0.10-fold variation to control), while ETX presented no significant cytotoxicity at all studied concentrations. This result corroborates the previous test results, suggesting that CAF may induce cellular toxicity to 3T3-L1 preadipocytes at 100 µM. Interestingly, ETX seems to be safer at higher concentrations, which is a promising feature for drug design and application. However, even if ETX did not show any cytotoxicity at all conditions tested, our main objective was to compare the effects of ETX with CAF in the adipocyte metabolism and oxidative profile. Thus, the highest concentration of 100 µM was not tested in subsequent assays.

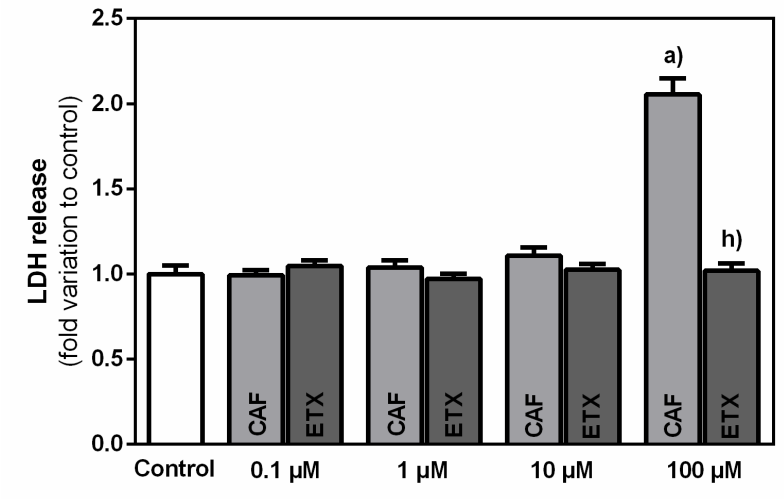

Figure 11 - Released lactate dehydrogenase (LDH) by 3T3-L1 preadipocytes after 24 h incubation with increasing concentrations of caffeine (CAF) or 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX). Results are expressed as fold variation to control and mean ± SEM (n = 5 for each condition). Significantly different results ($p<0.05$) are indicated as: a) relative to control; h) relative to 100 µM CAF.

4.3. ETX enhances metabolism towards energy expenditure in 3T3-L1 preadipocytes

The metabolic profile of 3T3-L1 preadipocytes after incubation with ETX or CAF (0, 0.1, 1 and 10 μM) was evaluated through analysis of the metabolites present in the medium by ^{1}H-NMR. Thus, samples of medium were collected and the following compounds were quantified: glucose, pyruvate, and glutamine (Figure 12), lactate, alanine, and acetate (Figure 13). These metabolites are involved in important cellular pathways for energy production and are responsible for cellular maintenance and function.

4.3.1. ETX increases glucose, pyruvate, and glutamine consumption

Glucose is known to be the primary nutrient for cellular maintenance and function. This simple sugar is the primary substrate for ATP production through a process designated glycolysis (Berg et al., 2002). CAF is known to regulate glucose metabolism in cells (Ojuka et al., 2002). This natural occurring methylxanthine is reported to modulate glucose metabolism towards energy production (Lopes-Silva et al., 2015). However, we observed that CAF at the studied concentrations did not increase glucose consumption or significantly alter the metabolic profile of 3T3-L1 preadipocytes (Figure 12A). Cells incubated with ETX, increased glucose consumption, although not enough to reach statistical significance (as compared with the control conditions). On the other hand, when comparing the same concentration of both compounds we observed that glucose consumption was significantly increased by ETX (101.60 ± 16.77 μmol/10^6 cells) as compared with CAF (49.66 ± 10.51 μmol/10^6 cells) at the concentration of 10 μM. Moreover, when comparing ETX with CAF at the concentrations of 0.1 μM (79.45 ± 5.46 μmol/10^6 cells and 56.68 ± 11.10 μmol/10^6 cells, respectively) and 1 μM (101.90 ± 21.65 μmol/10^6 cells and 70.46 ± 8.61 μmol/10^6 cells, respectively) we observed an increase in glucose consumption by ETX, though not enough to reach statistical significance.

Glycolysis culminates with the conversion of glucose into pyruvate, producing energy in the form of ATP (Berg et al., 2002). Pyruvate can be used in different pathways. For instance, pyruvate can be directly and reversibly reduced to lactate by LDH or, in the presence of L-glutamate, be converted to L-alanine and α-ketoglutarate by alanine transaminase. On the other hand, pyruvate may enter the mitochondria to be converted to acetyl-CoA by pyruvate dehydrogenase and be used in the citric acid cycle or Krebs cycle (Lehninger et al., 2008). The obtained results showed that CAF did not increase the consumption of pyruvate (Figure 12B). ETX increased pyruvate consumption, although not enough to reach statistical significance when compared to the control group. However, when comparing the same concentration of both compounds we observed that pyruvate consumption was significantly increased by ETX (12.11 ± 2.98 μmol/10^6 cells) as compared

with CAF (4.73 ± 1.03 µmol/10^6 cells) at the highest concentration (10 µM). Furthermore, when comparing ETX with CAF at the concentrations of 0.1 µM (7.17 ± 0.49 µmol/10^6 cells and 5.57 ± 1.37 µmol/10^6 cells, respectively) and 1 µM (9.48 ± 2.09 µmol/10^6 cells and 6.62 ± 0.75 µmol/10^6 cells, respectively) we observed an increase in pyruvate consumption by ETX, although not enough to reach statistical significance.

Glutamine is an amino acid that can be converted to glutamate and subsequently to α-ketoglutarate, a substrate used in the citric acid cycle or, as described above, to produce pyruvate (Plaitakis et al., 2017). It was observed that CAF did not increase glutamine consumption (Figure 12C). On the other hand, ETX increased glutamine consumption, though not enough to reach statistical significance when compared to the control group. However, when comparing the same concentration of both compounds we observed that glutamine consumption was significantly increased by ETX (28.07 ± 4.39 µmol/10^6 cells) as compared with CAF (13.68 ± 2.78 µmol/10^6 cells) at the highest concentration (10 µM). Additionally, when comparing ETX with CAF at the concentrations of 0.1 µM (20.70 ± 1.19 µmol/10^6 cells and 16.53 ± 3.91 µmol/10^6 cells, respectively) and 1 µM (27.47 ± 5.94 µmol/10^6 cells and 19.57 ± 2.05 µmol/10^6 cells, respectively) it was observed an increase in glutamine consumption by ETX, although not enough to reach statistical significance.

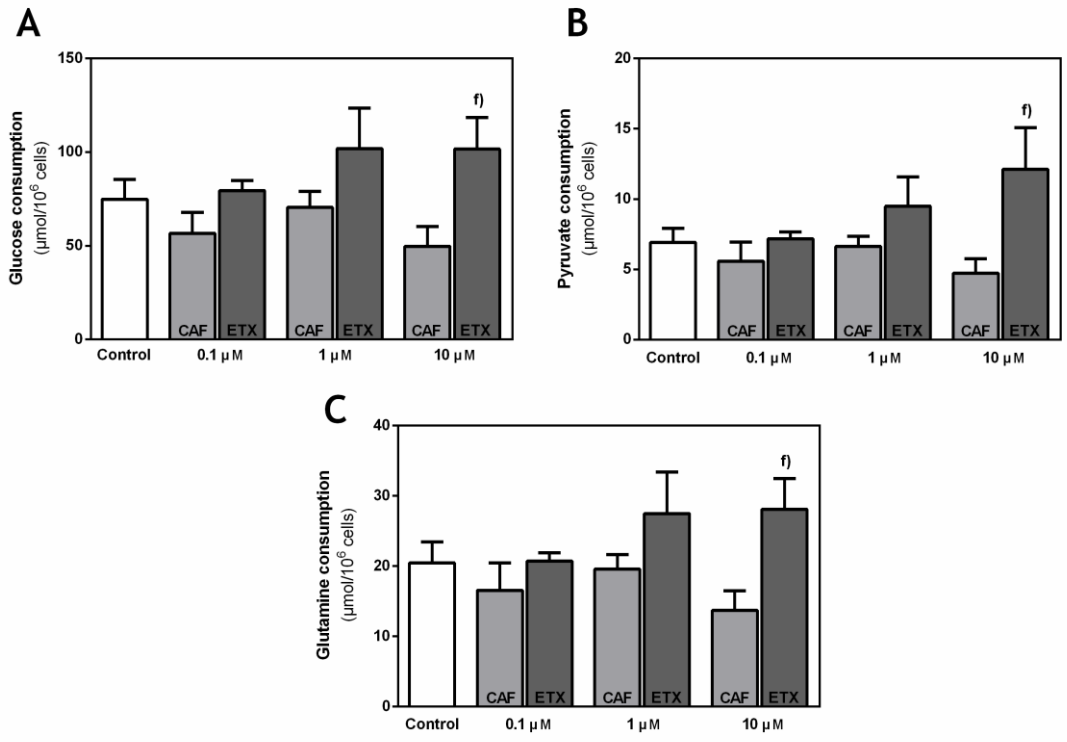

Figure 12 - Consumption of glucose **(A)**, pyruvate **(B)**, and glutamine **(C)** in 3T3-L1 preadipocytes after 24 h incubation with increasing concentrations of caffeine (CAF) or 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX). All metabolites were quantified through analysis of the extracellular medium by ^{1}H-NMR. Results are expressed as µmol/10^6 cells and mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are indicated as: f) relative to 10 µM CAF.

4.3.2. ETX increases lactate, acetate, and alanine production

Lactate is the direct product of the reversible pyruvate reduction by LDH. We observed that CAF only slightly increased lactate production at 1 μM when compared to the control group, whereas all the other studied concentrations did not increase lactate production (Figure 13A). However, ETX increased lactate production in all studied concentrations, although not enough to reach statistical significance. On the other hand, when comparing the same concentration of both compounds we observed that lactate production was significantly increased by ETX (38.85 ± 5.87 μmol/10^6 cells) as compared with CAF (22.03 ± 4.59 μmol/10^6 cells) at the concentration of 10 μM. Additionally, when comparing ETX with CAF at the concentrations of 0.1 μM (34.52 ± 4.07 μmol/10^6 cells and 22.64 ± 3.83 μmol/10^6 cells, respectively) and 1 μM (41.14 ± 8.24 μmol/10^6 cells and 32.16 ± 4.19 μmol/10^6 cells, respectively) we observed an increase in lactate production by ETX, although not enough to reach statistical significance.

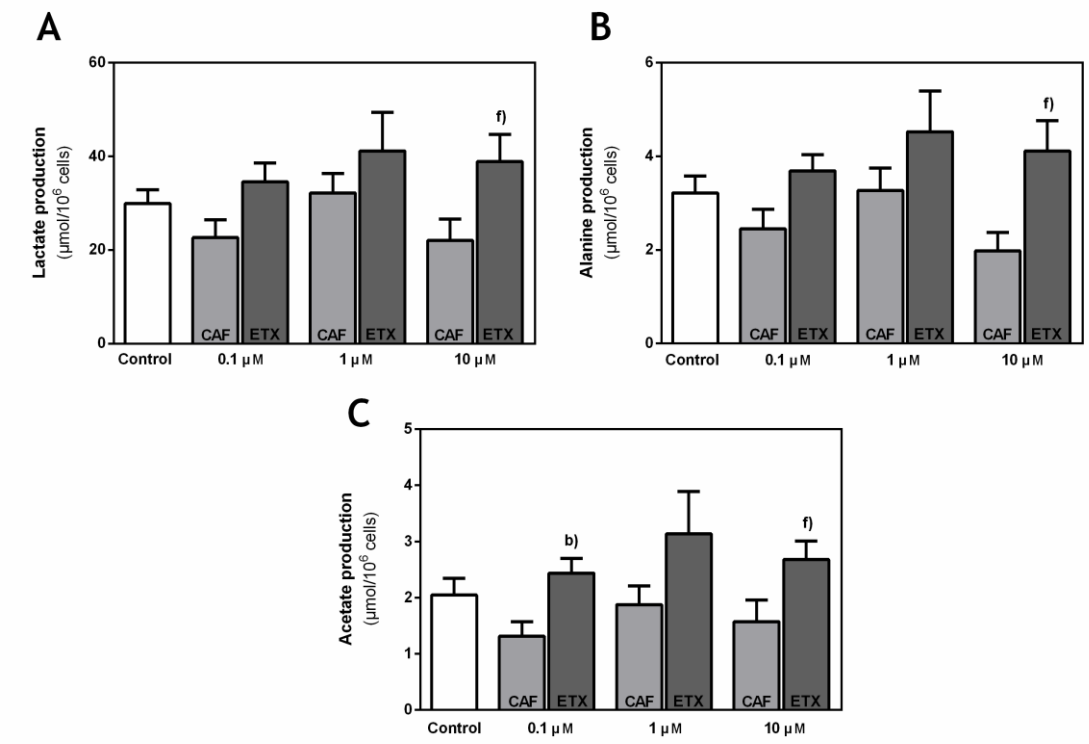

Figure 13 - Production of lactate **(A)**, alanine **(B)**, and acetate **(C)** in 3T3-L1 preadipocytes after 24 h incubation with increasing concentrations of caffeine (CAF) or 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX). All metabolites were quantified through analysis of the extracellular medium by ^{1}H-NMR. Results are expressed as μmol/10^6 cells and mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are indicated as: b) relative to 0.1 μM CAF; f) relative to 10 μM CAF.

Alanine is an amino acid that can be produced from pyruvate. In the presence of L-glutamate, pyruvate can be converted to L-alanine and α-ketoglutarate by alanine transaminase. Our results showed that ETX increased alanine production in all studied

concentrations, although not enough to reach statistical significance when compared with the control group (Figure 13B). On the other hand, CAF did not increase alanine production. However, when comparing the same concentration of both compounds we observed that alanine production was significantly increased by ETX (4.11 ± 0.65 μmol/10^6 cells) as compared with CAF (1.98 ± 0.40 μmol/10^6 cells) at the highest concentration (10 μM). Additionally, when comparing ETX with CAF at the concentrations of 0.1 μM (3.69 ± 0.34 μmol/10^6 cells and 2.45 ± 0.42 μmol/10^6 cells, respectively) and 1 μM (4.53 ± 0.87 μmol/10^6 cells and 3.27 ± 0.48 μmol/10^6 cells, respectively) we observed an increase in alanine production by ETX, although not enough to reach statistical significance.

As described above, pyruvate may enter the mitochondria to be converted to acetyl-CoA by pyruvate dehydrogenase and be used in the citric acid cycle or Krebs cycle. However, acetyl-CoA can enter the acetate switch, leading to acetate and ATP production (Wolfe, 2005). We observed that ETX increased acetate production in all studied concentrations, although not enough to reach statistical significance when compared with the control group (Figure 13C). On the other hand, CAF did not increase acetate production. However, when comparing the same concentration of both compounds we observed that acetate production was significantly increased by ETX when compared with CAF at the 0.1 μM (2.44 ± 0.26 μmol/10^6 cells and 1.31 ± 0.26 μmol/10^6 cells, respectively) and 10 μM (2.68 ± 0.33 μmol/10^6 cells and 1.57 ± 0.39 μmol/10^6 cells, respectively). Additionally, when comparing ETX with CAF at 1 μM (3.14 ± 0.75 μmol/10^6 cells and 1.88 ± 0.33 μmol/10^6 cells, respectively) we observed an increase in acetate production by ETX, although not enough to reach statistical significance.

4.5. ETX decreases protein oxidation in 3T3-L1 preadipocytes

High metabolic activity can lead to increased oxidative stress (OS) in cells. OS happens when the ratio between prooxidants and antioxidants is high, leading to injury in cells, tissues, and organs. This type of damage plays an important role in the pathophysiology of several diseases or conditions, such as obesity (Weber et al., 2015). Thus, it is important to study the oxidative profile of cells after exposition to CAF and the ETX. For that purpose, the oxidative profile of 3T3-L1 preadipocytes after incubation with increasing concentrations of CAF or ETX (0, 0.1, 1 and 10 μM) was evaluated through analysis of protein oxidation, lipid peroxidation and the tyrosine residues nitration (Figure 14).

Protein carbonyl content represents an irreversible form of protein modification due oxidative damage. This oxidative damage to proteins results in modification of a wide range of amino acids, which can lead to protein malfunction and cellular damage (Weber et al., 2015). For this reason, it is commonly used as a biomarker for protein oxidation (Dias et al., 2015). Our results point out that 3T3-L1 preadipocytes exposed to ETX decreased the protein carbonyl content at 0.1 μM (0.44 ± 0.09-fold variation to control), 1 μM (0.41 ± 0.06-fold

variation to control), and 10 µM (0.49 ± 0.08-fold variation to control), although not enough to reach statistical significance (Figure 14A). On the other hand, while CAF at 0.1 µM decreased protein carbonyl content (0.37 ± 0.08-fold variation to control) without reaching statistical significance, it significantly increased protein carbonyl content at 1 µM (2.69 ± 0.42-fold variation to control) and 10 µM (2.25 ± 0.43-fold variation to control). Moreover, when comparing the same concentration of both compounds we observed that protein oxidation was significantly decreased by ETX when compared with CAF at 1 and 10 µM.

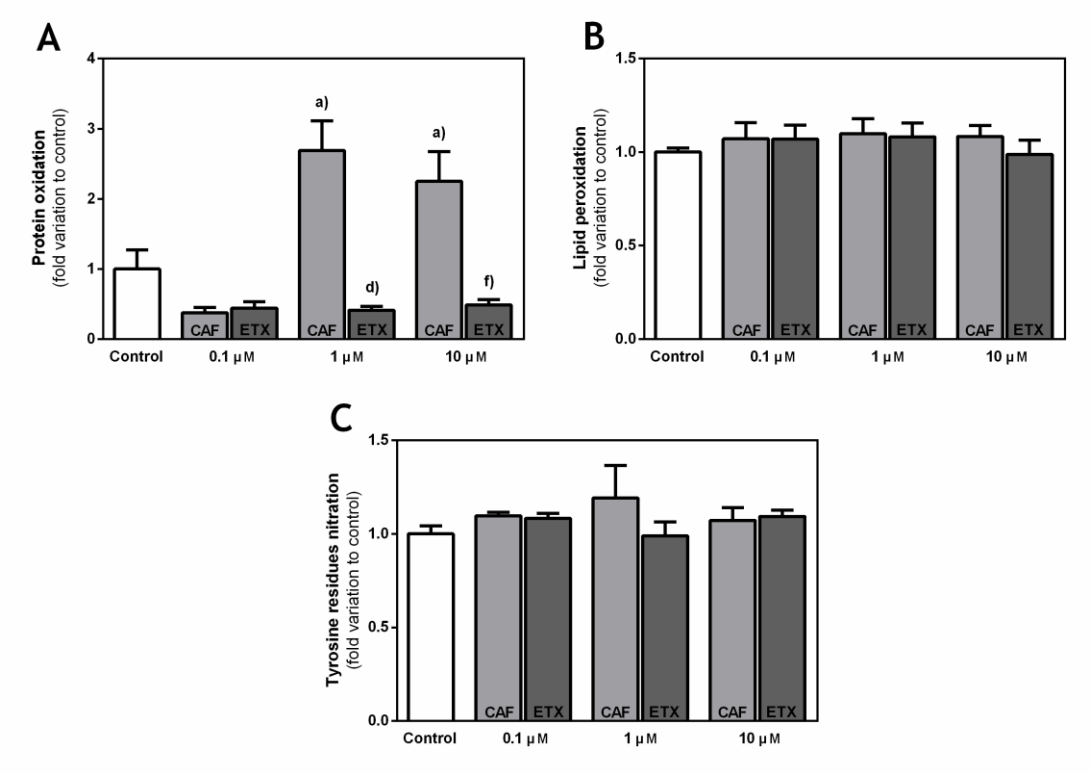

Figure 14 - Characterization of the oxidative profile in 3T3-L1 cells after 24 h incubation with increasing concentrations of caffeine (CAF) or 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX). **(A)** Protein oxidation was analysed through protein carbonyl content quantification. **(B)** Lipid peroxidation was analysed through 4-hydroxynonenal (4-HNE) quantification. **(C)** Tyrosine residues nitration was analysed through 3-nitrotyrosine quantification. Results are expressed as fold variation to control and mean ± SEM (n = 6 for each condition). Significantly different results ($p<0.05$) are indicated as: a) relative to control; d) relative to 1 µM CAF; f) relative to 10 µM CAF.

Lipid peroxidation results in the damage of the lipids constituting the membrane. The destruction of membrane lipids may be harmful *per si*, although the end-products of the reactions are also dangerous for cells and tissues (Ayala et al., 2014). We observed no significant alterations in lipid peroxidation in 3T3-L1 preadipocytes exposed to CAF when compared with non-exposed cells at 0.1 µM (1.07 ± 0.09-fold variation to control), 1 µM (1.10 ± 0.08-fold variation to control), or 10 µM (1.08 ± 0.06-fold variation to control) (Figure 14B). Moreover, no significant alterations were observed on 3T3-L1 exposed to ETX when compared with the control group (1.07 ± 0.08-fold variation to control, 1.08 ± 0.07-fold variation to control, and 0.99 ± 0.08-fold variation to control, respectively).

The nitration of tyrosine residues in proteins is considered a biomarker for the disruption of nitric oxide signaling and metabolism towards pro-oxidant processes. The incorporation of a nitro group to tyrosine can result in structural and functional changes, which contributes to the loss of homeostasis (Radi, 2013). We observed no significant alterations in the tyrosine residues nitration in 3T3-L1 preadipocytes exposed to CAF when compared with non-exposed cells at 0.1 μM (1.10 ± 0.02-fold variation to control), 1 μM (1.19 ± 0.17-fold variation to control), or 10 μM (1.07 ± 0.07-fold variation to control) (Figure 14C). Additionally, no significant alterations were observed on 3T3-L1 exposed to ETX when compared with the control group (1.08 ± 0.03-fold variation to control, 0.99 ± 0.07-fold variation to control, and 1.09 ± 0.03-fold variation to control, respectively).

V. Discussion

V. Discussion

Methylxanthines are pharmacologically active compounds commonly present in the human diet worldwide. These phytochemicals exhibit several biological actions that could be beneficial for the treatment of several disease conditions. One of the most promising is the perspective of methylxanthines use for obesity management, through their ability to increase energy expenditure, modulate adipose tissue metabolism, stimulate lipolysis, and inhibit adipogenesis (Dulloo, 2011). Based on the chemical structure of methylxanthines, several new molecules have been synthetized. One synthetic xanthine analogue, 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX), exhibits high A_1R affinity (Van der Walt & Terre'Blanche, 2015) and we hypothesized that this new molecule may be a potential candidate for anti-obesity drug design. Therefore, we tested the effect of ETX in 3T3-L1 adipocyte cell line glycolytic and oxidative profile. For comparative purposes, we used the most well studied methylxanthine, caffeine (CAF). Despite we experienced a low efficiency in the adipocyte differentiation protocol, our results were still promising.

The first step was to evaluate if ETX could induce cytotoxicity at the chosen concentrations (0.1 to 100 μM). We observed that ETX did not show any cytotoxicity at all conditions tested, which is a promising feature for drug design and application. On the other hand, CAF significantly induced cytotoxicity at 100 μM. This high concentration represents the plasma levels that can be attained by a high coffee consumption (Magkos & Kavouras, 2005). CAF is reported to present toxic effects to humans in plasma concentrations near 200 μM (Fredholm, 1985), while it is considered fatal at 1 mM range (Anderson et al., 1999). Even if ETX did not induce any cytotoxicity at all conditions tested, our main objective in this project was to compare the effects of ETX with CAF in the metabolism and oxidative profile of the adipose tissue. Thus, we decided to exclude the highest concentration (100 μM) from the subsequent assays.

Methylxanthines are reported to modulate glucose metabolism and increase energy expenditure (Lopes-Silva et al., 2015). Accordingly, we observed that ETX increased the consumption of glucose, pyruvate, and glutamine. Additionally, ETX also increased the production of lactate, alanine, and acetate. Overall, ETX increased glycolysis and modulated metabolism towards energy production, although at the studied concentrations it was not sufficient to reach statistical significance (as compared with the control conditions). One possible explanation for the metabolism modulation may be linked with the A_1R antagonism. Methylxanthines are described as non-specific antagonists of AR, being able to block these receptors and inhibit, in a competitive manner, the role of adenosine in the cells (Chen & Chern, 2011). It was reported that the blockade of the A_1R in cardiac muscle cells enhanced glycolysis, increasing glucose uptake and lactate production (Gao et al., 1997). Moreover, similar results were described in hippocampal cells (Duarte et al., 2016). However, the

mechanisms behind the A_1R activity to stimulate the glucose uptake and energy production are yet to be completely understood. One possibility is that these effects may be caused by the increased cAMP production resulting from the A_1R blockade. When this receptor is not active, the adenylyl cyclase is stimulated to produce cAMP, which activates PKA (Dhalla et al., 2009). In the muscle, this enzyme phosphorylates phosphofructokinase-2 (PFK-2), activating it and stimulating glycolysis (Rider et al., 2004). Thus, increased levels of cAMP may lead to enhanced glycolysis. On the other hand, in the liver increased levels of cAMP induce the opposite result, since PKA inactivates the PFK-2 leading to glycolysis inhibition (Pilkis et al., 1995). This differential effect occurs due to the existence of different isoforms, the L (liver) and the M (muscle) isozymes (Rider et al., 2004). Concerning adipose tissue, mature adipocytes are reported to express both the L and M isozymes, but the regulation of their expression is not fully understood. For instance, there is evidence that the M isozyme activity is modulated by insulin and its overexpression results in increased glycolysis (Atsumi et al., 2005). Regarding L isozyme, its expression and activity is yet to be elucidated. On the other hand, preadipocytes only express the M isozyme (Bruni et al., 1999). Since ETX is a potent A_1R antagonist, the blockade of this receptor may lead to the activation of M-PFK-2 and increased glycolysis and energy production. However, we observed that CAF did not increase glucose consumption or alter preadipocytes metabolism at concentrations from 0.1 up to 10 μM. This fact may be explained by the concentrations used in this study. The inhibitory constant (K_i) of a molecule reflects its binding affinity and the smaller the K_i, the smaller the concentration that is needed to inhibit the activity of the enzyme or receptor. For instance, if the K_i is higher than the maximal plasma concentration of a given drug, that compound is not likely to inhibit the activity of the enzyme or receptor. The concentrations chosen (0.1, 1 and 10 μM) are below the CAF K_i for A_1R, which is about 40-50 μM (Magkos & Kavouras, 2005), and might be insufficient to trigger the inhibitory response. On the other hand, ETX is described as much more potent. The ETX K_i is 0.164 μM, which is equivalent as being 206-fold more potent than CAF. While the 1-methyl group present in CAF is the great responsible for its affinity to A_1R, the 1,3,7-triethyl-substitions associated with substitutions at position 8 with aryl or cycloalkyl groups were reported as promising to enhancing this affinity (Van der Walt & Terre'Blanche, 2015). Thus, in comparison to CAF, the same concentrations of ETX have stronger inhibitory effects for A_1R. In fact, when comparing both compounds at the same concentration, we observed that ETX significantly increased glucose, pyruvate, and glutamine consumption at 10 μM, lactate and alanine production at 10 μM, and acetate production at 0.1 and 10 μM (as compared with CAF). Our results suggest that ETX may modulate preadipocytes metabolism towards energy expenditure much more efficiently and at much lower concentrations than CAF. Both features highlight the potential of ETX as an excellent candidate to address obesity.

Obesity is associated with increased OS in human and mice. Increased OS in adipose tissue can impair metabolism, resulting in metabolic complications (Furukawa et al., 2004). The main reason behind OS is the accumulation of ROS. By definition, ROS are singlet electron

intermediates formed during the partial reduction of oxygen to water, such as superoxide, hydrogen peroxide and hydroxyl radical. Superoxide may also interact with nitric oxide to produce reactive nitrogen species (RNS), like peroxynitrite, nitrogen dioxide and dinitrogen trioxide, between others (Kanaan & Harper, 2017). The primary sources of ROS production are the mitochondria, plasma membrane, endoplasmic reticulum and peroxisomes (Ayala et al., 2014). While mitochondrial and cellular functions may be compromised by the accumulation of ROS, under normal physiological conditions these species are important signalling molecules (Kanaan & Harper, 2017). However, when the production is higher than the cellular protective antioxidant systems, it might result in OS and several forms of cellular damage.

CAF is usually reported as an antioxidant and associated with reduced OS biomarkers (Grucka-Mamczar et al., 2009). Moreover, it has been described as a protective agent against cellular damage (Ofluoglu et al., 2009) and efficient radical scavenger *in vitro* (Shi et al., 1991). This protective effect against OS conferred by CAF was previously associated with AR, which are reported to regulate the formation of free radicals (Gołembiowska & Dziubina, 2012). However, methylxanthines may act as both antioxidant and prooxidant according to the dose (Azam et al., 2003). In our study, we observed that CAF at 1 and 10 μM significantly increased protein carbonyl content, a biomarker for protein oxidation. This oxidative damage to proteins results in modification in amino acids, which can lead to protein malfunction and cellular damage (Weber et al., 2015). Our results suggest that CAF may act as a prooxidant at certain concentrations and conditions, which may exacerbate the damage caused by OS. In fact, other studies also reported CAF as a prooxidant compound (Gulcin, 2008). For instance, 5 μM of CAF was able to increase protein oxidation in human Sertoli cells, increasing OS-induced damage (Dias et al., 2015). Moreover, at higher concentrations (500 μM) it was also observed increased protein oxidation. Thus, it is suggested that CAF prooxidant or antioxidant effects are concentration-dependent. On the other hand, we observed that all the studied concentrations of ETX decreased protein oxidation. Moreover, when comparing both compounds at the same concentration, we observed that ETX significantly decreased protein carbonyl content. These results illustrate that ETX may promote an antioxidant environment and have a protective role against cellular OS, which is advantageous for the development of anti-obesity approaches. However, more data is needed to draw further conclusions.

In sum, our results show that ETX is safe, once it does not induce cytotoxicity even at high concentrations. Moreover, ETX is a modulator of cell metabolism towards energy expenditure. Additionally, ETX promoted an antioxidant environment reducing protein oxidation and protecting cells against OS-induced damage. These results illustrate that ETX is an excellent candidate for anti-obesity drug design and application. Nevertheless, further studies are required to elucidate its anti-obesity potential. For instance, it is important to study its lipolytic activity in adipocytes and adipose tissue. If this molecule has a lipolytic activity, the mechanistic pathways behind this action should also be explored and characterized. Moreover, further studies are needed to elucidate the role of ETX in oxidative stress-related processes.

VI. Conclusions

VI. Conclusions

Methylxanthines are pharmacologically active compounds that have been used in the human diet for centuries. Methylxanthines display several biological actions that could be beneficial for the treatment of several disease conditions. One of the most promising is the perspective of methylxanthines use for obesity management in virtue of major unmet treatment needs, due to its increasing prevalence and lack of effective pharmacological tools. The cornerstones for obesity treatment are lifestyle interventions aiming to reduce caloric intake and increase energy expenditure trough physical exercise. Methylxanthines can stimulate lipolysis and inhibit adipogenesis through several molecular mechanisms, thus contributing to fat depletion and weight loss. These compounds antagonize adenosine receptors, stimulate the release of neural catecholamines, inhibit phosphodiesterases and decrease the expression of important transcription factors. Some new compounds have been synthetized based on xanthine chemical structure, enhancing those effects. We hypothesized that one of those innovative compounds, 8-(3-phenylpropyl)-1,3,7-triethylxanthine (ETX), may have a promising anti-obesity potential, due to its high adenosine receptor affinity. Our study aimed to characterize the modulation conferred by this new molecule in the metabolic and oxidative profile of adipocytes in order to evaluate its pharmacological potential to treat obesity and related complications. For this purpose, the anti-obesogenic potential of ETX was evaluated in mouse cell line 3T3-L1, using synthetic caffeine for comparative purposes. As the cells differentiation was not entirely successful, we evaluated the effects of both compounds in preadipocytes and the results were quite promising. ETX exhibited no cytotoxicity at all the studied concentrations (0.1, 1, 10, and 100 μM), where caffeine induced some cytotoxicity at the highest concentration (100 μM). While being safer at higher concentrations, ETX could modulate preadipocytes metabolism, significantly increasing glucose, pyruvate, and glutamine consumption at 10 μM, lactate and alanine production at 10 μM, and acetate production at 0.1 and 10 μM (as compared with caffeine). These results suggest that the ETX can modulate the cellular metabolism towards energy expenditure much more efficiently and at much lower concentrations than caffeine. Concerning the oxidative profile, ETX seems to promote an antioxidant environment and decrease protein oxidation, while caffeine showed prooxidant activity at higher concentrations. This data suggests that ETX may have a protective role against oxidative stress, which is advantageous for obesity treatment.

In sum, ETX appears as an excellent candidate for anti-obesity drug design and application. However, further data is required to elucidate the anti-obesity potential of this new compound. For instance, it is important to study its lipolytic activity in adipocytes and adipose tissue. If this new molecule has a lipolytic activity, the mechanistic pathways behind this action should be explored. Additionally, more studies are needed to elucidate its role in oxidative stress-related processes.

VII. Bibliography

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