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MASTER IN LEGAL MEDICINE

IN VIVO HEPATOTOXICITY OF THE NEW DRUGS OF ABUSE 3,4-DIMETHYLMETHCATHINONE (3,4-DMMC) AND METHYLONE

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Candidature dissertation of Master in Legal Medicine submitted to Institute of Biomedical Sciences Abel Salazar (ICBAS) of University of Porto

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Author's declaration

The author states to have afforded a major contribution to the technical execution of the work, acquisition of data, analysis and interpretation of the results and preparation of the published or under publication works included in this dissertation.

Abstract

3,4-Dimethylmetcathinone (3,4-DMMC) and methylone are new psychoactive substances belonging to the group of synthetic cathinones, whose abuse has recently increased by virtue of their stimulant, euphoric and empathogenic properties. As the popularity of these novel drugs increased, the number of reported intoxications, including some deaths, have been also proportionally reported in the scientific literature. Nevertheless, there is still a lack of systematic studies concerning their *in vivo* toxicological effects in the target organs. Thus, the current study aimed at contributing to the elucidation i) of the effects elicited by 3,4-DMMC and methylone in Wistar rats, and ii) of the underlying causes of toxicity, through the measurement of the alterations in the energetic content and in oxidative stress parameters in the liver, an important target of cathinones' toxicity.

For this purpose, adult female Wistar rats, weighing 250–300 g, were injected i.p. with saline (0.9% NaCl) or two doses (20 mg/Kg and 40 mg/Kg) of 3,4-DMMC or methylone. A group of animals treated with 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg was also included for comparison. During the test period, animals were kept in metabolic cages and their behaviour carefully monitored. After 1 h or 24 h, rats were anaesthetized and total blood collected from the inferior vena cava into EDTA tubes. After euthanasia, the liver was excised, weighed, and washed with 0.9% NaCl solution. A tissue aliquot was collected for histological examination. The remaining organ was homogenized with an Ultra-Turrax® homogenizer (1:4 m/v) in ice cold 100 mM phosphate buffer (pH 7.4), and used for analysis. Homogenate aliquots were added of NaOH 2 M (1:1) and used for the determination of protein content by the Lowry method; or added with ice-cold 10% perchloric acid (1:1), centrifuged at 3,200 g for 10 min at 4 ^{o}C, and the supernatants used for the quantification of adenosine triphosphate (ATP) by luciferin-luciferase assay and of reduced glutathione (GSH) and oxidised glutathione (GSSG) contents by the 5,5-dithio-bis-(2-nitrobenzoic acid)-GSSG reductase recycling assay. For measurement of antioxidant enzymes activities, homogenates were added with 0.2% Triton X-100 (1:1), centrifuged at 3,200 g for 10 min at 4 ^{o}C, and the supernatants used for the determination of glutathione-S-transferase (GSTs), selenium-dependent glutathione peroxidase (GPx), and glutathione redutase (GR). Total blood was centrifuged at 1,600 g for 15 min at 4 ^{o}C, and plasma used for further biochemical analysis.

Methylone increased overall animal locomotion, salivation, temperature and piloerection. Piloerection and hyperthermia were also observed in animals treated with 3,4-DMMC, which adopted a defensive posture and showed signs of confusion and imbalance. Methylone at 40 mg/Kg induced a significant increase of relative liver weight ($p < 0.05$), after 1 h. Overall, energetic content in the liver significantly decreased ($p < 0.05$)

after exposure to both drugs at 40 mg/Kg for 1 h, and to methylone at 20 mg/Kg for 24 h. The antioxidant GSH/GSSG balance was reduced for all test conditions, with exception of rats exposed to methylone at 20 mg/Kg for 24 h. While 3,4-DMMC and methylone reduced GST activity both after 1 h (at 40 mg/Kg) and 24 h (at 20 mg/Kg), GPx and GR activities were significantly reduced in rats exposed for 1 h to 3,4-DMMC at 20 mg/Kg and methylone at 40 mg/Kg. No significant liver histological alterations were observed after drug exposures for 24 h. Methylone and 3,4-DMMC also triggered changes in plasma biochemical markers indicative of liver damage (alanine transaminase, lactate dehydrogenase, alkaline phosphatase, γ-glutamyl transpeptidase and aspartate transaminase), but the extent of these alterations were highly dependent on the exposure time and dose and/or drug tested.

To our knowledge, this is the first *in vivo* animal study evaluating stress parameters induced by exposure to 3,4-DMMC and methylone. These data will help elucidate the toxicological effects of these drugs on their main targets, which is essential to assist the medical examiner in forensic investigations.

Resumo

A 3,4-dimetilmetcatinona (3,4-DMMC) e a metilona são novas substâncias psicoativas pertencentes ao grupo das catinonas sintéticas, cujo abuso aumentou recentemente devido às suas propriedades estimulantes, eufóricas e empatogénicas. À medida que a popularidade destas novas substâncias aumentou, o número de intoxicações, incluindo algumas mortes, também tem sido proporcionalmente relatado na literatura científica. No entanto, ainda há falta de estudos sistemáticos sobre os seus efeitos toxicológicos *in vivo* nos órgãos-alvo. Assim, o presente estudo teve como objetivo contribuir para a elucidação i) dos efeitos provocados pela 3,4-DMMC e metilona em ratazanas Wistar; e ii) das causas subjacentes à toxicidade, através da medição das alterações do conteúdo energético e dos parâmetros de stress oxidativo no fígado, um importante alvo da toxicidade das catinonas.

Para este fim, fêmeas de ratazanas Wistar adultas, com cerca de 250-300 g, foram administradas i.p. com solução salina (NaCl a 0,9%) ou duas doses (20 mg/Kg ou 40 mg/Kg) de 3,4-DMMC ou metilona. Um grupo de animais tratados com 3,4-metilenodioximetanfetamina (MDMA) a 20 mg/Kg também foi incluído para comparação. Durante o período de teste, os animais foram mantidos em gaiolas metabólicas e o seu comportamento foi cuidadosamente monitorizado. Após 1 h ou 24 h, os ratos foram anestesiados e o sangue total colhido da veia cava inferior em tubos de EDTA. Após a eutanásia, o fígado foi retirado, pesado e lavado com solução de NaCl a 0,9%. Foi retirado um fragmento de tecido para exame histológico. O restante órgão foi homogeneizado com um homogeneizador Ultra-Turrax® (1:4 m/v) em tampão de fosfatos a 100 mM (pH 7,4) e utilizado para análise. Às alíquotas de homogeneizado foi adicionado NaOH a 2 M (1:1) e de seguida utilizadas para a determinação do teor de proteínas pelo método de Lowry; ou adicionado ácido perclórico a 10% (1:1), centrifugado a 3,200 *g* durante 10 min a 4 °C, e os sobrenadantes foram utilizados para a quantificação da adenosina trifosfato (ATP) pelo ensaio de luciferina-luciferase e para quantificação da glutationa reduzida (GSH) e glutationa oxidada (GSSG) pelo ensaio da 5,5-ditio-bis- (ácido 2-nitrobenzoico)-GSSG redutase. Para a medição das atividades das enzimas antioxidantes, foi adicionado aos homogeneizados Triton X-100 a 0,2% (1:1), foram centrifugados a 3,200 g durante 10 min a 4 °C, e os sobrenadantes foram utilizados para determinação da glutationa-S-transferase (GSTs), glutationa peroxidase (GPx) e glutationa redutase (GR). O sangue total foi centrifugado a 1,600 *g* por 15 min a 4 ºC, e o plasma foi utilizado posteriormente para análises bioquímicas.

A metilona aumentou a locomoção geral do animal, salivação, temperatura e piloereção. A piloereção e hipertermia também foram observadas em animais tratados

com 3,4-DMMC, que adotaram uma postura defensiva e apresentaram sinais de confusão e desequilíbrio. A metilona a 40 mg/Kg induziu um aumento significativo do peso relativo do fígado (p<0,05), após 1 h. No geral, o conteúdo energético no fígado diminuiu significativamente (p <0,05) após a exposição a ambas as drogas a 40 mg/Kg, após 1 h, e à metilona a 20 mg/Kg, após 24 h. O balanço antioxidante GSH/GSSG foi reduzido para todas as condições testadas, com exceção dos ratos expostos a metilona a 20 mg/Kg, após 24 h. Enquanto a 3,4-DMMC e a metilona reduziram a atividade da GST após 1 h (a 40 mg/Kg) e 24 h (a 20 mg/Kg), as atividades da GPx e GR foram significativamente reduzidas em ratos expostos após 1 h à 3,4-DMMC a 20 mg/Kg e à metilona a 40 mg/Kg. Não foram observadas alterações histológicas significativas do fígado após 24 h da exposição das drogas. A metilona e a 3,4-DMMC também desencadearam alterações nos marcadores bioquímicos plasmáticos indicativos de dano hepático (alanina transaminase, lactato desidrogenase, fosfatase alcalina, γ-glutamil transpeptidase e aspartato transaminase), mas a extensão dessas alterações foi altamente dependente do tempo de exposição e dose e/ou droga testada.

Para nosso conhecimento, este é o primeiro estudo *in vivo* em animais a avaliar os parâmetros de stresse induzidos pela exposição à 3,4-DMMC e metilona. Estes dados ajudarão a elucidar os efeitos toxicológicos destas substâncias nos seus principais alvos, o que é essencial para auxiliar o médico legista em investigações forenses.

Palavras-chave: 3,4-Dimetilmetcatinona (3,4-DMMC). Metilona. Catinona sintética. Nova substância psicoativa (NSP). Tocixidade. *In vivo.*

General Index

Table Index

List of Abbreviations

3,4-DMMC, 3,4-Dimethylmethcathinone

4-MMC, Mephedrone or 4-methylmetcathinone

5-HT, Serotonin

α-PVP, α-Pyrrolidinopentiophenone

γ-GT, γ-Glutamyl transpeptidase

ALP, Alkaline phosphatase

ALT, Alanine transaminase

AST, Aspartate transaminase

ATP, Adenosine triphosphate

BSA, Bovine serum albumin

COMT, Catechol-O-methyltransferase

DA, Dopamine

DAT, Dopamine transporter

EMCDDA, European monitoring centre for drug and drug addiction

GPx, Glutathione peroxidase

GR, Glutathione reductase

GSH, Reduced glutathione

GSSH - Oxidized glutathione

GSTs, Glutathione S-transferases

HMMC, 3-Methoxy-4-hydroxymethcathinone or 3-MeO-4-OH-MC

LDH, Lactate dehydrogenase

MAO, Monoamine oxidase

MDMA, 3,4-Methylenedioxymethamphetamine or "ecstasy"

MDMC, 3,4-Methylenedioxy-N-methylcathinone or methylone

NA, Noradrenaline

NADPH, Nicotinamide adenine dinucleotide phosphate reduced form

NAT, Noradrenaline transporter

NPS, New psychoactive substances

ROW, Relative organ weight

SERT, Serotonin transporter

VMAT, Vesicular monoamine transporter

Chapter I - General Introduction

1.1 Introduction

In recent years, the production and consumption of new psychoactive substances (NPS) have undergone a major expansion in the drug abuse market. These NPS, which became very popular among young people, mainly in recreational environments such as parties and at clubs and other nightlife venues, have been known in the market by terms such as 'legal highs', 'designer drugs', 'bath salts', and 'plant fertilisers' (Coppola and Mondola, 2012; McGraw and McGraw, 2012; Paillet-Loilier et al., 2014; Rosenbaum et al., 2012). Many of these psychotropic substances have firstly been synthesized as a legal alternative to obtain the stimulant effects of illicit drugs such as 3,4-methylendioximentamphetamine (MDMA, commonly known as *ecstasy*) and cocaine, but in the meantime they were also banned as a result of their increased consumption and toxicological effects (Fowble et al., 2018; Richeval et al., 2018).

Among the most prevalent NPS are synthetic cathinones, being the second largest group of new drugs monitored by European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), with 130 substances reported in total by the end of 2017, including 12 that were first detected that year (EMCDDA, 2018). Other data that supports the growth of synthetic cathinones in the NPS market is the constant increase in the number of seizures, tripling to 1.3 tonnes in 2014 from the previous year. Most seizures occurred in Europe and in East and Southeast Asia (UNODC, 2016). In 2016, seizures of these substances accounted for a total of almost 1.9 tonnes, making synthetic cathinones the most seized NSP in that year (EMCDDA, 2018).

Synthetic cathinones are molecules derived from cathinone, a natural psychostimulant found in the *Khat* plant (*Catha Edulis*), which is grown mainly in East Africa and the Arabian Peninsula (El-Menyar et al., 2015; Fowble et al., 2018; Richeval et al., 2018; UNODC, 2018; Valente et al., 2014). Due to their structural similarity to amphetamine, they are also characterized by their psychostimulant effects (Feyissa and Kelly, 2008; Katz et al., 2014; Valente et al., 2014). The majority of these substances are manufactured in China and South East Asia and then shipped to distributors, who are in charge of packaging, selling and, eventually, tampering (common adulterants include paracetamol, caffeine and piperazines) (German et al., 2014; Valente et al., 2014).

The most popular synthetic cathinone among consumers is mephedrone or 4-methylmetcathinone (4-MMC), which is also one of the most reported drugs to the EMCDDA since the onset of NPS, and was the first synthetic cathinone to have a risk assessment report. Along with 4-MMC, one of the first synthetic cathinones reported to the competent authorities was methylone (βk-MDMA or 3,4-methylenedioxy-N-methylcathinone, MDMC), identified in the drug market in late 2004 in the Netherlands

(Bossong et al., 2005), but first synthesized by Alexander Shulgin in 1996 and patented back then as an antidepressant and antiparkinson (Bossong et al., 2005; Cottencin et al., 2014; Kelly, 2011).

With the increasing popularity of these new derivatives and the ease with which these substances could be acquired, several cases of poisoning and deaths related to the consumption of these substances have been reported (Adamowicz et al., 2014; Batisse et al., 2014; Borek and Holstege, 2012; Carbone et al., 2013; Frohlich et al., 2011; Pearson et al., 2012; Zaami et al., 2018) culminating in the legal framing of many of these derivatives, including 4-MMC. The phenomenon of 'emerging drugs' is very dynamic and as legal control was exercised over some cathinones whose popularity and abuse have disseminated, new variants were synthesized by molecular alterations that are sometimes very subtle (bioisosterism). Therefore, following their ban in 2010, a new generation of drugs further emerged as an alternative to these recently controlled cathinones, including: 4-methylethcathinone (4-MEC), α-pyrrolidinopentiophenone (α-PVP) and 3,4-dimethylmethcathinone (3,4-DMMC) (Katz et al., 2014; Valente et al., 2014; Zaami et al., 2018; Zawilska and Wojcieszak, 2013). The drug 3,4-DMMC was first identified in Hungary in October 2010 (EMCDDA, 2010), and in Portugal in 2012 through the EU Early Warning System (EU-EWS).

1.2 Chemistry

Synthetic cathinones are related to the phenylethylamine family, being chemically a β-ketophenylethylamine. Their chemical structure is very similar to that of amphetamines (Figure 1), the difference being an additional β-keto group on the amino alkyl chain, thus similar toxicological effects are produced (Valente et al., 2014).
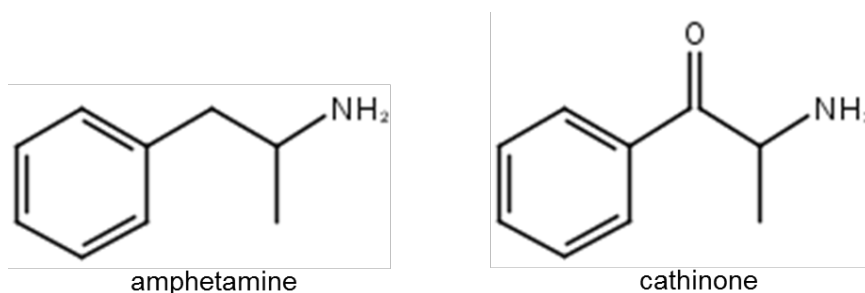
Figure 1. Chemical structure of amphetamine and natural cathinone.

The cathinone derivatives have a similar structure to that of natural cathinone, being synthesized by the addition of different substituents at different locations of the molecule (Valente et al., 2014). In addition to the ketone group, synthetic cathinones may have other functional groups, and their presence or absence classifies the compound in one of four groups (Figure 2), *i.e.* i) N-alkylated cathinones [*e.g.* buphedrone, 4-MMC, 3,4-DMMC and flephedrone (4-FMC or 4-fluoromethcathinone)]; ii) cathinones containing a 3,4-methylenedioxy group attached to the benzene ring (*e.g.* butylone and methylone), being structurally similar to MDMA; iii) cathinones characterized by the substitution of the primary amine by N-pyrrolidine (*e.g.* α-PVP and pyrovalerone); and, lastly, cathinone derivatives which exhibit the chemical characteristics of the two preceding groups, *i.e.* N-pyrrolidine-substituted synthetic cathinones with a 3,4-methylenedioxy ring [*e.g.* 3,4-methylenedioxypyrovalerone (MDPV)] (Valente et al., 2014).
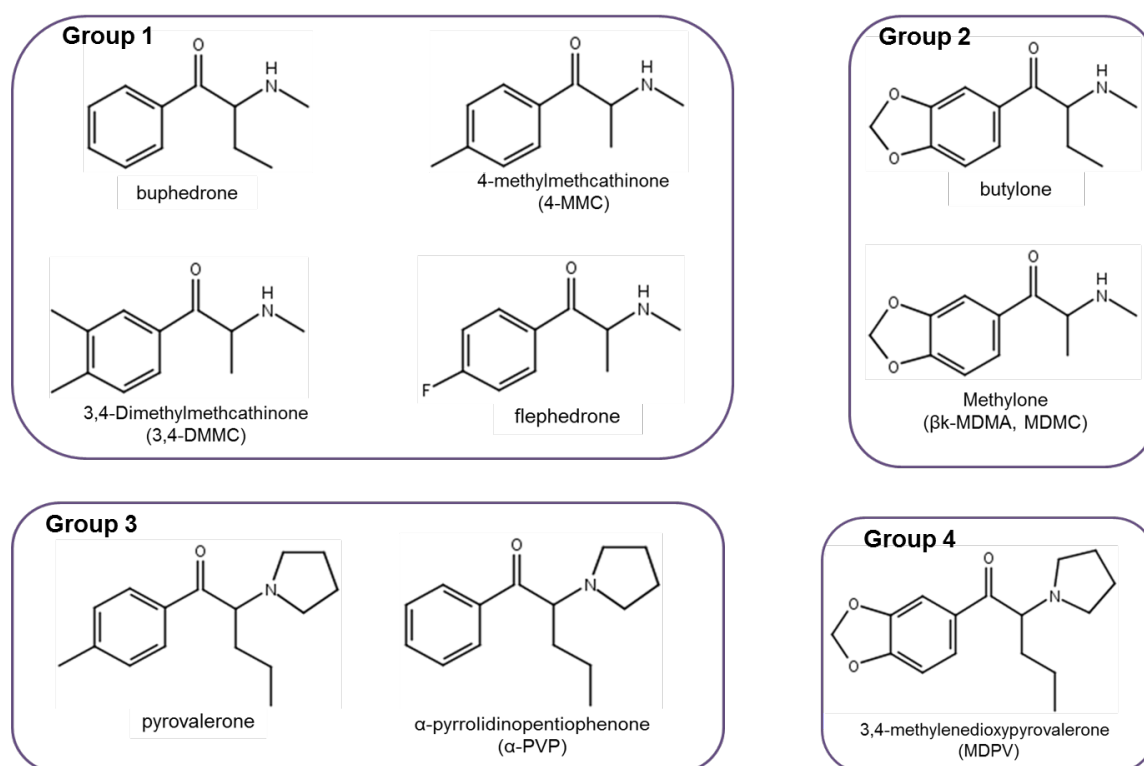
Figure 2. Some synthetic cathinones divided into four distinct groups according to their chemical structure. Group 1 is composed by N-alkylated cathinones; group 2 by cathinones containing a 3,4-methylenedioxy group attached to the benzene ring; in group 3, cathinones are characterized by the substitution of the primary amine by N-pyrrolidine; and, in group 4, cathinones exhibit both the N-pyrrolidine and 3,4-methylenedioxy ring substitutions

Compared to amphetamines, the presence of the ketone group is responsible for an increase in the polarity of synthetic cathinones, decreasing their ability to cross the

blood-brain-barrier. Therefore, there is a need for consumers to use higher doses to obtain compensatory effects, and in some cases there is even a need to repeat the dose administration (Kelly, 2011; Prosser and Nelson, 2012; Valente et al., 2016a). On the other hand, analogues exhibiting the pyrrolidine ring in the amino group show higher lipophilicity, facilitating the brain uptake, and leading to earlier and more intense peak effects (Coppola and Mondola, 2012; Gaspar et al., 2018; Valente et al., 2016a).

1.3 Patterns of use

According to the EMCDDA, in 2013, seizures of 3.4-DMMC and methylone in Europe accounted for 2 and 7%, respectively, of all cathinones apprehended (EMCDDA, 2015), which demonstrates their widespread abuse. These drugs are often sold on the Internet as 'bath salts' or 'plant fertilisers', usually acquired in the form of white or brownish powders, and can also be presented in capsules or tablets, or in crystal form to simulate the real bath salts (Coppola and Mondola, 2012; McGraw and McGraw, 2012; Paillet-Loilier et al., 2014; Rosenbaum et al., 2012). They are usually administered orally and through nasal insufflation, with some users reporting a characteristic odour and taste (Coppola and Mondola, 2012; Dargan et al., 2011; Rosenbaum et al., 2012). Intravenous and rectal administration have also been described (Fowble et al., 2018; Richeval et al., 2018; Valente et al., 2014), as well as combination of multiple pathways in a single session. Most consumers use these substances in combination with two or more cathinone derivatives, also mixing them with other types of psychoactive substances, caffeine, alcohol and tobacco (Tyrkko et al., 2013; Zawilska and Wojcieszak, 2013). The doses of synthetic cathinones consumed can vary greatly, depending on the potency of their effects and the route of administration used (Kelly, 2011; Prosser and Nelson, 2012). It impossible to determine a "safe" dose, as negative side effects may occur for all doses taken and often the same dose lead to different results in different individuals (Prosser and Nelson, 2012; Schifano et al., 2011).

1.4 Pharmacokinetics

After absorption, cathinone derivatives undergo an extensive phase I metabolism (Kelly, 2011) catalysed mainly by the CYP2D6 isoenzyme of cytochrome P450, at the hepatic level. Previous studies have additionally shown that synthetic cathinones can be

metabolized in multiple pathways, which vary according to their molecular structure (Kamata et al., 2006; Valente et al., 2014; Zaitsu et al., 2009; Zaitsu et al., 2011).

The metabolic pathways of synthetic N-alkylated cathinones, such as mephedrone and 3,4-DMMC, include N-demethylation, reduction of the β-ketone, hydroxylation and oxidation. The resulting metabolites may also be the product of the subsequent acetylation and/or glucorunidation reactions (Shima et al., 2012; Uralets et al., 2014; Valente et al., 2014). For example, detailed study of 3,4-DMMC metabolism showed the production of three main urinary metabolites, 3,4-dimethylcathinone, β-hydroxy-DMMC, β-hydroxy-DMC and other putative metabolites of unknown structure (Figure 3). In addition, partial conjugation of the parent drug and/or metabolites is a possibility, since the concentration of metabolites increased following urine hydrolysis (Shima et al., 2012; Tyrkko et al., 2013).
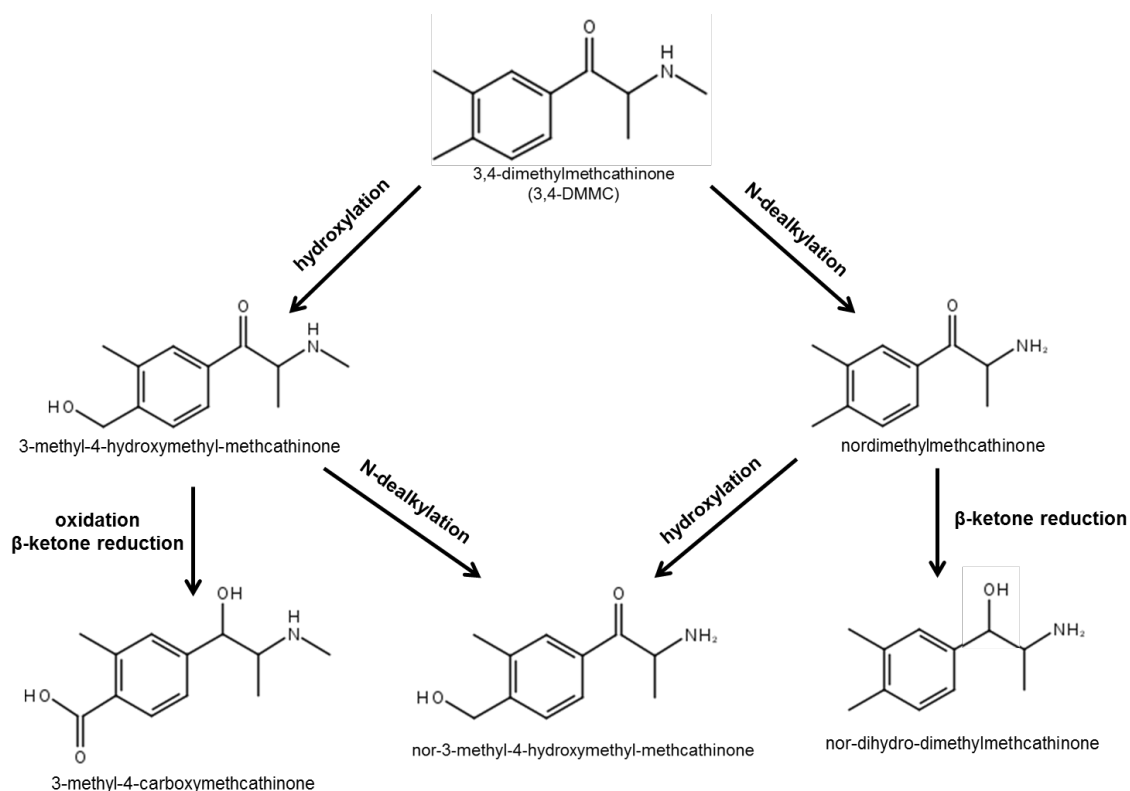
Figure 3. Phase I metabolism of 3,4-dimethylmethcathinone (3,4-DMMC) in rats and humans. Adapted from Rouxinol (2019b).

The metabolism of cathinones containing the 3,4-methylenedioxy group, such as methylone, includes the reduction of the β-ketone, demethylenation followed by O-methylation mediated by catechol-O-methyltransferase (COMT), and N-dealkylation (Figure 4) (Valente et al., 2014; Zaitsu et al., 2011). Studies with methylone identified the

main urinary metabolites as 3,4-methylenedioxycathinone (MDC), 3-methoxy-4-hydroxymethcathinone (3-MeO-4-OH-MC or HMMC) and 3-hydroxy-4-methoxymethcathinone (3-OH-4-MeO-MC) (Kamata et al., 2006; Silva and Martínez, 2016; Zaitsu et al., 2011). Kamata et al. (2006) (2006)(2006)(2006)concluded that the metabolite HMMC is the most abundant in rats and humans urine, so its detection/quantification may be essential to prove and study the consumption of these substances. The resulting metabolites can be conjugated with sulphate or glucuronide and then excreted in the urine, along with the non-metabolized parent drug (Coppola and Mondola, 2012; Valente et al., 2014; Zaitsu et al., 2011).
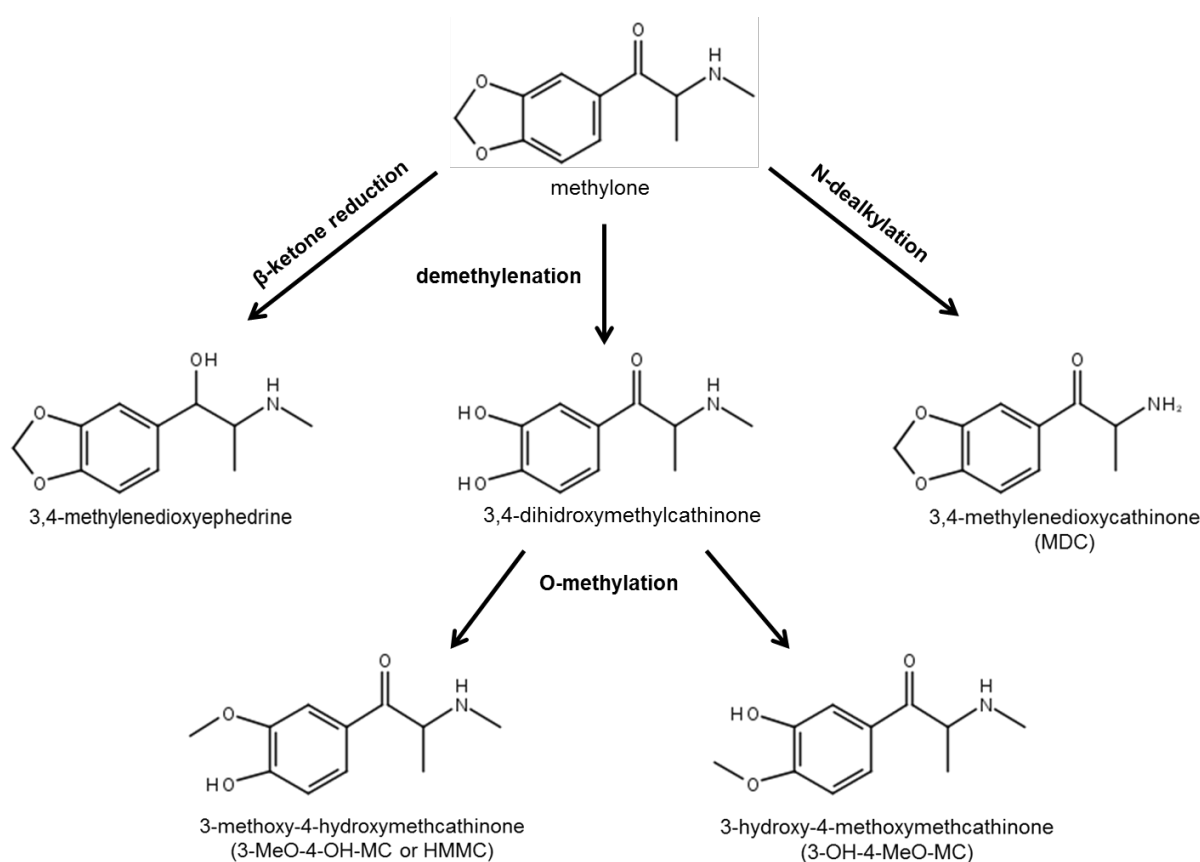
Figure 4. Phase I metabolism of methylone in rats and humans. Adapted from Kamata et al. (2006) and Valente et al. (2014).

1.5 Pharmacodynamics

Neurotransmitters, including noradrenaline (NA), dopamine (DA) and serotonin (5-HT), are contained in storage vesicles present in neuronal cells until they fuse with the cell

membrane. They are then released into the synaptic cleft, so they can elicit their physiological effects. After acting, the neurotransmitters are recaptured into the cell and stored again in vesicles or degraded by specialised enzymes (McGraw and McGraw, 2012).

Like amphetamines, synthetic cathinones have a stimulating and sympathomimetic effect on the central nervous system, as they promote an increased concentration of catecholamines in the synaptic cleft. This process is the result of two main mechanisms (Figure 5). One of the mechanisms involves the drug acting as a competitive inhibitor of monoamine transporters, competing with substrates for transporters, leading to a decrease in their reuptake and consequently to an increase in their concentration in the synaptic cleft. This mechanism of action is similar to that induced by cocaine and amphetamines. The second mechanism suggests that the drug causes premature release of neurotransmitters from storage vesicles. This release can be mediated by destabilization of the vesicles, increasing the pH, or by inhibition of the vesicular monoamine transporter (VMAT), which is located in the vesicular membrane and is responsible for the uptake of the neurotransmitters into the vesicles. This mechanism is also identical to that induced by amphetamines (Coppola and Mondola, 2012; Katz et al., 2014; McGraw and McGraw, 2012; Prosser and Nelson, 2012; Valente et al., 2014; Zawilska and Andrzejczak, 2015). In addition, these drugs are also capable of partially inhibiting the activity of monoamine oxidase (MAO), which is a regulatory enzyme responsible for the metabolism of monoamines that may be in excess in the synaptic cleft, having greater affinity for the isoform MAO-B, the main enzyme responsible for the degradation of DA (Coppola and Mondola, 2012; McGraw and McGraw, 2012). Of note, the methyl group present in the carbon α of synthetic cathinones prevents the degradation of these compounds by MAO (Kelly, 2011; Valente et al., 2014).
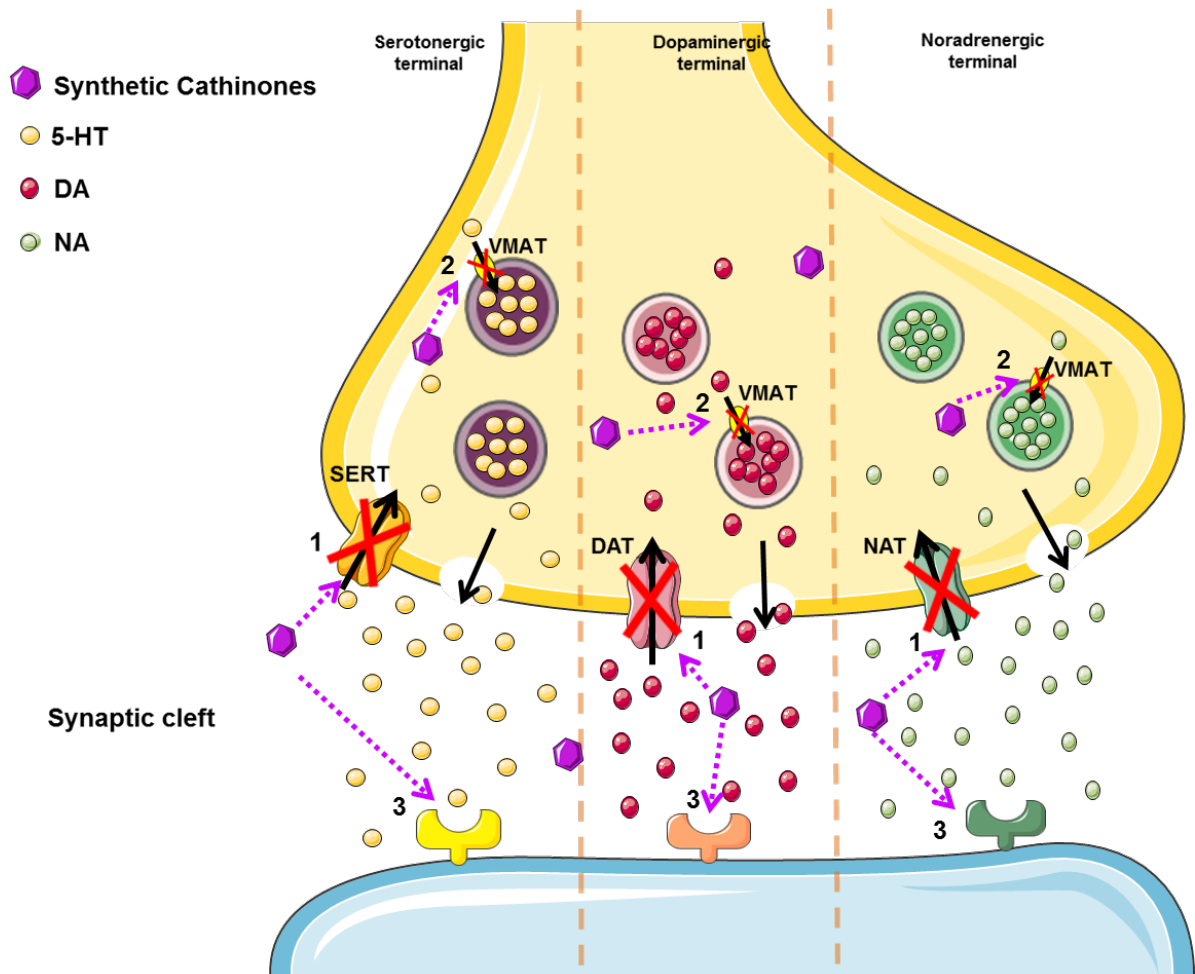
Figure 5. Representative scheme of the mechanism of action of synthetic cathinones. **1)** Synthetic cathinones act as a competitive inhibitors of monoamine transporters of serotonin (SERT), dopamine (DAT) and noradrenaline (NAT), and **2)** cause premature release of neurotransmitters from storage vesicles mediated by destabilization of the vesicles, or by inhibition of the vesicular monoamine transporter (VMAT). **3)** In addition, the drugs might also directly activate post-synaptic neurotransmitter receptors, contributing for dopaminergic, serotonergic, and/or noradrenergic overstimulation. *5-HT,* serotonin; *DA,* dopamine; *NA,* noradrenaline**.**

It is important to note that these substances have different affinities for the different monoamine transporters and receptors, and therefore different drugs may have different physiological effects (Valente et al., 2014). For example, methylone is, as previously mentioned, a β-keto analogue of MDMA, and studies show that this cathinone is as potent as MDMA in its inhibitory effects of DA and NA transporters (DAT and NAT, respectively) but three times less potent than MDMA in inhibiting 5-HT transporters (SERT). Therefore, these substances do not produce the same effects (Bossong et al., 2005). On the other hand, due to the structural similarity, the inhibition profile of the monoamine transporters

by 3,4-DMMC is similar to that of mephedrone (Calinski et al., 2019; Kelly, 2011; Simmler et al., 2013).

1.6 Biological effects and toxicity

The specific effects caused by the action of cathinones are sometimes difficult to assess, as they are often consumed in combination with other hallucinogenic or psychotropic agents, tobacco and alcohol (EMCDDA, 2011; Prosser and Nelson, 2012). Nevertheless, the overall symptoms elicited in humans following ingestion of synthetic cathinones are in conformity with their mechanism of action. Among the main pleasant effects of synthetic cathinone reported by consumers are increased empathy, euphoria, increased energy, motivation and increased libido. However, some consumers also report adverse effects such as panic attacks, tachycardia, hyperthermia, insomnia, vomiting, headaches, depression, short-term memory problems, dizziness, tremors, tolerance and dependence (Coppola and Mondola, 2012; Feyissa and Kelly, 2008; Lindsay and White, 2012; Penders et al., 2012; Prosser and Nelson, 2012; Valente et al., 2014). Of note, hyperthermia can potentiate all drug negative effects and is frequently responsible for complications that contribute to a fatal outcome, including multiple organ failure (Carvalho et al., 2012; Valente et al., 2014). Overall, the intoxication effects described are consistent with sympathomimetic toxicity, similar to cocaine or amphetamine intoxication, the main complications described being acute kidney injury, rhabdomyolysis, cardiovascular collapse, intracranial haemorrhage, and liver failure (Coppola and Mondola, 2012; Prosser and Nelson, 2012; Valente et al., 2014; Warrick et al., 2012).

1.7 Hepatotoxicity

Cases of acute or fulminant liver failure have been described in intoxications with synthetic cathinones (Borek and Holstege, 2012; Frohlich et al., 2011; Pearson et al., 2012), and hepatotoxicity have also been further demonstrated *in vitro* for several cathinones, including 3,4-DMMC and methylone (Araujo et al., 2015; Dias da Silva et al., 2019; Gaspar et al., 2018; Luethi et al., 2017; Valente et al., 2016b).

Due to the similarities with amphetamines, it is expected that many of the toxicological mechanisms of synthetic cathinones will be shared with the classic drugs, including those causing hepatotoxicity (Valente et al., 2016b). In addition, as ingestion is one of the main routes of administration for synthetic cathinones, the first-pass effect

makes the liver more susceptible to toxic damage, particularly when reactivity of the metabolites is expected. Many mechanisms of liver toxicity have been proposed, such as the direct formation of reactive oxygen and nitrogen species and the oxidation of released catecholamines that further promote oxidative stress and contribute to mitochondrial dysfunction and apoptosis (Araujo et al., 2015; Carvalho et al., 2012; Carvalho et al., 2010; Dias da Silva et al., 2019; Valente et al., 2016a; Valente et al., 2014), thus contributing to liver damage associated with these compounds (Luethi et al., 2017).

Of note, hyperthermia has been associated with the consumption of different cathinone derivatives (Borek and Holstege, 2012; Frohlich et al., 2011; Garrett and Sweeney, 2010; Levine et al., 2013; Lusthof et al., 2011; Warrick et al., 2012), and is already vastly demonstrated that the increase of temperature potentiates the hepatotoxic effects related to oxidative stress of phenylethylamines (Carvalho et al., 2012; Dias da Silva et al., 2013).

1.8 Treatment

Treatment for patients with prolonged exposure to synthetic cathinones should include a control plan along with psychotherapy (Karila et al., 2016), and should immediately begin by alleviating the psychotic effects. In cases of acute poisoning, opioids and benzodiazepines are used as first-line treatment to control agitation and reduce panic attacks (Calinski et al., 2019; Rouxinol, 2019b). However, the possible pharmacological interactions must be taken into account, since the consumption of cathinones is often associated with the consumption of antidepressants, other hallucinogenic or psychotropic agents, sedatives, alcohol and caffeine (Calinski et al., 2019; Prosser and Nelson, 2012).

1.9 Lethal cases

There are several reports of fatal intoxications related to the consumption of NPS (Barrios et al., 2016; Carbone et al., 2013; Cawrse et al., 2012; McIntyre et al., 2013; Pearson et al., 2012; Shimizu et al., 2007; Usui et al., 2014; Warrick et al., 2012; Zaami et al., 2018). However, in many cases, laboratory tests reveal the presence of multiple drugs of abuse, which makes it difficult to evaluate the causal agent of death (Coppola and Mondola, 2012; Prosser and Nelson, 2012). Furthermore, the detection of these new substances and their metabolites in biological samples before and after death does not necessarily mean that their consumption was the cause of death.

Only one fatal case was reported implicating the consumption of 3,4-DMMC (Usui et al., 2014). In this case, a man was found dead by his parents in his apartment with a disposable syringe next to his arm, along with plastic packets containing a fine white powder. After analysis, the substance present in the packaging and syringe was identified as 3,4-DMMC. Due to the absence of other fatal reports, it was not possible to draw conclusions about potentially toxic and/or lethal concentrations for 3,4-DMMC. Warrick et al. (2012) reported the first fatal case associated with the consumption of methylone together with butylone. In this case, the victim was 24 years old and presented severe symptoms of sympathomimetic intoxication, hyperthermia, tachycardia, tachypnoea, hypertension, diaphoresis, hyperreflexia, tremors and coma. Despite the efforts, the patient developed failures in the multiple organ systems and died. Several other fatal cases associated with methylone consumption have been reported (Barrios et al., 2016; Frohlich et al., 2011; Pearson et al., 2012; Zaami et al., 2018) leading some authors to argue that methylone concentrations equal or above 0.5 mg/L in peripheral blood are sufficient to result in death due to the property of the drug to increase body temperature and elicit other sympathomimetic symptoms (Pearson et al., 2012).

Chapter II – Objectives

Research into the toxicity of NPS is always imperative because these substances are marketed without testing their safety. Synthetic cathinones represent the majority of the NPS that come onto the drug market, and every time a substance is banned, new derivatives are synthesised to fill the gap. Due to their chemical and structural similarity to classical amphetamines and to first generation cathinones, identical toxicokinetic and toxicodynamic mechanisms can be anticipated for the new generation of synthetic cathinones. However, the exuberant toxicity induced by some of these novel derivatives, such as 3,4-DMMC and methylone, also suggests the possibility of some differences in toxicological mechanisms.

As oral ingestion is one of the main routes of administration of this type of stimulants, it is natural that the liver represents one of the main targets for the toxicity of NPS, largely due to the first pass effect. In addition, due to the important role of the liver in metabolism, this phenomenon may imply the generation of more toxic metabolites that may exert cytotoxic effects at this level, compromising the good functioning of the organ and, therefore, the general health of the user.

This project was intended to provide new insights into the understanding of the *in vivo* target organ toxicity of 3,4-DMMC and methylone. For such purpose, the impairment of antioxidant, energetic and other hepatic biochemical parameters induced by these drugs was evaluated, 1 h and 24 h after acute administration of two drug doses (20 mg/Kg or 40 mg/Kg) to female Wistar rats.

Chapter III - Material and Methods

3.1 Chemicals

Methylone and 3,4-DMMC (HCl salts) were acquired online at *sensearomatics.net* (website currently unavailable) on March 2013. MDMA (HCl salt) was extracted and purified from high purity MDMA tablets provided by the Portuguese Criminal Police Department, at REQUIMTE/Toxicology Laboratory, Biological Sciences Department of Faculty of Pharmacy, University of Porto. Chemical purity and identity of all drugs were verified by nuclear magnetic resonance and mass spectrometry methodologies. Analytical data were consistent with the assigned structures with about 99% purity. All reagents used were of analytical grade and, unless stated otherwise, obtained from Sigma Aldrich (Saint Loui, Missouri).

3.2 Animals

This *in vivo* study was performed at the highest standards of ethics after approval by the local Ethical Committee for the Welfare of Experimental Animals (University of Porto-ORBEA; project 251/2018) and by the national authority *Direção Geral de Alimentação e Veterinária* (DGAV). Housing and all experimental procedures were performed by investigators accredited for laboratory animal use and complied with the Portuguese and European legislation (law DL 113/2013, Guide for Animal Care; Directives 86/609/EEC and 2010/63/UE). Female adult Wistar rats weighing 150–300 g were used. The animals were kept in sterile facilities under controlled temperature (20 ± 2 °C), humidity (40–60%), and lighting (12 h-light/dark cycle) conditions, and fed with sterile standard rat chow and tap water *ad libitum*. On the day of the experiments, the vaginal smears of the female rats were collected and examined unstained under a microscope to identify the estrous cycle (proestrus, estrus, metestrus or diestrus) through the analysis of the proportion among nucleated epithelial cells, non-nucleated cornified cells and leukocytes (Marcondes et al., 2002; Nah et al., 2011). All animals used under the same experiment were synchronised.

3.3 Drug challenge

On the day of the experiments, the animals (at least six animals per group; the exception was methylone at 40 mg/Kg, with only three animals tested) were weighed and placed separately in polyethylene cages with metallic mesh at the top, having access to water and food *ad libitum.* The animals were injected i.p., at a maximum volume of 0.5 mL/300 g

body weight, with saline (0.9% NaCl), 20 mg/Kg MDMA, 20 or 40 mg/Kg 3,4-DMMC, or 20 or 40 mg/Kg methylone solution (all prepared in saline).

The effects of MDMA, 3,4-DMMC and methylone were studied after 1 h of administration. During the experimental period, animal behaviour was carefully monitored. After treatment, anaesthesia was induced by an i.p. injection of a combination of 20 mg/Kg xylazine (Rompun® 2 %, Bayer HealthCare, Germany) and 100 mg/Kg ketamine (Clorketam® 1000, Vétoquinol, France) and intrarectal temperature was immediately assessed using a digital thermometer. Anaesthesia was maintained through inhalation of isoflurane vapour (IsoVet® 1000 mg/g, B. Braun VetCare, Germany). Total blood was collected from the inferior vena cava into EDTA tubes and after euthanasia the liver was excised, rinsed with saline and weighted. In addition, brain, heart, and kidneys were also collected to analysis (these results will not be discussed in the present dissertation but are presented in Supplementary data). The relative organ weight (ROW), an indicator of tissue harm, was calculated for each animal as the percentage of organ weight to the total body weight at the time of sacrifice. Organs were homogenized (1:4 m/v) in ice-cold 100 mM KH_2PO_4 buffer (pH 7.4) with an Ultra-Turrax® homogenizer. Blood was centrifuged at 1,600 g for 15 min at 4 ºC, and plasma transferred into microcentrifuge tubes. All organ homogenates and plasma samples were stored at -80 ºC until further biochemical analysis being performed.

The effects of 3,4-DMMC and methylone at 20 mg/Kg were also studied after 24 h of administration. In this case, the animals were kept in metabolic cages and their behaviour carefully monitored during the experimental period. Food and water intake were also monitored. After treatment, the animals were anaesthetised and blood collected as described. The blood samples were centrifuged at 1,600 g for 15 min at 4 ºC, and plasma transferred into microcentrifuge tubes for further biochemical analysis. In addition to liver, also brain, heart, lungs and kidneys were excised, rinsed and weighted. An aliquot of each organ was collected for histological examination and the remaining tissue homogenized as described (again, only data concerning the liver will be discussed in the present dissertation; all the remaining results are included in Supplementary data). Samples were kept at -80 ºC until further analysis.

3.4 Determination of protein

Organ homogenates were added of 2 M NaOH (1:1) for the determination of protein, which was performed by the Lowry et al. (1951) method. Briefly, the samples were further diluted with 1 M NaOH (1:200 for liver; 1:100 for brain; 1:150 for heart and kidney). Fifty

microliters of each sample, standard, or blank were transferred into a 96-well plate, in triplicate, and added of 100 µL reagent A (190 mM Na_2CO_3, 700 µM $KNaC_4H_4O_6.4H_2O$, and 700 µM $CuSO_4.5H_2O$, extemporaneously prepared). The plate was incubated in the dark for 10 min, at room temperature, followed by the addition of 100 µL of Folin and Ciocalteu's phenol reagent (from Merck, Darmstadt, Germany) previously diluted 15 times in purified water. The plate was incubated for 10 min, under light protection at room temperature, and the absorbance measured at 750 nm using a multiple well plate reader (Power Wave X^{TM}, BioTek Instruments, Inc.). Results were compared to a standard curve (31.25–500 µg/mL of bovine serum albumin, BSA).

3.5 Determination of total adenosine triphosphate (ATP)

Cellular adenosine triphosphate (ATP) levels were quantified by a bioluminescent luciferase-based assay (Dias da Silva et al., 2017). To precipitate proteins in the samples, the organ homogenates were added of 10% $HClO_4$ (1:1), centrifuged at 13,000 g for 10 min at 4 °C, and the supernatants used after further dilution with 5% $HClO_4$ (1:20 for liver; 1:10 for brain, heart and kidneys). Samples, standards, and blank were then neutralized with 0.76 M $KHCO_3$ (1:1) and centrifuged at 13,000 g for 10 min at 4 °C. Seventy-five microliters of each supernatant were transferred into a 96-well plate and added of 75 µL of luciferin-luciferase solution (0.15 mM luciferin; 30,000 light units luciferase/mL; 10 mM $MgSO_4$; 50 mM glycine; 1 mM Tris; 0.55 mM EDTA; 1% BSA). The intensity of the emitting light was measured using a luminescence plate reader (BioTek Instruments, Vermont, USA). The results were compared to an ATP standard curve performed in each experiment, and normalized to the total amount of protein in the samples.

3.6 Determination of reduced glutathione (GSH) and oxidized glutathione (GSSG)

The levels of total glutathione (tGSH) were determined by the 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB)–oxidized glutathione (GSSG) reductase recycling assay (Dias da Silva et al., 2017). Briefly, organ homogenates were prepared as described for the ATP determination (dilutions in 5% $HClO_4$ were 1:200 for liver; 1:20 for brain and heart; and 1:2 for kidneys). Then, 100 µL of the sample, standard and blank supernatants were transferred into a 96-well plate, followed by the addition of 65 µL of reagent solution (72 mM phosphate buffer, 0.69 mM NADPH and 4 mM DTNB). The plates were incubated for 15 min at 30 °C in a multi-well plate reader (Power Wave X^{TM}, BioTek Instruments, Inc.).

Then, 40 µL of 10 U/mL glutathione reductase (GR) solution were added and the absorbance immediately read in kinetic mode, every 10 sec, during 3 min, at 415 nm, to accompany the formation of 5-thio-2-nitrobenzoic acid. The results of the samples were compared with a glutathione (GSH) standard curve, performed in each experiment, and normalized to the protein content of each sample.

The same protocol was used for GSSG quantification (dilutions in 5% $HClO_4$ were 1:30 for liver; 1:10 for heart; no dilution for brain and kidneys) with the additional step of incubating the samples with 10 µL of 2-vinylpyridine for 1 h at 4 °C with shaking, previous to samples, standards and blank neutralization with 0.76 M $KHCO_3$. The results of the samples were compared with a GSSG standard curve, performed in each experiment, and normalized to the protein content of each sample.

The intracellular GSH was calculated using the formula GSH = tGSH – 2 x GSSG.

3.7 Determination of enzymatic activities

Organ homogenates were added with 0.2% Triton X-100 (1:1) to disrupt cell membranes, centrifuged at 13,000 g for 10 min at 4 °C, and the supernatants used to determine the enzymatic activities.

3.7.1 Glutathione reductase (GR)

For the determination of GR, the Calberg and Mannervik (1985) method was used, which is based on the principle of GR catalysing the NADPH-dependent reduction of GSSG to GSH. Briefly, 50 µL of sample supernatants were placed in a 96-well plate after dilution with 0.1% Triton X-100 (1:5 for liver and kidneys; no dilution for heart and brain), and added of 100 µL of phosphate buffer (200 mM KH_2PO_4; 2 mM EDTA; pH 7.0), 30 µL of 10 mM GSSG and 90 µL of deionized H_2O. After addition of 30 µL of 1 mM NADPH (prepared in 10 mM TRIS-HCl; pH 7.0), the absorbance of the samples was immediately read in a multiwell plate reader (Power Wave X^{TM}, BioTek Instruments, Inc.) set at 340 nm, in a kinetic mode, every 60 sec, for 5 min, at 30 °C. The enzyme activity was calculated by using a NADPH extinction coefficient of 6.22 $mM^{-1}.cm^{-1}$ and expressed as units of GR per ng of protein.

3.7.2 Glutathione peroxidase (GPx)

The activity of glutathione peroxidase (GPx) was determined by monitoring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GR, which catalyses the reduction of GSSG formed by GPx, according to the technique described by Flohé and Günzler (1984). Briefly, 30 µL of the sample supernatants were placed in a 96-well plate after dilution with 0.1% Triton X-100 (1:10 for liver; 1:2 for heart; 1:5 for kidneys; no dilution for brain), and added with 90 µL of 100 mM $Na_2HPO_4.2H_2O$ (pH 7.0), 30 µL of 2.4 U/mL GR, 30 µL of 10 mM GSH, 30 µL of 10 mM NaN_3, and 30 µL of 10 mM Na_2EDTA. After incubation for 10 min at room temperature, 30 µL of 1.5 mM NADPH (prepared in 0.1% $NaHCO_3$) and 30 µL of 1.5 mM H_2O_2 were added, and the absorbance immediately read in a multiwell plate reader (Power Wave X^{TM}, BioTek Instruments, Inc.) set at 340 nm, every 60 sec, during 5 min, at 30 ºC. The enzyme activity was calculated by using a NADPH extinction coefficient of 6.22 $mM^{-1}.cm^{-1}$ and expressed as units of GPx per ng of protein.

3.7.3 Glutathione S-transferases (GSTs)

Glutathione S-transferases (GSTs) are a group of interrelated enzymes that catalyse the conjugation of glutathione with various hydrophobic compounds displaying electrophilic centres. This determination was based on the formation of a GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB), *i.e.* S-(2,4-dinitrophenyl)glutathione (G-S-DNP) (Habig et al., 1974). Briefly, 250 µL of phosphate buffer (100 mM $Na_2HPO_4.2H_2O$; 1 mM EDTA; pH 7.0; at 30 °C) were added to each well of a 96-well plate, followed by 20 µL of sample, 15 µL of 20 mM GSH and 15 µL of 20 mM CDNB (prepared in ethanol). The absorbance was read in a multiwell plate reader (Power Wave X^{TM}, BioTek Instruments, Inc.) set at 340 nm, in a kinetic mode, every 60 sec, for 5 min, at 30 °C. The GST activity was calculated by using a G-S-DNP extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ and expressed as units GST per ng of protein.

3.8 Other biochemical analyses

The following biochemical parameters were determined in a Prestige 24i® Automated Chemistry Analyser (Costa et al., 2015), using 3 µL of plasma for each analysis, which

was performed in triplicate: substrates (urea, creatinine, uric acid, lactate, glucose), enzymes [creatine kinase (CK) and creatine kinase isoenzyme CK-MB, γ-glutamyl transpeptidase (γ-GT), lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), pseudocholinesterase, and amylase], proteins (albumin and total protein), lipids (cholesterol and triglycerides) and electrolytes (calcium, magnesium, iron, and sodium). The principles of the methods used in these determinations are present in Table 1. Given their relevance for the evaluation of the hepatic damage, only results of ALT, LDH, ALP, γ-GT and AST determinations will be discussed in the present dissertation (all the other results are presented in the Supplementary data).

Table 1. Principles and methods of the biochemical parameters analysed.

Parameter	Principle of the method	Detection
Lactate	Lactate is oxidized by lactate oxidase to pyruvate and H_2O_2, which reacts with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline in the presence of peroxidase, forming a red compound	Photometrical detection at 546 and 700 nm
Glucose oxidase	Glucose oxidase catalyses the oxidation of glucose to gluconate, producing H_2O_2, which reacts with a chromogenic oxygen acceptor, phenol-4-aminophenazone, in the presence of peroxidase	Photometrical detection at 505 nm
Creatinine	Creatinine reacted with alkaline picrate, forming a red complex	Photometrical detection at 492 nm
Urea	Urea reacted with o-phthalaldehyde in acid medium forming a coloured complex	Photometrical detection at 510 nm
Uric acid	Uric acid is oxidized by uricase to allantoin and H_2O_2, which reacts with 4-aminophenazone and 2,4-dichlorophenol sulfonate, in a reaction catalysed by peroxidase, to form a red quinoneimine compound	Photometrical detection at 520 nm
Calcium	The quantification was based on the formation of a calcium coloured complex with o-cresolphthalein in alkaline medium	Photometrical detection at 570 nm
Iron	Iron was dissociated from a transferring-iron complex in a weakly acid medium. The released iron was reduced to ferrous form by ascorbic acid and formed a coloured complex with FerroZine	Photometrical detection at 562 nm
Magnesium	Magnesium forms a colourful complex when reacts with magon sulfonate in alkaline solution	Photometrical detection at 546 nm
Sodium	Determined enzymatically via sodium dependent-galactosidase activity with o-nitrophenyl-β-galactoside as the substrate and the coloured o-nitrophenyl as the product	Photometrical detection at 405 nm
Cholesterol	Production of a colourful complex in the presence of peroxidase	Photometrical detection at 505 nm
Triglycerides	Triglycerides are converted to glycerol and free fatty acids by lipoprotein lipase. Glycerol was converted to glycerol-3-phosphate and ADP by glycerol kinase and ATP. Glycerol-3-phosphate was then converted by glycerol phosphate dehydrogenase to dihydroxyacetone phosphate and hydrogen peroxide. In the last reaction, hydrogen peroxide reacted with 4-aminophenazone and p-chlorophenol in the presence of peroxidase to give a red colored	Photometrical detection at 505 nm
ALP	ALP catalyses the hydrolysis of p-nitrophenyl phosphate at pH 10.4, releasing phosphate and p-nitrophenol	Photometrical detection at 405 nm
Amylase	Amylase hydrolysis of 2-chloro-4-nitrophenyl-α-D-maltotrioside to release 2-chloro-4-nitrophenol and forms 2-chloro-4-nitrophenylα-D-maltoside, maltotriose and glucose. The rate of 2-chloro-4-nitrophenol formation is proportional to the activity of amylase present in the sample	Photometrical detection at 405 nm

γ-GT, γ-glutamyl transpeptidase; *ALP,* alkaline phosphatase; *ALT,* alanine transaminase; *AST,* aspartate transaminase; *CK,* creatine kinase; *LDH,* lactate dehydrogenase.

Table 2. Continued.

Parameter	Principle of the method	Detection
Pseudocholinesterase	Pseudocholinesterase catalyses the hydrolysis of butyrylthio-choline to butyrate and thiocholine. Thiocholine reacts with 5,5′-dithiobis-2-nitrobenzoic acid to form 5-mercapto-2-nitro-benzoic acid	Photometrical detection at 405 nm
CK	CK catalyses the reversible transfer of a phosphate group from phosphocreatine to ADP. This reaction is coupled to those catalysed by hexokinase and glucose-6-phosphate dehydrogenase, with formation of NADPH	Photometrical detection at 340 nm
γ-GT	γ -GT catalyses the transfer of the γ -glutamyl group from γ -glutamyl-p-nitroanilide to the acceptor glycylglycine, forming 2-nitro-5-aminobenzoic acid	Photometrical detection at 405 nm
AST	AST catalyses the reversible transfer of an amino group from aspartate to α-ketoglutarate, forming glutamate and oxalacetate. The oxalacetate produced is reduced to malate by malate dehydrogenase, using NADH as the cofactor	Photometrical detection at 340 nm
ALT	ALT catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate, forming glutamate and pyruvate The pyruvate produced was reduced to lactate by LDH using NADH as the cofactor.	Photometrical detection at 340 nm
LDH	LDH activity was measured by the reduction of pyruvate to lactate by LDH using NADH as the cofactor	Photometrical detection at 340 nm
Total proteins	Proteins react with copper salts, in the presence of iodide (antioxidant) and at alkaline pH, to give rise to an intense violet-blue complex	Photometrical detection at 546 nm
Albumin	Albumin, at a slightly acid pH, produces a colour change of bromocresol green from yellow-green to green-blue	Photometrical detection at 605 nm

γ-GT, γ-glutamyl transpeptidase; *ALP,* alkaline phosphatase; *ALT,* alanine transaminase; *AST,* aspartate transaminase; *CK,* creatine kinase; *LDH,* lactate dehydrogenase.

3.9 Histological analysis

Liver, kidneys, heart, lungs and brain were cut into slices with no more than 3 mm of thickness and representative slices of all organ regions were systematically selected and sampled for light microscopy. The organs were fixed for 48 h in commercial 4% buffered formalin (Klinipath, Duiven, the Netherlands) for histopathological analysis. After fixation, tissues were dehydrated through a series of graded ethanol solutions (70.0–99.8%; Panreac AppliChem, Darmstadt, Germany), cleared in xylene (BDH–Prolabo, VWR Chemicals, Dublin, Ireland), and embedded in paraffin (Merck, Darmstadt, Germany). Each organ was sectioned (microtome Leica RM 2255, Wetzlar, Germany) into 3 µm thin sections, which were stained with hematoxylin–eosin and Masson's trichrome (Rodrigues et al., 2017), after mounting in silane-coated microscope slides (Nuova Aptaca, Canelli, Italy). Lung tissues were also stained with periodic acid-Schiff with diastase digestion (PAS-D) (Fu and Campbell-Thompson, 2017).

3.10 Statistical analysis

Data obtained were presented as mean ± standard error of the mean (SEM) and statistically processed using GraphPad Prism® software, version 6.07 (San Diego, CA, USA). The normality of data distribution was analysed using the Kolmogorov-Smirnov test and statistical comparisons between groups were performed using the non-parametric Kruskal-Wallis test.

Chapter IV – Results

4.1 Animals treated with 3,4-methylenedioxymethamphetamine (MDMA), methylone and 3,4-dimethylmethcathinone (3,4-DMMC) presented characteristic altered behaviour

In all experiments performed, the behaviour of the animals was carefully monitored. The main observations are compiled in the Table 2. Hyperthermia, piloerection, increased locomotion in the open field, stereotyped circling around the arena walls, increased saliva spreading and disorientation were common effects to all drugs. In a pilot assay, it was observed that all these stress indicators were intensified for group-housed rats, so further experiments were conducted in drug-treated individually caged animals. Of relevance, 3,4-DMMC-treated animals usually adopted a defensive posture and a characteristic upwards erect tail; diarrhoea was also observed in three of the animals treated for 24 h. Behavioural features of animals administered with MDMA and methylone were very similar, and the same was observed for the impairment in thermoregulation, with both drugs inducing temperature increases up to 40.3 °C.

By virtue of our previous experience with the drug, MDMA was never tested at 40 mg/Kg. On the other hand, it was observed that upon dose escalation from 20 mg/Kg to 40 mg/Kg for 3,4-DMMC and methylone, all behavioural alterations were anticipated and intensified. In this regard, given the severe animal discomfort observed in the experiments with methylone at the highest dose (n=3), this treatment was interrupted and not included in further experiments. By precaution, 3,4-DMMC at 40 mg/Kg was also not assayed for 24 h.

No alterations were observed in food and water intake patterns between treated animals and controls in the 24 h-experiments.

Table 3. Main alterations induced by 3,4-methylenedioxymethamphetamine (MDMA), methylone and 3,4-dimethylmethcathinone (3,4-DMMC) in Wistar rats.

	Behavioural effects
MDMA	Changes in locomotion; imbalance; hyperactivity; stereotyped behaviour; intense salivation; piloerection; hyperthermia; redness in oral mucosal and paws (vasodilatation); prone and transient extension of the body; vocalization upon touch.
3,4-DMMC	Piloerection; adoption of a characteristic defensive posture; signs of confusion; imbalance; stereotyped behaviour; hyperthermia; redness in oral mucosal and paws (vasodilatation); characteristic sounder type tail; vocalization upon touch; hallucination-type behaviour; diarrhoea.
Methylone	Increased overall animal locomotion; changes in locomotion (crawl); intense salivation; piloerection; hyperactivity; stereotyped behaviour; hyperthermia; prone and transient extension of the body ; redness in oral mucosal and paws (vasodilatation); imbalance; signs of confusion.

4.2 Methylone at 40 mg/Kg disturbs the relative liver weight, 1 h after administration

After administration of the drugs, no statistically significant differences to controls were observed in the ratio between liver weight and animal weight. The exception was methylone administered at 40 mg/Kg, as can be seen in Figure 6. As referred, due to the high animal discomfort, this dose was only tested for 1 h, in three animals. While, the mean ROW for controls was 3.79 ± 0.07, a significantly higher value was observed for methylone at 40 mg/Kg (4.22 ± 0.07; p<0.05). The remaining treatments elicited results close to controls (3.41 ± 0.19 for 20 mg/Kg MDMA; 3.30 ± 0.09 for 20 mg/Kg 3,4-DMMC; 3.74 ± 0.43 for 20 mg/Kg methylone; 3.61 ± 0.16 for 40 mg/Kg 3,4-DMMC).

Also, no disturbances on the relative liver weight were observed for animals treated for 24 h with 20 mg/Kg of 3,4-DMMC (3.36 ± 0.16) or methylone (3.21 ± 0.09), compared to controls (3.49 ± 0.09). The ROW results for brain, kidneys and heart are presented in Supplementary data.
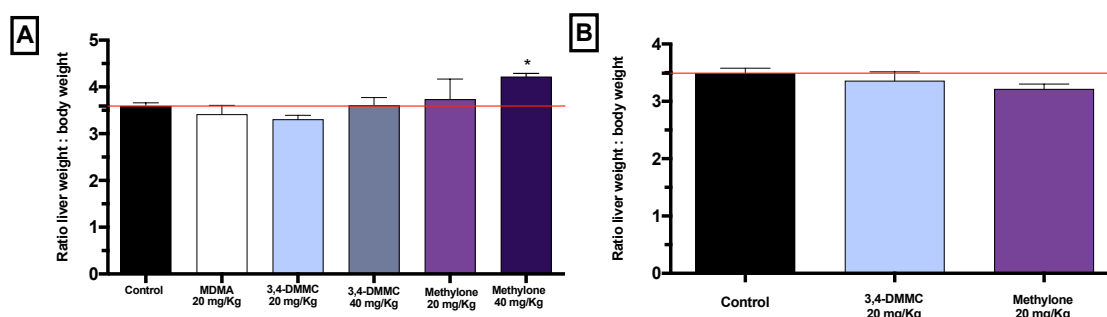
Figure 6. Effect on relative organ weight of 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg and 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 or 40 mg/Kg in Wistar rats, 1 h (A) or 24 h (B) after i.p. drug administration. Results are presented as mean ± standard error of the mean. *p<0.05, *vs*. control.

4.3 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone decrease intracellular adenosine triphosphate (ATP)

Following drug administration, a decrease in ATP levels was observed for all drugs, as shown in Figure 7. However, after 1 h of administration, statistically significant differences to controls (0.51 ± 0.04 µmol/mg; set as 100%) were only observed for 3,4-DMMC (75.09 ± 8.62%; p<0.05) and methylone (52.45 ± 2.91%; p<0.0001) at 40 mg/Kg; although for 20 mg/Kg methylone, a clear decrease tendency was already observed (82.78 ± 6.34%). The observed decrease in ATP levels seems to be dose-dependent for 3,4-DMMC and

methylone, however, a statistically significant difference between both doses was only observed for methylone (p<0.01; Figure 7A).
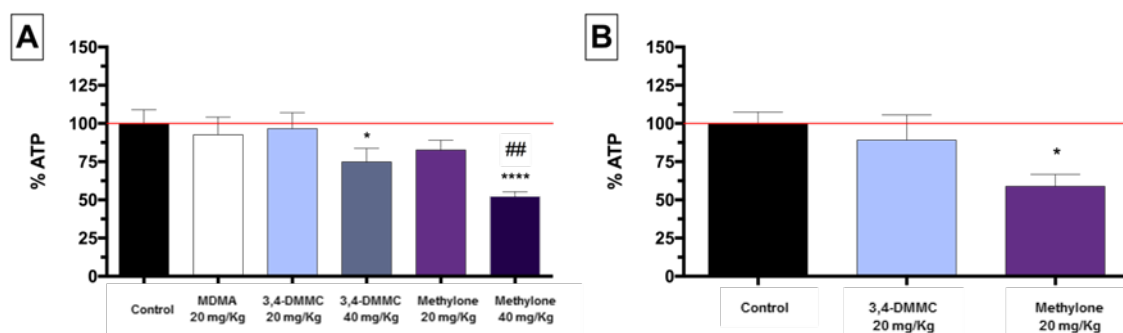
Figure 7. Alterations in adenosine triphosphate (ATP) levels induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h (A) or 24 h after i.p. drug administration (B). Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; ****p<0.0001, *vs*. control. ##p<0.01, *vs*. methylone at 20 mg/Kg.

Decreased ATP levels were still observed 24 h after administration of 20 mg/Kg 3,4-DMMC and methylone. However, statistically significant differences to controls (0.52 ± 0.03 µmol/mg; set as 100%) were only observed for methylone (59.02 ± 7.63%; p<0.05) (Figure 7B).

4.4 Administration of 3,4-methylenedioxymethamphetamine (MDMA), 3,4-dimethylmethcathinone (3,4-DMMC) and methylone disrupts ratio of reduced to oxidized glutathione (GSH/GSSG)

After 1 h of administration, GSH/GSSG ratio significantly decreased for all drug treatments (p<0.05; Figure 8A). The exception was 40 mg/Kg 3,4-DMMC (13.06 ± 0.52), which showed values close to that obtain for controls (14.55 ± 0.67). In animals treated with 20 mg/Kg 3,4-DMMC or methylone, the mean ratios of GSH/GSSG were 10.89 ± 0.21 and 11.59 ± 0.64, respectively, values that were close to that observed for the reference drug, MDMA, at the same dose (9.98 ± 0.39). The decrease in GSH/GSSG ratio appears to be dose-dependent for methylone, as at 40 mg/Kg the ratio decreased to 5.77 ± 0.51 (p<0.0001).
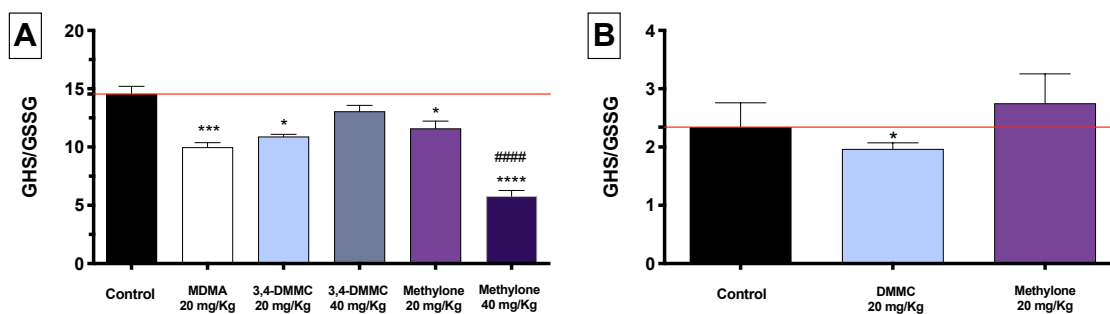
Figure 8. Alterations in the ratio of reduced to oxidized glutathione (GSH/GSSG) induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h (A) or 24 h (B) after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; ***p<0.001; ****p<0.0001, *vs.* control. ####p<0.0001, *vs.* methylone at 20 mg/Kg.

Twenty-four hours after administration, a significant decrease in GSH/GSSG ratio was also observed for 20 mg/Kg 3,4-DMMC (1.96 ± 0.11; p<0.05; Figure 8B), compared to controls (2.34 ± 0.42). The same does not apply to 20 mg/Kg methylone (2.75 ± 0.50), which elicited a slight increase in the GSH/GSSG ratio.

4.5 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone disrupted glutathione peroxidase (GPx) activity

As can be seen in Figure 9, 1 h after drug administration, a significant decrease in GPx activity was observed for 20 mg/Kg 3,4-DMMC (76.87 ± 2.45%; p<0.01) and for 40 mg/Kg methylone (63.76 ± 2.89%; p<0.0001), as compared to controls (40.25 ± 2.12 U/ng, set as 100%). This value obtained for 20 mg/Kg 3,4-DMMC was significantly different from that observed for MDMA at the same dose (98.96 ± 5.59%; p<0.01). Although statistically significant differences between the doses tested (20 mg/Kg *vs.* 40 mg/Kg) were observed for 3,4-DMMC (p<0.001) and methylone (p<0.0001), a dose-dependent decrease in GPx activity was only shown for methylone after 1 h-treatments (Figure 9A).

Figure 9. Alterations in glutathione peroxidase (GPx) activity induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h (A) or 24 h (B) after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of mean. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$, *vs.* control. ###$p < 0.001$; ####$p < 0.0001$, *vs.* same drug at 20 mg/Kg. &&$p < 0.01$, *vs.* 20 mg/Kg MDMA.

As represented in Figure 9B, 24 h after administration, a significant decrease in GPx activity was observed for 20 mg/Kg of methylone (84.39 ± 5.16%; $p < 0.05$), compared to controls (67.18 ± 6.74 U/ng; set as 100%). For 20 mg/Kg 3,4-DMMC, a reversion on the effect observed at 1 h was observed after 24 h.

4.6 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone changed glutathione S-transferases (GSTs) activity

As observed in Figure 10, animals treated with 40 mg/Kg 3,4-DMMC or methylone showed a significant decrease in GST activity, 1 h after administration (88.39 ± 6.30% and 71.86 ± 4.02%, respectively; $p < 0.01$), as compared to controls (0.10 ± 0.01 U/ng; set as 100%). No alterations were observed for animals treated with 20 mg/Kg 3,4-DMMC, methylone or the reference drug, MDMA. In addition, as the mean GST activities for animals injected with 40 mg/Kg of 3,4-DMMC or methylone were significantly lower ($p < 0.01$) than those for the 20 mg/Kg administration, this effect seems to be dose-dependent (Figure 10A).
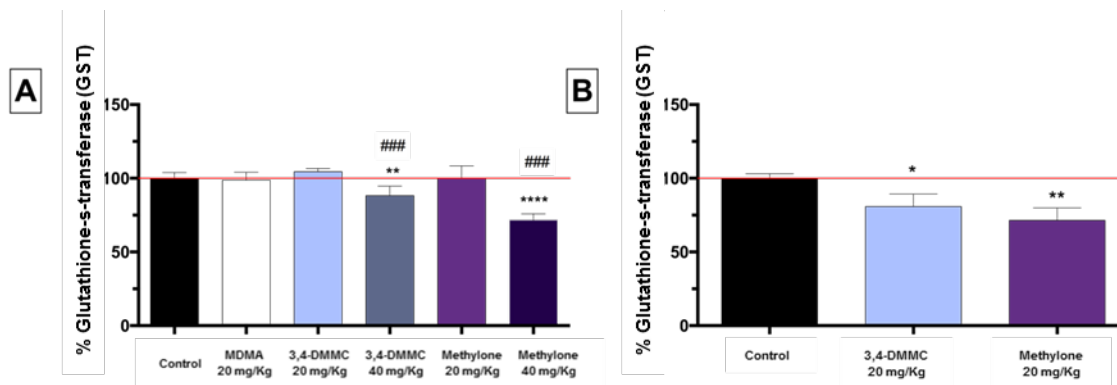
Figure 10. Alterations in glutathione S-transferase (GST) activity induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h (A) or 24 h (B) after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of mean. *p<0.05; **p<0.01; ****p<0.0001, *vs.* control. ###p<0.001, *vs.* same drug at 20 mg/Kg.

Twenty-four hours after cathinones administration, a significant decrease in GST activity was also observed (p<0.05). Accordingly, animals treated with 20 mg/Kg 3,4-DMMC and methylone had mean GST activities of 81.01 ± 8.26% and 71.64 ± 8.29%, respectively, which was significantly lower than the observed for controls (0.17±0.01 U/ng protein; set as 100%; Figure 10B).

4.7 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone changed glutathione reductase (GR) activity

As observed in Figure 11A, 1 h after drug administration, a significant decrease in GR activity was observed for 20 mg/Kg 3,4-DMMC (72.55 ± 3.68%; p<0.0001) and 40 mg/Kg methylone (83.37 ± 3.56%; p<0.05), compared to controls (8.53 ± 0.45 U/ng; set as 100%). This value obtained for 3,4-DMMC was significantly different than that for MDMA at the same dose (109.30 ± 6.45%; p<0.0001). In addition, there was a significant difference in the activities of this enzyme between both doses of 3,4-DMMC administered (p<0,001), as shown in Figure 11A.

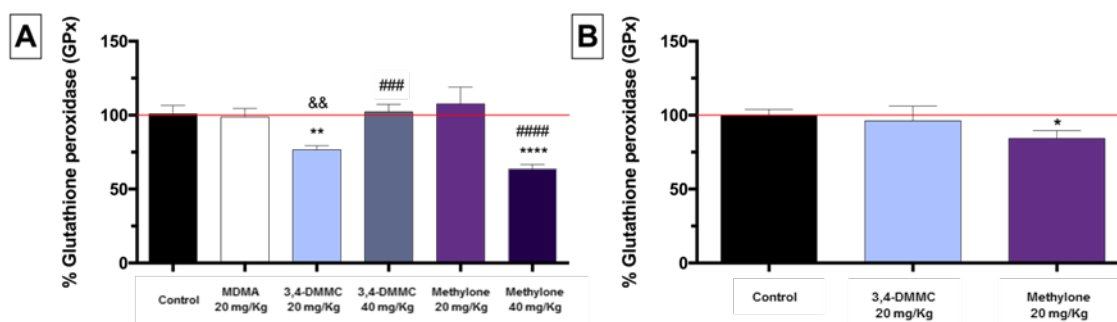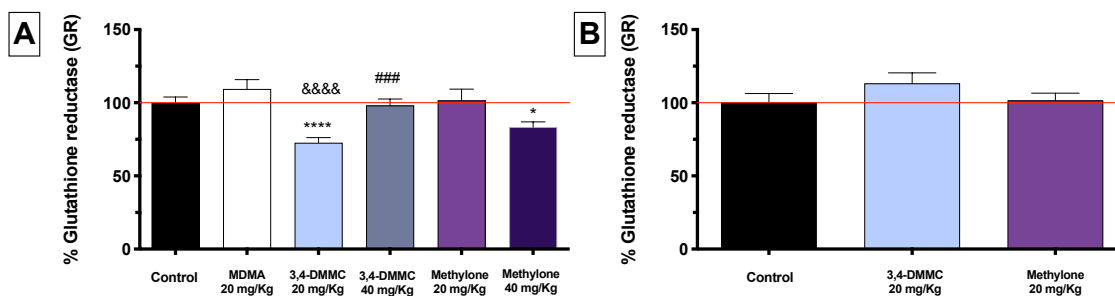**Figure 11.** Alterations in glutathione reductase (GR) activity induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h (A) or 24 h (B) after i.p. drug administration. Values are presented as percentage compared to the control ± standard error of mean. *$p<0.05$; ****$p<0.0001$, *vs.* control. ###$p<0.001$, *vs.* same drug at 20 mg/Kg. &&&&$p<0.0001$ *vs.* 20 mg/Kg MDMA.

Nevertheless, as depicted in Figure 11B, the disruption of GR activity observed at 1 h for 20 mg/Kg 3,4-DMMC was already reversed after 24 h, with a slight, non-significant increase in GR activity observed (113.10 ± 7.35 %), as compared to controls (12.37 ± 0.85 U/ng protein; set as 100%).

4.8 3,4-Methylenedioxymethamphetamine (MDMA), 3,4-dimethylmethcathinone (3,4-DMMC) and methylone induced changes in plasma biochemical markers indicative of hepatic injury

Drug-induced changes in plasma biochemical markers are shown in Table 3. As observed, 1 h after drug administration, a decrease in plasma albumin concentration of the treated animals was observed relative to controls (3.49 ± 0.13 g/dL). In animals treated with 20 mg/Kg of 3,4-DMMC or methylone the average albumin concentration was 2.85 ± 0.37 g/dL and 2.53 ± 0.30 g/dL, respectively. These values were lower than those observed for the reference drug, MDMA, at the same dose (3.19 ± 0.35 g/dL). In addition, as the average albumin concentrations for animals injected with 40 mg/Kg of 3,4-DMMC or methylone (1.93 ± 0.86 g/dL and 0.26 ± 0.26 g/dL, respectively) were lower than those for the 20 mg/Kg administration, this effect seems to be dose-dependent. After 24 h of drug administration, the values observed for the treated animals were 2.62 ± 0.38 g/dL and 1.94 ± 0.53 g/dL, for 20 mg/Kg of 3,4-DMMC and methylone, respectively, which were close to those obtained for control animals (2.44 ± 0.87 g/dL).

After 1 h of drug administration a decrease in plasma ALT concentration can be observed in animals treated with 40 mg/Kg 3,4-DMMC (5.05 ± 1.31 U/L) and methylone at 20 mg/Kg (17.75 ± 3.78 U/L), compared to control animals (35.99 ± 5.85 U/L). For

methylone at 40 mg/Kg, values were below the limit of quantification of the method. Compared to ALT plasma concentrations of animals treated with 20 mg/Kg methylone, animals treated with the reference drug, MDMA, at the same dose (31.99 ± 2.46 U/L), presented values significantly higher ($p<0.05$). After 24 h-administrations, a decrease in plasma ALT concentration was observed in animals treated with 20 mg/Kg of 3,4-DMMC (3.57 ± 1.58 U/L) or methylone (8.83 ± 5.14 U/L), compared to control animals (13.38 ± 5.92 U/L).

Both cathinones tested, *i.e.* 3,4-DMMC and methylone, led to a decrease in plasma levels of LDH, 1 h after administration. The mean plasma LDH concentration was 310.70 ± 130.80 U/L for the control animals, while in animals injected with 20 mg/Kg of 3,4-DMMC or methylone, the mean plasma LDH concentrations were 175.70 ± 42.23 U/L and 81.15 ± 18.38 U/L, respectively. These values were lower to that observed for the reference drug, *i.e.* MDMA, at the same dose (420.80 ± 128.30 U/L). The observed decreases in plasma LDH seem to be dose-dependent, since at 40 mg/Kg the values obtained for 3,4-DMMC and methylone were 43.25 ± 18.48 U/L and 12.40 ± 12.40 U/L, respectively. Decreased LDH plasma levels were still observed 24 h after administration of 20 mg/Kg methylone (59.87 ± 22.61 U/L) relative to controls (128.00 ± 37.38 U/L). Regarding 3,4-DMMC at 20 mg/Kg (137.20 ± 38.00 U/L), it seems that the effect observed at 1 h was reversed after 24 h, as a small increase in plasma LDH levels was observed.

One hour after drug administration, a decrease in plasma ALP concentration was observed in all treated animals. For control animals, the mean plasma ALP concentration was 3.65 ± 1.59 U/L, while in animals treated with 20 mg/Kg of 3,4-DMMC, methylone or MDMA were 3.09 ± 1.15 U/L, 2.36 ± 0.76 U/L and 1.53 ± 0.66 U/L, respectively. The decrease in plasma ALP concentrations seems to be dose-dependent, since at 40 mg/kg the values obtained for 3,4-DMMC (0.40 ± 0.40 U/L) and methylone were lower. For methylone at 40 mg/Kg, values were again below the limit of quantification of the method. Twenty-four hours after drug administration, there was still a decrease in plasma ALP concentrations for 3,4-DMMC at 20 mg/Kg (0.85 ± 0.85 U/L), relative to ALP concentration in controls (1.90 ± 1.90 U/L), but a reversal of the effect for methylone at 20 mg/Kg was observed, as a small increase in plasma ALP concentration was observed (2.33 ± 1.36 U/L).

An increase in plasma γ-GT concentration was observed in animals treated for 1 h with 40 mg/Kg methylone (2.90 ± 1.20 U/L; $p<0.05$), compared to the control animals (0.48 ± 0.31 U/L). In animals treated with 3,4-DMMC, MDMA or 20 mg/Kg methylone the mean plasma γ-GT concentration values were close to those obtained for control animals. Decreased plasma concentrations of γ-GT were observed 24 h after administration for 20

mg/Kg 3,4-DMMC or methylone (2.45 ± 2.45 U/L and 0.85 ± 0.56 U/L, respectively), compared to controls (2.98 ± 2.98 U/L).

After 1 h of drug administration, a decrease in AST plasma concentration was observed in animals treated with 3,4-DMMC at 20 or 40 mg/Kg (19.80 ± 5.07 U/L and 6.87 ± 4.66 U/L, respectively) and with 40 mg/Kg methylone (4.70 ± 4.70 U/L), compared to controls (29.67 ± 6.35 U/L). A decrease in AST plasma concentrations was also observed in animals treated with 20 mg/Kg 3,4-DMMC or methylone (40.72 ± 8.28 U/L) compared to MDMA (50.70 ± 9.24 U/L). In addition, this drug-induced effect seems to be dose-dependent, since for animals treated with 40 mg/Kg 3,4-DMMC or methylone, the plasma AST concentrations were lower than those obtained for animals treated with a dose of 20 mg/Kg. Twenty-four hours after drug administration, animals treated with 20 mg/Kg of 3,4-DMMC or methylone presented values of 13.50 ± 3.25 U/L and 21.47 ± 8.12 U/L, respectively, that were close to those obtained in controls (18.72 ± 5.95 U/L).

Table 4. Alterations in plasma biomarkers of hepatic injury induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, methylone and 3,4-dimethylmetcatinone (3,4-DMMC) at 20 or 40 mg/Kg, 1 h or 24 h after i.p. drug administration. Values are presented as mean ± standard error of mean. *$p<0.05$; **$p<0.01$, *vs.* control.

		Control	MDMA 20 mg/Kg	3,4-DMMC 20 mg/Kg	3,4-DMMC 40 mg/Kg	Methylone 20 mg/Kg	Methylone 40 mg/Kg
Albumin (g/dL)	1 h	3.49±0.13	3.19±0.35	2.85±0.37	1.93±0.86	2.53±0.30	0.26±0.26 **
	24 h	2.44±0.87	-	2.62±0.38	-	1.94±0.53	-
ALT (U/L)	1 h	35.99±5.85	31.99±2.46	39.28±20.20	5.05±1.31 *	17.75±3.78	0 *
	24 h	13.38±5.92	-	3.57±1.58	-	8.83±5.14	-
LDH (U/L)	1 h	310.70±130.80	420.80±128.30	175.70±42.23	43.25±18.48	81.15±18.38 *	12.40±12.40 *
	24 h	128.00±37.38	-	137.20±38.00	-	59.87±22.61	-
ALP (U/L)	1 h	3.65±1.59	1.53±0.66	3.09±1.15	0.40±0.40 *	2.36±0.76	0
	24 h	1.90±1.90	-	0.85±0.85	-	2.33±1.36	-
γ-GT (U/L)	1 h	0.48±0.31	1.11±0.58	0.49±0.23	0.55±0.55	0.76±0.35	2.90±1.20
	24 h	2.98±2.98	-	2.45±2.45	-	0.85±0.56	-
AST (U/L)	1 h	29.67±6.35	50.70±9.24	19.80±5.07	6.87±4.66	40.72±8.28	4.70±4.70
	24 h	18.72±5.95	-	13.50±3.25	-	21.47±8.12	-

γ-GT, γ-glutamyl transpeptidase; ***ALP,*** alkaline phosphatase; ***ALT,*** alanine transaminase; ***AST,*** aspartate transaminase; ***LDH,*** lactate dehydrogenase.

4.9 3,4-Dimethylmethcathinone (3,4-DMMC) and methylone did not trigger liver histological alterations after 24 h of administration

No histological modifications occurred in the liver of animals treated with 20 mg/Kg 3,4-DMMC or methylone (Figure 12).
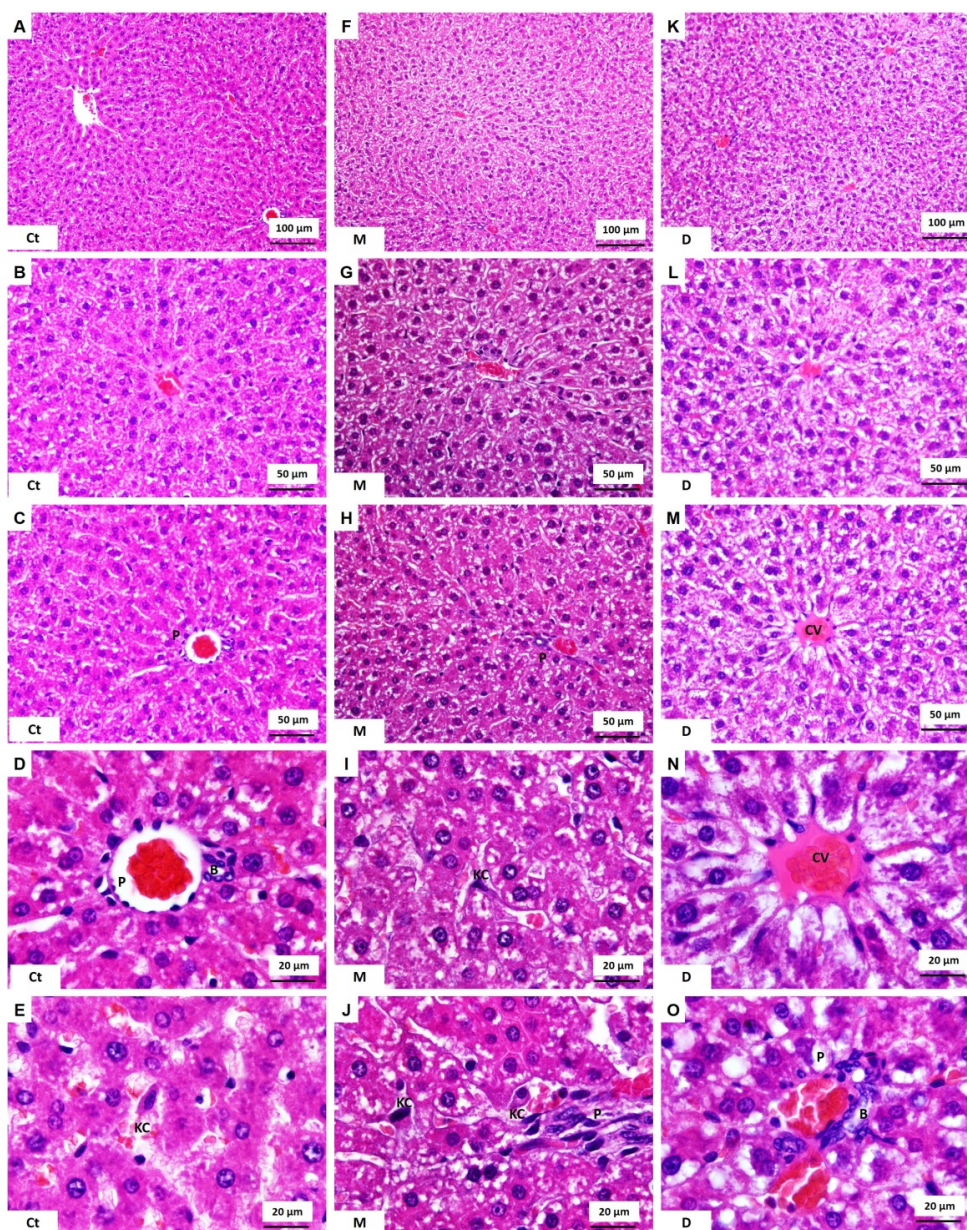
Figure 12. Light microscopy photomicrographs of hematoxylin–eosin stained sections of Wistar rat liver, 24 h after i.p. administration of saline (Ct) or 20 mg/Kg methylone (M) or 3,4-DMMC (D). No pathologic features were observed for any treatment group. In higher magnification views, portal regions (P) and central veins (CV) are evident. Hepatocytes morphology is normal as it is the transition of their staining from dark to light between zones 1 and 3 due to glycogen accumulation and cytoplasmic clearing of centrilobular hepatocytes. Kupffer cells (KC) project into the sinusoid lumen are more frequent in periportal regions. *B,* bile ductulus.

Chapter V – Discussion

Methylone and 3,4-DMMC are psychoactive stimulants that have been traditionally advertised to recreational drug users as relatively safe drugs, in spite of the related fatalities reported due to the hyperthermia, serotonin syndrome, and multi-organ system failure (Barrios et al., 2016; Frohlich et al., 2011; Pearson et al., 2012; Usui et al., 2014; Zaami et al., 2018). Since *in vivo* toxicological information at the target organs remain scarce for these drugs, mainly by virtue of the absence of reports on the clinical and pathological features achieved in fatal and non-fatal intoxications, we have focused our research on the detrimental outcomes of 3,4-DMMC and methylone in the rat liver.

The liver is one of the main targets of synthetic cathinones, including 3,4-DMMC and methylone (Cawrse et al., 2012; Frohlich et al., 2011; Kovacs et al., 2012; Pearson et al., 2012; Shimomura et al., 2016), and recent evidence has demonstrated that these drugs bioaccumulate in the organ even in situations of parental administration (Rouxinol, 2019b). An additional factor that renders the liver more susceptible to the drugs toxicity is the metabolic competence of the organ, either due to the oxidative burst that arise from the activity of the enzymes of metabolism or to the potential of many of the resulting metabolites to exhibit higher toxicity than the parent drug (*e.g.* metabolites of methylone) (Luethi et al., 2019; Martinez-Clemente et al., 2013; Pedersen et al., 2013). In fact, there is a relevant number of reports on the liver injury or abnormal liver function as clinical adverse effects associated with the abuse of methylone (Kovacs et al., 2012; Pearson et al., 2012) and mephedrone (Holliman et al., 2011; Kasick et al., 2012; Schifano et al., 2011), a cathinone whose structure is closely related to that of 3,4-DMMC.

To provide further knowledge on the *in vivo* liver effects of methylone and 3,4-DMMC, in the present study two drug doses were administered i.p*.* to Wistar rats, *i.e.* 20 mg/Kg and 40 mg/Kg, and several endpoints were further evaluated, including behavioural effects (1 h and 24 h), liver histopathological alterations (24 h), hepatic energetic status (1 h and 24 h), liver content of antioxidant molecules and activity of oxidative stress-related enzymes (1 h and 24 h), and several plasma biomarkers indicative of hepatic damage (1 h and 24 h). Of note, the two doses tested are in line with those used in previous animal experiments with both methylone (Lopez-Arnau et al., 2014; Stefkova et al., 2017) and 3,4-DMMC (Rouxinol, 2019a).

Following drug administration, 3,4-DMMC and methylone dose-dependently increased overall locomotion and rectal temperature of animals, which is in line with results herein obtained for MDMA, and with previous reports in rats for methylone (Stefkova et al., 2017), other cathinones (Horsley et al., 2018; Sichova et al., 2017) and amphetamines (Carvalho et al., 2012). These effects, which peaked approximately at 1 h

and were totally abolished after 24 h, reflect two well-acknowledged acute clinical consequents of the abuse of these synthetic stimulants (Karila and Reynaud, 2011; Valente et al., 2014). In what concerns hyperthermia, body temperatures as high as 42 °C were reported in intoxicated users after methylone administration (Pearson et al., 2012). In agreement with this finding, some thermolytic behaviours in response to heat stress were also observed in the current study in drug-exposed animals, such as vasodilatation in oral mucosal and paws, prone and transient extension of the body, and intense salivation. In fact, at mild increase of body temperature, passive means of thermolysis, such as radiation and convection of heat from the animals to the environment facilitated by vasodilatatory mechanisms, could ensure the thermal equilibrium, as indicated by the redness in the rat paws, nose and mouth mucosa. Then, further temperature increase in the anterior hypothalamus/pre-optic region is responsible for the body extension of the animal, which can be regarded as one of the most important and prevalent thermolytic responses across mammalians, as it reduces metabolism and facilitates heat conduction (Roberts and Mooney, 1974). Although quantitative examinations were not herein performed, relaxation and typical extension of the body could often be observed in drug-administered rats. With the escalation of the body temperature, salivary cooling controlled by the autonomic nervous system came at play. The intense saliva spreading observed in the vascularized surfaces of the 3,4-DMMA and methylone-treated animals offers an important and effective means of heat loss through evaporative cooling (Obal et al., 1979). This is, for instance, the rat thermolytic primary response needed for survival at environment temperatures above 36 °C (Damas, 1995), in which the submaxillary glands are predominantly involved. Nevertheless, when body temperature reaches levels as high as 40 °C, evaporative heat loss from saliva is ameliorated by a rise of saliva flow through stimulation of secretion of the other salivary glands (Obal et al., 1979). This is in line with the severe hypersalivation observed in animals treated with methylone and MDMA. Piloerection is also a physiological reaction of the sympathetic autonomic nervous system that is present in situations of pain or discomfort, such those observed in our study (Benedek et al., 2010). Of note, the behavioural and exuberant temperature effects reported in the present work were even higher for rat group-housed conditions, suggesting that hyperpyrexia and serotonin syndrome are especially potentiated when drugs are used in crowded settings, such those typical of the abuse (*e.g.* rave parties, dancing night clubs, etc.). In addition, as toxicity was so marked at the highest dose, drug testing for 24 h was not performed in animals treated at 40 mg/Kg.

In what concerns liver effects, methylone at 40 mg/Kg was able to significantly increase ROW, after 1h, indicating liver compromise and a proinflammatory status of the organ (Carvalho et al., 2012; Carvalho et al., 2010; Yamamoto et al., 2010).

Methylone is structurally close to MDMA, which has been demonstrated to induce in humans hepatic inflammatory processes associated with increased infiltration of lymphocytes, neutrophils and eosinophils (Andreu et al., 1998; Dykhuizen et al., 1995; Ellis et al., 1996; Fidler et al., 1996; Khakoo et al., 1995). In addition, MDMA-induced alteration in inflammatory markers (tumor necrosis factor (TNF)-alpha and interleukin (IL)-10) was also observed in animals and *in vitro (Connor, 2004; Connor et al., 2005).*

Based on our results, the occurrence of liver injury might heavily rely on the drug dose administered, since histological examination of the organs of animals treated with 20 mg/Kg methylone or 3,4-DMMC for 24 h denoted no signs of injury. This is also supported by data showing that energetic levels of the liver were only significantly lessened at 1 h when both cathinones were tested at the highest dose (40 mg/Kg), although prolonged exposure at a lower dose of methylone (24 h; 20 mg/Kg) was also able to elicit ATP fall. These results are also supported by *in vitro* evidence for 3,4-DMMC (Roque Bravo, 2016), but conflicting data exist for methylone (Valente et al., 2016a) in primary rat hepatocytes.

Energetic impairment may be a consequent of the acute oxidative/nitrosative stress induced by these drugs (evidenced by the decline of GSH/GSSG ratio at 1 h), as reactive species are able to interfere with the mitochondrial transport chain (Dias da Silva et al., 2017), disrupting ATP synthesis. On the other hand, mitochondrial dysfunction may itself exacerbate production of prooxidant species through the uncoupling of oxidative phosphorylation. Other factors that may worsen the generation of reactive species is drug-induced hyperthermia (da Silva et al., 2014) and the increase in neurotransmitters efflux and further oxidation of biogenic amines (Carvalho et al., 2010). In the particular case of methylone, metabolic activation might constitute an important source of prooxidants. It has been postulated that, similarly to what occurs for MDMA, methylone-derived catecholic toxicants display high inherent reactivity. So, if circulating concentrations of these oxidative metabolites become excessive, and concurrent saturation of COMT occurs, then enzymatic, cellular and autoxidative mechanisms may lead to the formation of quinones, which are highly redox active molecules. Quinones enter in redox cycling, originating electrophilic semiquinone radicals that lead to intense production of reactive species (da Silva et al., 2014). The role of the oxidative metabolites in MDMA-elicited hepatotoxicity was already established in freshly isolated rat hepatocytes (Carvalho et al., 2004a; Carvalho et al., 2004b) and similar mechanisms might be at play for methylone.

The most abundant and important non-enzymatic antioxidant intracellular defence against reactive species and electrophilic xenobiotics is GSH, which prevents

cell damage induced by ROS/RNS. The role of this antioxidant becomes even more important in the liver due to the relevance of the drug-metabolizing processes in the organ. Hepatocytes exposed to excessive or sustained oxidative stress accumulate GSSG with reduction of the GSH/GSSG ratio. On the other hand, since evidence supports that methylone can also suffer extensive hepatic metabolism into catechols, further being oxidised to unstable orthoquinones (Valente et al., 2017b), disruption of thiol redox homeostasis might potentially be explained by the formation of conjugates of GSH with these reactive metabolites, resembling what occurs with MDMA (Carvalho et al., 2012). These glutathione-S-yl-adducts might still be redox active, and thus readily being oxidised and reduced by a second molecule of glutathione, resulting in 2,5-bis-(glutathione-S-yl)-conjugates (Carvalho et al., 2012). In this process, GST, a phase II metabolic enzyme, is a pivotal player as it catalyse the conjugation of GSH via a sulfhydryl group to electrophilic metabolites, facilitating their detoxification.

Both methylone and 3,4-DMMC diminished GST activity, either at 40 mg/Kg when tested for 1 h or at 20 mg/Kg for 24 h. As far as we concerned, this is the first time that alterations on GST functionality are reported for these cathinones. Previous works examining this effect for amphetamines are controversial, with some studies demonstrating no notable imbalances in the enzymatic activity, in spite of an overproduction of reactive species and oxidative damage, as confirmed by the significant increase of protein carbonylation and DNA fragmentation (Parolini et al., 2016); while others present increased expression of GST and GPx (yet with no information on the activity of the enzyme) (Antolino-Lobo et al., 2011; Carvalho et al., 2001; Hsieh et al., 2015). The data we present might be justified by the direct inhibition of the enzyme by 3,4-DMMC and methylone (or respective metabolites), but also by the drug-induced perturbation of the metabolism of some endogenous compounds, such as bilirubin, which is itself known to inhibit the GST activity (Fukai et al., 1989). There is only study conducted in Swiss albino male mice supporting our findings, in which the authors observed the dose-dependently decrease of the activity of GST (at 0.25 and 0.5 mg/Kg, once daily for 15 days) and also of GPx and GR (at 0.5 mg/Kg, once daily for 15 days) (Safhi et al., 2014). In accordance, also herein GPx and GR activities were significantly reduced in rats exposed for 1 h to 3,4-DMMC and methylone. While GPx main biological role is to protect the organism from oxidative damage by reducing lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water, GR catalyses the thiol reduction of GSSG to GSH, in a mechanism that is critical for the resistance to oxidative stress and maintenance of the reducing environment of the cell (Couto et al., 2016). On the other

hand, the GPx expression was increased in Swiss-CD1 mice administered with mephedrone (25 mg/Kg, four times in one day, every 2 h), but no information on the activity of the enzyme is available. *In vitro* exposure of human neuronal SK-N-SH cells to α-pyrrolidinononanophenone at 20–50μM also decreased cellular levels of GR activity (Matsunaga et al., 2017), but no further information was found in the literature on alterations induced by synthetic cathinones in activity of these antioxidant enzymes.

Overall, complex cross talk between levels of GSSG and GSH and other thiols, and antioxidant enzymes such as GST, GPx and GR determine the most appropriate circumstances for redox control within the hepatocyte or for the activation of programmed cell death, which was previously demonstrated *in vitro* for methylone (Valente et al., 2017a; Valente et al., 2016a; Valente et al., 2017b).

Other signs of hepatocellular damage evidenced in the present study include increased plasma levels of γ-GT and AST for methylone and MDMA, after 1 h; and of LDH for MDMA at 1 h. These alterations were however statistically non-significant in the majority of the cases. In addition, unexpected reduction of plasma ALT, albumin and ALP were also observed, deserving further investigation, as the majority of the laboratory results of patients intoxicated with methylone indicate significant increased lactate, liver transaminases, creatinine, myoglobin, creatine kinase and clotting times, and decreased pH, glucose and calcium (Pearson et al., 2012).

Chapter VI – Conclusions

Our work may advance the knowledge regarding the toxicological effects of methylone and 3,4-DMMC in vivo. Summing up, herein was demonstrated that:

➢ Methylone and 3,4-DMMC induced hyperpyrexia, piloerection, increased locomotion, stereotyped circling, intense saliva spreading and disorientation. All these stress indicators were intensified in group-housed rats, suggesting that crowded settings, such those typical of the abuse (*e.g.* rave parties, dancing night clubs, etc.), potentiate hyperthermia and serotonergic syndrome;

➢ No significant liver histological alterations were observed after drug exposures for 24 h. Nevertheless, after 1 h, methylone at 40 mg/Kg induced a significant increase of the relative liver weight, suggesting a proinflammatory status of the organ.

➢ Overall, energetic content in the liver significantly decreased after exposure to both drugs at 40 mg/Kg for 1 h, and to methylone at 20 mg/Kg for 24 h.

➢ The antioxidant GSH/GSSG balance was reduced for all test conditions, with exception of rats exposed to methylone at 20 mg/Kg for 24 h.

➢ These alterations were accompanied by changes in oxidative stress-related enzymes, which were never reported before for these drugs. While 3,4-DMMC and methylone reduced GST activity both after 1 h (at 40 mg/Kg) and 24 h (at 20 mg/Kg), GPx and GR activities were significantly reduced in rats exposed for 1 h to 3,4-DMMC at 20 mg/Kg and methylone at 40 mg/Kg.

➢ Methylone and 3,4-DMMC also triggered changes in plasma biochemical markers (ALT, LDH, ALP, γ-GT and AST). Although the extent of these alterations highly rely on the exposure time and dose and/or drug tested. These alterations which are not compatible with liver damage, deserve further investigation.

To our knowledge, this is the first in vivo animal study evaluating stress parameters induced by exposure to 3,4-DMMC and methylone in rats. These data will certainly help elucidate the toxicological effects of these drugs on their main targets, which is essential to help clinicians find possible therapeutic solutions for this drug poisoning, but in particular to assist pathologists to establish the cause of death in fatal forensic investigations.

Chapter VII – References

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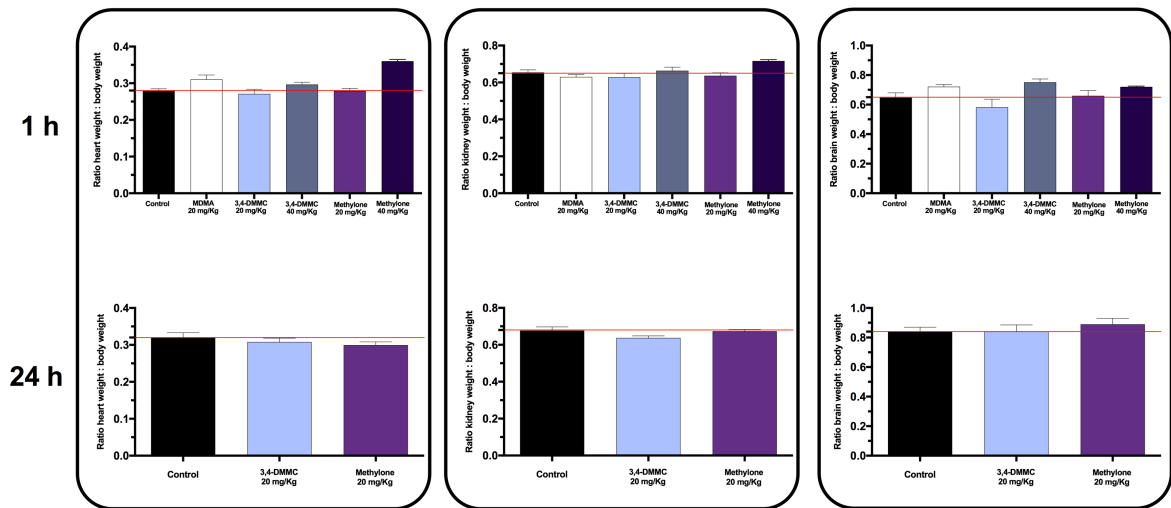
Supplementary Data

Figure S1. Effect on relative organ weight of 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and 3,4-dimethylmethcathinone (3,4-DMMC) and methylone at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Results are presented as mean ± standard error of mean. ****$p<0.0001$, *vs.* control.
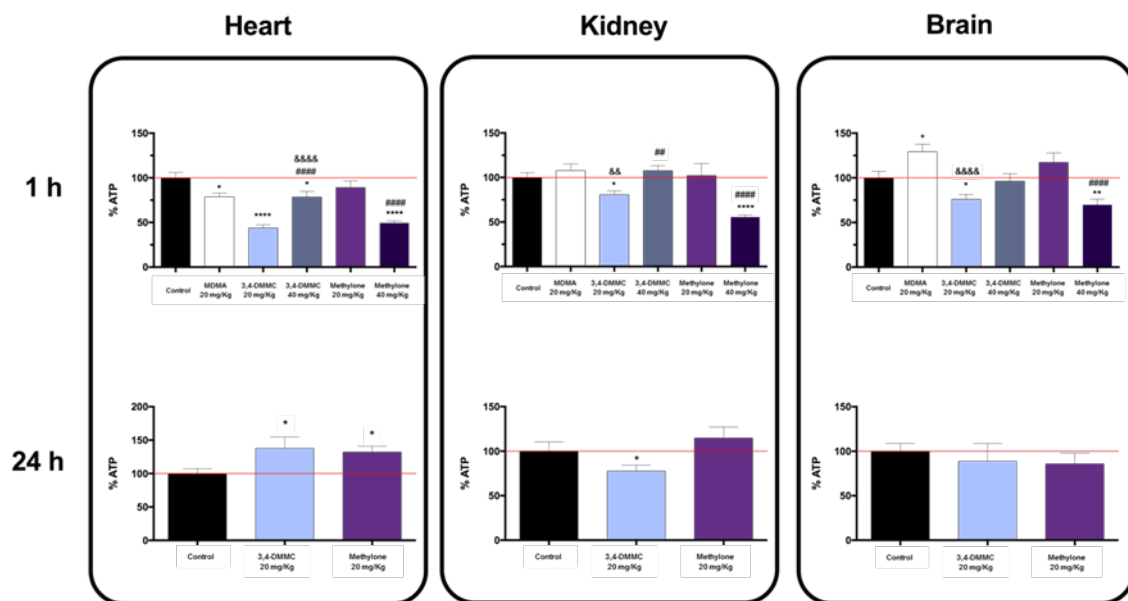

Figure S2. Alterations in adenosine triphosphate (ATP) levels induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *$p<0.05$; **$p<0.01$; ****$p<0.0001$, *vs.* control. ##$p<0.01$; ####$p<0.0001$, *vs.* same drug at 20 mg/Kg. &&$p<0.01$; &&&&$p<0.0001$, *vs.* 20 mg/Kg MDMA.
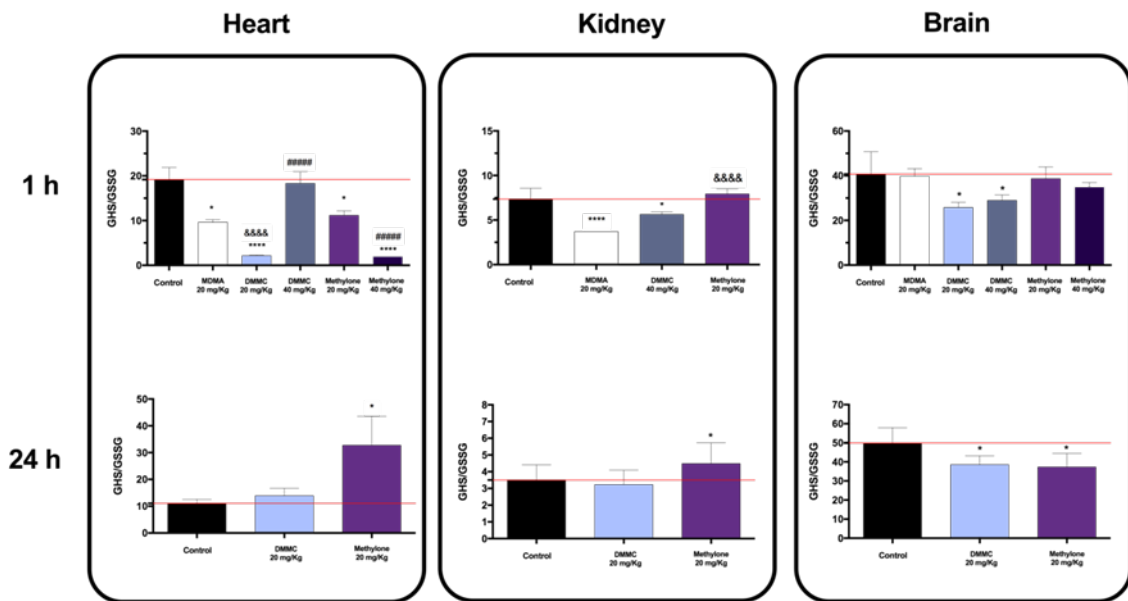
Figure S3. Alterations in the ratio of reduced to oxidised glutathione (GSH/GSSG) induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; ****p<0.0001, *vs.* control. ####p<0.0001, *vs.* same drug at 20 mg/Kg. &&&&p<0.0001, *vs.* 20 mg/Kg MDMA.

Figure S4. Alterations in reduced glutathione (GSH) levels induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; **p<0.01; ****p<0.0001, *vs.* control. #p<0.05; ##p<0.01, *vs.* same drug at 20 mg/Kg. &&p<0.01, *vs.* 20 mg/Kg MDMA.
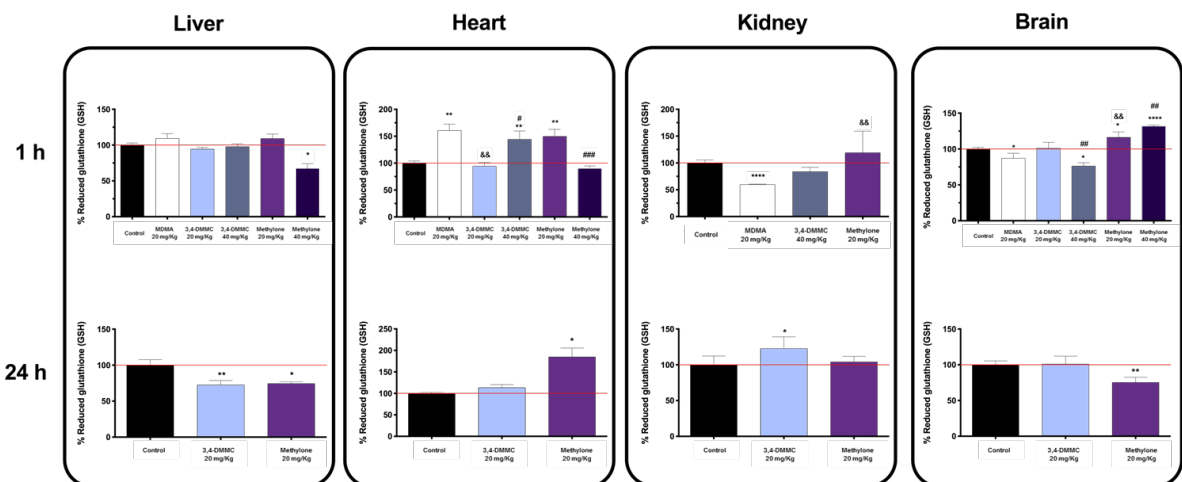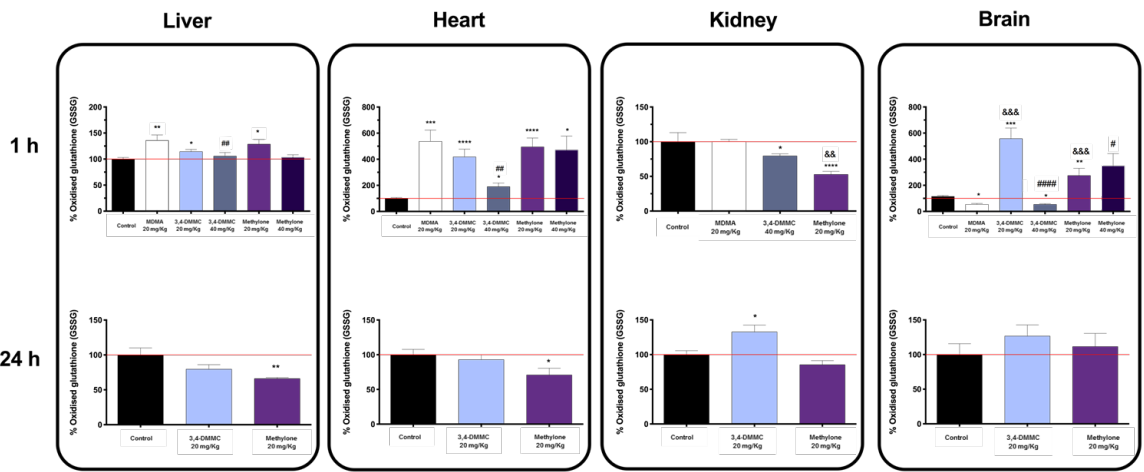
Figure S5. Alterations in oxidised glutathione (GSSG) levels induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of mean. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, *vs.* control. [#]p<0.05; [##]p<0.01; [####]p<0.0001, *vs.* same drug at 20 mg/Kg. [&&]p<0.01; [&&&]p<0.001, *vs.* 20 mg/Kg MDMA.

Figure S6. Alterations in glutathione peroxidase (GPx) activity induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, *vs.* control. [#]p<0.05; [##]p<0.01; [###]p<0.001; [####]p<0.0001, *vs.* same drug at 20 mg/Kg. [&]p<0.05; [&&]p<0.01; [&&&&]p<0.0001, *vs.* 20 mg/Kg MDMA.
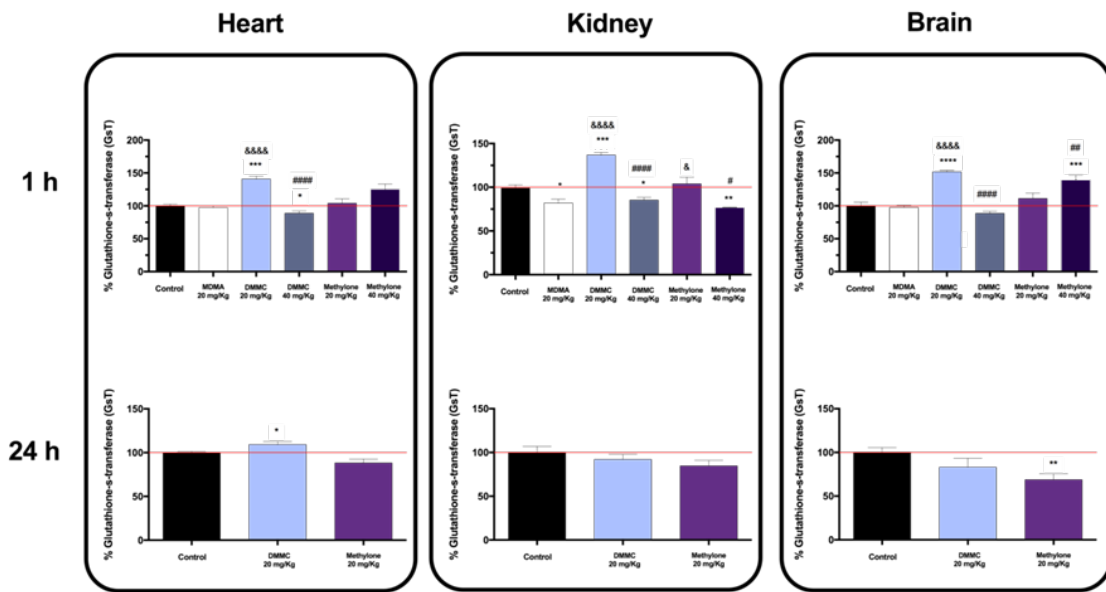
Figure S7. Alterations in glutathione S-transferase (GST) activity induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, *vs.* control. #p<0.05; ##p<0.01; ####p<0.0001, *vs.* same drug at 20 mg/Kg. &p<0.05; &&&&p<0.0001, *vs.* 20 mg/Kg MDMA.
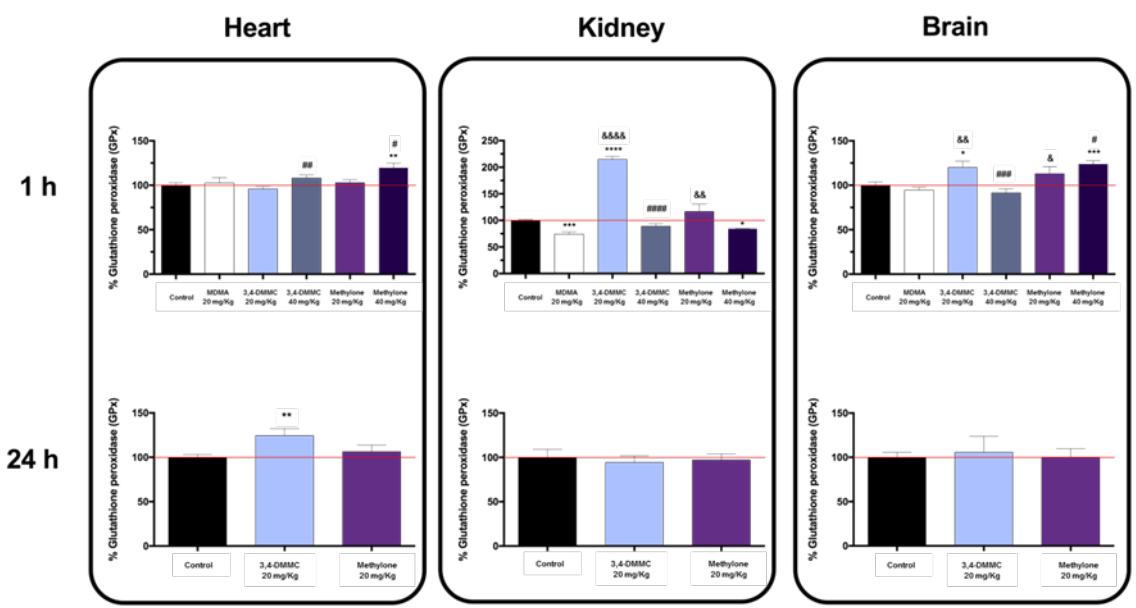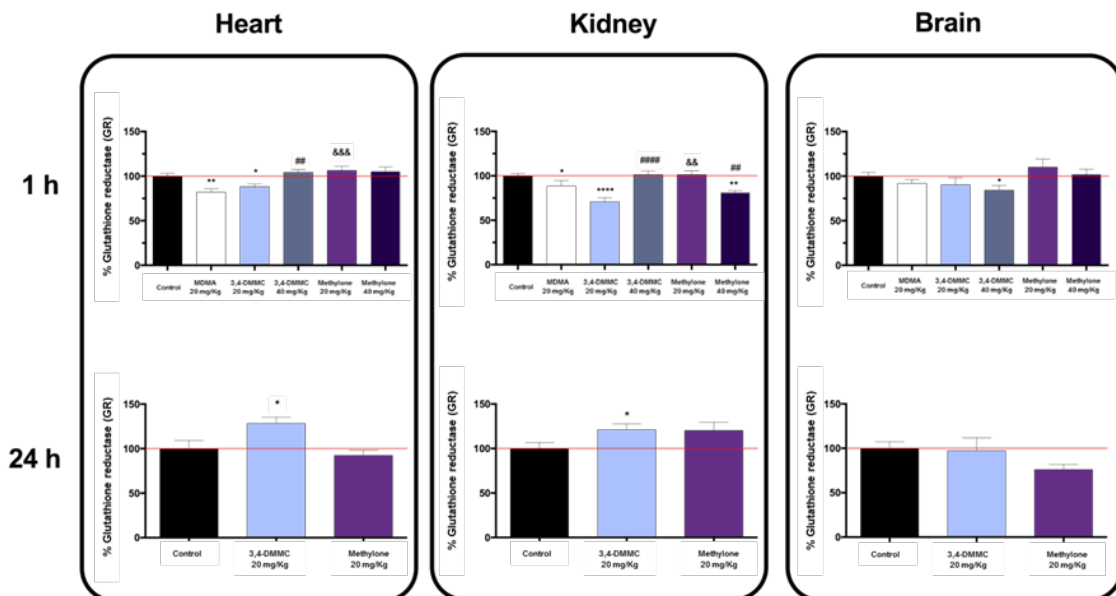

Figure S8. Alterations in glutathione reductase (GR) activity induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, and methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg in Wistar rats, 1 h or 24 h after i.p. drug administration. Values are presented as mean percentage of controls ± standard error of the mean. *p<0.05; **p<0.01; ****p<0.0001, *vs.* control. ##p<0.01; ####p<0.0001, *vs.* same drug at 20 mg/Kg. &&p<0.01; &&&p<0.001, *vs.* 20 mg/Kg MDMA.

Table S1. Alterations in the oxidative parameters induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, methylone and 3,4-dimethylmethcathinone (3,4-DMMC) at 20 or 40 mg/Kg, 1 h or 24 h after i.p. drug administration. Values are presented as mean ± standard error of the mean.

		Control	MDMA 20 mg/Kg	3,4-DMMC 20 mg/Kg	3,4-DMMC 40 mg/Kg	Methylone 20 mg/Kg	Methylone 40 mg/Kg
ATP ((µmol/mg protein)							
Liver	1 h	0.51±0.04	0.52±0.11	0.48±0.06	0.32±0.04	0.38±0.04	0.41±0.05
	24 h	0.52±0.03	-	0.47±0.06	-	0.31±0.03	-
Heart	1 h	1.28±0.15	0.88±0.15	0.38±0.04	0.71±0.05	0.87±0.11	1.03±0.31
	24 h	0.58±0.03	-	0.78±0.05	-	0.77±0.04	-
Kidneys	1 h	0.60±0.06	0.64±0.11	0.54±0.08	0.61±0.10	0.64±0.14	0.57±0.15
	24 h	0.46±0.03	-	0.34±0.02	-	0.52±0.03	-
Brain	1 h	1.95±0.16	2.84±0.41	0.97±0.04	1.71±0.24	1.81±0.21	3.89±0.64
	24 h	0.91±0.07	-	0.78±0.10	-	0.77±0.07	-
GSH (nmol/mg protein)							
Liver	1 h	17.26±1.15	19.72±3.90	19.68±0.81	16.62±1.90	17.97±1.15	13.56±5.82
	24 h	29.74±2.49	-	22.17±2.18	-	25.56±3.45	-
Heart	1 h	5.61±0.44	6.38±0.63	6.10±1.38	6.25±0.88	6.21±0.35	4.60±0.55
	24 h	5.44±0.38	-	6.22±0.72	-	9.13±1.42	-
Kidneys	1 h	0.05±0.01	0.06±0.02	0.03±0.01	0.07±0.01	0.05±0.01	0.13±0.05
	24 h	0.04±0.01	-	0.05±0.01	-	0.04±0.01	-
Brain	1 h	2.39±0.21	2.26±0.35	2.00±0.40	1.69±0.21	2.35±0.26	3.66±0.52
	24 h	1.39±0.08	-	1.42±0.17	-	1.05±0.10	-

ATP, adenosine triphosphate; *GPx,* Glutathione peroxidase; *GR,* Glutathione reductase; *GSH,* Glutathione reduced; *GSSH,* Oxidized Glutathione*; GSTs,* Glutathione S-transferases.

Table S1. Continued.

		Control	MDMA 20 mg/Kg	3.4-DMMC 20 mg/Kg	3.4-DMMC 40 mg/Kg	Methylone 20 mg/Kg	Methylone 40 mg/Kg
GSSG (nmol/mg protein)							
Liver	1 h	1.40±0.14	2.51±0.58	1.66±0.09	1.68±0.20	2.10±0.27	2.37±0.02
	24 h	13.94±2.08	-	11.12±1.30	-	9.24±0.28	-
Heart	1 h	0.71±0.20	0.76±0.27	0.44±0.13	0.45±0.11	1.28±0.26	1.42±0.80
	24 h	0.51±0.06	-	0.48±0.05	-	0.36±0.07	-
Kidneys	1 h	0.01±0.002	0.01±0.001		0.01±0.001	0.01±0.001	0.01±0.001
	24 h	0.01±0.001	-	0.01±0.001	-	0.01±0.001	-
Brain	1 h	0.08±0.01	0.06±0.01	0.03±0.001	0.06±0.01	0.07±0.01	0.13±0.03
	24 h	0.03±0.01	-	0.04±0.01	-	0.03±0.01	-
GPx (U/ng protein)							
Liver	1 h	40.25±2.12	40.89±2.81	42.52±8.55	39.75±2.39	40.99±6.03	24.69±1.78
	24 h	67.18±6.74	-	60.67±5.63	-	54.22±3.32	-
Heart	1 h	10.11±0.40	11.08±1.56	11.30±1.52	11.09±0.53	10.35±0.40	10.36±0.54
	24 h	17.26±0.61	-	21.45±1.18	-	18.24±1.04	-
Kidneys	1 h	16.95±1.65	15.34±3.05	17.91±1.88	15.61±2.32	15.03±1.48	20.44±0.05
	24 h	19.51±1.79	-	18.47±1.36	-	19.01±1.29	-
Brain	1 h	2.94±0.18	2.68±0.19	2.88±0.40	2.65±0.22	3.29±0.25	3.01±0.43
	24 h	3.81±0.27	-	3.93±0.58	-	3.85±0.38	-

ATP, adenosine triphosphate; *GPx,* Glutathione peroxidase; *GR,* Glutathione reductase; *GSH,* Glutathione reduced; *GSSH,* Oxidized Glutathione; *GSTs,* Glutathione S-transferases.

Table S1. Continued.

		Control	MDMA 20 mg/Kg	3,4-DMMC 20 mg/Kg	3,4-DMMC 40 mg/Kg	Methylone 20 mg/Kg	Methylone 40 mg/Kg
GR (U/ng protein)							
Liver	1 h	8.53±0.45	9.14±0.89	9.39±1.69	7.52±0.39	7.90±0.55	7.79±0.24
	24 h	12.37±0.85	-	13.86±0.72	-	12.46±0.51	-
Heart	1 h	1.44±0.06	1.23±0.07	1.42±0.16	1.55±0.10	1.53±0.06	1.46±0.16
	24 h	2.11±0.19	-	2.70±0.14	-	1.94±0.10	-
Kidneys	1 h	10.20±1.00	5.28±0.70	8.82±2.38	9.72±1.07	9.72±1.07	11.92±1.15
	24 h	9.16±0.61	-	11.13±0.60	-	11.02±0.78	-
Brain	1 h	2.55±0.12	2.34±0.23	2.31±0.31	2.21±0.21	2.90±0.24	2.54±0.36
	24 h	6.71±0.53	-	6.42±0.83	-	5.12±0.39	-
GST (U/ng protein)							
Liver	1 h	0.10±0.01	0.08±0.01	0.12±0.01	0.07±0.01	0.09±0.01	0.07±0.01
	24 h	0.17±0.01	-	0.13±0.01	-	0.12±0.01	-
Heart	1 h	0,06±0.01	0.06±0.01	0.07±0.01	0.06±0.01	0.06±0.01	0.07±0.01
	24 h	0.09±0.01	-	0.10±0.01	-	0.08±0.01	-
Kidneys	1 h	0.07±0.01	0.08±0.03	0.05±0.01	0.07±0.02	0.07±0.01	0.04±0.001
	24 h	0.07±0.01	-	0.07±0.01	-	0.06±0.01	-
Brain	1 h	0.06±0.01	0.06±0.01	0.09±0.01	0.05±0.01	0.07±0.01	0.09±0.01
	24 h	3.45±0.73	-	3.30±0.77	-	2.62±0.55	-

ATP, adenosine triphosphate; *GPx,* Glutathione peroxidase; *GR,* Glutathione reductase; *GSH,* Glutathione reduced; *GSSH,* Oxidized Glutathione; *GSTs,* Glutathione S-transferases.

Table S2. Alterations in plasma biochemical markers induced by 3,4-methylenedioxymethamphetamine (MDMA) at 20 mg/Kg, methylone and 3,4-dimethylmetcatinone (3,4-DMMC) at 20 or 40 mg/Kg, 1 h or 24 h after i.p. drug administration. Values are presented as mean ± standard error of the mean.

		Control	MDMA 20 mg/Kg	3,4-DMMC 20 mg/Kg	3,4-DMMC 40 mg/Kg	Methylone 20 mg/Kg	Methylone 40 mg/Kg
Total proteins (g/L)	1 h	52.94±3.41	46.85±5.22	43.98±5.36	29.13±10.12	39.76±5.12	11.34±1.51
	24 h	33.07±11.30	-	37.29±5.75	-	33.35±7.57	-
Cholesterol (mg/dL)	1 h	68.43±4.92	47.89±7.73	53.87±6.88	26.53±8.61	44.36±5.30	31.2±8.00
	24 h	37.42±12.64	-	59.65±16.71	-	39.03±7.95	-
Glucose oxidase (mg/dL)	1 h	378.1±18.07	400.50±30.40	368.30±49.44	220.30±58.77	277.10±31.02	173.90±138.30
	24 h	171.80±51.76	-	251.5±30.08	-	174.9±29.35	-
Creatinine (mg/dL)	1 h	0.26±0.15	0.17±0.08	0.61±0.24	0.28±0.16	1.11±0.58	0.31±0.31
	24 h	0.002±0.002	-	0.11±0.07	-	1.24±1.23	-
Uric acid (mg/dL)	1 h	2.30±0.53	3.81±0.98	3.06±0.89	0.50±0.19	1.43±0.53	8.70±8.70
	24 h	1.32±0.91	-	2.98±0.64	-	1.03±0.34	-
Urea (mg/dL)	1 h	73.16±41.2	31.10±3.28	20.33±4.56	17.50±1.98	19.08±3.71	25.70±25.7
	24 h	11.56±5.36	-	15.35±1.78	-	10.68±2.46	-
Triglycerides (mg/dL)	1 h	651.30±250.90	120.80±20.88	704.50±205.00	531.30±410.80	806.10±185.90	469.90±258.50
	24 h	576.7±327.10	-	251.80±114.90	-	662.70±244.00	-
Amylase (U/L)	1 h	721.3±27.02	462.30±65.78	504.80±78.44	142.70±47.98	420.10±67.39	95.60±95.60
	24 h	290.90±95.68	-	322.20±55.42	-	249.30±63.52	-
CK (U/L)	1 h	63.33±16.70	195.70±56.51	79.72±20.69	28.23±8.84	90.76±25.84	66.45±66.45
	24 h	40.26±14.37	-	74.03±26.05	-	32.72±11.3	-

CK, creatine kinase; *CK-MD,* creatine kinase isoenzyme.

Table S2. Continued.

		Control	MDMA 20 mg/Kg	3,4-DMMC 20 mg/Kg	3,4-DMMC 40 mg/Kg	Methylone 20 mg/Kg	Methylone 40 mg/Kg
CK-MB (U/L)	1 h	119.20±28.44	151.90±53.27	92.22±25.58	29.20±9.69	94.68±25.86	67.77±59.79
	24 h	56.28±19.09	-	68.19±20.88	-	42.83±19.92	-
Magnesium (mg/dL)	1 h	0.79±0.24	0.47±0.06	0.53±0.08	0.69±0.25	0.46±0.07	0
	24 h	0.46±0.16	-	0.50±0.16	-	0.59±0.11	-
Calcium (mg/dL)	1 h	0.14±0.09	0	0.28±0.11	0.47±0.47	0.14±0.06	0
	24 h	0.06±0.06	-	0.38±0.14	-	0.34±0.13	-
Iron (ug/dL)	1 h	18.46±7.07	18.16±11.33	366.00±236.40	411.20±400.2	868.10±844.70	2696.00±2696.00
	24 h	18.60±4.97	-	27.58±12.60	-	104.50±95.51	-
Sodium (mmo/L)	1 h	92.64±12.92	31.77±17.40	83.92±20.59	33.60±12.93	81.04±13.60	67.00±38.60
	24 h	32.22±19.47	-	46.18±4.29	-	56.75±10.33	-
Lactate (mg/dL)	1 h	23.22±6.18	66.46±24.43	33.09±11.51	8.31±4.79	17.7±4.30	0
	24 h	22.59±8.80	-	45.68±9.70	-	26.33±8.63	-
Pseudocholinesterase (U/L)	1 h	7451.00±7321.00	399.50±154.30	1317.00±1077.00	1123.00±991.50	1057.00±602.30	570.60±570.60
	24 h	1197.00±408.20	-	2713.00±1975.00	-	1865.00±1174.00	-

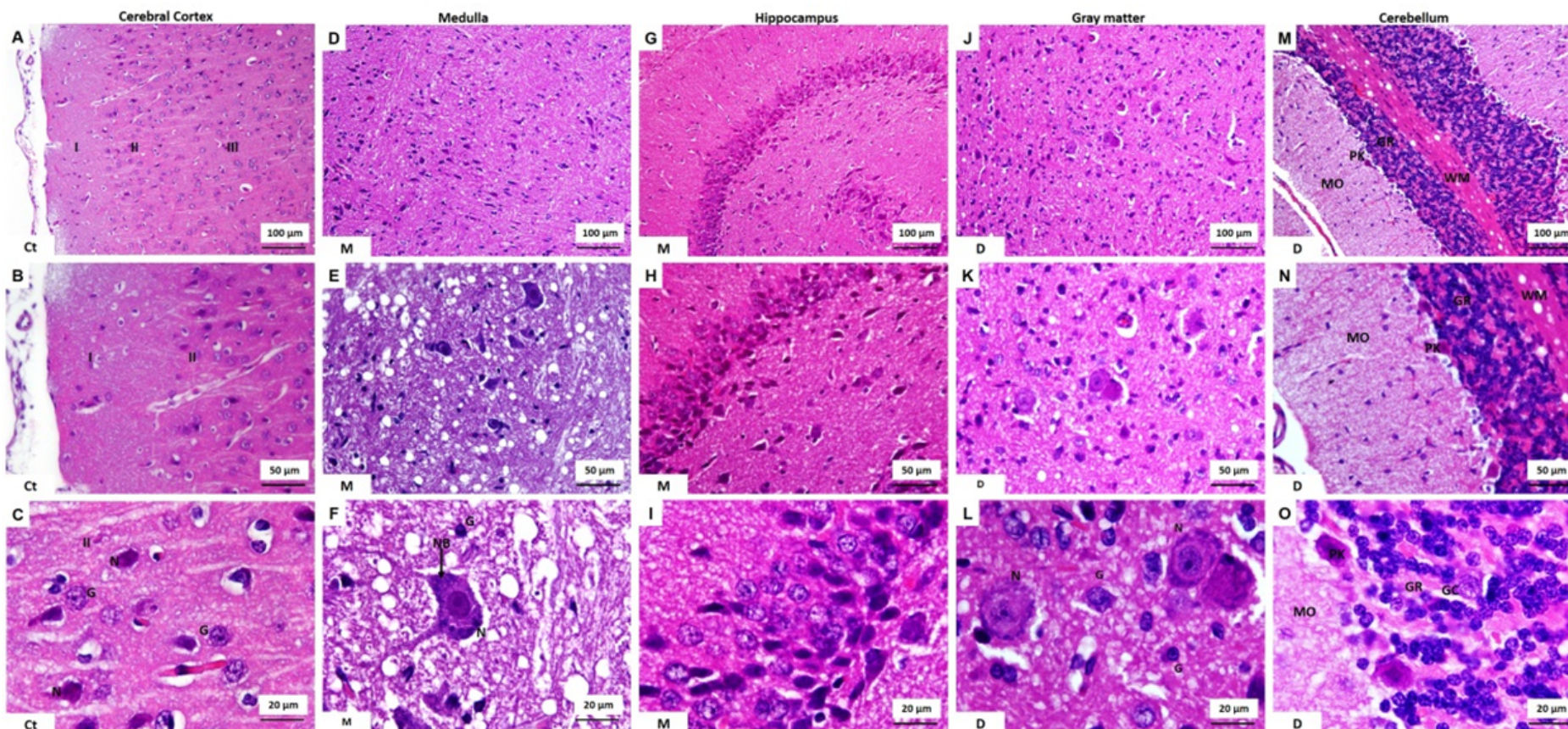*CK,* creatine kinase; *CK-MD,* creatine kinase isoenzyme.

Figure S9. Light microscopy photomicrographs of hematoxylin–eosin stained sections of Wistar rat brain, 24 h after i.p. administration of saline (Ct), 20 mg/Kg methylone (M) or 3,4-DMMC (D). Since no morphologic changes occur in any brain region, the photomicrographs chosen are from animals of different treatment groups. The most representative regions are illustrated, namely cerebral cortex (A, B, C), medulla (D, E, F), hippocampus (G, H, I), gray matter (J, K, L) and cerebellum (M, N, O). *I,* cerebral cortex molecular layer; *II,* cerebral cortex external granular cell layer; *III,* cerebral cortex external pyramidal cell layer; *G,* glial cell; *GC,* Golgi cell; *GR,* cerebellum granular layer; *MO,* cerebellum molecular layer; *N,* cellular body neurons; *NB,* Nissl bodies; *PK,* cerebellum Purkinje layer; *WM,* white matter.
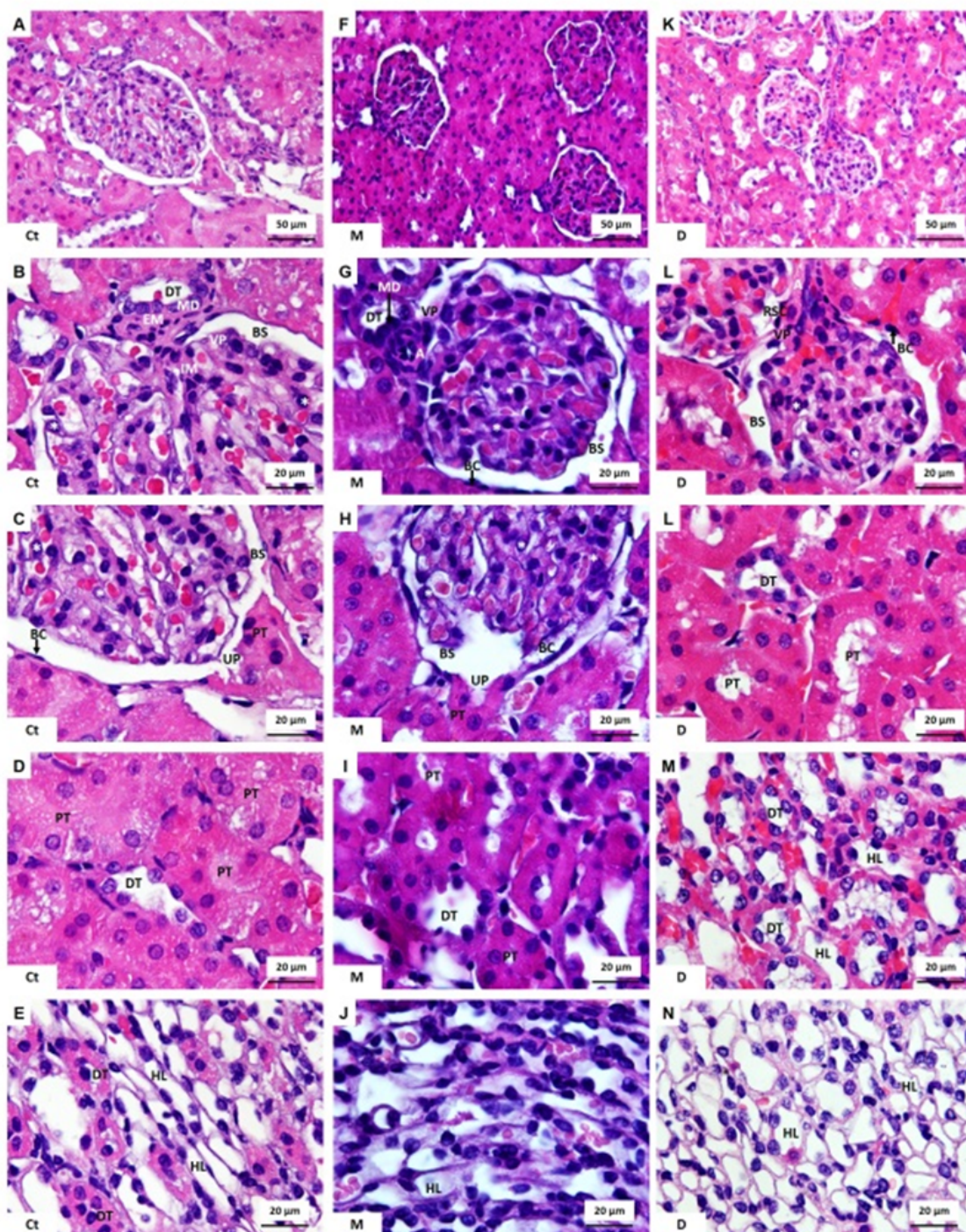
Figure S10. Light microscopy photomicrographs of hematoxylin–eosin stained sections of Wistar rat kidney, 24 h after i.p. administration of saline (Ct), 20 mg/Kg methylone (M) or 3,4-DMMC (D). No histopathologic alterations were found in rat kidney after the drug treatment. ***A,*** arteriole; ***BC,*** Bowman's capsule; ***BS,*** Bowman's space; ***DT,*** distal tubule; ***EM,*** extraglomerular mesangial cells; ***IM,*** intratraglomerular mesangial cells; ***HL,*** Henle loops; ***MD,*** macula densa;****,*** podocytes; ***PT,*** proximal tubule; ***UP,*** urinary pole of renal corpuscle; ***VP,*** vascular pole of renal corpuscle; ***RSC,*** renin-secreting cell.
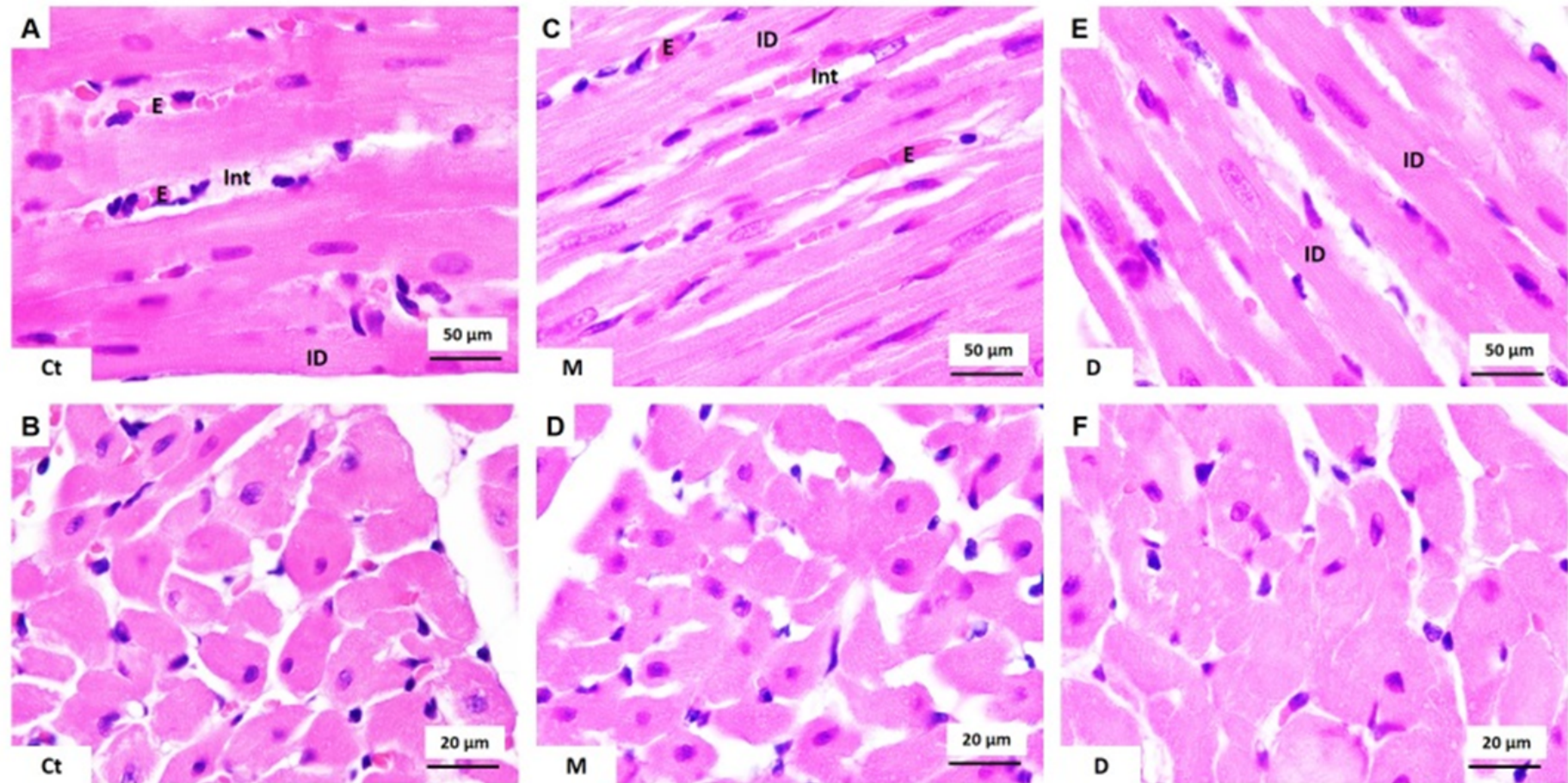
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Figure S11. Light microscopy photomicrographs of hematoxylin–eosin stained sections of Wistar rat myocardium, 24 h after i.p. administration of saline (Ct), 20 mg/Kg methylone (M) or mg/Kg 3,4-DMMC (D). Most of the area is occupied by the large cardiomyocyte eosinophilic cytoplasm. In A, C and E photomicrographs, cardiomyocytes are oriented longitudinally, and the faint cross-striations as well as the intercalated discs (ID) are evident. Between cardiomyocytes is a delicate interstitium (Int) containing fibroblasts and capillaries lined by endothelial cells.
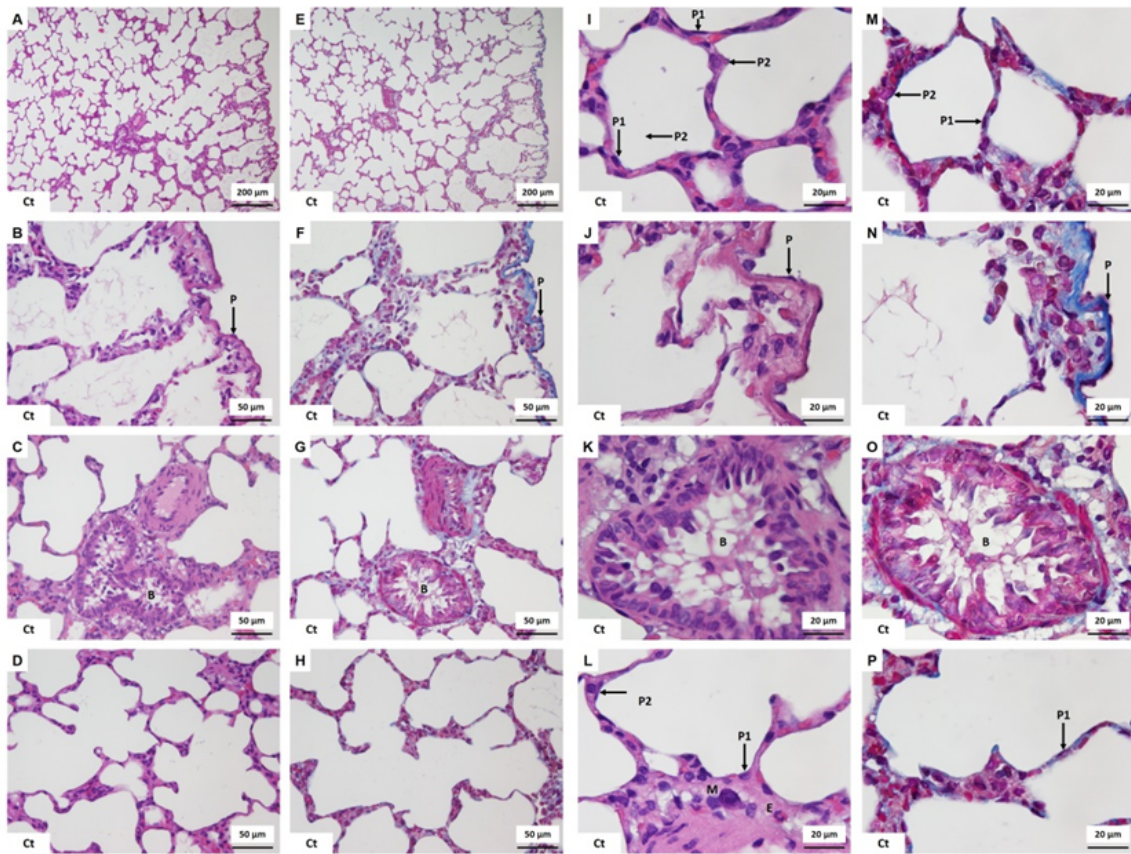
Figure S12. Light microscopy photomicrographs of hematoxylin–eosin stained sections (A, B, C, D, I, J, K, L) and Masson's trichrome stained sections (E, F, G, H, M, N, O, P) of Wistar rat lung, 24 h after i.p. administration of saline (Ct). The pleura (P, visceral pleura) of the rat lung is very thin. The alveolar wall is composed of a thin epithelial layer of squamous cells, either type I pneumocytes (P1) or squamous alveolar cells, interspersed with cuboidal cells known as type II pneumocytes (P2). Alveolar septum is the connective tissue and capillary network intervening between two apposed alveolar walls. Loose connective tissue contains elastic and reticular fibers, fibroblasts, myofibroblasts, pericytes, resident macrophages, lymphocytes, mast cells (M), and a scarce eosinophils (E). *B,* bronchiole.
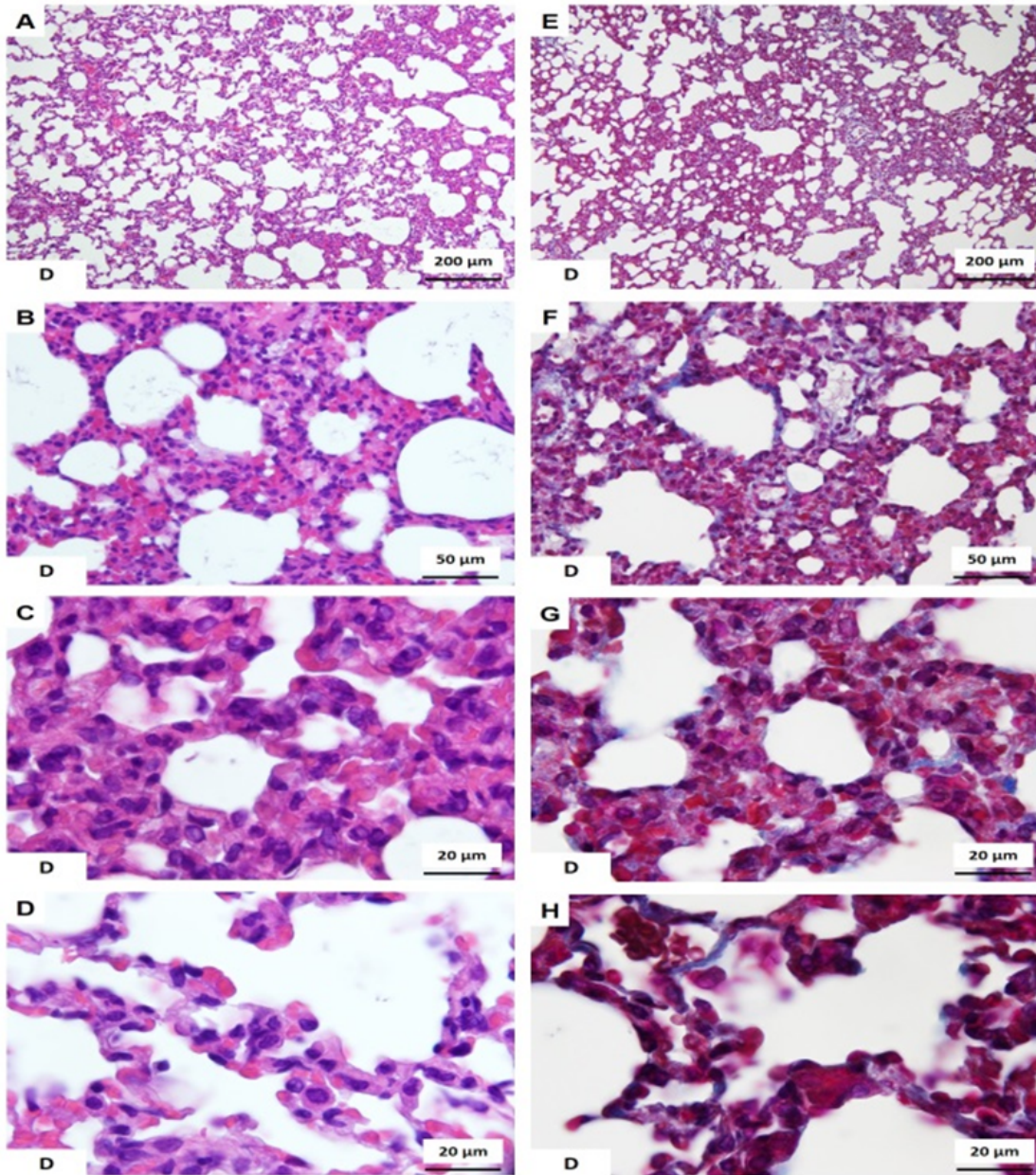
Figure S13. Light microscopy photomicrographs of hematoxylin–eosin stained sections (A, B, C, D) and Masson's trichrome stained sections (E, F, G, H) of Wistar rat lung, 24 h after i.p. administration of 20 mg/Kg 3,4-DMMC (D). The photomicrographs show thicker alveolar septa attributable to higher volumes of interstitial cells. Masson's trichrome stain confirms that collagen fibers are rare. In higher magnification photomicrographs (C, D, G and H) moderate pulmonary interstitial and alveolar hyperemia is observed.
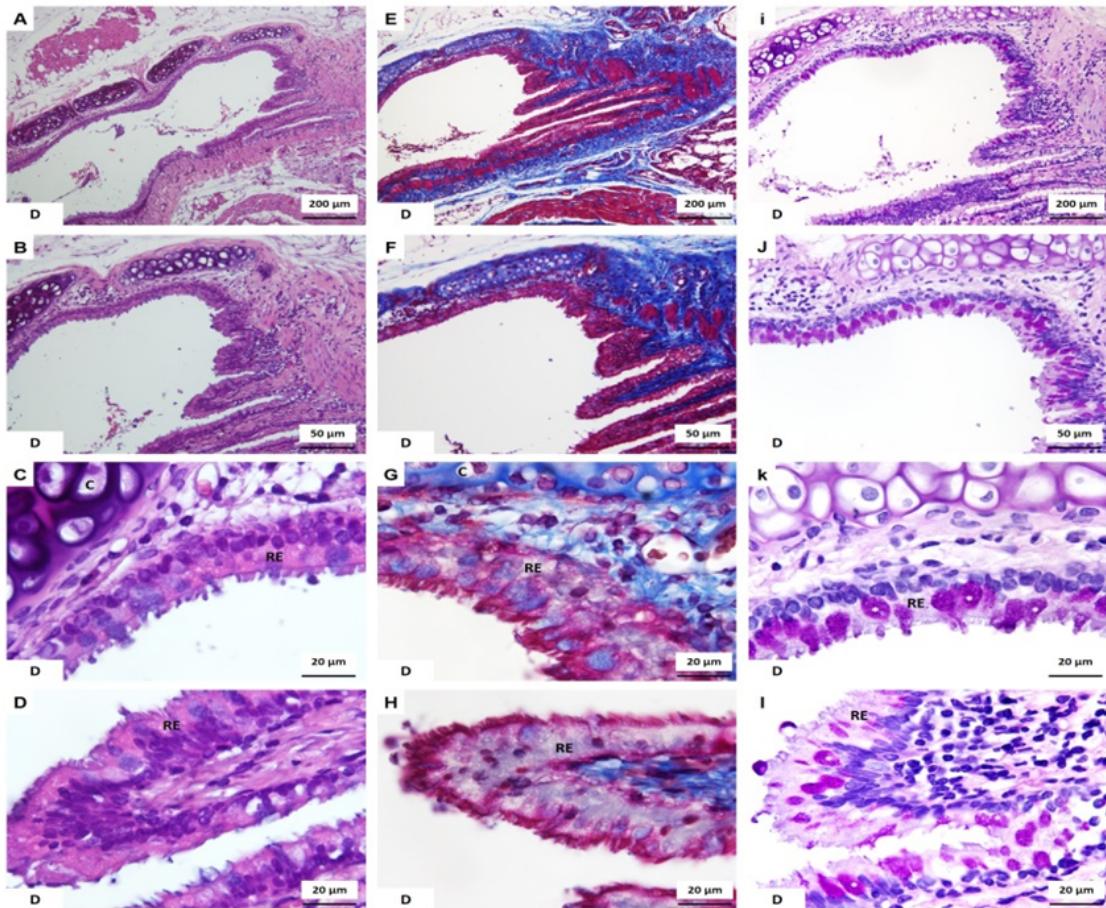
Figure S14. Light microscopy photomicrographs of hematoxylin–eosin stained sections (A, B, C, D), Masson's trichrome stained sections (E, F, G, H) and periodic acid-Schiff (PAS) with diastase (I, J, K, L) of Wistar rat bronchi, 24 h after i.p. administration of 20 mg/Kg 3,4-DMMC (D). Mucin was specifically identified in goblet cells (*) using the PAS staining procedure since the glycogen, which is also PAS-positive, was digested with diastase and washed out. *C,* cartilage; *RE,* respiratory pseudostratified ciliated epithelium.