

**RESEARCH INTO THE TOXICOLOGICAL
MECHANISMS OF TRAMADOL AND TAPENTADOL:
CLINICAL AND FORENSIC ASPECTS**

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Tese de Doutoramento em Biomedicina

Apresentada à Faculdade de Medicina da Universidade do Porto

Porto, 2018

The candidate performed the experimental work supported by a PhD grant (SFRH/BD/104795/2014) of “Fundação para a Ciência e Tecnologia”.

The REQUIMTE and the IINFACTS - Institute of Research and Advanced Training in Health Sciences and Technologies, Department of Sciences, University Institute of Health Sciences (IUCS), CESPU - provided the facilities and logistical support for the experimental work.



*Dissertação de candidatura ao grau
de Doutor em Biomedicina apresentada
à Faculdade de Medicina da
Universidade do Porto*

*Dissertation thesis for the degree of
Doctor of Philosophy in Biomedicine
submitted to the Faculty of
Medicine of Porto University*

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Doutor Ricardo Jorge Dinis Oliveira, professor auxiliar convidado da Faculdade de Medicina da Universidade do Porto e orientador da tese;

Doutor Ricardo Jorge Leal Silvestre, investigador principal do Instituto de Investigação em Ciências da Vida e Saúde da Universidade do Minho.

Aos meus Pais, Conceição e Manuel, e Irmã, Carolina.

To my Parents, Conceição e Manuel, and Sister, Carolina.

Ao Tiago.

To Tiago.

AUTHOR'S DECLARATION

Under the terms of the Decree-Law nº 74/2006, of March 24th, it is hereby declared that the following original articles were prepared in the scope of this dissertation.

PUBLICATIONS

Articles in international peer-reviewed journals

Theoretical Background

- I. **Faria J**, Barbosa J, Queiros O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative pharmacology and toxicology of Tramadol and Tapentadol. **European Journal of Pain** 2018; 22(5): 827-844.

Original Research

- II. **Faria J**, Barbosa J, Queiros O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells. **Toxicology** 2016;359-360: 1-10.
- III. **Faria J**, Barbosa J, Leal S, Afonso LP, Lobo J, Moreira R, Queiros O, Carvalho F, Dinis-Oliveira RJ. Effective analgesic doses of tramadol or tapentadol induce brain, lung and heart toxicity in Wistar rats. **Toxicology** 2017;385: 38-47.

Abstracts in international peer-reviewed journals

Original Research

- I. **Faria J**, Barbosa J, Carvalho F, Queiros O, Moreira R, Dinis-Oliveira RJ. An in vitro comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells. **Toxicology Letters** 2015, Volume 238 (Suppl. 2), Page S310.

Presentations in congresses

Oral communications

- I. **Faria J**, Barbosa J, Carvalho F, Queiros O, Moreira R, Dinis-Oliveira RJ. Toxicological effects of tramadol and tapentadol: a comparative study. VI Workshop IINFACTS, 1 July 2016, Paredes, Portugal.
- II. **Faria J**, Barbosa J, Carvalho F, Queiros O, Moreira R, Dinis-Oliveira RJ. An in vitro comparative study of the toxicological effects of tramadol and tapentadol in a panel of cell lines. APCF.SPECAN 2016, 12 and 13 February 2016, Porto, Portugal.
- III. **Faria J**, Barbosa J, Queiros O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Tramadol and tapentadol cause bioenergetic crisis and necrosis in the SH-SY5Y cell model. XLVII Reunião Anual da Sociedade Portuguesa de Farmacologia (SPF), 2-4 February 2017, Coimbra, Portugal.
- IV. Barbosa J, **Faria J**, Queiros O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Acute administration of tramadol and tapentadol induces dose-dependent toxicity in Wistar rats, affecting both metabolizing and target organs. XLVII Reunião Anual da Sociedade Portuguesa de Farmacologia (SPF), 2-4 February 2017, Coimbra, Portugal.
- V. Barbosa J, **Faria J**, Carvalho F, Queiros O, Moreira R, Dinis-Oliveira RJ. Acute administration of tramadol and tapentadol induces dose-dependent toxicity in Wistar rats. VII Workshop IINFACTS, 20 July 2017, Paredes, Portugal.

Poster communications

- I. **Faria J**, Barbosa J, Carvalho F, Queiros O, Moreira R, Dinis-Oliveira RJ. An in vitro comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells. EUROTOX 2015, 13-16 September 2015, Porto, Portugal.
- II. Barbosa J, **Faria J**, Queiros O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative toxicity of tramadol and tapentadol in Wistar rats. I Encontro Anual do DCB da FFUP, 19 July 2016, Porto, Portugal.
- III. Barbosa J, **Faria J**, Carvalho F, Queiros O, Moreira R, Dinis-Oliveira RJ. Tramadol and tapentadol comparative toxicity: *in vivo* insights. EUROTOX 2016, 4-7 September 2016, Seville, Spain.

Under the terms of the referred Decree-Law, the author declares that he afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

ACKNOWLEDGMENTS / AGRADECIMENTOS

O trabalho apresentado nesta tese não seria possível sem o apoio de várias pessoas, que direta ou indiretamente contribuíram para a sua realização. Neste sentido, não posso deixar de expressar o meu mais sincero agradecimento.

Ao meu orientador, Professor Ricardo Dinis-Oliveira, em primeiro lugar por ter aceitado orientar o meu trabalho, mesmo sem nos conhecermos previamente. Agradeço também todos os ensinamentos transmitidos, o exemplo científico e humano, a disponibilidade constante, assim como a sua boa disposição e otimismo, que foram fundamentais.

Às minhas coorientadoras, Professoras Roxana Moreira e Odília Queirós, por sempre terem apoiado e encorajado a minha progressão académica. Agradeço igualmente a confiança e as oportunidades de trabalho conjunto que me proporcionaram desde 2006, e por me terem permitido aprender muito com o Vosso exemplo.

Ao Professor Félix Carvalho, que mesmo não fazendo parte da equipa oficial de orientação, foi fundamental ao longo deste trabalho. Agradeço o rigor científico que sempre me transmitiu, assim como as ideias de trabalho e reflexões que teve nas várias reuniões conjuntas. Agradeço igualmente a cuidadosa revisão dos artigos publicados.

À Professora Sandra Leal agradeço todas as palavras de apoio e incentivo, e por ter tornado possível a realização da análise histológica.

À minha amiga Vanessa Nascimento agradeço a ajuda durante o sacrifício dos animais. A tua boa disposição e alegria são contagiantes, em todos os momentos.

À minha querida amiga Joana Barbosa não há palavras para descrever, nem tão pouco para agradecer a amizade, a ajuda e incentivo inesgotáveis ao longo destes anos. A Professora Roxana apresentou-nos no início dos nossos estágios de licenciatura, e estávamos longe de prever o quão longa seria a nossa jornada de partilha de bancada de laboratório. Felizmente a nossa amizade há muito que transpôs a porta do laboratório. Obrigada por tudo.

À Andrea, pela amizade, pela partilha de tantas horas no laboratório e pelo exemplo de simplicidade e generosidade.

À Diana pela sua boa-disposição e companheirismo, que foram sem dúvida importantes.

À Manu agradeço as animadas conversas sobre toxicologia e química, que tornaram os almoços conjuntos inesquecíveis.

Agradeço à Fundação para a Ciência e a Tecnologia pela bolsa de doutoramento que me atribuiu, sem a qual este trabalho não teria sido possível.

Agradeço ao REQUIMTE e ao IINFACTS/CESPU, que disponibilizaram os seus laboratórios para realização deste projeto, assim como a todos os colaboradores destas instituições.

Aos meus Pais e à Carolina agradeço por sempre terem confiado em mim e apoiado as minhas decisões. Foi (é) fundamental o vosso amor.

Ao Tiago, meu amigo, companheiro e marido, por estar comigo em todos os momentos e compreender a minha falta de tempo e de paciência em alguns (vários) momentos. O teu apoio, confiança e amor são fundamentais.

ABSTRACT

Tramadol and tapentadol are synthetic, structurally related and commonly prescribed opioids for the treatment of moderate to severe pain. Tramadol is one of the most prescribed opioids, while tapentadol is a more recently marketed drug. The clinical interest of these opioids is associated with their high analgesic efficiency, combined with the low incidence of adverse effects. Regarding their mechanism of action, both are mu-opioid receptor agonists and neurotransmitter reuptake inhibitors. The synergetic interactions of these mechanisms of action increase the analgesic activity and minimize the adverse effects. Despite the structural and mechanistic similarity between both drugs, tapentadol is considered an update over tramadol. Tramadol inhibits noradrenaline and serotonin reuptake and requires metabolization to exert full pharmacological activity, whereas tapentadol does not require metabolic activation and its action is predominantly through inhibition of noradrenaline reuptake.

Although tramadol and tapentadol are effective in pain relief and show a low rate of adverse reactions, the incidence of poisoning cases, including fatal intoxications, as well as cases of addiction, have been increasing in parallel with the increase in their consumption. However, the toxicity mechanisms are not fully understood yet, and few studies are available on the comparison of adverse events and toxicological profiles between these two opioids. Concerning tapentadol in particular, literature is still scarce, since it is a more recently marketed drug. In this sense, the main objective of the present work was to perform a comparative evaluation of tramadol and tapentadol acute toxicity, as well as to contribute to the clarification of the mechanisms underlying toxicity.

Considering the central action of these opioids, one specific objective was the *in vitro* evaluation of potential neurotoxicity, using the SH-SY5Y neuroblastoma cell line as a model. SH-SY5Y undifferentiated cells were exposed to increasing tramadol and tapentadol concentrations up to 600 μ M. Upon tramadol/tapentadol incubation for 48 hours, cell viability, cell death mechanisms, mitochondrial integrity, metabolic alterations and oxidative stress damage were assessed. The results obtained were analysed and compared with non-treated cells (control), and a comparative discussion of the toxicological damage triggered by tramadol and tapentadol was performed. SRB and MTT assays have shown that tapentadol caused a higher decrease in biomass and a in mitochondrial metabolic activity, comparing to tramadol. However, both opioids caused toxicity and decreased cell viability, as analysed through the trypan blue

exclusion method. Concerning oxidative stress damage, after exposure to tramadol or tapentadol no alterations in lipid peroxidation or protein oxidation were observed, suggesting that the toxicity resulting from the exposure to both opioids was probably not due to oxidative damage. On the other hand, a bioenergetic crisis, more evident when the cells were treated with tapentadol, characterized by an increase in intracellular glucose levels, together with a decrease in ATP content and an increase in lactate production, was observed as a result of the changes in the expression of genes that encode for energetic metabolism enzymes (such as LDH (lactate dehydrogenase), NDUF51 (Fe-S protein 1 of NADH dehydrogenase), CKB (creatine kinase B), PDK3 (pyruvate dehydrogenase kinase 3) and ALDOC (aldolase C)). Therefore, the results showed that, in this *in vitro* cell model, tapentadol caused higher toxicity, leading to a bioenergetic crisis and increased cell death, when compared to tramadol. In addition, necrosis was the main mechanism of death after exposure to both opioids, and no alterations in mitochondrial membrane potential or in cytochrome *c* release were observed, with only a minor increase in the activity of caspases being detected.

In addition to the *in vitro* assays, *in vivo* acute exposure assays were performed using male Wistar rats. *In vivo* assays have allowed a more complete and comprehensive study of the toxicological profile of these opioids. 10, 25 or 50 mg/kg tramadol and tapentadol doses (delivered in 1 ml of 0.9% NaCl), corresponding to a low effective analgesic dose, an intermediate dose and the maximum recommended daily dose, were intraperitoneally administered. A control group of animals, intraperitoneally injected with 1 ml of 0.9% NaCl, was included in the study. 24 hours after administration, biochemical alterations, as well as histological alterations and oxidative damage, were assessed in brain, lung and heart tissues. Regarding the analysis of biochemical alterations in serum samples, both drugs caused an increase in the AST/ALT (aspartate aminotransferase/alanine aminotransferase) ratio, as well as an increase in the activity of LDH (lactate dehydrogenase), CK (creatine kinase) and CK-MB (creatine kinase-MB isoform) enzymes. An increase in protein oxidation, in pulmonary and cardiac tissues, was observed. In the histological analysis, alterations in brain cortex samples were observed, such as the presence of swollen neurons, lower definition of nuclear membranes and the presence of degenerated neurons. In lung tissue, the presence of intra-alveolar hemorrhage and alveolar collapse was observed. Cardiac toxicity was also observed, characterized by the presence of inflammatory infiltrates and loss of cardiomyocyte striation, which, together with the biochemical

changes detected, suggested that both opioids cause cardiac damage. Taken as a whole, the *in vivo* results showed that tapentadol led to more prominent toxic effects than the exposure to tramadol.

In conclusion, the results of the present study showed that tramadol and tapentadol cause toxicity, even in an acute exposure context, with tapentadol causing more evident toxic damage in the models used. The results presented are an important starting point, which should be complemented with chronic exposure assays, as well as with simultaneous exposure assays using other drugs. The understanding of the molecular and biochemical alterations triggered by opioids will allow the recognition and better clarification of adverse reactions, and it represents an additional tool for the evaluation of intoxication cases due to exposure to these opioids.

The results within this thesis underline the recommendation for a careful and conscious tramadol and tapentadol prescription, and that opioid selection should be adjusted individually in order to optimize therapeutic strategies and minimize adverse effects.

Keywords: Toxicity assays, neurotoxicity, pneumotoxicity, cardiotoxicity, acute exposure, *in vivo*, *in vitro*, opioids, tapentadol, tramadol.

RESUMO

O tramadol e o tapentadol são opioides sintéticos, estruturalmente relacionados e amplamente prescritos para o tratamento da dor moderada a severa. O tramadol é atualmente um dos opioides mais prescritos no mundo, ao passo que o tapentadol é um fármaco de introdução mais recente no mercado. O interesse clínico destes dois opioides encontra-se associado à sua elevada eficácia analgésica, aliada à baixa incidência de efeitos adversos. No que concerne ao seu mecanismo de ação, ambos combinam o agonismo dos recetores mu-opioides com a inibição da recaptação de neurotransmissores. Estes mecanismos de ação interagem de forma sinérgica, aumentando a atividade analgésica e minimizando os efeitos adversos. Apesar das semelhanças estruturais e dos seus mecanismos de ação, o tapentadol é considerado uma atualização em relação ao tramadol. O tramadol, que atua através da inibição da recaptação de noradrenalina e de serotonina, requer metabolização para exercer atividade farmacológica plena, enquanto o tapentadol não requer ativação metabólica e atua principalmente através da inibição da recaptação de noradrenalina.

Embora, tal como referido anteriormente, tanto o tramadol como o tapentadol sejam efetivos no tratamento da dor, e apresentem uma baixa taxa de reações adversas reportadas, a incidência de intoxicações, inclusive de intoxicações fatais, assim como de casos de dependência, tem aumentado paralelamente ao aumento do seu consumo. No entanto, os mecanismos subjacentes à toxicidade observada não são ainda totalmente conhecidos, e existe pouca informação disponível sobre a comparação dos eventos adversos e perfis toxicológicos entre estes dois opioides. Em particular para o tapentadol, a literatura é ainda escassa, devido ao facto de ser um fármaco mais recentemente introduzido no mercado. Neste sentido, o principal objetivo do presente trabalho foi o de efetuar uma avaliação comparativa da toxicidade resultante da exposição aguda a tramadol e a tapentadol, assim como contribuir para o esclarecimento dos mecanismos subjacentes à toxicidade.

Considerando a ação destes opioides ao nível do sistema nervoso central, um dos objetivos específicos do trabalho compreendeu a avaliação *in vitro* da eventual neurotoxicidade, utilizando como modelo a linha celular de neuroblastoma SH-SY5Y. Expuseram-se células indiferenciadas desta linha a concentrações crescentes de tramadol e tapentadol, até 600 μ M. Após 48 horas de exposição, avaliaram-se a viabilidade celular, os mecanismos de morte associados à diminuição da viabilidade

celular, assim como eventuais alterações mitocondriais, metabólicas e o aumento do stresse oxidativo. Os resultados obtidos foram analisados comparativamente aos obtidos em células não expostas a opioide (controlo), e foi posteriormente efetuada uma discussão comparativa dos danos toxicológicos desencadeados pelo tramadol e tapentadol. Após a realização de ensaios de SRB e MTT, observou-se que o tapentadol causou uma maior diminuição da biomassa, assim como da atividade metabólica. Contudo, ambos os opioides causaram toxicidade e diminuição da viabilidade celular, analisada através de ensaios de marcação ao azul de tripano. No que concerne ao aumento de stresse oxidativo, não se observaram alterações na peroxidação lipídica ou na oxidação proteica, após a exposição a tramadol ou tapentadol, pelo que a toxicidade resultante da exposição a estes opioides não deverá ser, predominantemente, uma consequência de danos oxidativos. Por outro lado, observou-se a existência de uma crise bioenergética caracterizada pelo aumento dos níveis intracelulares de glucose, aliado à diminuição dos níveis de ATP e aumento dos níveis de lactato, como resultado de alterações da expressão de genes que codificam enzimas do metabolismo energético (tais como: LDH (lactato desidrogenase), NDUFS1 (Fe-S proteína 1 do complexo NADH desidrogenase), CKB (creatina cinase B), PDK3 (piruvato desidrogenase cinase 3) e ALDOC (aldolase C)). Neste contexto, os resultados obtidos permitiram concluir que, no modelo utilizado, o tapentadol causou uma maior toxicidade, promovendo uma crise bioenergética e uma maior morte celular, comparativamente ao tramadol. Verificou-se ainda que o principal mecanismo de morte, após a exposição a ambos os opioides, foi a necrose, não se tendo observado alterações no potencial de membrana mitocondrial, nem o aumento da libertação do citocromo c, assim como se observou apenas um ligeiro aumento na atividade das caspases.

Após a conclusão dos ensaios *in vitro*, realizaram-se ensaios de exposição aguda *in vivo*, utilizando ratos Wistar machos. Os ensaios *in vivo* permitiram um estudo mais abrangente e completo do perfil toxicológico destes opioides. Nestes ensaios, administraram-se por via intraperitoneal doses de 10, 25 ou 50 mg/Kg de tramadol e tapentadol (preparadas em 1 ml de solução salina de NaCl a 0,9%), que correspondem, respetivamente, à dose analgésica frequentemente prescrita, uma dose intermédia, e à dose máxima diária recomendada. Um grupo de animais controlo foi incluído no estudo, ao qual se administrou, por via intraperitoneal, 1 mL de solução salina de NaCl a 0,9%. Vinte e quatro horas após a administração, avaliou-se a existência de alterações bioquímicas, assim como alterações histológicas e danos oxidativos nos tecidos

cerebral, pulmonar e cardíaco. No que concerne a alterações bioquímicas analisadas no soro, ambos os fármacos levaram a um aumento da razão AST/ALT (aspartato transaminase/alanina transaminase), assim como ao aumento da atividade das enzimas LDH (lactato desidrogenase), CK (creatina cinase) e CK-MB (creatina cinase-isoforma MB). Nos tecidos pulmonar e cardíaco, observou-se um aumento de oxidação proteica. Na análise histológica, no tecido cerebral observaram-se alterações, tais como a presença de neurónios inchados, células com membrana nuclear mal definida, assim como a presença de células mortas. No tecido pulmonar, verificou-se a presença de hemorragia intra-alveolar e colapso alveolar. Foi também observada toxicidade cardíaca, caracterizada pela presença de infiltrados inflamatórios e perda da estriação dos cardiomiócitos, que, em conjunto com as alterações bioquímicas observadas, permitiram concluir que estes opioides causam dano cardíaco. A análise combinada de todos os resultados obtidos *in vivo* permitiu concluir que o tratamento com tapentadol conduziu a efeitos mais tóxicos do que a exposição a tramadol.

Em conclusão, os resultados do presente trabalho demonstraram que o tramadol e o tapentadol apresentam toxicidade, mesmo num cenário de exposição aguda, sendo o tapentadol mais tóxico nos modelos utilizados. Destaca-se ainda que os resultados obtidos constituem um importante ponto de partida que deve ser complementado com ensaios de exposição crónica a ambos os opioides, assim como com ensaios de exposição simultânea a outras drogas/fármacos. A compreensão das alterações moleculares e bioquímicas desencadeadas pelos opioides permitirá reconhecer e melhor explicar as reações adversas, e constitui uma ferramenta adicional para a avaliação de casos de intoxicação decorrentes da exposição a estes compostos.

Neste contexto, os resultados obtidos sublinham a necessidade de uma prescrição cuidadosa e consciente de tramadol e tapentadol, devendo a escolha do opioide ser ponderada individualmente, de modo a otimizar estratégias terapêuticas e minimizar efeitos adversos.

Palavras-chave: Ensaios de toxicidade, neurotoxicidade, pneumotoxicidade, cardiotoxicidade, exposição aguda, *in vivo*, *in vitro*, opioides, tapentadol, tramadol.

ABBREVIATIONS LIST

5-HT, serotonin;
ALDOC, aldolase C;
ALT, alanine aminotransferase;
AST, aspartate aminotransferase;
AST/ALT, aspartate aminotransferase/alanine aminotransferase;
ATP, adenosine triphosphate;
BUN, blood urea nitrogen;
CAT, catalase;
cDNA, complementary DNA (Deoxyribonucleic acid);
CK-MB, creatine kinase isoform MB;
CKB, creatine kinase B;
C_{max}, maximum serum concentration;
CNECV, National Council of Ethics for the Life Sciences;
CNS, central nervous system;
CO₂, carbon dioxide;
COX-2, cyclooxygenase-2;
Cu-Zn, copper-zinc;
CYP450, cytochrome P450;
DAPI, 4',6-diamidino-2-phenylindole;
DNPH, 2,4-dinitrophenylhydrazine;
ECL, enhanced chemiluminescence;
ER, extended release;
FBS, fetal bovine serum;
FDA, United States Food and Drug Administration;
GFR, glomerular filtration rate;
GLUTs, glucose transporters;
GSH, reduced glutathione;
H&E, hematoxylin and eosin;
H₂O₂, hydrogen peroxide;
HED, human equivalent dose;
IC₅₀, half maximal inhibitory concentration;
IMS, Intercontinental Marketing Services;

INCB, International Narcotics Control Board;
 ip, intraperitoneal;
 IR, immediate release;
 LD₅₀, median lethal dose;
 LDH, lactate dehydrogenase;
 LPO, lipid peroxidation;
 M1, *O*-desmethyltramadol;
 M2, *N*-desmethyltramadol;
 M3, *N,N*-didesmethyltramadol;
 M4, *O,N,N*-tridesmethyltramadol;
 M5, *O,N*-didesmethyltramadol;
 MAOIs, monoamine oxidase inhibitors;
 MDA, malondialdehyde;
 MDMA, 3,4-methylenedioxymethamphetamine – “ecstasy”;
 MMP, mitochondrial membrane potential;
 Mn-SOD, manganese-dependent superoxide dismutase;
 MOR, μ -opioid receptors;
 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium;
 NA, noradrenaline;
 NaCl, sodium chloride;
 NAD⁺, oxidized nicotinamide adenine dinucleotide phosphate;
 NADH, reduced nicotinamide adenine dinucleotide phosphate;
 NDUFS1, Fe-S protein 1 of NADH dehydrogenase;
 NEAA, non-essential amino acids;
 NHS, National Health Service;
 NSAIDs, non-steroidal anti-inflammatory drugs;
 PAS, periodic acid-Schiff;
 PDH, pyruvate dehydrogenase;
 PDK3, pyruvate dehydrogenase kinase 3;
 PI, propidium iodide;
 PMs, poor metabolizers;
 PubMed, U.S. National Library of Medicine;
 RADARS, Researched Abuse, Diversion and Addiction-Related Surveillance;
 RNA, ribonucleic acid;

ROS, reactive oxygen species;
SD, standard deviation;
SNRIs, serotonin-noradrenaline reuptake inhibitors;
SOD, superoxide dismutase;
SRB, sulforhodamine B;
SS, serotoninergic syndrome;
SSRIs, serotonin reuptake inhibitors;
TBARS, thiobarbituric acid-reactive substances;
TCAs, tricyclic antidepressants;
TMRE, tetramethylrhodamine ethyl ester perchlorate;
UGT, UDP-glucuronosyltransferase;
UMs, ultra-rapid metabolizers;
 α_2 -AR, α_2 -adrenergic receptor;
 $\Delta\Psi_m$, mitochondrial membrane potential.

OUTLINE OF THE THESIS

The present thesis is organized in four main parts:

PART I

1. GENERAL INTRODUCTION

An introduction to contextualize the state of the art, as well as to provide a general overview about tramadol and tapentadol pharmacological and toxicological profiles. This part is presented as a review paper.

2. GENERAL AND SPECIFIC OBJECTIVES

The general and specific objectives of the thesis are provided.

PART II – ORIGINAL RESEARCH

Part II is divided in two chapters, corresponding to the original articles published during the thesis period. For each report, information concerning the journal and date of publication is provided.

PART III

This section it is divided in three major points:

1. INTEGRATED OVERVIEW OF THE PERFORMED STUDIES

The studies undertaken are integrated in a harmonized manner;

2. CONCLUSIONS

The main conclusions of this thesis are summarized;

3. DIRECTIONS FOR FUTURE RESEARCH

The prospects for future studies are presented.

PART IV

The references used in PART III are listed.

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PART I

1. GENERAL INTRODUCTION

REVIEW ARTICLE

Comparative pharmacology and toxicology of tramadol and tapentadol

Reprinted from the European Journal of Pain, 22(5): 827-844

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REVIEW ARTICLE

Comparative pharmacology and toxicology of tramadol and tapentadolJ. Faria^{1,2,3}, J. Barbosa^{1,2,3}, R. Moreira¹, O. Queirós¹, F. Carvalho², R.J. Dinis-Oliveira^{1,2,3}¹ Department of Sciences, IINFACTS, Institute of Research and Advanced Training in Health Sciences and Technologies, University Institute of Health Sciences (IUCS), CESPU, CRL, Gandra, Portugal² Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy UCIBIO-REQUIMTE, University of Porto, Porto, Portugal³ Department of Public Health and Forensic Sciences, and Medical Education, Faculty of Medicine, University of Porto, Porto, Portugal**Correspondence**

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Funding sources

This work was supported by grants from CESPU TramTap-CESPU-2016 and Chronic-TramTap_CESPU_2017 and project NORTE-01-0145-FEDER-000024, supported by Norte Portugal Regional Operational Programme NORTE 2020, under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Juliana Faria is a PhD fellowship holder from FCT (SFRH/BD/104795/2014). Ricardo Dinis-Oliveira acknowledges Fundação para a Ciência e a Tecnologia (FCT) for his Investigator Grant (IF/01147/2013).

Conflict of interest

None declared.

Accepted for publication

17 January 2018

doi:10.1002/ejp.1196

Abstract

Moderate-to-severe pain represents a heavy burden in patients' quality of life, and ultimately in the society and in healthcare costs. The aim of this review was to summarize data on tramadol and tapentadol adverse effects, toxicity, potential advantages and limitations according to the context of clinical use. We compared data on the pharmacological and toxicological profiles of tramadol and tapentadol, after an extensive literature search in the US National Library of Medicine (PubMed). Tramadol is a prodrug that acts through noradrenaline and serotonin reuptake inhibition, with a weak opioid component added by its metabolite *O*-desmethytramadol. Tapentadol does not require metabolic activation and acts mainly through noradrenaline reuptake inhibition and has a strong opioid activity. Such features confer tapentadol potential advantages, namely lower serotonergic, dependence and abuse potential, more linear pharmacokinetics, greater gastrointestinal tolerability and applicability in the treatment of chronic and neuropathic pain. Although more studies are needed to provide clear guidance on the opioid of choice, tapentadol shows some advantages, as it does not require CYP450 system activation and has minimal serotonergic effects. In addition, it leads to less side effects and lower abuse liability. However, *in vivo* and *in vitro* studies have shown that tramadol and tapentadol cause similar toxicological damage. In this context, it is important to underline that the choice of opioid should be individually balanced and a tailored decision, based on previous experience and on the patient's profile, type of pain and context of treatment.

Significance: This review underlines the need for a careful prescription of tramadol and tapentadol. Although both are widely prescribed synthetic opioid analgesics, their toxic effects and potential dependence are not completely understood yet. In particular, concerning tapentadol, further research is needed to better assess its toxic effects.

1. Introduction

The use, misuse and abuse of analgesics have increased throughout the last decades (Compton and Volkow, 2006; Manchikanti et al., 2010; Compton et al., 2015; Kaye et al., 2017). Prescription opioids, the mainstay of chronic pain treatment, have largely

contributed to this phenomenon, leading to the so-called opioid epidemic and a public health crisis associated with an increase in the number of opioid-related morbidity and mortality (Burgess and Williams, 2010; Manchikanti et al., 2010; Mercadante

et al., 2012; Meneghini et al., 2014; Compton et al., 2015; Mercadante, 2015; Crow, 2016; Just et al., 2016; Agarwal et al., 2017; Han et al., 2017; Pezalla et al., 2017). Besides the typical unwanted side effects caused by opioids, including bowel dysfunction and constipation, vomiting, nausea, itching, sex hormone dysfunction (hypogonadism), somnolence, sleep and concentration difficulties (which sometimes lead to treatment discontinuation), other effects related to tolerance, dependence and addiction potential, as well as possible fatal overdose, represent additional troubling concerns (Burgess and Williams, 2010; Manchikanti et al., 2010; Mercadante et al., 2012, 2014; Gunther et al., 2017; Ventura et al., 2017). Therefore, the demand for the development of new analgesic formulations with nonexistent or reduced side effects and abuse potential has never been so urgent (Burgess and Williams, 2010; Crow, 2016; Gunther et al., 2017; Pezalla et al., 2017; Vadivelu et al., 2017).

Tramadol and tapentadol are two centrally acting, fully synthetic opioids with an atypical mechanism of action, as they combine μ -opioid receptor (MOR) agonism with neurotransmitter (serotonin (5-HT) and/or noradrenaline (NA) reuptake inhibition (Barbosa et al., 2016). They are two synthetic and structurally related opioids. Tramadol was first synthesized in 1962, having then been tested for 15 years and made available to the foreign market, for pain treatment, in 1977, under the name Tramal (Grond and Sablotzki, 2004; Vadivelu et al., 2017). However, it was not until 1994 and 1995 that it was registered as a new molecular entity in the United Kingdom and in the United States, respectively (Grond and Sablotzki, 2004; Kissin, 2010). In turn, tapentadol was developed in the late 1980s, aiming at a new class of analgesics, retaining MOR agonism and NA reuptake inhibition, while having minimal 5-HT effect (Knezevic et al., 2015; Langford et al., 2016; Vadivelu et al., 2017). The tapentadol hydrochloride immediate release (IR) formulation obtained approval by the United States Food and Drug Administration (FDA) in 2008, for the management of moderate-to-severe acute pain, while an extended release (ER) formulation was approved for the treatment of moderate-to-severe chronic pain and neuropathic pain in 2011 and 2012, respectively (Mercadante et al., 2014; Knezevic et al., 2015; Chang et al., 2016).

In spite of the partial share of mechanism of action, tapentadol is, in some ways, regarded as an upgrade over tramadol – the limitations of tramadol have been used as a starting point for the

development of tapentadol. Nevertheless, there is few information available on the comparison of their adverse events and toxicological profiles, mainly due to the more limited history of tapentadol in the market. With this review, we aimed to summarize data on tramadol and tapentadol opioid and nonopioid-related adverse effects, toxicity, and potential advantages and limitations according to the context of clinical use.

2. Methodology

An extensive literature search was performed using text and structural queries for tramadol, tapentadol and data concerning their toxicity and abuse potential in the US National Library of Medicine (PubMed), with no restriction of publication date or language. The publications retrieved were additionally screened as referrals to others. Bibliographical references concerning *in vitro* and *in vivo* (human and non-human) studies were considered.

3. Chemical remarks

Tramadol, (1*RS*, 2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclo-hexanol, is a synthetic 4-phenyl-piperidine; as codeine, it has a 3-methoxy group substitution on the phenolic moiety (Fig. 1), which explains the weak affinity for opioid receptors (Dayer et al., 1994), and has *O*-demethylation as a metabolic step, producing metabolites with higher pharmacological activity (Grond and Sablotzki, 2004; Raffa et al., 2012). The dimethylaminomethyl moiety of tramadol resembles the methylated ring nitrogen of morphine and codeine, composing a fundamental part of the pharmacophore that interacts with the MOR and monoamine transporters (Grond and Sablotzki, 2004; Raffa et al., 2012).

Tapentadol, 3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]-phenol, was developed from the structures of morphine, tramadol and its metabolite *O*-desmethyltramadol (M1), in the search for an active parental compound, composed of only one enantiomer displaying both MOR and NA activities, and devoid of cytochrome P450 (CYP450) metabolism (Chang et al., 2016; Langford et al., 2016). This led to the opening of tramadol cyclohexane ring and to the replacement of the tertiary hydroxyl group (Chang et al., 2016). The result is a *meta*-substituted phenol ring with an ethyl and an aminopropyl residue at C9, located in opposite sides of the plane defined by the aromatic ring (Arjunan et al., 2015; Knezevic et al., 2015; Chang et al., 2016). C8 and

C9 are two stereogenic centres, leading to four possible diastereomers, from which the (*R,R*) isomer is currently the used form in the clinics (Fig. 2) (Arjunan et al., 2015; Knezevic et al., 2015). Tapentadol is structurally the closest chemical compound to tramadol in clinical use (Arjunan et al., 2015).

4. Formulations and pharmacokinetic profile comparison

Tramadol is delivered through different routes of administration (such as oral, intranasal, rectal and intravenous), in IR and ER formulations (Grond and

Sablitzki, 2004). Oral formulations are the preferred route used in the clinics (Leppert, 2009). Nevertheless, in severe pain, after surgery, intravenous formulations represent a more appropriate option (Grond and Sablotzki, 2004). In turn, tapentadol is available in oral formulations only, both for IR and ER (Tzschentke et al., 2007; Singh et al., 2013).

Following oral administration, tramadol is rapidly and almost completely absorbed and distributed in the body, with 20–30% undergoing first-pass metabolism (Grond and Sablotzki, 2004). Its bioavailability achieves 68–84%, while its plasma protein binding is 20% (Grond and Sablotzki, 2004; Barbosa

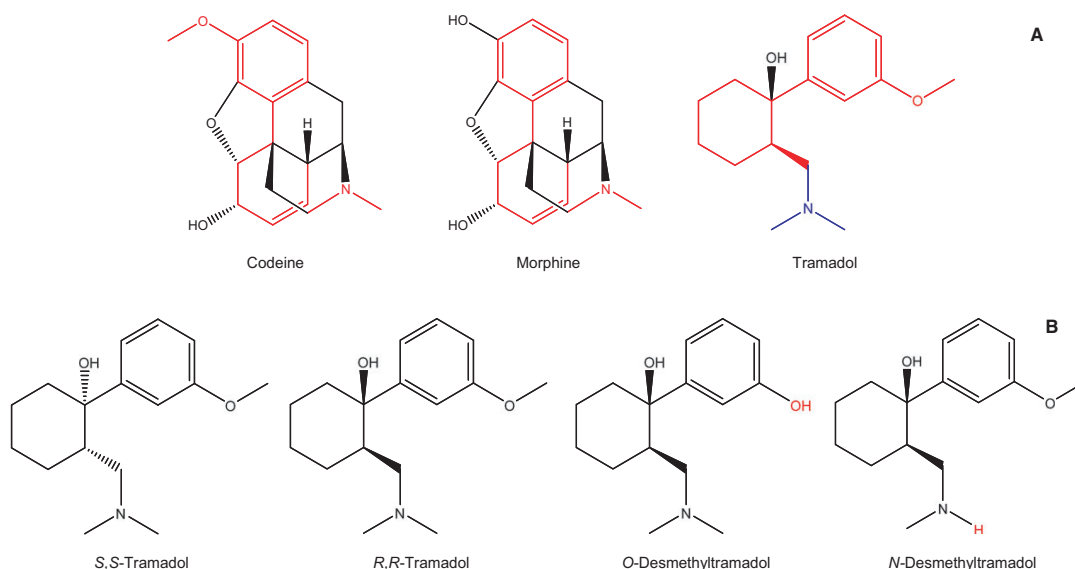


Figure 1 Molecular structures of codeine, morphine and tramadol with their common structure in red, and the dimethylaminomethyl moiety of tramadol, a fundamental part of the pharmacophore, in blue (A). Molecular structures of both tramadol enantiomers and its main metabolites (B). O-desmethyltramadol (M1) is produced via CYP2D6, while N-desmethyltramadol (M2) is produced via CYP2B6 and CYP3A4.

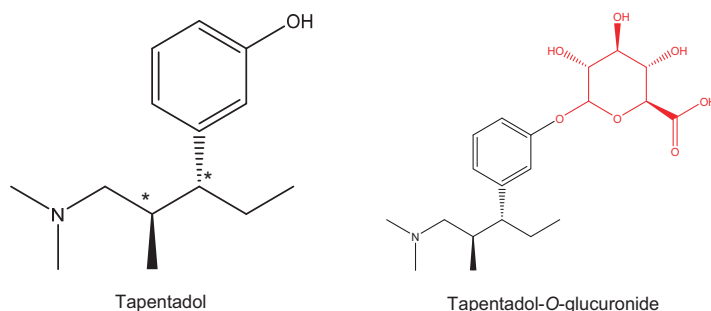


Figure 2 Molecular structures of tapentadol and its main metabolite, tapentadol-O-glucuronide, produced via UGT1A9- and UGT2B7-mediated glucuronidation. C8 and C9, the two stereogenic centres, are evidenced with asterisks.

et al., 2016). It is extensively metabolized in the liver, through six metabolic pathways, from which *O*-demethylation, *N*-demethylation and cyclohexyl oxidation are the major routes, resulting in 7 *O*-desmethyl/*N*-desmethyl and hydroxycyclohexyl metabolites (Grond and Sablotzki, 2004; DePriest et al., 2015; Barbosa et al., 2016; Wu et al., 2017). CYP450-mediated phase I reactions yield 14 metabolites, from which M1 stands out for its prominent pharmacological activity. CYP2D6 mediates M1 formation, while CYP2B6 and CYP3A4 are responsible for *N*-desmethyltramadol (M2) production (Grond and Sablotzki, 2004; DePriest et al., 2015; Barbosa et al., 2016; Wu et al., 2017). Phase II reactions produce 12 metabolites, including seven glucuronides and five sulphonates (Grond and Sablotzki, 2004; DePriest et al., 2015; Barbosa et al., 2016; Wu et al., 2017). Tramadol elimination is mainly urinary (90%), and the M1, M2, *N,N*-didesmethyltramadol (M3), *O,N,N*-tridesmethyltramadol (M4) and *O,N*-didesmethyltramadol (M5) metabolites are mostly detected in urine upon oral administration (Grond and Sablotzki, 2004; Wu et al., 2017).

In turn, tapentadol is a nonracemic molecule that does not require metabolic activation via the CYP450 system (Bourland et al., 2010; Hartrick and Rozek, 2011; Barbosa et al., 2016). As for tramadol, its absorption is fast and complete, undergoing first-pass metabolism and plasma protein binding (20%), with an estimated bioavailability of 32%, upon extensive glucuronidation (Hartrick and Rozek, 2011). While tapentadol is subject to phase I oxidative reactions that include its *N*-demethylation (13%) via CYP2C9 and CYP2C19 and its hydroxylation (2%) via CYP2D6, its metabolism mainly comprises phase II glucuronidations (55%) via UGT1A9 and UGT2B7, as well as sulphonations (15%) (Kneip et al., 2008; Bourland et al., 2010; Coulter et al., 2010; Hartrick and Rozek, 2011; Kemp et al., 2013; Singh et al., 2013; DePriest et al., 2015; Barbosa et al., 2016; Bairam et al., 2017). Elimination occurs mainly through the kidney (99%) (Hartrick and Rozek, 2011).

CYP2D6 is a polymorphic enzyme; due to the considerable dependence of tramadol pharmacological activity on the bioactivation by CYP2D6, and considering the genetic variability affecting this enzyme, it becomes clear that tramadol pharmacokinetics is highly dependent on the individual genotype. Indeed, CYP2D6 ultra-rapid metabolizers (UMs) have an increased risk of side effects, with this phenotype having been associated with cases of toxicity. On the other hand, the CYP2D6 poor metabolizer (PM)

phenotype was associated with less tramadol analgesic efficacy, with lower M1 concentrations and increased tramadol half-life (Poulsen et al., 1996; Lassen et al., 2015; Barbosa et al., 2016). This is particularly relevant considering that the frequency of PMs is 7–10% in Caucasians, 2–7% in Hispanics, 0–5% in Asia (0–5%) and more variable in the African population (0–19%), depending on the region (Lassen et al., 2015). Concerning the frequency of UMs, the highest values are in Black Ethiopians (16%) and Saudi Arabians (20%), while the lowest values are among North Europeans (0.8–3.6%) and American Caucasians and Blacks (4.3 and 4.5%, respectively); the frequency in Mediterranean Europeans corresponds to 7–10% (Lassen et al., 2015). In contrast, for UGT2B7, no polymorphisms have been reported as clinically relevant, and this is not a very polymorphic enzyme (Lassen et al., 2015).

Tramadol and tapentadol metabolic pathways have been extensively reviewed elsewhere (Barbosa et al., 2016). A comparative summary of relevant features of the pharmacokinetics of both drugs is provided in Table 1.

5. Pharmacodynamic profile comparison

Besides enhancing their analgesic effects, tramadol and tapentadol dual mechanism of action significantly increases tolerability and reduces the adverse effects associated with classical opioids.

Tramadol is marketed as a racemate composed of (+)-tramadol and (–)-tramadol. While (–)-tramadol is mostly responsible for the inhibition of NA reuptake, (+)-tramadol is more potent in the inhibition of 5-HT reuptake (Duthie, 1998; Grond and Sablotzki, 2004; Leppert, 2011; Barbosa et al., 2016) (Fig. 3). In turn, MOR agonism is mainly ensured by the (+)-enantiomer of the M1 metabolite and, to a lesser extent, by the parental compound ((+)-tramadol) (Lai et al., 1996; Duthie, 1998; Gillen et al., 2000; Grond and Sablotzki, 2004; Leppert, 2011; Barbosa et al., 2016). The (–)-M1 enantiomer is responsible for NA reuptake inhibition (Duthie, 1998; Grond and Sablotzki, 2004; Leppert, 2011; Barbosa et al., 2016).

Tapentadol shows moderate MOR agonist activity, pronounced NA reuptake inhibition and minimal 5-HT effect (Table 2) (Tzschentke et al., 2007; Hartrick and Rozek, 2011; Meske et al., 2014; Barbosa et al., 2016), thereby minimizing the risk of serotonin syndrome (Tzschentke et al., 2007; Hartrick and Rozek, 2011; Giorgi et al., 2012; Steigerwald et al., 2013; Meske et al., 2014; Barbosa et al., 2016). NA inhibits the transmission of nociceptive impulses by

Table 1 Comparative summary of main tramadol and tapentadol pharmacokinetic aspects (Dayer et al., 1994, 1997; Matthiesen et al., 1998; Wade and Spruill, 2009; Singh et al., 2013; Mercadante et al., 2014; Arjunan et al., 2015; Knezevic et al., 2015; McNaughton et al., 2015; Barbosa et al., 2016; Chang et al., 2016; Langford et al., 2016; Agarwal et al., 2017; Pergolizzi et al., 2017; Vadivelu et al., 2017; Wu et al., 2017).

	Tramadol	Tapentadol
Route(s) of administration/doses	- Oral IR: 50 mg tablets/capsules; 37.5 mg tramadol + 325 mg acetaminophen tablets/capsules - Oral ER: 50, 100, 150, 200, 300 mg tablets/capsules - Intramuscular: 50 mg/mL suspension - Rectal IR: 100 mg suppositories	- Oral IR: 50, 75, 100 mg tablets - Oral ER: 50, 100, 150, 200, 250 mg tablets
Recommended daily dose (mg)	100–300	50–100
Maximum daily dose (mg)	400	600–700
Absorption	Intestinal	Intestinal
Onset of action for IR formulations	60 min	30 min
Plasma peak	- Oral, IR: 1–2 h (racemic tramadol); 3 h (M1) - Oral, ER: 4.9 h - Intramuscular: 30–45 minutes - Rectal: 3 h	- Oral, IR: 1–1.5 h - Oral, ER: 3–6 h
Metabolism	- Hepatic - Main reactions: Phase I CYP2D6-mediated O-demethylation to M1 - Minor reactions: CYP2B6- and CYP3A4-mediated N-demethylation to M2	- Hepatic - Main reactions: Phase II glucuronidation reactions, through UGT1A9 and UGT2B7, to tapentadol-O-glucuronide (55%) - Minor reactions: CYP2D6-mediated oxidation to hydroxytapentadol (2%), CYP2C9- and CYP2C19-mediated oxidation to N-desmethyltapentadol (13%)
Main metabolites	- M1 - M2	Tapentadol-O-glucuronide
Active metabolite	M1	Active parental compound
Bioavailability	68–84%	32%
Plasma protein binding	20%	20%
Elimination half-life	5–6 h (racemic tramadol) 8–9 h (M1)	~4 h for IR formulations 5–6 h for ER formulations
Excretion	- Renal (90%): M1 (16%); M5 (15%); M2 (2%); metabolite conjugates; unchanged tramadol (10–30%) - Faecal (10%)	- Renal (99%): conjugated (glucuronide, sulphate) form (69%); other metabolites (27%); unchanged tapentadol (3%) - Faecal (1%)
Drug–drug interactions	CYP2D6-mediated metabolism increases susceptibility to drug interactions	Absence of significant CYP450-mediated metabolism decreases liability to drug interactions
Interindividual variability	CYP450 genetic polymorphisms determine different metabolizer phenotypes and, thus, analgesic efficacy	No evidences of polymorphisms significantly affecting metabolism and analgesic efficacy

M1, O-desmethyiltramadol; M2, N-desmethyiltramadol; IR, immediate release; ER, immediate release; CYP450, cytochrome P450; UGT, UDP-glucuronosyltransferase.

activating α_2 -adrenergic receptors on pain fibres in the Central Nervous System (CNS). By blocking NA reuptake, tapentadol thus prolongs its effects at the terminal endings of interneurons and descending inhibitory fibres, suppressing pain transmission (Vadivelu et al., 2017) (Fig. 4). Combined with its MOR agonism, which is associated with the modulation of ascending pathways, these complementary effects lead to a high level of analgesia, compensating the

lower MOR binding affinity (Knezevic et al., 2015; Chang et al., 2016; Vadivelu et al., 2017). In fact, the synergistic effect of both mechanisms of action explains why tapentadol is only about threefold less potent than morphine, although its MOR affinity is 18-fold lower (Knezevic et al., 2015; Chang et al., 2016; Vadivelu et al., 2017). Table 2 comparatively lists some important pharmacodynamic features of both opioids.

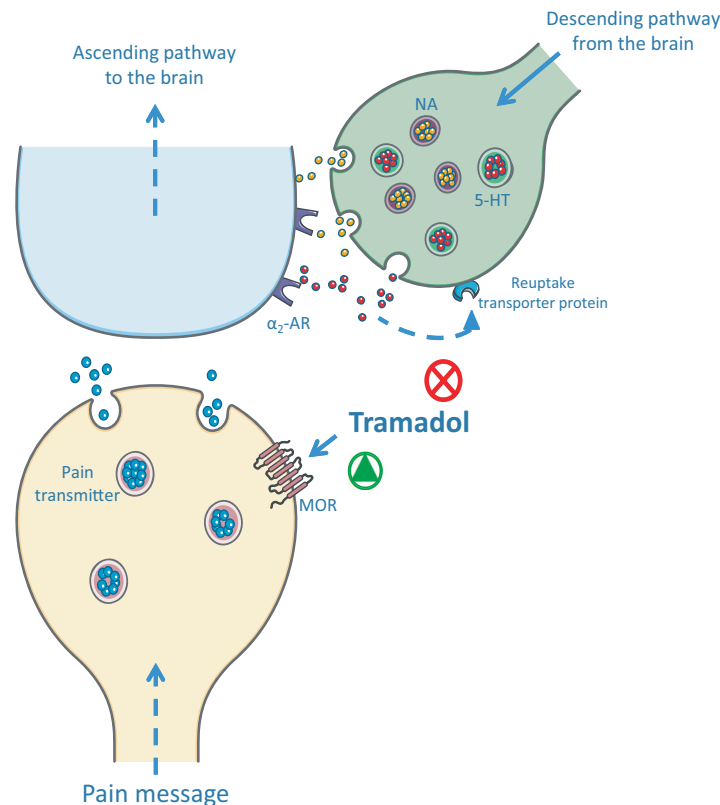


Figure 3 Tramadol dual mechanism of action. Tramadol acts through μ -opioid receptor (MOR) agonism, and both NA and 5-HT reuptake inhibition. NA and 5-HT inhibit the transmission of nociceptive impulses by activating α_2 -adrenergic receptors. Pain neurotransmitters glutamate and substance P bind the respective receptors, modulating primary afferent pathways of pain stimulation. NA: noradrenaline; 5-HT: serotonin; MOR: μ -opioid receptor; α_2 -AR: α_2 -adrenergic receptor.

6. Clinical applications and efficacy

Tramadol and tapentadol provide an opiate-sparing effect through their atypical and dual mechanism of action, which is an advantage over classical opioids, particularly in chronic treatment, where opioid receptor downregulation takes place (Giorgi, 2012). As such, given the duality and synergy of their mechanisms of action (Grond and Sablotzki, 2004; Tzschentke et al., 2007) and the resulting fewer side effects and lower addiction potential, tramadol and tapentadol are typically recommended for the treatment of moderate to severe pain. Table 3 illustrates main tramadol and tapentadol clinical applications (Dayer et al., 1994, 1997; Matthiesen et al., 1998; Wade and Spruill, 2009; Singh et al., 2013; Mercadante et al., 2014; Arjunan et al., 2015; Knezevic et al., 2015; McNaughton et al., 2015; Barbosa et al.,

2016; Chang et al., 2016; Langford et al., 2016; Agarwal et al., 2017; Pergolizzi et al., 2017; Vadivelu et al., 2017; Wu et al., 2017).

The analgesic efficacy of tramadol has been verified in the treatment of acute and chronic situations. It is usually used in cancer pain. For instance, Grond and colleagues have verified the clinical efficacy of tramadol in the treatment of moderate oncological pain, when nonopioids fail to be effective (Grond et al., 1999). Nonetheless, it is also used in nonmalignant pain, such as in osteoarthritis pain (Grond and Sablotzki, 2004; Leppert, 2009). Cepeda and co-authors, in a meta-analysis, reported less adverse effects for osteoarthritic pain patients receiving tramadol or tramadol/paracetamol compared to participants who received placebo (Cepeda et al., 2007). Several randomized, double-blind studies evaluating

Table 2 Comparative summary of main tramadol and tapentadol pharmacodynamic aspects (Dayer et al., 1994, 1997; Matthiesen et al., 1998; Wade and Spruill, 2009; Raffa et al., 2012; Singh et al., 2013; Mercadante et al., 2014; Arjunan et al., 2015; Knezevic et al., 2015; McNaughton et al., 2015; Barbosa et al., 2016; Chang et al., 2016; Langford et al., 2016; Agarwal et al., 2017; Pergolizzi et al., 2017; Vadivelu et al., 2017; Wu et al., 2017).

Class	Tramadol Opioid agonist	Tapentadol Opioid agonist
Mechanism of action	• MOR agonism ((+)-M1) • 5-HT reuptake inhibition ((+)-tramadol) • NA reuptake inhibition ((-)-tramadol)	• MOR agonism • Strong NA reuptake inhibition • Weak 5-HT reuptake inhibition
MOR binding affinity (compared to that of morphine)	Weak (1/6000)	Strong (1/18)
MOR K _i binding affinity (μM)	• Racemic tramadol = 2.4 • (+) Tramadol = 1.3 • (-) Tramadol = 24.8 • (+) M1 = 0.0034 • (-) M1 = 0.24	0.16
NA transporter K _i binding affinity (μM)	Racemic tramadol = 14.6	8.8
5-HT transporter K _i binding affinity (μM)	Racemic tramadol = 1.19 (+) Tramadol = 0.87	5.28
Practical equianalgesic dose to 10 mg morphine (mg)	50	25
Equianalgesic dose conversion factor (opioid dose ÷ factor = morphine dose)	÷5	÷2.5

M1, O-desmethyltramadol; MOR, μ-opioid receptor; 5-HT, serotonin; NA, noradrenaline.

the efficacy of tramadol and comparator drugs in post-operative situations, such as those following orthopedic, abdominal and gynaecological laparoscopic surgery, have clearly evidenced the clinical efficacy of tramadol (Grond and Sablotzki, 2004; Leppert, 2009).

In some nociceptive and neuropathic pain situations, tramadol is a good approach for moderate pain treatment (Sindrup et al., 1999a,b; Leppert, 2009). However, concerning neuropathic pain, Duehmke et al. (2017) underline the lack of information about tramadol use in this context, in addition to the inadequate studies available. These authors suggested a bias towards the increase in apparent tramadol benefits, so the conclusions may not reliably reflect the real scenario (Duehmke et al., 2017). A systematic review and meta-analysis by Finnerup et al. (2015), concerning neuropathic pain treatment in adults from January 1966 to April 2013, reported tramadol and tapentadol number needed to treat as 4.7 and 10.2, respectively. The combination between tramadol and nonopioid drugs, such as paracetamol, dipyrrone and ketorolac, increases analgesia without increasing the risk of serious adverse events (Grond and Sablotzki, 2004; Leppert, 2009; Sawaddiruk, 2011). However, it is imperative to monitor tramadol analgesic efficacy and its potential adverse effects; in this context, the titration of tramadol dose is of particular importance (Grond and Sablotzki, 2004; Leppert, 2009).

Tapentadol ER showed to be safe for patients with hypertension, a frequent condition in subjects with

chronic pain. In this situation, no significant changes in heart rate or blood pressure were detected (Biondi et al., 2014), a clinical condition for which tapentadol is a possible option for pain treatment, in opposition to paracetamol, NSAIDs and COX-2 inhibitors, which have been associated with increases in blood pressure (Schnitzer, 2006; Sudano et al., 2010; Snowden and Nelson, 2011; Biondi et al., 2014). Serotonin-noradrenaline reuptake inhibitors (SNRIs) are also associated with hypertension (Stahl et al., 2005; Biondi et al., 2014), for which tapentadol is suggested to present increased cardiovascular safety, and to be a better option than tramadol in such situations. Tapentadol IR formulations were also shown to be better than similar oxycodone forms in the treatment of postsurgical pain, as they were associated with lower rates of gastrointestinal adverse events and higher cost savings in postsurgical hospital settings (Paris et al., 2013; Mercadante et al., 2014). In a review by Candiotti and Gitlin, tapentadol therapy was also reported to show an improved gastrointestinal tolerability profile, with low incidence of constipation (7% and 10%, in the treatment with tapentadol ER 100 mg and 200 mg, respectively) (Candiotti and Gitlin, 2010). However, other studies suggest that acute administration of tapentadol may not have significant advantages in gastrointestinal motor dysfunction, delaying gastric and small bowel transit, similarly to oxycodone (Jeong et al., 2012). In turn, a network meta-

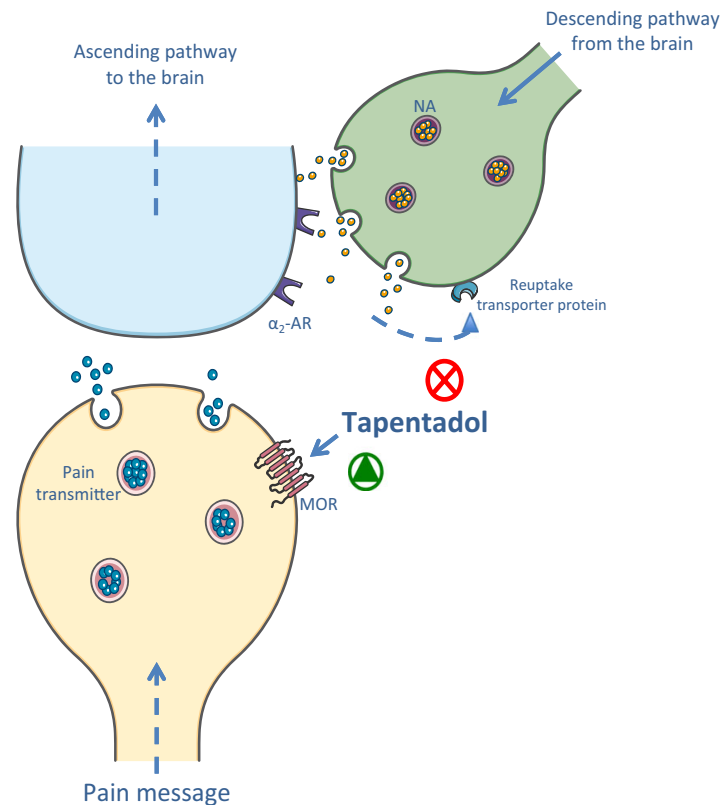


Figure 4 Tapentadol dual mechanism of action. Tapentadol acts through μ -opioid receptor (MOR) agonism and NA reuptake inhibition. NA inhibits the transmission of nociceptive impulses by activating α_2 -adrenergic receptors. Pain neurotransmitters glutamate and substance P bind the respective receptors, modulating primary afferent pathways of pain stimulation. NA: noradrenaline; MOR: μ -opioid receptor; α_2 -AR: α_2 -adrenergic receptor.

analysis of randomized-controlled trials showed tapentadol to have the best tolerability profile when compared to oxycodone-naloxone, tramadol, oxycodone, fentanyl, morphine, hydromorphone, buprenorphine and oxymorphone, as it leads to the lowest rates of adverse events, constipation and trial withdrawal, ranking third for the 'patient satisfaction' criteria only (Meng et al., 2017). In a prospective open-label study on tapentadol efficacy and tolerability in patients with moderate-to-severe cancer pain, pain intensity significantly decreased from baseline, with quality of life improvement and no changes in adverse effects; the tendency to increase the dose was low and independent of sex, age and pain mechanism (Mercadante et al., 2012). Nonetheless, the number of enrolled patients was low and further, larger studies are required (Mercadante et al., 2012). Moreover, tapentadol might be useful for its antihyperalgesic effects, such as those

observed in hyperexcitation states of patients undergoing consecutive unsuccessful opioid trials (Schroder et al., 2010). Importantly, *in vitro* and *in vivo* studies showed that tapentadol causes lower inhibition of hippocampal neurogenesis than morphine, as well as other classical opioids, arising as an attractive alternative for the treatment of neuropathic pain (Meneghini et al., 2014), which has a particularly complex pathophysiology (Burgess and Williams, 2010). Tapentadol noradrenergic component has been suggested to potentially counteract the negative MOR-mediated effects on hippocampal neurogenesis, with its antiapoptotic and proneurogenic effects being mediated by adrenergic receptors (Meneghini et al., 2014; Bortolotto and Grilli, 2017). The increasing role of the noradrenergic component observed over time in persistent neuropathic pain models further reinforces tapentadol suitability for the treatment of neuropathic pain (Langford et al.,

Table 3 Comparative summary of main tramadol and tapentadol clinical and toxicological aspects (Dayer et al., 1994, 1997; Matthiesen et al., 1998; Wade and Spruill, 2009; Singh et al., 2013; Mercadante et al., 2014; Arjunan et al., 2015; Knezevic et al., 2015; McNaughton et al., 2015; Barbosa et al., 2016; Chang et al., 2016; Langford et al., 2016; Agarwal et al., 2017; Pergolizzi et al., 2017; Vadivelu et al., 2017; Wu et al., 2017).

	Tramadol	Tapentadol
Main indications	Acute and chronic, moderate to severe forms of: • Postoperative pain • Odontological pain • Cancer pain • Musculoskeletal pain • Adjuvant to NSAID therapy in osteoarthritis	Acute and chronic, moderate to severe forms of: • Postoperative pain • Musculoskeletal pain • Neuropathic pain (e.g. associated with diabetic peripheral neuropathy) • Mixed pain states
Main contraindications	• Concomitant use of MAOIs and/or tricyclic antidepressants • Renal and/or hepatic failure • Pregnancy • Lactation • CYP2D6-defective subjects	• Impaired pulmonary function • Paralytic ileus • Concomitant use, within a 14 day-window, of MAOIs • Safety in pregnancy, lactation, children < 18 years old, hepatic and renal commitment has not been fully established
Main complications	• Serotonin syndrome • Respiratory depression • Seizures • Nausea and vomiting • Dizziness • Drowsiness • Fatigue • Increased sweating • Constipation	• Respiratory depression • Coma • Nausea and vomiting • Dizziness • Lethargy and somnolence
Median lethal dose (LD ₅₀ , rat, oral) (mg/kg)	300	980

NSAIDs, nonsteroidal anti-inflammatory drugs; MAOIs, monoamine oxidase inhibitors.

2016). Owing to the combination of two modes of action, tapentadol also seems to be a good option for the treatment of mixed pain, as nociceptive and neuropathic pain arise from different pathogenic mechanisms (Borsook et al., 2014; Coluzzi et al., 2017). Moreover, considering the functional and structural neuroplasticity changes and pain modification pathways that accompany pain chronification, tapentadol emerges as a promising therapeutic alternative for chronic pain (Borsook et al., 2014; Coluzzi et al., 2017).

6.1 Paediatric use

In this context, an important point is the paediatric use of tramadol and tapentadol and their safety. Tsutaoka et al. also compared the use of tramadol and tapentadol in children aged <6 years old, with tapentadol showing a significantly higher risk for severe outcomes, and this higher risk was suggested as a consequence of tapentadol having a higher MOR binding affinity than tramadol. However, in this

study, the number of children exposed to tapentadol (groups <6 years, 6 to 12 years and 13 to 19 years) was substantially lower than that of children exposed to tramadol (Tsutaoka et al., 2015), thereby limiting the comparative analysis and the conclusions on tapentadol toxic effects. However, Borys et al. studied tapentadol toxicity in 104 children, from which 62 had no effects, 34 had minor, six had moderate and two had major effects with life-threatening events (Borys et al., 2015). In this study, the side effects reported were similar to other opioid analgesics, including drowsiness, lethargy, nausea, vomiting, miosis, tachycardia, respiratory depression, dizziness/vertigo, coma, dyspnoea, pallor, oedema, hives/welts, slurred speech, pruritus and hallucinations/delusions (Borys et al., 2015).

To evaluate tramadol toxicity in children, a retrospective assessment of 7334 cases, of children aged <6 years and tramadol acute ingestion only, was performed (Stassinis et al., 2017). Only one death was observed, while in most of the children, no effects were observed (84.8%); minor effects were observed

in 12.6% of the sample, moderate in 2.2% and major in 0.4% only (Stassinis et al., 2017). The most frequent symptoms were drowsiness and vomiting, and the most severe were respiratory depression, detected in 36 children exposed to 225 mg tramadol median dose (minimum dose 7.9 mg/kg), and seizures, observed in 24 children exposed to 525 mg tramadol median dose (minimum dose 4.8 mg/kg), but these effects were rarely detected (Stassinis et al., 2017). In turn, Friedrichsdorf et al. studied the efficacy and side effects of tramadol compared to codeine/paracetamol, in 84 children aged 4–15 years, after tonsillectomy, concluding that both treatments were efficient in pain management, with no significant differences (Friedrichsdorf et al., 2015). These authors defend tramadol use in children in postoperative settings due to its good safety profile and low potential for side effects (Friedrichsdorf et al., 2015). In this study, codeine/paracetamol caused more oversedation, while tramadol treatment caused more itching in the postoperative period (Friedrichsdorf et al., 2015). In another study, Bedirli et al. evaluated tramadol and fentanyl efficacy and safety in children during upper gastrointestinal endoscopy. Tramadol led to a lower frequency of adverse effects, a shorter recovery time, and better hemodynamic and respiratory stability. Accordingly, this study suggests that tramadol provides sedation as efficiently as fentanyl, but with better safety and tolerance (Bedirli et al., 2012).

Although oral and intravenous tramadol formulations are effective and well tolerated in children with postoperative pain (Grond and Sablotzki, 2004), more studies are needed to ascertain its efficacy. In fact, it should be interpreted with caution, as there are few studies available and some methodological problems may be present, such as the use of different validated and nonvalidated pain scales, with different pain triggers (Schnabel et al., 2015). Moreover, it should be emphasized that the exposure to tramadol and tapentadol by children aged <6 years old was mainly unintentional, and that dose data for the same responses were not confirmed in laboratory assays (Borys et al., 2015; Tsutaoka et al., 2015; Stassinis et al., 2017). More studies are required to establish safety, clinical effects and toxic ranges in paediatric patients, as well as to assess potential differences between IR and ER forms.

6.2 Veterinary use

Concerning veterinary use, tramadol and tapentadol clinical efficacy is uncertain and controversial, but

tapentadol appears as more promising because it is a single and active enantiomer, has linear kinetics, with no CYP450 induction/inhibition and showing negligible 5-HT reuptake inhibition (Giorgi, 2012). In fact, in a study developed by Kögel et al., the acute treatment with tramadol, morphine and tapentadol in Beagle dogs was compared, showing that, in this experimental model, tramadol did not promote antinociception, in contrast to tapentadol or morphine (Kogel et al., 2014). Lower M1 concentrations were detected, suggesting decreased tramadol metabolism, and explaining the absence of tramadol antinociception (Kogel et al., 2014). Hence, in these animals, treatment with tapentadol arises as a better option (Kogel et al., 2014). However, in cats with osteoarthritis, tramadol showed to be safe and efficient, with the most common adverse events being mydriasis, euphoria and sedation (Monteiro et al., 2017). Thus, tramadol may be a good approach for osteoarthritic treatment in cats (Monteiro et al., 2017). Additionally, in this model, tapentadol shows some disadvantages, such as a higher bioavailability due to the defective glucuronidation observed in cats, and a very low oral bioavailability (Giorgi, 2012). In dogs, tapentadol showed to be rapidly absorbed after oral administration, although with a low bioavailability (4.4%); dose-dependent adverse effects, such as salivation and sedation, were detected mostly after intravenous administration (Giorgi et al., 2012). Tapentadol effects were also assessed in goats after intravenous and intramuscular administration, and comprised tremors, ataxia, and severe hair loss 3 days after administration (Lavy et al., 2014). Tapentadol absorption was fast and with a short half-life (1.29 h), after intramuscular injections, which also led to a quite high bioavailability (Lavy et al., 2014). However, more studies are required to evaluate tramadol and tapentadol safety in veterinary medicine.

7. Toxicity

In parallel with tramadol and tapentadol extensive prescription and widely recognized efficacy in pain treatment, several cases of toxicity, as well as death, have been reported. In this context, some studies were performed after tramadol or tapentadol exposure. In humans, after oral administration of a single dose of 100 mg, tramadol and tapentadol therapeutic blood concentrations in adults are approximately 300 µg/L and 2.45 µg-eq/mL, respectively (Grond and Sablotzki, 2004; Terlinden et al., 2007).

Symptoms of tramadol intoxication typically start 4 hours after administration, being reported for

doses above 500 mg (Spiller et al., 1997). For higher doses (>800 mg), the risk of coma and respiratory depression is increased (Spiller et al., 1997), and further accentuated when tramadol is taken in combination with other psychoactive substances, such as ethanol and CNS depressants. Tramadol side effects are varied; however, the most common events are nausea, followed by vertigo, dizziness, vomiting, tiredness, constipation, sweating, dry mouth and sedation (Grond and Sablotzki, 2004; Shadnia et al., 2008; Beakley et al., 2015) (Table 3). Other side effects, also reported to be secondary to tramadol exposure, include angioedema, exacerbated anticoagulant effect, serotonin syndrome, CNS depression, seizures and tachycardia (Beakley et al., 2015). Another tramadol side effect is delayed ejaculation; however, the mechanisms involved are not clearly identified yet (Abdel-Hamid et al., 2016). Nonetheless, the long-term action on the monoaminergic system has been suggested as a cause for sexual dysfunction (Barber, 2011). In such situation, the prolonged action on the monoaminergic system is the possible cause of side effects involving sexual dysfunction and weight increase (Barber, 2011). Tramadol efficacy is dependent on its metabolism, through CYP450, to M1, which is 200-300 times more potent than the parental compound in MOR activation. Therefore, its effectiveness differs among different subjects (Giorgi, 2012). In this context, the genetic variability contributes to the susceptibility to serotonin syndrome (Beakley et al., 2015) and should be considered in a toxicological context. In PMs, with lower CYP2D6 activity, (+)-tramadol levels increase (Raffa, 2008; Barbosa et al., 2016), thereby promoting serotonin reuptake inhibition and increasing the risk of serotoninergic syndrome (Grond and Sablotzki, 2004; Beakley et al., 2015). On the other hand, in UM subjects with higher CYP2D6 activity, an increase in M1 levels can lead to more side effects, such as respiratory depression (Stamer et al., 2008; Orliaguet et al., 2015), in particular in renal insufficiency patients who exhibit a decrease in renal clearance, which contributes to increased toxicity (Stamer et al., 2008). In this context, a higher frequency of UMs in Southern Europeans and Northern Africans may increase tramadol adverse effects (Kirchheiner et al., 2008). Serotonin syndrome is particularly important when patients are being treated with multiple drugs that collectively increase serotonin levels, such as selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) (Goeringer et al., 1997; Sansone and

Sansone, 2009), which considerably increase the potential for side effects and drug interactions (Beakley et al., 2015).

Several studies with animal models documented tramadol-induced toxicity in different organs, such as liver, kidney, brain, heart and lung (Atici et al., 2005; Samaka et al., 2012; Ezzeldin et al., 2014; Ghoneim et al., 2014; Saleem et al., 2014; Awadalla and Salah-Eldin, 2016; Barbosa et al., 2017; Faria et al., 2017). Tramadol toxicity was observed through biochemical and histological analyses. Several alterations were detected, such as increased aminotransferase activity, creatinine and blood urea nitrogen (BUN) (Atici et al., 2005; Saleem et al., 2014; Barbosa et al., 2017; Faria et al., 2017); increased oxidative stress markers (e.g. malondialdehyde levels and decreased antioxidant activity) (Ghoneim et al., 2014; Awadalla and Salah-Eldin, 2016; Barbosa et al., 2017; Faria et al., 2017) and histopathological alterations (e.g. necrosis, vacuolization, mononuclear cell infiltration) (Atici et al., 2005; Samaka et al., 2012; El Fatoh et al., 2014; Ezzeldin et al., 2014; Ghoneim et al., 2014; Saleem et al., 2014; Awadalla and Salah-Eldin, 2016; Barbosa et al., 2017; Faria et al., 2017). One study, concerning human exposure, reports the case of a 19-year-old male patient with a history of tramadol abuse for 6 months, who was found unconscious with multiple organ dysfunction syndrome. In this situation, toxicological analyses only detected toxic tramadol levels (blood concentration of 9.5 mg/L), and medical analyses described several alterations, comprising deep coma, acute respiratory distress syndrome, electrocardiogram with sinus tachycardia, as well as hepatic, cardiac and renal dysfunction (Wang et al., 2009), in accordance with nonclinical *in vitro* and *in vivo* acute exposure studies (Faria et al., 2016, 2017; Barbosa et al., 2017). Hepatic dysfunction was characterized by increased alanine and aspartate aminotransferase activities and BUN levels (Wang et al., 2009). Lung X-rays analysis showed diffuse bilateral alveolar opacities, compatible with pulmonary oedema (Wang et al., 2009). Acute liver failure, as well as nonfatal hepatobiliary dysfunction, has also been reported for tramadol (Loughrey et al., 2003; Randall and Crane, 2014). Similarly, myocardial damage is reported as an autopsy finding in a death caused by tramadol (Mannocchi et al., 2013), as well as increased creatine kinase (CK) and CK-MB activities (Wang et al., 2009). In this context, tramadol treatment, when performed after surgical coronary revascularization, was reported to aggravate myocardial injury (Wagner et al., 2010).

Nephrotoxicity has been also implied in tramadol intoxications, as an increase in creatinine, BUN and potassium levels were observed (Wang et al., 2009).

The most common side effects of tapentadol comprise nausea, vomiting, dizziness, headache and somnolence (Table 3). The incidence of constipation is lower upon treatment with tapentadol than with other opioids, which decreases the rates of treatment discontinuation (Hartrick and Rozek, 2011). Given that tapentadol presents considerably higher opioid receptor activation, it may imply lower toxicity. Additionally, as already mentioned, tapentadol has a minimal serotonin effect, lowering the potential for serotonin syndrome induction (Barbosa et al., 2016).

Regarding human studies, Kemp et al. reported a death after tapentadol intravenous injection, with tapentadol concentrations reaching 1.05 and 3.20 mg/L in femoral and heart blood, respectively (Kemp et al., 2013). Larson and co-authors also reported two deaths following tapentadol exposure, with drug concentrations of 0.77 mg/L in femoral blood and 1.65 mg/kg in the liver, in one of the cases, and 0.26 mg/L in femoral blood and 0.52 mg/kg in the liver, in the other case (Larson et al., 2012). Besides these findings, foam cone was observed in the nose and mouth, and pulmonary congestion was detected in an autopsy context, for which tapentadol might be regarded as a possible lung damage-triggering factor (Kemp et al., 2013; Faria et al., 2017). Pulmonary alterations (namely pulmonary oedema) were also reported in a case of tapentadol and oxycodone intoxication, where tapentadol concentrations reached 1.1 mg/L in peripheral blood, 1.3 mg/L in central blood, 9.9 mg/kg in the liver, 0.94 mg/L in vitreous humour, and 88 mg/L in urine (Cantrell et al., 2016). Another fatal intoxication by tapentadol was reported by Franco et al., who suggested its acute ingestion; the events responsible for death included respiratory and CNS depression and serotonin syndrome, with tapentadol concentration in cardiac blood being as high as 6600 ng/mL, more than 20 times the higher limit of its therapeutic range (Franco et al., 2014). These authors also reported autolytic changes in kidney, suggestive of tapentadol nephrotoxicity (Franco et al., 2014).

Fewer studies have been performed concerning tramadol and tapentadol comparative toxicity. In the sequence of a comparative study of tramadol and tapentadol toxicity in Wistar rats, Faria et al. (Faria et al., 2017) concluded that both opioids cause brain, pulmonary and cardiac toxic damage following a single exposure, at typical effective doses, intermediate

doses and maximum recommended daily doses, with tapentadol causing comparatively more toxic damage. Following the same experimental approach, Barbosa and co-workers reported tramadol and tapentadol to lead to hepatotoxicity and nephrotoxicity, with the latter inducing more toxic damage (Barbosa et al., 2017). In a study by Tsutaoka et al. (Tsutaoka et al., 2015), the authors have compared several cases, at different ages, of tapentadol or tramadol single medication. While tapentadol elicited more toxic clinical effects and more severe outcomes (namely greater risk of respiratory depression, coma, drowsiness/lethargy and hallucinations), tramadol caused more seizures and vomiting events. The causes of seizures, after tramadol exposure, are unclear, although some studies, using different models, have associated tramadol antidepressant activity with seizures (Reeves and Cox, 2008; Reichert et al., 2014). Besides the adverse reactions discussed so far, more severe toxicological effects, with even more serious implications, have been reported, including respiratory depression, serotonin syndrome (Sansone and Sansone, 2009; Pilgrim et al., 2011; Beakley et al., 2015) and fatal intoxications (Tjaderborn et al., 2007; Pilgrim et al., 2011; Larson et al., 2012; Costa et al., 2013; Kemp et al., 2013; Pinho et al., 2013; Franco et al., 2014; Cantrell et al., 2016).

Considering the number and the diversity of the above-mentioned studies, it becomes clear that the comparison between both drugs is far from being straightforward. Given that tapentadol is a more recently marketed drug, the number of studies reporting its toxic effects is more limited, which makes direct comparisons with tramadol more difficult at this level. Also, tramadol and tapentadol advantages and limitations are highly dependent on the context of use. Indeed, if patients with a polymorphic CYP2D6 isoenzyme are concerned, the use of tapentadol will be a more appropriate option. Therefore, the choice of opioid to be prescribed should be based on the individual patient characteristics and aims of the treatment.

8. Abuse liability

Tramadol has a dual mechanism of action, presenting a low abuse potential, although it is not completely devoid of risk. Indeed, given that tramadol inhibits serotonin reuptake and promotes dopamine release, and as the levels of these neurotransmitters are involved in the regulation of mood, its potential for addiction should be considered (Barber, 2011). Shadnia et al. (Shadnia et al., 2008) analysed 114

cases of intentional tramadol intoxications at the Loghman-Hakim Hospital Poison Center, in Iran. Most cases concerned young adults, upon oral administrations, and with cardiopulmonary arrest being the cause of death. However, and according to other studies, tramadol mortality was low (Musshoff and Madea, 2001; Shadnia et al., 2008). Tramadol co-administration with other drugs was detected in only 28.95% of all cases. The rationale for this study was the fact that, in Iran, tramadol overdose has been one of the most frequent causes of drug intoxication, and the most common reason for admission into the Loghman-Hakim Hospital Poison Center (Shadnia et al., 2008). Iranian adolescents were found to use tramadol in combination with ethanol, cannabis and *ecstasy* (Shadnia et al., 2008; Nazarzadeh et al., 2014). The use of one drug is associated with the propensity to use other drugs, but there are other possibilities to explain the higher use of tramadol in Iran, such as the family background and social perceptions about tramadol safety, as it is a prescription medication (Nazarzadeh et al., 2014). The higher rates of tramadol intoxication in Iran may be related to the higher frequency of UMs in Saudi Arabians (20%), as M1 levels increase in this phenotype. Genotyping could help in the interpretation of intoxication cases, as well as in the reduction of their frequency in these countries (LLerena et al., 2014; Lassen et al., 2015). In turn, tramadol use is also common among the Egyptian adolescent population, from which one-third of all users had drug-related problems (Bassiony et al., 2015). Although tramadol users report a sense of happiness deriving from its consumption, it is also known to cause cognitive alterations (Bassiony et al., 2015). Randall and Crane, in a review, analysed several cases of tramadol deaths in Northern Ireland from 1996 to 2012 (Randall and Crane, 2014). A total of 127 cases were reported, from which 49% concerned the combination of tramadol with other drugs/medicines and 23% the intake of tramadol alone (Randall and Crane, 2014). These authors also reported a 10% increase in tramadol-related deaths, representing 40% of all drug-related deaths in 2011, although few changes in prescribing have been documented (Randall and Crane, 2014). In turn, Tjaderborn and co-authors studied fatal unintentional tramadol intoxications in Sweden, in the period comprised between 1995 and 2005. In all cases, other drugs were detected in addition to tramadol, although in seven cases tramadol was the only substance detected at toxic concentrations, while eight cases (47% of the total) were associated with a history of

tramadol abuse (Tjaderborn et al., 2007). These authors alert to risks of tramadol prescription, particularly for subjects with abuse history (Tjaderborn et al., 2007).

In this context, according to the Intercontinental Marketing Services (IMS) Kilochem data, tramadol worldwide consumption increased 42% between 2006 and 2012 (Abdel-Hamid et al., 2016). In line with this, tramadol was included in the annual report for 2013 by the International Narcotics Control Board (INCB), and five countries reported a significant risk of tramadol abuse (Abdel-Hamid et al., 2016). In several countries, tramadol use and abuse are serious problems, and numerous countries, such as Iran, Venezuela, Sweden, Ukraine, Egypt, Australia, Brazil, Japan, China, the United Kingdom and the United States, have put tramadol under national control (Lanier et al., 2010; Ojha and Bhatia, 2010; Babalonis et al., 2013). In developed countries, the increase in prescription is a problem. For instance, in the United Kingdom, tramadol prescription had a 50% increase, as well as 77% increase in tramadol-related deaths, between 2009 and 2011 (Verri et al., 2015).

Considering that MOR activation is responsible for opioid-related euphoria and mood alterations, and that tapentadol has lower MOR affinity than classical opioids (showing, for instance, 18-fold less affinity than morphine), its risk of abuse liability is potentially lower (Tzschentke et al., 2007; Arjunan et al., 2015). In fact, several data suggest that tapentadol abuse is infrequently reported, with the support for this fact being its dual mechanism of action (Butler et al., 2015; Pergolizzi et al., 2017). Consistently with this, prescription volume-adjusted relative risk for tapentadol ER was the lowest compared to analogous ER formulations, including those of fentanyl, oxycodone, morphine, oxycodone and tramadol, with the exception of hydromorphone. Also, tapentadol IR abuse prevalence was lower than all comparators except fentanyl IR (Butler et al., 2015; Pergolizzi et al., 2017). In turn, to assess tapentadol tolerance liability, equianalgesic doses of tapentadol and morphine were administered in rat tail-flick models, showing that the loss of the antinociceptive effect was delayed for tapentadol, while rapid onset for morphine (Tzschentke et al., 2007; Pergolizzi et al., 2017). Cepeda et al. (Cepeda et al., 2013a), in a cohort study using two claims databases, concluded that patients treated with tapentadol IR have a lower risk of receiving an abuse diagnosis and developing abuse/dependence than with oxycodone IR, although they may happen. A comparative study on

the likeliness to obtain opioid prescriptions from multiple prescribers – a phenomenon known as doctor shopping – also concluded that the risk is substantially lower for tapentadol than for oxycodone (Cepeda et al., 2013b), as well as the risk of abuse (Cepeda et al., 2014). Likewise, the Researched Abuse, Diversion and Addiction-Related Surveillance ('RADARS') system found tapentadol rates of abuse and diversion to be much lower than those for oxycodone or hydrocodone in the first 2 years following the initial release and marketing of its IR forms in the United States (Dart et al., 2012). Although there are reports of nonmedical diversion of tapentadol by college students, through chewing and swallowing and inhalation, besides intact swallowing, this was considered to be the result of a brief experimentation period following introduction (Dart et al., 2014). In turn, population-based rates and drug availability of the diversion of tapentadol ER were slightly lower than that of tapentadol IR, and significantly lower than those of other Schedule II opioid medications (Dart et al., 2016). Another study analysed the messages posted in online forums for drug abusers, between January 2011 and September 2012, focusing on the amount of discussion and endorsement for tapentadol abuse, in comparison with other drugs (McNaughton et al., 2015). In this study, the number of posts on tapentadol was significantly lower than on all other compounds, although slightly higher than that on tramadol; these authors also suggest that the dual mechanism of action may be associated with a lower abuse liability (McNaughton et al., 2015). Also, a study comparing the effects after tapentadol, tramadol and hydromorphone administration concluded that positive subject-rated effects (reported as 'good effects' and 'like the drug') occur earlier and dissipate faster for tapentadol than for tramadol or hydromorphone, with no negative side effects associated, which may contribute to tapentadol potential abuse (Stoops et al., 2013).

Therefore, additional studies are necessary in order to evaluate tramadol and tapentadol safety and minimize their side effects, as well as to evaluate their potential for abuse and dependence. Although the difference in the number of studies concerning tramadol and tapentadol abuse potential only reflects the difference in the length of their marketing periods, it hinders a truly fair comparison between both drugs and, as such, the demonstration of an absolute advantage of tapentadol over tramadol. Taken together, and despite the low rate of adverse events and the seemingly lower abuse liability provided by tapentadol, the results from the studies performed in

this field substantiate the need to carefully balance the prescription of both drugs.

9. Concluding remarks and future prospects

Pain physiology is complex and, as a primary survival mechanism, shows remarkable redundancy and involves the overlap of numerous sensory pathways. In this context, the concept of 'mixed pain' gains sense and validates the search for poly-pharmacological approaches, as well as for multifunctional and multitarget drugs. Mixed MOR agonists and monoamine reuptake inhibitors, such as tramadol and tapentadol, engage different CNS pathways, enhancing analgesia and reducing undesirable adverse effects.

Despite their structural and pharmacodynamic similarities, it should be emphasized that tramadol and tapentadol have different MOR affinities (Table 2), which justifies their inclusion in different classes. In fact, tramadol belongs to class II, according to the WHO Guideline Cancer Pain Relief, being meant for mild-to-moderate pain treatment, while tapentadol belongs to class III, regarding the treatment of moderate to severe pain. Besides, while tramadol is a Schedule 4 drug (with a lower abuse potential and physical and psychological dependence than Schedule 3 drugs), tapentadol belongs to Schedule 2, being accepted for medical use in several countries, but with severe restrictions. While tramadol is available as an analgesic since the late 1970s, tapentadol has been approved in the late 2000s. In spite of providing an overall low rate of adverse events, tramadol was shown to have a considerable dependence and abuse potential. Given the contribution of the MOR component to dependence and abuse, and considering the more pronounced NA reuptake inhibition component of tapentadol, it shows comparable analgesic efficacy to other strong opioids, but considerably less side effects and abuse potential. Its minimal serotonergic effect, along with evidences of enhanced gastrointestinal tolerability and no negative effects on neurogenesis, makes it a promising alternative for the treatment of chronic and neuropathic pain. Moreover, unlike tramadol and oxycodone, tapentadol does not depend on the CYP450 system to produce more active metabolites, thereby circumventing interindividual differences in metabolizer phenotypes, providing linear and predictable pharmacokinetics and minimizing the need for dose adjustments. However, recent *in vitro* and *in vivo* studies using tramadol and tapentadol

equimolar concentrations have reinforced available data on the toxicological potential of tramadol and showed that the toxicological potential of tapentadol is at least comparable. Although tapentadol seems to entail lower abuse, addiction and diversion liability, it has been associated with more severe outcomes, when compared to tramadol, in the clinical setting. Therefore, additional *in vitro* and *in vivo*, randomized double-blind controlled studies are required to establish tapentadol efficacy, safety and long-term abuse liability. Likewise, tapentadol safety on the paediatric population and in patients with renal and hepatic commitment needs to be further determined. In order to help patient rotation to or from tapentadol, the establishment of more dose-conversion ratios would also be useful.

Accordingly, further research on tapentadol is needed to gather long-term efficacy and safety data to reach an equitable comparison between this drug and tramadol and other opioids. We believe that such information will accumulate in the next years, along with the increasing clinical experience that is expected for tapentadol within the same period, given its potential advantages over comparator drugs.

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PART I

2. GENERAL AND SPECIFIC OBJECTIVES OF THE DISSERTATION

The overall aim of the present thesis was to comparatively study the toxic effects associated with tramadol and tapentadol exposure, using *in vitro* and *in vivo* models. While tramadol is one of the most prescribed opioids worldwide, tapentadol is a more recent drug, associated with a higher analgesic effect than tramadol, but has raised many doubts about its toxicity and dependence among the scientific community. In fact, very few studies on its toxic effects and safety have been performed, which difficult the comparative analysis of safety between both drugs.

With this thesis, an increase in the knowledge in the opioid toxicology field is aimed, by providing new data on the molecular and mechanistic aspects of tramadol and tapentadol toxicity. It is also expected to provide an insight into the damage in target organs: brain, lung and heart, as well as about alterations on biochemical parameters. A comparative analysis of the results obtained upon exposure to both drugs might contribute to a more conscious prescription and consumption, besides helping in the identification and explanation of intoxication and fatal intoxication cases.

The hypothesis derived from these general objectives supported the specific goals of the original research corresponding to the two chapters of this thesis, as described below:

CHAPTER I

In order to assess the potential neurotoxicity of tramadol and tapentadol, an *in vitro* model of nervous system cells was used. Nervous cells were acutely exposed to both opioids. The studies were addressed considering four aspects:

1. A comparative evaluation of the potential neurotoxicity of both opioids;
2. The evaluation of cell viability and the mechanisms of death promoted by each drug;
3. The role of oxidative stress and mitochondrial integrity in cell toxicity;
4. The assessment of the metabolic alterations.

CHAPTER II

In order to complement the *in vitro* approach, *in vivo* studies, using male Wistar rats, were performed to analyse the effects induced by an acute exposure to effective (analgesic) doses. The analyses were performed in blood samples and in brain, lung and heart specimens. The methodologies used aimed at the evaluation of:

1. The analysis of the potential biochemical changes in serum and brain samples;
2. The assessment of the oxidative stress damage in brain, lung and heart tissues;
3. The evaluation of the potential histopathological alterations in brain, lung and heart tissues.

PART II

1. ORIGINAL RESEARCH

CHAPTER I

Comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells

Reprinted from Toxicology, 359-360: 1-10

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Comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells



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ARTICLE INFO

Article history:

Received 18 April 2016

Received in revised form 12 June 2016

Accepted 14 June 2016

Available online 15 June 2016

Keywords:

Tramadol

Tapentadol

Neurotoxicity

In vitro

Bioenergetics

ABSTRACT

Opioid therapy and abuse are increasing, justifying the need to study their toxicity and underlying mechanisms. Given opioid pharmacodynamics at the central nervous system, the analysis of toxic effects in neuronal models gains particular relevance. The aim of this study was to compare the toxicological effects of acute exposure to tramadol and tapentadol in the undifferentiated human SH-SY5Y neuroblastoma cell line. Upon exposure to tramadol and tapentadol concentrations up to 600 μ M, cell toxicity was assessed through evaluation of oxidative stress, mitochondrial and metabolic alterations, as well as cell viability and death mechanisms through necrosis or apoptosis, and related signalling. Tapentadol was observed to trigger much more prominent toxic effects than tramadol, ultimately leading to energy deficit and cell death. Cell death was shown to predominantly occur through necrosis, with no alterations in membrane potential or in cytochrome *c* release. Both drugs were shown to stimulate glucose uptake and to cause ATP depletion, due to changes in the expression of energy metabolism enzymes.

The toxicity mechanisms in such a neuronal model are relevant to understand adverse reactions to these opioids and to contribute to dose adjustment in order to avoid neurological damage.

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1. Introduction

Tramadol and tapentadol are centrally acting, synthetic opioid analgesics widely used in the treatment of moderate to severe pain (Hartrick and Rozek 2011; Pinho et al., 2013; Zhou 2009). Tramadol is the most commonly prescribed opioid and tapentadol use is increasing (Meske et al., 2013; Raffa et al., 2012). Although tapentadol does not require metabolic activation, tramadol owes most of its analgesic effect to its main active metabolite, *O*-desmethyiltramadol (M1), although the parent compound exhibits some pharmacological activity (Grond and Sablotzki 2004; Hartrick and Rozek 2011). Their clinical interest is mostly due to

their low incidence of side effects, when compared with classical opioids such as heroin and morphine (Hartrick and Rozek 2011). Nevertheless, respiratory depression and fatal intoxications have been reported for these compounds (Costa et al., 2013; Dinis-Oliveira et al., 2012a; Dinis-Oliveira et al., 2012b; Larson et al., 2012; Pinho et al., 2013; Ryan and Isbister 2015), and brain lesions were found in one subject with a history of tramadol abuse in a study that analysed 28 subjects (Boostani and Derakhshan 2012). Although serotonin syndrome is often associated with tramadol, a recent study by Ryan and Isbister did not detect serotonin toxicity in overdose cases with tramadol (Ryan and Isbister 2015; Sansone and Sansone 2009). However, when excessive use or co-administration of other drugs (particularly antidepressants) take place, the risk of serotonin toxicity is considerable (Sansone and Sansone 2009).

Chronic use of tramadol has been associated with red neuron degeneration in rat brains, which has been raised as a possible explanation for cerebral dysfunction (Atici et al., 2004). This fact

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emphasizes the need for the analysis of the effects in humans, particularly concerning neurotoxicity and central nervous system dysfunction upon tramadol and tapentadol administration, similarly to what happens with other drugs of abuse of this class. Neurotoxicity has been claimed to be due to oxidative stress, mitochondrial changes, apoptosis and neurogenesis inhibition, as suggested by proteomic assays with zebrafish brain (Zhuo et al., 2012). Since tramadol presents positive charge at physiological pH, it may accumulate within negatively charged cell compartments such as mitochondria, eventually causing mitochondrial dysfunction, similarly to what happens with cocaine derivatives (Cunha-Oliveira et al., 2008). As the neurotoxic mechanisms of tramadol and tapentadol are not fully understood, the present work aims to study the *in vitro* toxic effects of these opioids in a human neuronal model, the SH-SY5Y cell line, namely by assessing oxidative stress, mitochondrial and metabolic alterations, as well as cell viability through necrosis or apoptosis, and related signalling.

2. Materials and methods

2.1. Cell culture

SH-SY5Y cells (ATCC, Manassas, VA, USA) were maintained in 25 cm² flasks, being regularly observed through microscopy. Cells were routinely subcultured to new flasks, by trypsinization [Trypsin EDTA 1 × (ScienCell)], when they were approximately 70–90% confluent. SH-SY5Y cells were cultured in complete growth medium consisting of DMEM with 1% (v/v) GlutaMAXTM and 4.5 g/L glucose (Lonza), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco Invitrogen), 1% NEAA (Non-Essential Amino Acids, Gibco Invitrogen), 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma-Aldrich). Cells were maintained in a humidified incubator (Heraeus Hera Cell), at 37 °C and 5% CO₂ atmosphere. Before each assay, after seeding, cells were incubated for 24 h in the humidified incubator, allowing them to stabilize and adhere, before exposure to tramadol/tapentadol.

2.2. Drug preparation

Stock solutions of tramadol hydrochloride (Sigma-Aldrich) and tapentadol hydrochloride (Deltacolon) were freshly prepared in sterile water, with the appropriate dilutions (5 concentration levels) being made in complete medium. Cells were exposed to different drug concentrations (up to 600 µM) for 48 h at 37 °C and 5% CO₂.

2.3. Cytotoxicity assays

Cytotoxicity was assessed through MTT and SRB (sulforhodamine B) assays. Cell suspensions with a final density of 1.5×10^5 cells/ml were prepared in growth medium containing 5% FBS and lacking antibiotic/antimycotic mixture and cells were seeded in 96 well-plates (1.5×10^4 cells/well) and exposed to tramadol and tapentadol. Non-exposed cells were used as controls, and wells without cells and with compound were used as blanks for each concentration.

2.3.1. MTT assay

MTT assay measures dehydrogenase activity, an indicator of metabolically active mitochondria, and thus of cell viability. After 48 h of exposure to tramadol and tapentadol at 37 °C and 5% CO₂, cells were incubated for 4 additional hours with 200 µl of DMEM containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich). After incubation, the cell culture medium was removed, and formazan crystals were then

solubilized by adding 100 µl of solubilization solution [(89% (v/v) isopropanol, 10% (v/v) Triton X-100, 1% (v/v) of HCl 0.37% (w/v)]. Upon homogenization, absorbance values were read at 550 nm in a plate reader (Biotek Synergy 2) and retrieved using Gene5 software (Biotek). Dose-response curves were plotted using GraphPad Prism[®] version 5.0c. Results were presented as the percentage of cell viability *versus* concentration. All drugs were tested in 5 independent experiments, with each concentration tested in 6 replicates within each experiment.

2.3.2. SRB assay

The SRB assay is an indicator of cell density, based on the measurement of cellular protein content. Before cell exposure to tramadol/tapentadol, an identical, additional plate (T0 plate) was fixed with 50 µl 50% (w/v) pre-cold trichloroacetic acid and by incubating 1 h at 4 °C, in order to determine the initial amount of cells used in the assay. After 48 h of tramadol/tapentadol exposure at 37 °C and 5% CO₂, cells were fixed as described for the T0 plate. After fixation, the plates were washed 5 times with deionized water and air-dried at room temperature. Then, cell proteins were stained with 50 µl 0.4% (w/v) SRB (Sigma-Aldrich) in 1% (v/v) acetic acid, followed by a 30 min-incubation. SRB was then removed through 5 washings with 1% (v/v) acetic acid. The plates were again allowed to dry at room temperature. In order to solubilize protein/SRB complexes, 100 µl of 10 mM Tris buffer were added to each well. Absorbance of each well was measured at 515 nm in a plate reader (Biotek Synergy 2). Dose-response curves were plotted using GraphPad Prism[®] version 5.0c. Results were presented as the percentage of cell viability *versus* concentration. All drugs were tested in 5 independent experiments, with each concentration tested in 6 replicates within each experiment. The T0 plate was used to determine the amount of cells present by the time that the compound was added, and the mean absorbance value was subtracted to the absorbance value of the wells containing the compound, upon exposure.

2.4. Trypan blue assay

After tramadol/tapentadol incubation, cells were trypsinized and cell viability was determined through the trypan blue exclusion method. Non-exposed cells were used as controls.

2.5. TBARS and carbonyl groups

For the preparation of cell suspensions, cells were cultured in complete growth medium in T25 flasks until 70% of confluence. After exposure to tramadol/tapentadol, the medium was removed and cells were washed with PBS and collected through trypsinization. Non-exposed cells were used as controls. Culture medium was used to prepare blank samples. Cells were centrifuged (860g, 4 min) and the supernatant was removed. Cell pellets were lysed with lysis buffer [(50 mM Tris-HCl, pH 7.5; 30 mM NaCl; 0.5% (v/v) Triton X-100; 1 mM EDTA; protease inhibitor cocktail 1×)], incubated on ice for 20 min and then centrifuged at 19,330g for 15 min, 4 °C. The obtained supernatants were acidified with 10% (v/v) perchloric acid and used for TBARS (thiobarbituric acid-reactive substances) quantification, whereas the pellets were used for carbonyl group quantification. Lipid peroxidation (LPO) was evaluated using the well-known TBARS methodology (Buege and Aust 1978), with the results being expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein used. Protein carbonyl groups (ketones and aldehydes) were determined according to Levine et al. (Levine et al., 1994) and the results were expressed as nanomoles of 2,4-dinitrophenylhydrazine (DNPH) incorporated per milligram of protein.

2.6. Detection of apoptosis by annexin V-FITC apoptosis and TUNEL assays

Cell death by apoptosis was analysed using the TUNEL kit assay (DeadEnd™ Colorimetric TUNEL System, Promega) and through staining with annexin V-FITC and propidium iodide, PI (annexin V-FITC Detection Kit, Biotool). In these assays, cells were plated in 6-well plates (1.5×10^4 cells/well) with coverslips and exposed to tramadol and tapentadol for 48 h. The results were analysed under fluorescence microscopy in a Zeiss Spinning Disc AxioObserver Z.1 SD microscope, coupled to an AxioCam MR3 camera. Representative focus plans were chosen for image acquisition, which was performed using the AxioVision 4.8.2 software.

In the annexin V-FITC assay, after 48 h of tramadol/tapentadol exposure at 37 °C and 5% CO₂, cells were washed with cold PBS. Then, 5 µl annexin V-FITC, 5 µl PI and 90 µl binding buffer were added, followed by incubation at room temperature, in the dark, for 15 min. Cells were washed in PBS for 5 min, and coverslips were then mounted in 4',6-diamidino-2-phenylindole (DAPI). Positive control cells were treated with H₂O₂. Non-treated cells were used as negative controls.

In the TUNEL assay, after 48 h of tramadol/tapentadol exposure at 37 °C and 5% CO₂, cells were fixed through immersion in a 4% (v/v) paraformaldehyde solution in PBS, for 25 min, at 4 °C. Then, they were washed twice with PBS and permeabilized with a 0.2% (v/v) Triton X-100 solution in PBS, and washed again with PBS. Upon washing, cells were incubated with 100 µl of Equilibration Buffer for 10 min, and then incubated with rTdT Incubation Buffer (composition per standard 50 µl reaction: 45 µl Equilibration Buffer, 5 µl Nucleotide Mix, 1 µl rTdT Enzyme) for 1 h at 37 °C. The reaction was stopped by washing cells with 2 × SSC for 15 min, at room temperature. Coverslips were then washed with PBS, and mounted in DAPI. Cells treated with DNaseI (Fermentas) were the positive control. Non-exposed cells were used as negative controls.

2.7. Caspase activity assay

For the preparation of cell suspensions, cells were cultured in complete growth medium in 6 well-plates (2×10^5 cells/well). Incubation time and assay conditions were previously optimized. After incubation with tramadol/tapentadol for 48 h, the medium was removed and the cells were washed with PBS, and 150 µl of Glo Lysis Buffer (Promega) were added to each well. Cells were incubated for 5 min at room temperature. The lysates were collected and frozen at –20 °C. The results were normalized against protein content and the final results expressed as the percentage of the control (cells that had not been exposed to tramadol and tapentadol) from 5 independent experiments, tested in 2 replicates within each experiment, totalling 10 analyses for each concentration.

2.7.1. Caspase-3

Caspase-3 activity was determined as described by Barbosa et al., 2014 (Barbosa et al., 2014). 50 µl of each lysate was mixed with 200 µl assay buffer (100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 0.5 mM EDTA) and 5 µl of the caspase-3 peptide substrate Ac-DEVD-pNA (Sigma-Aldrich) (final concentration 80 µM), in 96 well-plates, followed by incubation at 37 °C for 24 h. Caspase-3 activity was determined at 405 nm, by quantifying the reaction product, using a microplate reader (Biotek Synergy 2).

2.7.2. Caspase-9

Lysates (10 µl) were mixed with 200 µl of assay buffer (100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 0.5 mM EDTA) in 96 well-plates, followed by incubation at 30 °C for 30 min. After incubation, the reaction was started by adding 10 µl of caspase-9

fluorogenic substrate N-acetyl-Leu-Glu-His-Asp 7-amido-4-trifluoromethylcoumarin (Sigma-Aldrich) (final concentration 180 µM). Fluorescence was determined using a microplate reader (Biotek Synergy 2) to 400 nm excitation and 500 nm emission, in a kinetic reaction for 5 min.

2.8. Measurement of intracellular ATP levels

Regarding the preparation of cell suspensions, cells were cultured in complete growth medium in 6 well-plates (2×10^5 cells/well). Upon incubation with tramadol/tapentadol, the medium was removed and the cells were washed with PBS and recovered through trypsinization. Cells were then centrifuged (860g, 4 min) and the supernatant removed. The pellets were lysed with lysis buffer [50 mM Tris-HCl, pH 7.5; 30 mM NaCl; 0.5% (v/v) Triton X-100; 1 mM EDTA; protease inhibitor cocktail 1×], incubated for 20 min on ice, and then centrifuged at 19,330g for 15 min, 4 °C. The supernatants obtained were frozen at –20 °C and used to quantify ATP. Cellular ATP levels were quantified with the ATP determination kit (Molecular Probes – Invitrogen) according to the manufacturer's instructions, and solutions have been protected from light. Bioluminescence was determined using a microplate reader (Biotek Synergy 2) with an appropriate black plate. Six calibration standards were prepared in order to plot a standard curve, and the background luminescence was subtracted. The results were normalized against protein content and the final results were expressed as the percentage of control (cells that had not been exposed to tramadol and tapentadol) from 5 independent experiments, with each concentration tested in 2 replicates within each experiment, totalling 10 analyses for each condition.

2.9. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$)

Assessment of mitochondrial integrity was performed by measuring tetramethylrhodamine ethyl ester perchlorate (TMRE) inclusion as previously described (da Silva et al., 2014). In order to prepare cell suspensions, cells were seeded in complete growth medium in 96 well-plates (1.5×10^4 cells/well). Cells were exposed to different drug concentrations (up to 600 µM), and the compounds were allowed to act for 48 h at 37 °C and 5% CO₂, in order to reproduce MTT and SRB assay conditions. Cells lacking compound exposure were used as controls. Upon exposure, culture medium was removed and replaced by fresh medium containing 2 mM TMRE (Sigma-Aldrich), followed by incubation in the dark, for 30 min at 37 °C. Then, the medium was removed and 0.2% BSA in HBSS was added to each well. Fluorescence was determined using a microplate reader (Biotek Synergy 2), at 544 nm excitation and 590 nm emission. The results were expressed as the percentage of control (cells lacking exposure to tramadol and tapentadol) from 5 independent experiments, with each concentration tested in 6 replicates within each experiment.

2.10. Protein quantification

Proteins were quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, using bovine serum albumin as standard.

2.11. Measurement of cytochrome c release

For the preparation of cell suspensions, cells were cultured in complete growth medium in T25 flasks. After exposure to tramadol/tapentadol for 48 h, the media were recovered and cells were washed with PBS, collected through trypsinization and combined with the recovery medium. This suspension was

centrifuged (2000g, 5 min) and the supernatant was removed. The pellet was lysed with lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, pH 7.4), followed by incubation for 15 min on ice, and then centrifuged at 15,000g for 15 min, 4 °C. The supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) were frozen at –20 °C. Cytochrome c levels in the cytosolic and mitochondrial fractions (20 µg protein) were analyzed by Western blot. The extracts were separated by SDS-PAGE, on a 12% acrylamide separating gel. Then, proteins were transferred (60 mA for 60 min) to a nitrocellulose membrane. Proteins were stained with Ponceau S in order to confirm a homogeneous loading. Membranes were blocked with 10% non-fat dried milk in TBST (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) at 4 °C for 1 h and probed with anti-cytochrome c primary antibody (sc-13560, Santa Cruz Biotechnology) (diluted 1:250 in TBST with 5% non-fat dried milk) overnight. Then, membranes were incubated for 1 h with peroxidase-conjugated secondary antibodies (diluted 1:2000 in TBST with 5% non-fat dried milk), and washed (3 times for 10 min). Bands were visualized by treating the immunoblots with ECL (enhanced chemiluminescence) and analyzed with the QuantityOne Software (Bio-Rad).

2.12. Biochemical parameters: glucose and lactate levels

Cell lysates were prepared as described in the “Measurement of intracellular ATP levels” section. Glucose and lactate intracellular levels were quantified by Prestige 24i automated analyzer (Tokyo Boeki) according to the manufacturer's instructions, using 2 appropriate calibrators, which were diluted to plot a standard curve with 5 points. A quality control was also used (Costa et al., 2015).

Results were normalized against total protein content and against the respective control (non-treated cells).

2.13. RNA extraction and cDNA synthesis

For the preparation of cell suspensions, cells were seeded in complete growth medium in 6 well-plates (2×10^5 cells/well). After cell exposure to different concentrations of tramadol and tapentadol during 48 h, total RNA was extracted using PureZol (Bio-Rad), with 500 µl PureZol/well, according to the manufacturer's instructions. cDNA was synthesized from 1 µg total RNA, using the High capacity cDNA Reverse Transcriptase kit (Applied Biosystems), according to the manufacturer's directions.

2.14. Real-time PCR

The primers used for the analysis of gene expression through qReal-Time PCR are described in Table 1. Each reaction mixture had a total volume of 25 µl, comprising 12.5 µl $2 \times$ SYBR Green mix (BioRad); 2 µl cDNA diluted 1:10; 0.25 µl forward primer; 0.25 µl reverse primer and 10 µl RNase-free H₂O. The PCR amplification program comprised an initial denaturation step at 95 °C for 3 min, followed by 55 denaturation cycles at 95 °C for

10 s, primer annealing at 52 °C for 20 s, extension at 72 °C for 20 s and plate read. Upon the cycling steps, the melt curve was performed from 55 °C to 95 °C, with 0.5 °C increments for 5 s, followed by a plate read. Lactate dehydrogenase (LDH), Fe-S protein 1 of NADH dehydrogenase (NDUFS1), creatine kinase B (CKB), pyruvate dehydrogenase kinase 3 (PDK3) and aldolase C (ALDOC) genes were analyzed, and 18 S ribosomal RNA was used as housekeeping gene. Results were normalized against those of non-exposed cells.

2.15. Statistical analysis

Results were expressed as means $\pm$ SD. All analyses comprised at least 6 replicates performed in 3 independent assays. The graphics were plotted using GraphPad Prism® version 5.0c. Statistical data analysis was performed as an Analysis of Variance (ANOVA). Probability values of $p \leq 0.05$ were considered to be significant.

3. Results

3.1. Toxicity of tramadol and tapentadol in SH-SY5Y cells

Human cell line SH-SY5Y was exposed to tramadol and tapentadol. In order to understand what happens in extreme situations, such as intoxication and fatal cases, high concentrations of both opioids were used (Costa et al., 2013; Kemp et al., 2013; Larson et al., 2012; Shadnia et al., 2008). Indeed, in therapeutic doses (single oral dose of 100 mg) maximum serum concentrations (C_{max}) were approximately 1 µM for tramadol (Grond and Sablotzki 2004; Raffa et al., 2012) and 0.5 µM for tapentadol (Raffa et al., 2012). Other authors have used high opioid concentrations in similar studies, such as that of Perez-Alvarez et al., who exposed SH-SY5Y cells to methadone concentrations up to 1000-fold its C_{max} (Bruce et al., 2013; Perez-Alvarez et al., 2010). Regarding *postmortem* cases, tramadol and tapentadol concentrations are highly variable. Indeed, tramadol concentrations of 7.7 mg/L and 48.34 mg/L were reported, while 3.2 mg/L and 0.77 mg/L were found for tapentadol in *postmortem* blood samples (Costa et al., 2013; De Backer et al., 2010; Kemp et al., 2013; Larson et al., 2012). Thus, given the variability in maximum *postmortem* blood concentrations, our aim was to ascertain the damage extent in a broader concentration range. Upon exposure, toxic effects were evaluated through MTT and SRB assays (Fig. 1). The obtained results suggest that, within the concentrations and exposure period tested, tapentadol shows higher toxicity than tramadol. In fact, tapentadol has a more pronounced effect than tramadol on the decrease of metabolic activity (Fig. 1A), and cell biomass (Fig. 1B). These effects are also reflected in cell viability. This was observed both qualitatively, through morphologic alterations (from the increase in the amount of round, suspension cells in the culture), and quantitatively, through the trypan blue method, reflected on the significant increase in the amount of dead cells (Fig. 2).

Table 1
Primers used for Real-Time PCR amplification of each gene.

Gene	Forward Primer	Reverse Primer	Reference
LDH-A (lactate dehydrogenase)	AGCCCGATTCCGTTACCT	CACCAGCAACATTTCATCCA	Yu et al. (2014)
ALDOC (aldolase C)	GCGCTGTGTGCTGAAATCAG	CCACAATAGGCACAATGCCATT	Walczak-Drzewiecka et al. (2008)
NDUFS1 (Fe-S protein 1 of NADH dehydrogenase)	TCGGATGACTAGTGGTGTTA	TTATAGCCAAGGTCCAAAGC	Yang et al. (2014)
PDK3 (pyruvate dehydrogenase kinase 3)	TTAATAAGTCCGATGGCGC	TGAAGCATCCCTGGGTTCAC	Lu et al. (2008)
CKB (creatine kinase b)	CCTTCTCCACAGCCACAAC	CAGTCCGCTACAGCTC	Pfefferle et al. (2011)
18 S ribosomal RNA	CAACATCGATGGCGCGGGA	CCCCCTCTTGGTGAGGTC	

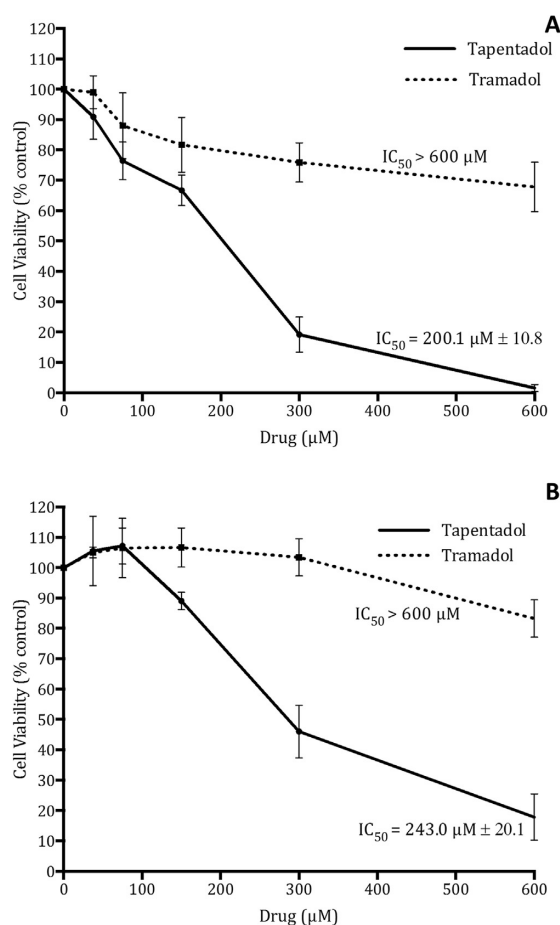


Fig. 1. MTT (A) and SRB (B) assays, upon exposure of SH-SY5Y cells to tramadol or tapentadol for 48 h. Results are expressed by means $\pm$ SD, from at least 5 independent assays, with each concentration tested in 6 replicates within each experiment.

3.2. Oxidative stress does not contribute to the toxic effects

In order to evaluate the mechanisms of toxicity that are elicited by tramadol and tapentadol, TBARS and protein carbonyl group levels were determined. As shown in Fig. S1A and B, no significant differences in oxidative stress parameters were found, comparatively to untreated cells, suggesting that the toxicity caused by tramadol and tapentadol is not predominantly caused by oxidative damage.

3.3. Necrosis is the main mechanism of cell death elicited by tramadol and tapentadol

In order to assess the mechanism of tramadol and tapentadol toxicity and cell death, different assays were performed. TUNEL assays (Fig. 3A) showed that tapentadol induces some signs of apoptosis. However, annexin V-FITC labelling (Fig. 3B) showed that necrosis is the main mechanism responsible for cell death following exposure to both drugs. Cytochrome *c* release assays (Fig. S2) showed no alterations in the presence of both drugs, when compared to non-exposed cells. Caspase activity quantification

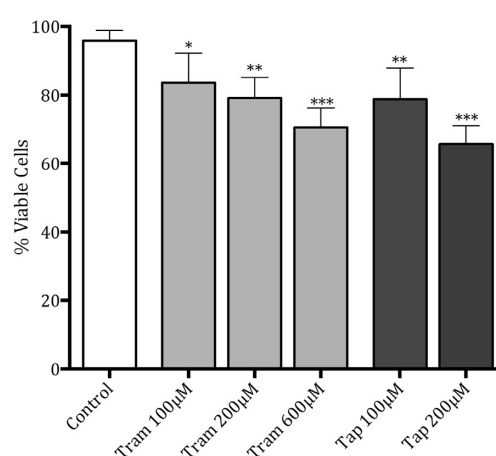


Fig. 2. Cell viability upon exposure to tramadol (Tram) and tapentadol (Tap), for 48 h. Cell viability was determined through the trypan blue assay, as the ratio between the number of living cells and the total amount of cells. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

(Fig. 4) corroborates these results, since only a slight increase was detected upon tapentadol exposure. In order to evaluate if tramadol and tapentadol lead to mitochondrial damage, the mitochondrial membrane potential was assessed (Fig. S3). No significant differences were found in the presence of tramadol or tapentadol, when compared to the control. Thus, the mitochondrial integrity is not affected by tramadol/tapentadol. These results are in accordance with the fact that no significant increase in cytochrome *c* release was detected and with the results pointing to necrosis as being the main mechanism of cell death triggered by tramadol/tapentadol (Fig. S2).

3.4. Tramadol and tapentadol induce changes in energetic metabolism

When SH-SY5Y cells were exposed to tramadol and tapentadol, an increase in intracellular lactate and glucose (Fig. 5), as well as a decrease in ATP levels (Fig. 6), were observed, comparatively to untreated cells, with this effect being dependent on drug dose. When toxic tramadol concentrations (600 μM) and tapentadol (200 μM) were used, significant increases in intracellular glucose and lactate were detected (Fig. 5). Glucose levels (Fig. 5A) increased 1.77 $\pm$ 0.16 fold after exposure to tramadol, and 1.78 $\pm$ 0.09 after exposure to tapentadol. Lactate levels (Fig. 5B) increased 1.51 $\pm$ 0.21 fold and 1.47 $\pm$ 0.30 fold after exposure to tramadol and tapentadol, respectively. At low tramadol/tapentadol concentrations (100 μM), there were no significant changes in glucose and lactate levels. According to these results, both tramadol and tapentadol were shown to promote a decrease in ATP levels, suggesting alterations in energetic metabolism. In ATP quantification, the results show that there is a general trend towards the decrease in total ATP content, particularly when cells are exposed to high tramadol (600 μM) and tapentadol (200 μM) concentrations – a decrease in the order of 24.6% $\pm$ 9.3 after tramadol exposure, and 42.0% $\pm$ 16.3 after tapentadol exposure. However, comparing the results obtained for the 200 μM concentration, tapentadol induced a higher reduction (to 42.0% $\pm$ 16.3) in ATP levels than tramadol (reduction to 73.3% $\pm$ 17.0). At low concentrations (100 μM), only tapentadol caused a significant decrease in ATP (reduction to 60.9% $\pm$ 16.4). Once tramadol and tapentadol were shown to increase extracellular glucose and lactate levels and to decrease ATP levels, some

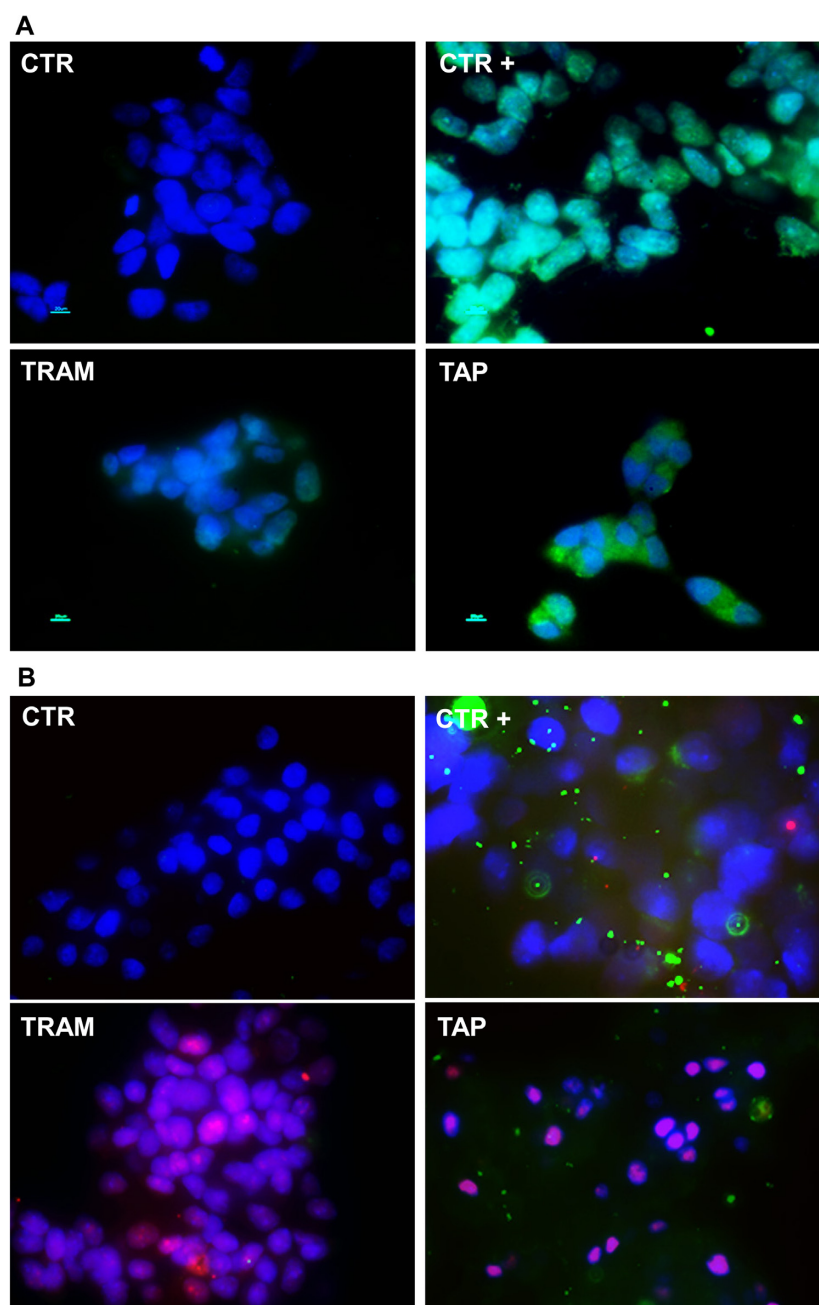


Fig. 3. TUNEL (panel A) and annexin V-FITC (panel B) labelling of SH-SY5Y cells upon exposure to 600 μ M tramadol (Tram) and 200 μ M tapentadol (Tap). DNase-treated cells and 50 μ M H_2O_2 were used as positive controls for apoptosis and necrosis, (panels A and B, respectively). DNA was stained with DAPI. DNA nick ends (panel A) and annexin V-FITC (panel B) were stained green, and necrotic cells were stained with and propidium iodide (panel B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assays were performed in order to clarify whether there were changes in the expression of energetic metabolism enzymes (Fig. 7). The results showed that cells exposed to both drugs presented a decrease in the expression of lactate dehydrogenase (LDH), Fe-S protein 1 of NADH dehydrogenase (NDUFS1) and

creatine kinase B (CKB), and an increase in the expression of pyruvate dehydrogenase kinase 3 (PDK3) and aldolase C (ALDOC). After exposure of the cells to 600 μ M tramadol or to 200 μ M tapentadol, LDH, NDUFS1 and CKB levels decreased upon tramadol/tapentadol exposure. PDK3 levels increased after

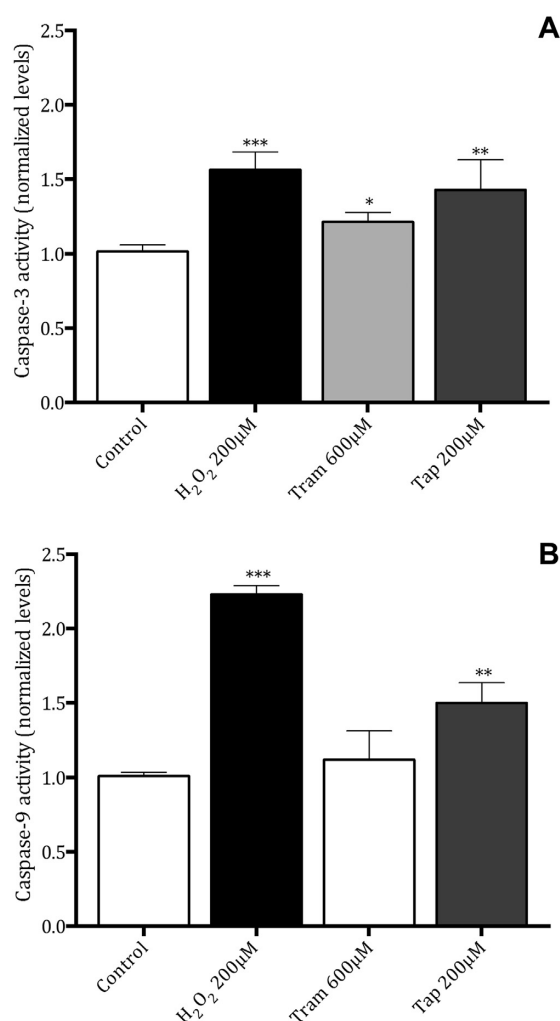


Fig. 4. Caspase 3 activity (A) and Caspase 9 activity (B) in SH-SY5Y cells exposed to 600 µM tramadol (Tram) and 200 µM tapentadol (Tap). 200 µM H₂O₂ was used as a positive control for apoptosis. Quantifications were normalized against the protein content of the extract. It was also normalized against the value obtained in the absence of opioid, for each assay, set as 1. Results are expressed as means ± SD, reflecting at least 10 replicates performed in 5 independent assays. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

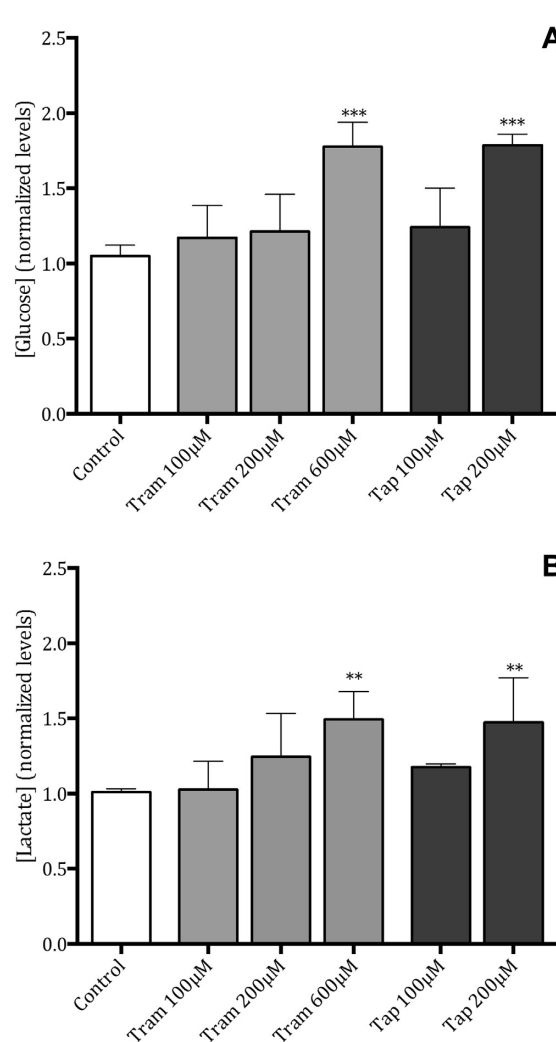


Fig. 5. Glucose (A) and lactate (B) levels in cell lysate after exposure to different tramadol and tapentadol doses. Glucose and lactate levels were normalized against the protein content of the extract. They were also normalized against the value obtained in the absence of opioid, for each cell line, set as 1. Results are expressed as means ± SD, reflecting at least 6 replicates performed 3 independent assays. ** $p < 0.01$, * $p < 0.05$.

tramadol/tapentadol exposure. ALDOC expression levels were only significantly altered after tapentadol exposure.

4. Discussion

Tramadol and tapentadol are widely prescribed synthetic opioid analgesics. Therefore, it is important to further evaluate its safety, in order to improve therapeutic strategies and prevent adverse effects, namely when these drugs are used in high doses. Few studies have been focused on the toxicological assessment of these opioids in neuronal cells, particularly concerning tapentadol, since it is a more recent drug that is often administered as a safer alternative to tramadol (Iyer et al., 2015). Indeed, this is, to our knowledge, the first study dealing with the comparative toxic potential of tramadol and tapentadol in an *in vitro* model of human

neuronal cells. In this work, we used the undifferentiated neuronal cell line SH-SY5Y, an *in vitro* model that exhibits many biochemical and functional characteristics of neurons (Valdiglesias et al., 2013; Xie et al., 2010), and that is frequently used in neurotoxicity studies (Di Daniel et al., 2005; Hong et al., 2003; Valdiglesias et al., 2013). In our study, cells were not differentiated; therefore, they do not have a dopaminergic phenotype. Tramadol moderately decreased cell biomass of the SH-SY5Y cell line, whereas tapentadol strongly decreased cell biomass at concentrations above 200 µM. In fact, as described by Caputi et al. (Caputi et al., 2014), low tapentadol concentrations (<100 µM) do not appear to cause toxicity in the SH-SY5Y cell line in MTT assays (Caputi et al., 2014). Although low concentrations did not lead to toxicity, changes in gene expression have been reported in similar conditions (Caputi et al., 2014), for which it is relevant to test the effects over a broader concentration

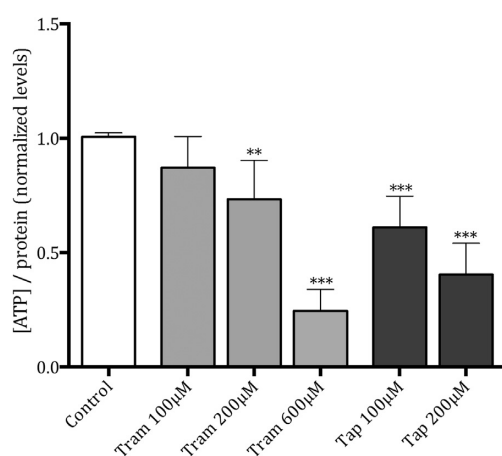


Fig. 6. ATP quantification on SH-SY5Y cells exposed to different tramadol (Tram) and tapentadol (Tap) concentrations, for 48 h. ATP quantification was normalized against the protein content of the extract. It was also normalized against the value obtained in the absence of opioid, set as 1. Results are expressed as means $\pm$ SD, 10 replicates performed in 5 independent assays. *** $p < 0.001$, ** $p < 0.01$.

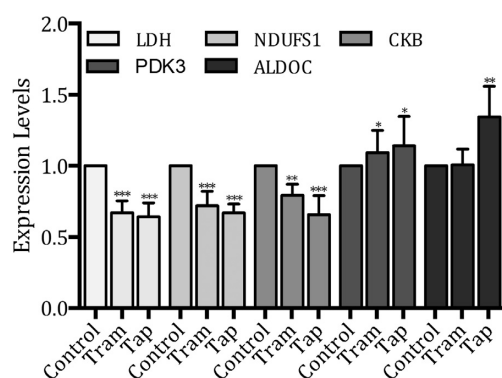


Fig. 7. Relative expression genes, upon exposure to tramadol (Tram) or tapentadol (Tap), for 48 h. The values were normalized against rRNA 18S gene expression. Expression levels were also normalized against the value obtained in the absence of opioid, set as 1 (100%). Results are expressed as means $\pm$ SD, reflecting at least 5 independent assays. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

range and evaluate the toxic effects. In the same way, fatal intoxications by tramadol (Clarkson et al., 2004; Costa et al., 2013; Shadnia et al., 2008) and tapentadol (Kemp et al., 2013; Larson et al., 2012) have been described, for which it is important to evaluate their toxicity, and its corresponding mechanisms. We demonstrate that higher opioid concentrations caused an increase in toxicity, as assessed by SRB and MTT assays, and corroborated by the viability assay. In order to evaluate the mechanisms of cell death induced by the exposure to tramadol/tapentadol, oxidative stress was estimated through quantification of TBARS and protein carbonyl group levels. Previously, an increase in oxidative stress, mitochondrial changes and apoptosis were suggested following proteomic assays with zebrafish brains after exposure to tramadol (Zhuo et al., 2012). However, our results suggest that lipid peroxidation is not an implicated mechanism of toxicity. Combined cell death analysis through TUNEL and annexin V-FITC assays indicated that necrosis is the predominant mechanism of cell death. Consistently with our results, Bonelli et al. (Bonelli et al.,

2005) did not find any tramadol influences on the induction of apoptosis in cerebrospinal fluid obtained from patients (Bonelli et al., 2005). Likewise, the decrease in ATP was consistent with the predominant mechanism of death being necrosis, since high ATP levels are required for apoptotic cell death (Tsujimoto 1997). In the same way, the analysis of cytochrome *c* release revealed no changes after exposure to tramadol/tapentadol, in comparison to the control. Concerning the caspase activity, the weak activation of the effector caspase-3 also suggests that a mechanism other than apoptosis is the main responsible for cell death. Nevertheless, as tapentadol has been shown to induce some apoptosis, caspase-9 activity was assessed. The activation of caspase-9 activity indicates that tapentadol may also cause some apoptosis. Some drugs, such as buprenorphine, accumulate in mitochondria, inhibiting β -oxidation and mitochondrial respiration, which cause a decrease in ATP and subsequent mitochondrial collapse (Berson et al., 2001). Similarly, studies with high methadone concentrations in SH-SY5Y cells have shown mitochondrial alterations, cytochrome *c* release, necrotic cell death and drastic ATP reduction (more than 60%) (Perez-Alvarez et al., 2010). Since tramadol has a positive charge in working conditions and at physiological pH (Pinho et al., 2013), it can accumulate within mitochondria, and the decrease of ATP and other toxic effects caused by tramadol and tapentadol may be caused by damage to the mitochondria. However, our results do not indicate mitochondrial damage, since changes in membrane potential or cytochrome *c* release were not observed. The assessment of mitochondrial membrane potential did not evidence any measurable modifications in $\Delta\psi_m$, even at highly cytotoxic concentrations, although ATP levels decreased significantly.

Considering the absence of effects in mitochondrial integrity, and the detection of an accentuated decrease in ATP, the levels of glucose and lactate were evaluated in order to ascertain changes in energetic metabolism. Glucose and lactate intracellular levels were shown to be increased, which indicates changes in the energetic metabolism. It is known that tramadol therapy is associated with an increased risk of hypoglycemia, sometimes requiring hospitalization (Fournier et al., 2015), and tramadol administration is associated with a hypoglycemic effect in type 2 diabetics (Choi et al., 2005). The exposure of neuronal cells to tramadol activates the insulin signalling pathway, increases insulin receptor expression, and consequently glucose uptake will be higher (Choi et al., 2005). Our results suggest that both tramadol and tapentadol stimulate glucose uptake, as an increase of intracellular glucose levels was detected. Moreover, the changes found in acute exposure may also be due to alterations in the expression levels of enzymes involved in energetic metabolism, leading to a bioenergetic crisis that is suggested by several authors as a consequence of the exposure to opioids (Perez-Alvarez et al., 2010). The exposure of mice to morphine promotes a decrease of the expression in 3 energetic metabolism enzymes (NDUFS, LDH2 and PDH) and a decrease in ATP levels, and these changes are associated with withdrawal jumping and memory impairment (Chen et al., 2007). In this sense, in the specific case of tramadol, proteomic studies indicate changes in several enzymes of energy metabolism, similarly to what happens with abuse drugs such as heroin, cocaine and methamphetamine (Zhuo et al., 2012). In accordance, our results have shown alterations in the expression of LDH, NDUFS1 and CKB enzymes involved in energetic metabolism. The activity of these enzymes is associated with ATP production, for which the reduction of their expression will contribute to the reduction of ATP showed in our results. NDUFS1 is a respiratory chain enzyme, one of the enzymes of oxidative phosphorylation in the mitochondria; LDH catalyzes the interconversion of pyruvate and lactate; and CKB catalyzes the interconversion of creatine and phosphocreatine. The decrease in the expression of these three genes is also detected in rats following exposure to morphine, and

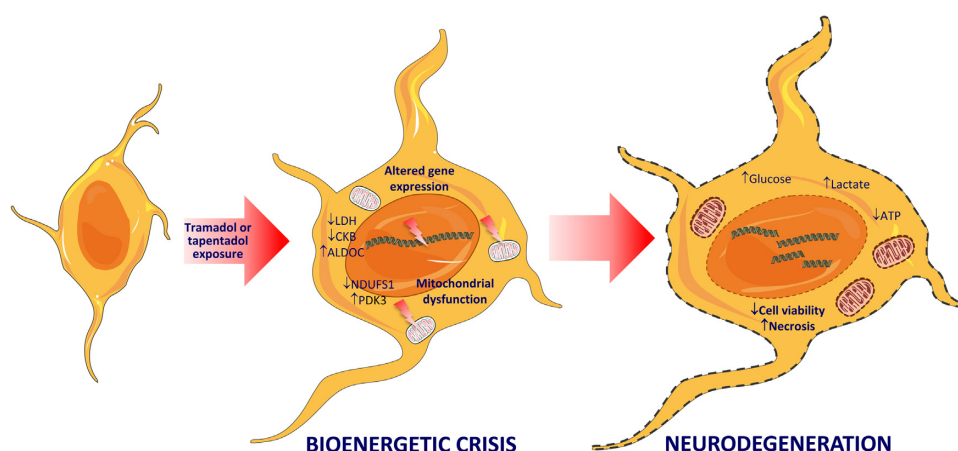


Fig. 8. Schematic illustration of the toxic acute effects induced by tramadol and tapentadol in the SH-SY5Y cell line. Exposure to both opioids leads to the alteration in the expression of glycolytic and mitochondrial enzymes (decrease in the expression of LDH, NDUFS1 and CKB, and increase in the expression of PDK3 and ALDOC), associated with increased intracellular glucose levels and decreased lactate and ATP concentrations. Cell viability decreases as a consequence of a bioenergetic crisis and necrotic cell death.

has been involved in the development of morphine dependence and memory deficits (Chen et al., 2007). In addition, the decrease in CK expression is also associated with brain damage and is closely associated with a neurodegenerative pathway (Streck et al., 2008). As for CK, it has been previously reported that tramadol leads to a decreased expression in zebrafish brain (Zhuo et al., 2012); however, for tapentadol there are no studies, at least to our knowledge. The decrease in the expression of NDUFS1, LDH and CKB, along with the increased uptake of glucose after tramadol and tapentadol exposure, denote a compromised energetic metabolism and cause a decrease in ATP production. Our results also show increased expression of PDK3 and ALDOC genes. Changes in the expression of these two genes had been previously suggested in proteomic assays in zebrafish brain after tramadol exposure (Zhuo et al., 2012). PDK is a kinase enzyme which acts to inactivate the pyruvate dehydrogenase (PDH) enzyme (PDH catalyses the conversion of pyruvate to acetyl-CoA), while ALDOC is a glycolytic enzyme, catalyzing the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate or dihydroxyacetone phosphate. The slight increase in the expression of ALDOC enzyme may be explained by the fact that the cell has reduced levels of ATP, as well as considerable intracellular amounts of glucose. Therefore, the cell increases the expression of ALDOC gene in order to further metabolize glucose through the glycolysis pathway, so that ATP production is enhanced, but subsequent conversion of pyruvate to acetyl CoA is conditioned (because of the increasing PDK expression that inactivates PDH). On the other hand, we detected a reduction of NDUFS1 expression, reflecting that mitochondrial respiration will be compromised. Thus, due to the increase in the expression of PDK and decrease in NDUFS1 expression, pyruvate will accumulate and will be converted into lactate, whose accumulation consequently inhibits the expression of LDH. In the future, it would be interesting to evaluate the expression of other enzymes involved in energetic metabolism, such as ATP synthase beta-chain, pyruvate dehydrogenase and phosphofructokinase. It would also be important to evaluate the possibility that tramadol and tapentadol cause changes on glucose transporters (GLUTs), and, as such, on glucose uptake, which would help to understand high intracellular glucose levels. In the same way, *in vivo* assays will be important in order to complement the results obtained *in vitro*. The combination of all changes detected shows that tramadol/tapentadol exposure elicits an energetic

disorder (Fig. 8). As energetic disorders are often associated with neurodegenerative diseases (Zhuo et al., 2012), so the abuse of these two opioids, in particular of tapentadol, must be controlled in order to prevent neurologic damage.

5. Conclusions

Taken together, the results of the present study demonstrate that tramadol and tapentadol exhibit neurotoxic effects *in vitro*, tapentadol being more toxic in the same concentrations and exposure times. Both drugs decreased cell viability and necrosis is the main mechanism of cell death. Tramadol-induced cell death does not involve apoptosis, while this mechanism is present for tapentadol. Both drugs decrease ATP intracellular levels and increase lactate and glucose levels, but no mitochondrial changes were observed. Cell exposure to tramadol or tapentadol causes changes in the expression of genes encoding for enzymes involved in glucose metabolism, leading to a bioenergetic crisis.

Acknowledgements

This work was supported by grants from CESPU (TETT-CESPU-2014); and the project NORTE-01-0145-FEDER-000024, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). FEDER Juliana Faria is a PhD fellowship holder from FCT (SFRH/BD/104795/2014). Ricardo Dinis-Oliveira acknowledges Fundação para a Ciência e a Tecnologia (FCT) for his Investigator Grant (IF/01147/2013).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2016.06.010>.

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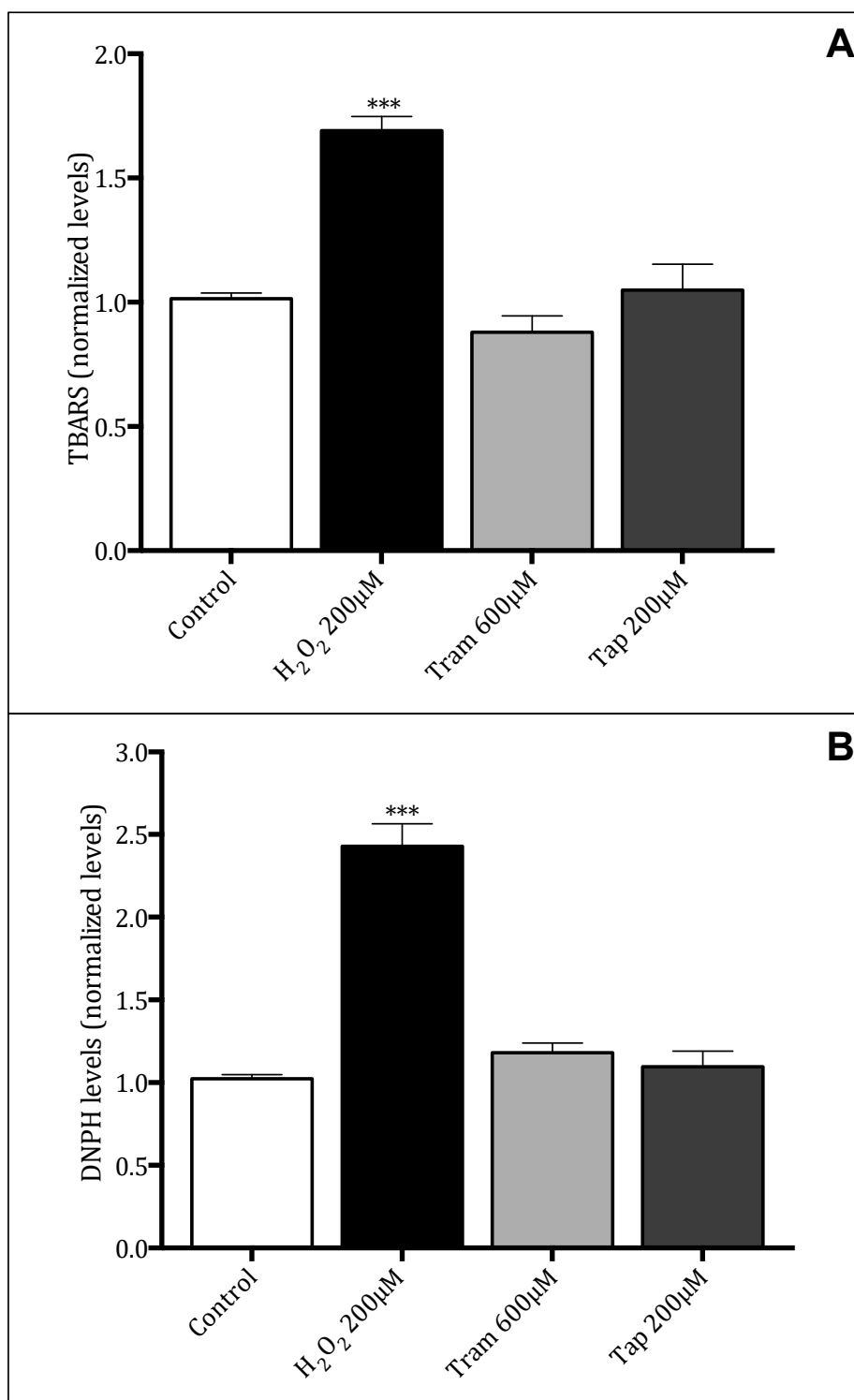
Figure S1

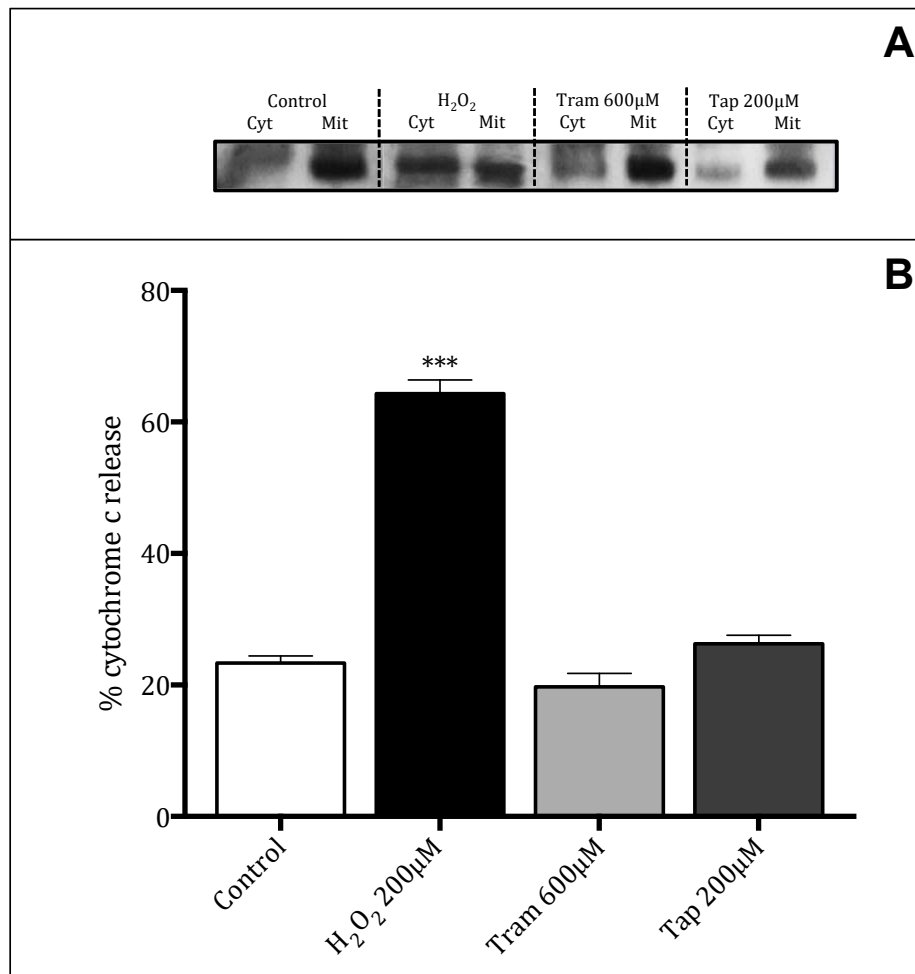
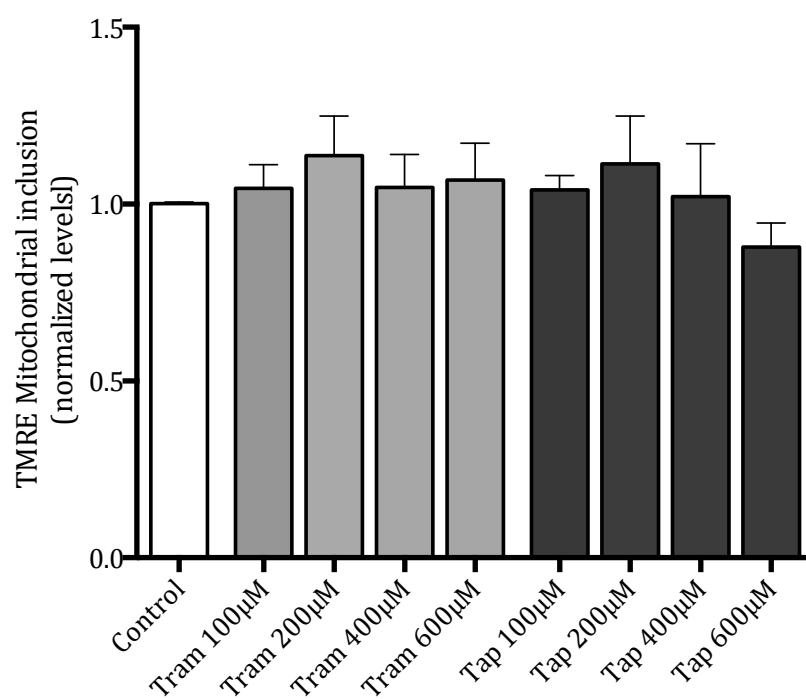
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Figure S3

CHAPTER II

Effective analgesic doses of tramadol or tapentadol induce brain, lung and heart toxicity in Wistar rats

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Effective analgesic doses of tramadol or tapentadol induce brain, lung and heart toxicity in Wistar rats



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ARTICLE INFO

Keywords:

Tramadol

Tapentadol

In vivo

Acute toxicity

Pharmacodynamics

ABSTRACT

Tramadol and tapentadol are extensively prescribed for the treatment of moderate to severe pain. Although these drugs are very effective in pain treatment, the number of intoxications and deaths due to both opioids is increasing, and the underlying toxic mechanisms are not fully understood. The present work aimed to study the potential biochemical and histopathological alterations induced by acute effective (analgesic) doses of tramadol and tapentadol, in Wistar rats. Forty-two male Wistar rats were divided into different groups: a control, administered with normal saline solution, and tramadol- or tapentadol-treated groups (10, 25 or 50 mg/kg – typical effective analgesic dose, intermediate and maximum recommended doses, respectively). 24 h after intraperitoneal administration, biochemical and oxidative stress analyses were performed in blood, and specimens from brain, lung and heart were taken for histopathological and oxidative stress studies. Both drugs caused an increase in the AST/ALT ratio, in LDH, CK and CK-MB activities in serum samples, and an increase in lactate levels in serum and brain samples. Oxidative damage, namely protein oxidation, was found in heart and lung tissues. In histological analyses, tramadol and tapentadol were found to cause alterations in cell morphology, inflammatory cell infiltrates and cell death in all tissues under study, although tapentadol caused more damage than tramadol.

Our results confirmed the risks of tramadol exposure, and demonstrated the higher risk of tapentadol, especially at high doses.

1. Introduction

Pain is a symptom associated with several pathologies, and opioids are currently used for the treatment of its moderate to severe forms. However, besides analgesia, the activation of μ -opioid receptors (MOR) also causes side effects such as central nervous system (CNS) depression, nausea, dependence and addiction (DePriest et al., 2015; Harrison et al., 1998; Kosten and George 2002). Hence, the use of non-classical opioids, which combine MOR agonist activity with monoamine reuptake blocking, has been seen to improve analgesia and decrease the side effects (Pergolizzi et al., 2012; Power 2011; Singh et al., 2013; Tzschentke et al., 2014; Vadivelu et al., 2010). Tramadol and tapenta-

dol are two synthetic opioid analgesics, with a dual mechanism of action – opioid agonist action and monoamine reuptake inhibition (Barbosa et al., 2016; Mercier et al., 2014).

Tramadol (1*RS*, 2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclo-hexanol is a racemic mixture of two enantiomers, (–)-tramadol and (+)-tramadol, that have two distinct but complementary mechanisms of action (Grond and Sablotzki 2004; Raffa et al., 2012). Tramadol is responsible for moderate MOR agonist activity, and noradrenaline (NA) and serotonin (5-HT) reuptake inhibition, and its analgesic activity is predominantly provided by the *O*-desmethyltramadol (M1) active metabolite (Duthie 1998; Gillen et al., 2000; Grond and Sablotzki 2004; Lai et al., 1996; Leppert 2011). Tapentadol (3-[(1*R*,2*R*)-

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3-(dimethylamino)-1-ethyl-2-methylpropylphenol) is a single and active molecule, without active metabolites (Bourland et al., 2010; Hartrick and Rozek 2011; Raffa et al., 2012). Tapentadol effect on the inhibition of 5-HT reuptake is less pronounced than that of tramadol, which reduces the risk of serotonin syndrome (Giorgi et al., 2012; Hartrick and Rozek 2011; Meske et al., 2014; Raffa et al., 2012; Steigerwald et al., 2013; Tzschentke et al., 2014).

Although tramadol and tapentadol have low incidence and intensity of side effects, addiction, respiratory depression and fatal cases have been reported (Clarkson et al., 2004; Costa et al., 2013; Dinis-Oliveira et al., 2012; Giorgi et al., 2012; Larson et al., 2012; Pinho et al., 2013; Ryan and Isbister 2015; Shadnia et al., 2008). Therefore, it is important to assess the potential toxicity of these compounds. Previous reports showed that acute gavage administration of tramadol LD₅₀ induced rat brain alterations, including brain congestion, edema and inflammatory cellular infiltrates (Samaka et al., 2012). Chronic use of tramadol in increasing doses was also associated with neuronal degeneration in the rat brain, which has a possible contribution to cerebral dysfunction (Atici et al., 2005, 2004; Awadalla and Salah-Eldin, 2016). Repeated tramadol administration has also been shown to cause lung damage (Awadalla and Salah-Eldin, 2016). Moreover, *in vitro* acute exposure assays in a human neuronal model showed that both compounds, tramadol and tapentadol, cause toxicity (Faria et al., 2016). However, concerning tapentadol there are no studies evaluating its toxicity in *in vivo* models. Additionally, myocardial damage may be associated with the inhibition of NA reuptake (Vadivelu et al., 2011), and therefore it is important to evaluate the potential cardiovascular damage after tramadol and tapentadol exposure. Hence, it is relevant to assess the toxic effects of different doses, including overdoses, particularly concerning neurotoxicity and central nervous system dysfunction, and lung and heart damage, upon tramadol and tapentadol administration, in order to evaluate their safety. Therefore, the present work aimed to study biochemical changes, oxidative damage and histopathological toxicity in brain, lung and heart of rats exposed to a single dose, corresponding to a typical effective analgesic dose, an intermediate dose and the maximum recommended daily dose, of tramadol or tapentadol.

2. Materials and methods

2.1. Animals

In this study, 42 male Wistar rats were used. Animals (aged 8 weeks) were obtained from the IBMC – i3S Animal House (Oporto, Portugal), weighing between 250 and 275 g. Animals were kept in standard laboratory conditions (12/12 h light/darkness, 22 ± 2 °C room temperature, 50–60% humidity) for at least 1 week (quarantine) before starting the assays. Animals were allowed access to tap water and rat chow *ad libitum* during the quarantine period. Animal experimentation was performed accordingly to the Portuguese Agency for Animal Welfare (general board of Veterinary Medicine), in compliance with the Institutional Guidelines and the European Convention. All experimentation was conducted in conformity with ethical and human principles of research, complying with the current Portuguese laws and in line with the recommendations of the National Council of Ethics for the Life Sciences (CNECV).

2.2. Tramadol and tapentadol exposure

Animals were divided into 7 groups of 6 animals each. The different groups were exposed to different tramadol hydrochloride (Sigma-Aldrich) or tapentadol hydrochloride (Deltaclon) doses, by intraperitoneal (ip) administration. Group I was used as control, receiving normal saline solution (0.9% NaCl); groups II, III and IV were injected with 10, 25 and 50 mg/kg tramadol, respectively; groups V, VI and VII were injected with 10, 25 and 50 mg/kg tapentadol, respectively. 10, 25 or

50 mg/kg correspond to a typical analgesic dose, an intermediate dose and the maximum recommended daily dose, respectively, as will be discussed in the “Results” section. Tramadol and tapentadol doses were delivered in a volume of 1 mL of normal saline. After ip administrations, animals were individually housed in metabolic cages, for 24 h, and during the exposure period they were provided with water *ad libitum*, but not food, and monitored.

2.3. Surgical procedures and sample collection

Twenty-four hours after administrations, rats were sacrificed through anesthesia with sodium thiopental (B. Braun Portugal, 60 mg/Kg, ip). Then, blood samples were drawn by cardiac puncture, using a heparinized needle. Blood was centrifuged (3000g, 4 °C, 10 min) and serum was separated and stored (–80 °C) for further biochemical analysis (aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), creatine kinase isoform MB (CK-MB), glucose, lactate and lactate dehydrogenase (LDH)). Brain cortex, lungs and heart were removed, dried, weighed and processed.

2.4. Tissue processing for biochemical analysis

Brain cortex, lung and heart samples were homogenized using an Ultra-Turrax® homogenizer with 1:4 (m/v) of ice-cold 50 mM phosphate buffer (KH₂PO₄ + Na₂HPO₄·H₂O), pH 7.4. Homogenates were centrifuged at 4000g, 4 °C, for 10 min. Supernatants were aliquoted and stored at –80 °C for protein, catalase (CAT) and superoxide dismutase (SOD) quantification and biochemical analyses (CK, lactate, LDH and proteins). 10% perchloric acid was added in a proportion of 1:1 to one aliquot of each homogenate. Then, samples were centrifuged at 13,000g, 4 °C, for 10 min. The supernatants were stored at –80 °C for the quantification of lipid peroxidation (LPO), and the pellets were used for quantification of carbonyl groups. Other aliquots of brain cortex, lungs and heart were homogenized, using an Ultra-Turrax® homogenizer, with 1:3 (m/v) of ice-cold isotonic buffer (300 mM sucrose, 10 mM HEPES, 2 mM EDTA) pH 7.9, and incubated for 15 min on ice, for further quantification of caspase 3 activity. After homogenization, the sample was vortexed and then centrifuged at 850g, 4 °C, for 10 min. The resulting supernatant was collected, and the pellets were resuspended in 500 µL ice-cold isotonic buffer, and again incubated for 15 min on ice and centrifuged at 14000g, 4 °C, for 10 min. The supernatant was collected and combined with the first supernatant. The supernatants were stored at –80 °C for protein and caspase 3 quantifications.

2.4.1. Biochemical analysis

2.4.1.1. Biochemical parameters. Biochemical parameters (AST, ALT, CK, CK-MB, lactate, LDH, glucose and proteins) were quantified in a Prestige 24i automated analyser (Tokyo Boeki) as previously described (Costa et al., 2015), using 2 appropriate calibrators, which were diluted to plot a standard curve with 5 points. A quality control was also used. Results were normalized against the control group, and also against protein content in brain samples.

2.4.1.2. Measurement of toxicity biomarkers. LPO was evaluated using the thiobarbituric acid-reactive substances (TBARS) methodology (Buege and Aust, 1978) and the results were expressed as nanomoles of malondialdehyde (MDA) equivalents per gram of protein. Protein carbonyl groups (ketones and aldehydes) were measured according to Levine et al. (Levine et al., 1994), with the results being expressed as nanomoles of 2,4-dinitrophenylhydrazine (DNPH) incorporated per gram of protein. CAT and SOD activity was determined according to the method of Aebi (Aebi, 1984) and of McCord and Fridovich (McCord and Fridovich, 1969), respectively. The results were expressed in enzyme units per gram of protein and were normalized against the control group. Caspase 3 activity was determined as described by Faria et al. (Faria et al., 2016), using caspase 3 peptide substrate Ac-DEVD-

pNA (Sigma-Aldrich) (to a final concentration of 80 μ M). Results were expressed in enzyme units per gram of protein. All results were normalized against the control group.

2.5. Tissue processing for histopathological examinations

Brain cortex, lung and heart samples were subjected to routine procedures for fixing, washing, dehydration and paraffin embedding as previously described by Dinis-Oliveira et al. (Dinis-Oliveira et al., 2006a, 2006b). Hematoxylin and eosin (H & E)-stained slides were prepared by standard methods and were analyzed by light microscopy, using 100 $\times$ and 600 $\times$ magnifications (Nikon Eclipse TE2000-U microscope equipped with a DXM1200F digital camera and controlled by Nikon ACT-1 software). Photos were taken from representative fields.

2.6. Statistical analysis

Results were expressed as means $\pm$ SD. The graphics were plotted using GraphPad Prism[®] version 7.0a. Statistical analyses were performed by analysis of variance (ANOVA). Post-hoc analysis was performed through Dunnett's multiple comparisons test. *P* values lower than 0.05 were considered as statistically significant.

3. Results

3.1. Biochemical alterations upon tramadol and tapentadol exposure

Wistar rats were exposed to different tramadol and tapentadol doses, in order to evaluate the potential toxicity and biochemical alterations caused by these opioids. Doses were selected in order to assess changes, after a single administration, when a typical effective dose and intermediate and maximum recommended doses are administered. Doses were established estimating the human equivalent dose (HED): (animal dose (mg/kg) = human equivalent dose (mg/kg) $\times$ 6.2) (Nafea et al., 2016; Nair and Jacob 2016; Reagan-Shaw et al., 2008), considering that an average human adult weighs 60 kg, as well as the LD₅₀ values reported (Matthiesen et al., 1998), concentrations reported in intoxication cases (Kemp et al., 2013), and the human recommended daily dose (Barbosa et al., 2016). Groups II and V were exposed to 10 mg/kg, which is an effective dose corresponding to a single dose of 100 mg in humans, and less than the maximum recommended daily dose (400 mg per day for tramadol, and 600–700 mg per day for tapentadol) (Barbosa et al., 2016; Matthiesen et al., 1998; Singh et al., 2013; Tzschentke et al., 2014). Indeed, 100 mg administered in a 60 kg-human correspond to a 1.67 mg/kg dose. Applying the HED calculation formula mentioned before, 1.67 mg/kg (human dose) is equal to 10.35 mg/kg (rat dose), when multiplied by a 6.2 factor. Groups IV and VII received the maximum recommended dose (50 mg/kg) for rats, through ip administration (Matthiesen et al., 1998). Groups III and VI received an intermediate dose (25 mg/kg).

Following exposure for 24 h, biochemical alterations were analyzed in serum samples (Fig. 1). The AST activity, AST/ALT ratio and LDH activity significantly increased when intermediate and higher doses of tramadol (25 mg/kg and 50 mg/kg) were administered, but not with the lower effective (analgesic) dose. After tapentadol exposure, AST and LDH activities also significantly increased after the exposure to the intermediate and maximum doses (25 mg/kg and 50 mg/kg), but the AST/ALT ratio only significantly increased with the intermediate dose (25 mg/kg). CK activity and lactate concentration significantly increased at the highest dose (50 mg/kg), with this increase being higher upon tapentadol exposure, and CK-MB activity also increased at the highest dose (50 mg/kg) of both. Glucose concentration did not change significantly after a 24-h exposure to the doses tested. The typical effective dose (10 mg/kg) did not lead to significant differences comparatively with the control group.

Besides blood, biochemical parameters were also analyzed in cerebral cortex samples (Fig. 2). LDH activity increased significantly after exposure to 50 mg/kg tapentadol, and lactate concentration increased significantly after exposure to 50 mg/kg tramadol and 25 and 50 mg/kg tapentadol. CK activity did not change significantly after exposure to the doses tested, for 24 h.

3.2. Tramadol and tapentadol increase protein oxidation in lung and heart tissues

In order to evaluate possible oxidative damage and activation of apoptosis, caused by an acute exposure to tramadol and tapentadol, the concentrations of protein carbonyl groups and LPO, as well as SOD, CAT and caspase 3 activities were analysed in brain cortex, lung and heart samples. In brain cortex samples taken from rats exposed to tramadol or tapentadol, oxidative damage was not detected (Fig. 3). Interestingly, at low doses of both compounds (10 or 25 mg/kg), there was a reduction in LPO, and exposure to tramadol caused a decrease in protein carbonyl groups at all doses tested. Thus, these compounds do not appear to induce brain lipid oxidative damage in an acute exposure, and possibly have a protective effect for lipid oxidation, especially at low doses. In lung samples (Fig. 3), analysed after rat exposure to tramadol or tapentadol, no alterations in LPO were detected. However, a significant increase in protein carbonyl groups was detected after exposure to 50 mg/kg tramadol and 25 and 50 mg/kg tapentadol. The results of oxidative damage evaluation in the heart, upon acute exposure to tramadol or tapentadol, show a significant increase in protein carbonyl groups at the highest doses (50 mg/kg tramadol and 25 and 50 mg/kg tapentadol) (Fig. 3). In the lower effective dose these compounds showed a protective effect. Regarding LPO, in heart samples no changes were detected in comparison to the control group. Concerning SOD and CAT, no changes were detected in their brain, heart and lung activity, compared to the control (data not shown).

Regarding the assessment of caspase 3 activity in brain cortex and lung samples, no increase was detected relative to the control for both opioids (Fig. 4). However, in heart samples from rats exposed to tramadol at all doses, a decrease in caspase 3 activity was detected compared to the control group. In heart samples from rats exposed to tapentadol, no changes were detected, with respect to the control group.

3.3. Tramadol and tapentadol cause neural degeneration and damage to the lung and heart

Brain cortex (Fig. 5), lung (Fig. 6) and heart (Fig. 7) samples of rats treated with tramadol or tapentadol, and the control group, were investigated histologically. All groups treated with tramadol or tapentadol were compared with negative control tissues (non-treated rats).

After treatment with 10 mg/kg tramadol in brain tissues, swollen neurons and vacuolization were observed (Fig. 5). The increment in tramadol dose (25 and 50 mg/kg) caused an increase in damage/histological changes. At the 25 mg/kg tramadol dose, degenerated neurons, with darker staining, were observed, as well as a lower definition of the nuclear membrane, which appeared irregular in shape. In parallel, smaller cells were observed, suggesting the occurrence of cell death. In addition, after administration of 50 mg/kg tramadol, there was an increased number of degenerated neurons, more darkly stained, and showing focal aggregation of the glial cells (focal gliosis). In animals treated with tapentadol, brain damage, observed through histology, was more pronounced and detected at all doses. After a 10 mg/kg tapentadol dose, swollen neurons and neurons with marked cytoplasmic vacuolization were observed, besides a lower definition of the nuclear membrane, appearing irregular in shape. In higher tapentadol doses (25 and 50 mg/kg), neuronal damage was more pronounced, with various neurons with darker staining, indicating neural degeneration, as well as glial activation with microglial proliferation.

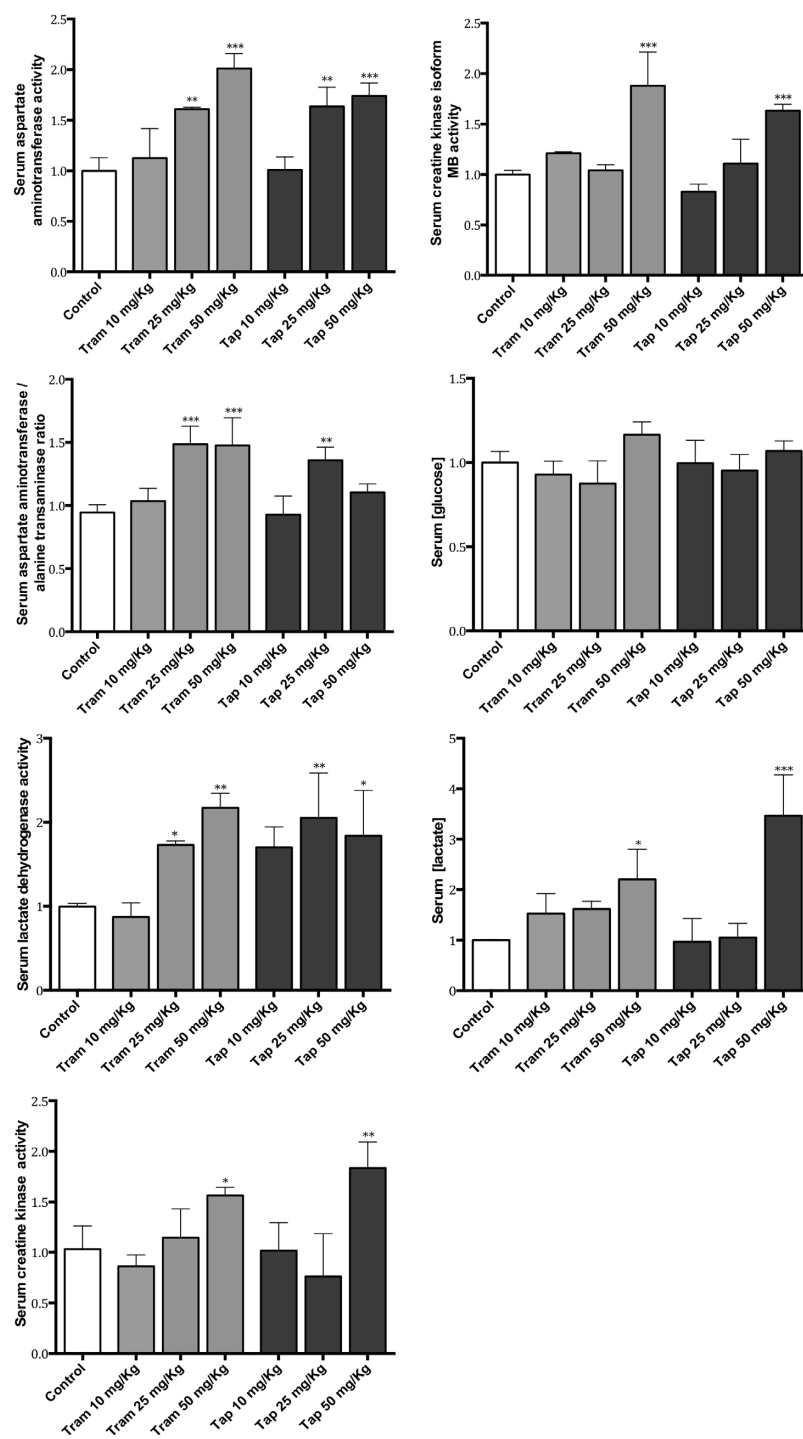


Fig. 1. Activities of: aspartate aminotransferase, aspartate aminotransferase/alanine transaminase ratio, lactate dehydrogenase, creatine kinase, creatine kinase isoform MB; and glucose and lactate concentrations in rat serum samples after exposure to tramadol or tapentadol for 24 h. Results were normalized against the value obtained in the absence of opioid (control group), set as 1. Results are expressed by means $\pm$ SD. *** p < 0.001, ** p < 0.01, * p < 0.05.

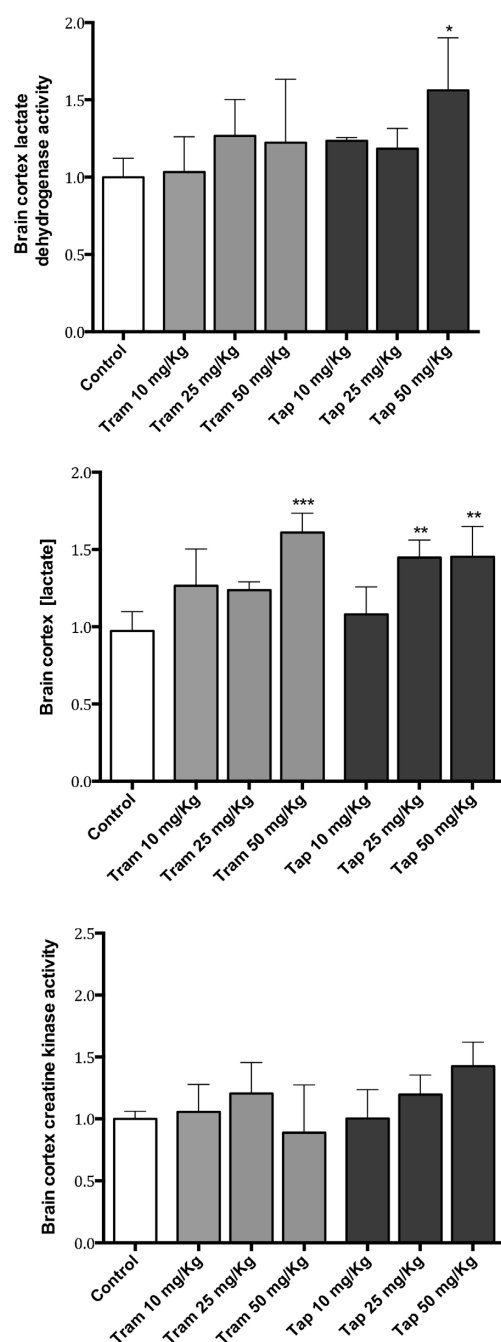


Fig. 2. Lactate dehydrogenase and creatine kinase activities, and lactate concentration in rat brain cortex samples after exposure to tramadol or tapentadol for 24 h. Quantifications were normalized against the protein content. They were also normalized against the value obtained in the absence of opioid (control group), set as 1. Results are expressed by means $\pm$ SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Lung histological analysis, after H & E staining, is shown in Fig. 6. Tramadol exposure causes lung injury, and the increase in its dose led to an increase in histological changes. After 10 mg/kg doses, interstitial changes (congestion) were observed; in addition, after 25 and 50 mg/kg

administration, intra-alveolar hemorrhage, alveolar collapse and destruction were observed. Acute exposure to tapentadol caused several alterations at all doses, such as inflammatory cell infiltrates, interstitial changes (hemorrhage) and, at higher doses (25 and 50 mg/kg), alveolar collapse was observed.

Fig. 7 shows the histological analysis of heart tissue samples. After treatment with 10 mg/kg tramadol, no alterations or damage were observed. Nevertheless, at intermediate and higher doses (25 and 50 mg/kg tramadol), altered cardiomyocytes and inflammatory cell infiltrates were observed, and loss of striation of cardiomyocytes was detected. In heart samples taken from rats exposed to tapentadol, alterations were observed at all doses. At the lowest dose (10 mg/kg tapentadol), loss of cardiomyocyte striation and inflammatory cell infiltrates were observed, and altered cardiomyocytes were also detected along with dose increase.

4. Discussion

The therapeutic use of opioids, namely tramadol and tapentadol, is increasing nowadays. Cases of toxicity have been reported, emphasizing the need to understand their toxicity mechanisms (Barbosa et al., 2016; Clarkson et al., 2004; Kemp et al., 2013). In this context, it is important to use animal models to study the toxic effects and particularly to study the possibility of brain damage, since these opioids target the CNS. Especially for tapentadol, this is the first study that evaluates rat brain, lung and heart alterations resulting from its exposure. Moreover, this is, to our knowledge, the first study dealing with the comparative brain, pulmonary and cardiac toxic potential of tramadol and tapentadol in an *in vivo* model.

The analysis of biochemical parameters in blood samples, following 24 h of exposure, showed changes in several parameters. AST is an important enzyme in amino acid metabolism, being found in several organs, such as heart, liver, muscle and brain. Serum AST/ALT ratio is commonly measured as a clinical biomarker for liver and muscle damage. Given the high AST/ALT activity ratio obtained following administration of tramadol and tapentadol, reflecting a higher increase in AST activity than in that of ALT, it is possible that muscle injury is happening. Increases in serum AST activity, upon exposure to tramadol in different dosages and periods, have already been described by different authors (Atici et al., 2005; El Fatoh et al., 2014; Ezzeldin et al., 2014; Saleem et al., 2014; Youssef and Zidan 2015). Increased AST activity relative to that of ALT was reported previously in a case of human tramadol intoxication (Wang et al., 2009). Increases in AST activity are also associated with the exposure to other opioids, such as morphine and heroin (Atici et al., 2005). In the highest tapentadol dose, we observed a higher increase in ALT activity, possibly denoting liver injury, in addition to muscle damage. Consequently, the AST/ALT ratio is lower than for other doses. However, for tapentadol there are no studies on the effect on serum AST levels. Thus, the results suggest that tramadol and tapentadol may cause muscle damage, most likely in the cardiac muscle, and with more pronounced effect at toxic doses. LDH is an enzyme that catalyzes the interconversion of lactate to pyruvic acid, as it converts NAD^+ to NADH and back. Tissue breakdown, such heart muscle, releases LDH; consequently, serum LDH activity can be measured as a tissue breakdown biomarker. Increased serum LDH activity, after exposure to tramadol, has been described by Atici et al. (Atici et al., 2005), although the increase was not significant. Other opioids, such as morphine and heroin, are associated with an increased LDH activity, as well as ALT and AST activities, after prolonged exposures (Atici et al., 2005; Borzelleca et al., 1994). Similarly, this increased LDH activity in serum also suggests the occurrence of cell lysis, and that the damage caused by tramadol and tapentadol is evident from 25 mg/kg. Although toxicity on metabolizing organs was not the focus of this study, the alterations in AST/ALT levels, as well in LDH activity, may additionally suggest liver toxicity, since these markers are not specific. Thus, such possibility cannot be ruled out and deserves to

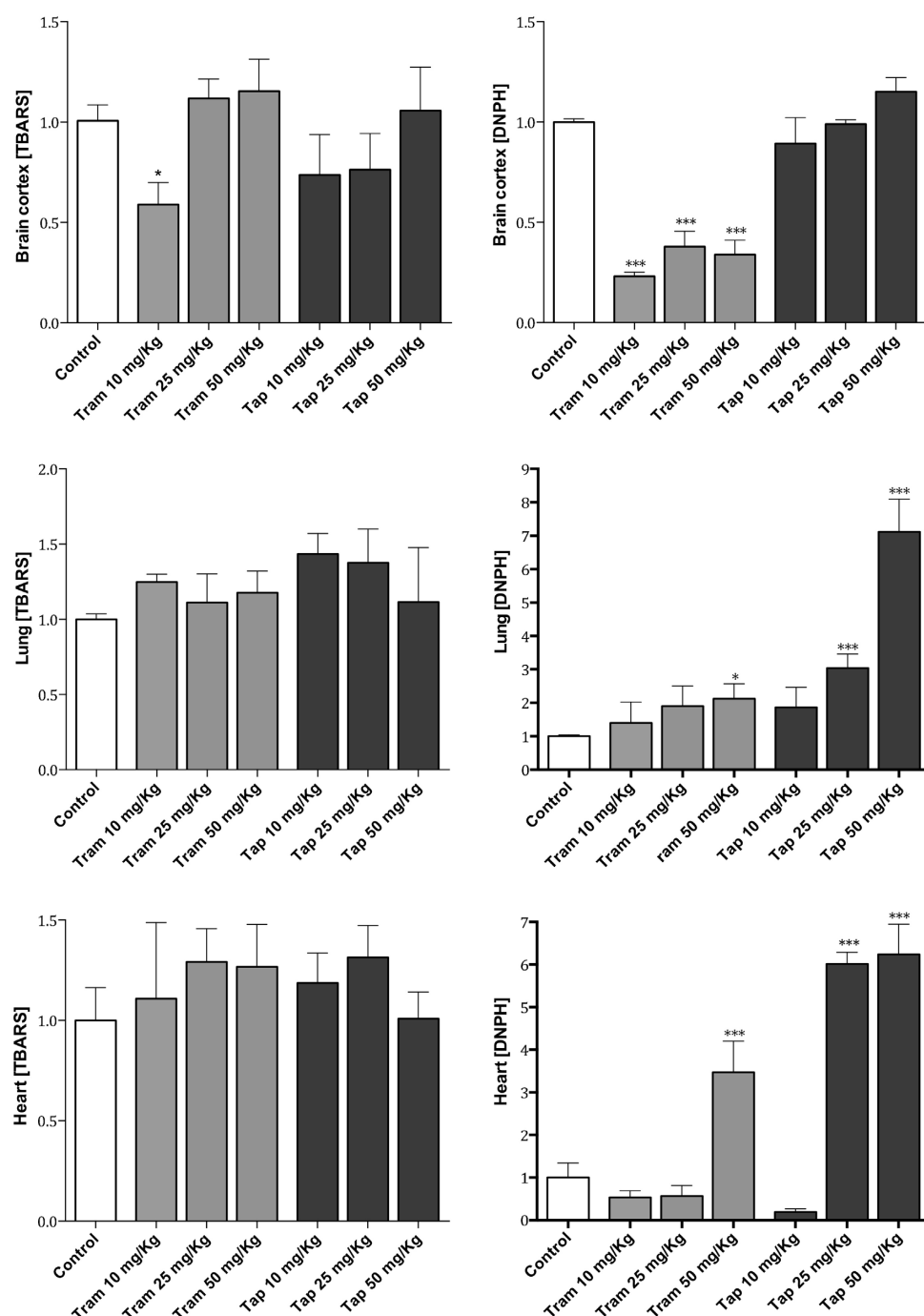


Fig. 3. Lipid peroxidation and protein carbonyl group concentrations in rat brain cortex, lung and heart samples after exposure to tramadol or tapentadol for 24 h. Quantifications were normalized against the protein content. They were also normalized against the value obtained in the absence of opioid (control group), set as 1. Results are expressed by means $\pm$ SD. *** $p < 0.001$, * $p < 0.05$.

be evaluated in future assays. In turn, CK catalyzes the interconversion of creatine and phosphocreatine. In the CK activity assay, the activity of all isoforms is measured, whereas CK-MB is a specific cardiac marker. Therefore, the increase in CK-MB activity, together with the increased

AST and LDH activities, suggest that tramadol and tapentadol cause damage to the cardiac muscle. Moreover, in situations of death from tramadol exposure, myocardial damage is reported as one of the autopsy findings (Mannocchi et al., 2013), and increased CK and CK-

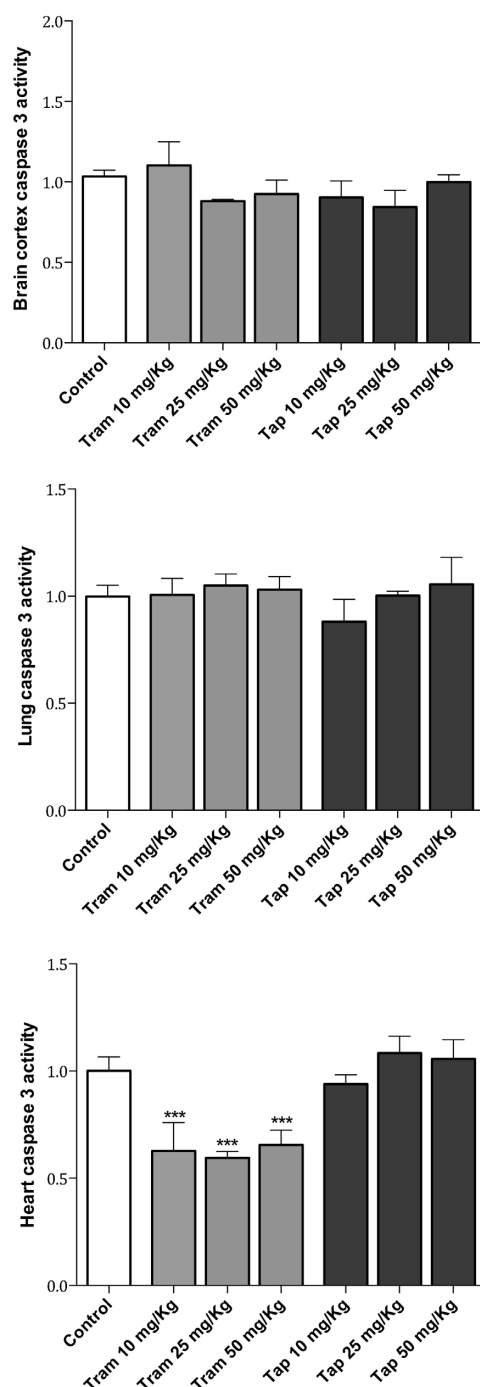


Fig. 4. Caspase 3 activity in brain cortex, lung and heart samples after exposure to tramadol or tapentadol for 24 h. Quantifications were normalized against the protein content. They were also normalized against the value obtained in the absence of opioid (control group), set as 1. Results are expressed by means $\pm$ SD. *** $p < 0.001$.

MB activities were described in tramadol overdose (Wang et al., 2009). Serum lactate concentrations increased after exposure to the highest doses. *In vitro* studies by our group have shown that an acute exposure

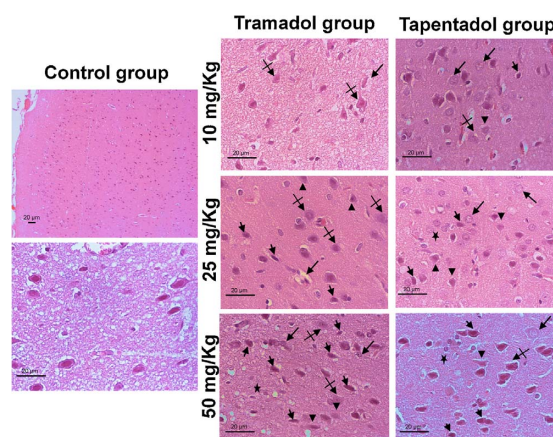


Fig. 5. Microscopy analysis, after staining with hematoxylin and eosin, of brain cortex samples from control Wistar rats and rats acutely exposed to different tramadol or tapentadol doses. After tramadol exposure, several cell alterations are observed, such as swollen neurons (crossed arrows) and vacuolization (long arrows), lower definition of nuclear membranes, appearing irregular in shape (arrow heads), degenerated neurons, with darker staining (short arrows) and aggregation of the glial cells (star). After exposure to tapentadol several cell alterations are observed, in all conditions, such as swollen neurons (crossed arrows) and marked cytoplasmic vacuolization (long arrows), lower definition of nuclear membrane, appearing irregular in shape (arrow heads), activation of glial cells (stars) and neurons with darker staining (short arrows), indicating neural degeneration. Magnifications 100 $\times$ and 600 $\times$; scale bar, 20 μ m.

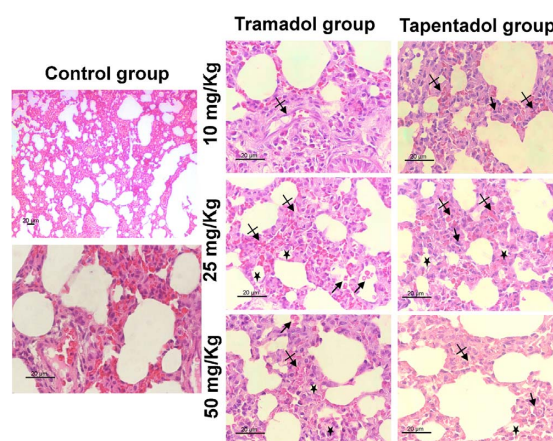


Fig. 6. - Microscopy analysis, after hematoxylin and eosin staining, of lung tissue samples from control Wistar rats and rats acutely exposed to different tramadol or tapentadol doses. After the 10 mg/kg tramadol dose, interstitial changes - congestion (crossed arrow) - were detected; at the 25 mg/kg dose, interstitial changes comprising congestion (crossed arrow), intra-alveolar hemorrhage (arrow) and alveolar collapse (star) were observed; in addition, at the higher tramadol dose (50 mg/kg), alveolar collapse and destruction (star) were observed. After exposure to tapentadol, several cell alterations are observed, in all conditions, such as interstitial changes - hemorrhage (crossed arrow) and inflammatory cell infiltrates (arrow). At 25 mg/kg and 50 mg/kg tapentadol doses, alveolar collapse (star) was observed. Magnifications 100 $\times$ and 600 $\times$; scale bar, 20 μ m.

to tramadol and tapentadol causes an increase in lactate levels as a result of alterations in the expression levels of enzymes involved in energetic metabolism (Faria et al., 2016). These changes, and the consequent bioenergetic crisis resulting from the exposure to opioids, including tramadol and tapentadol, have been suggested by several authors (Faria et al., 2016; Perez-Alvarez et al., 2010; Zhuo et al., 2012). In fact, changes in energetic metabolism and cell lysis cause an increase in lactate levels, which is more evident at the highest dose

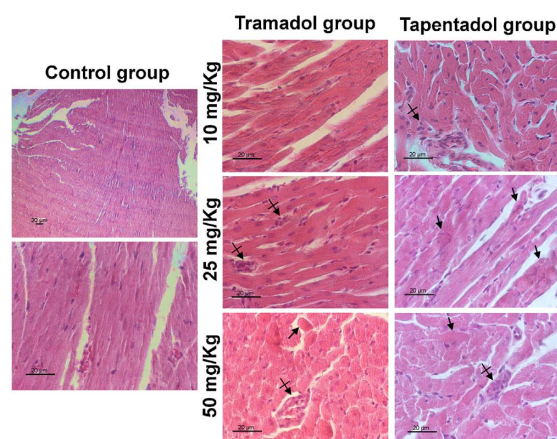


Fig. 7. Microscopy analysis, after hematoxylin and eosin staining, of heart tissue samples from control Wistar rats and rats acutely exposed to different tramadol or tapentadol doses. At 10 mg/kg tramadol dose, no alterations were detected. At the highest doses of tramadol (25 mg/kg and 50 mg/kg), some alterations were detected, such as altered cardiomyocytes (arrows), inflammatory cell infiltrates (crossed arrows) and loss of striation of cardiomyocytes. At the 10 mg/kg tapentadol dose, loss of striation of cardiomyocytes and inflammatory cell infiltrates were observed (crossed arrows); at the 20 mg/kg dose, altered cardiomyocytes (arrows) were observed; at 50 mg/kg doses, altered cardiomyocytes (arrows) and inflammatory cell infiltrates (crossed arrows) were observed. Magnifications 100 $\times$ and 600 $\times$; scale bar, 20 μ m.

analyzed (50 mg/kg).

In parallel, oxidative stress parameters were studied, and histological analyses were performed in brain, lung and cardiac tissues. Oxidative damage was detected in lung and cardiac muscle tissue, in which we detected a significant increase in protein carbonyl groups when tramadol and tapentadol highest dose (50 mg/kg) was used, as well as at tapentadol intermediate dose (25 mg/kg). These results, combined with the changes detected in biochemical parameters, suggest that tramadol and tapentadol cause heart damage, with tapentadol having more toxic effects. Regarding the activity of antioxidant enzymes, no significant changes were detected. Nevertheless, in assays with prolonged exposure to a low tramadol dose (1/5 of the LD₅₀ for 4 weeks), a significant increase in LPO in brain and testicular tissues has been found, as well as a significant decrease in the gene expression of antioxidant enzymes (e.g., Cu Zn SOD, Mn-SOD and CAT) (Ghoneim et al., 2014). Similarly, Awadalla and Salah-Eldin also detected an increase in LPO in serum samples, and a decrease in GSH, SOD and CAT expression, after chronic treatment with tramadol (Awadalla and Salah-Eldin, 2016).

It has been previously suggested that the inhibition of NA reuptake by tapentadol may potentially lead to adverse effects at the cardiovascular level (Vadivelu et al., 2011), although such effects are not always observed (McCarberg, 2007). In the case of heroin exposure, several papers analyzed cardiac pathology, though also with inconsistent results (Milroy and Parai, 2011). Histological analyses of samples collected from heroin users have shown an increase in the number of inflammatory cells in the myocardium in comparison to the control group, suggesting an activation of the cellular immune system (Dettmeyer et al., 2009). Accordingly, we also observed infiltrates of inflammatory cells at the highest dose of tramadol and at all tapentadol doses. However, some studies using experimental models suggest that the treatment with tramadol relieves myocardial injuries induced by ischemia reperfusion (Takhtfooladi et al., 2015). Even so, a study evaluating exposure to tramadol, after surgical coronary revascularization, concluded that tramadol may aggravate myocardial injury (Wagner et al., 2010), so in situations upon heart surgery the use of tramadol or tapentadol should be balanced.

In situations of death from opioid overdose, prominent intra-alveolar hemorrhage (Milroy and Parai, 2011) may be observed, and we also identified this finding following exposure to 25 and 50 mg/kg tramadol and at all doses of tapentadol. Similarly to our results, Samaka et al. described several lung alterations in albino rats after an acute tramadol LD₅₀ dose (Samaka et al., 2012). These authors identified interstitial changes (such as pulmonary congestion, hemorrhage and inflammatory cellular infiltrates) and alveolar changes (such as alveolar damage, wall thickening and destruction) (Samaka et al., 2012). Likewise, Awadalla and Salah-Eldin described several histopathological alterations in rat lung, after chronic tramadol treatment (such as inflammatory cellular infiltrates, congestion and intra-alveolar hemorrhage) (Awadalla and Salah-Eldin, 2016). In our study, we observed that tapentadol is more toxic to the lung than tramadol, for the same dose and exposure period, since it caused more marked damage at all doses. Thus, tramadol and tapentadol use may be a potential factor for lung function impairment, and in fact congested lung is an autopsy finding in deaths by tramadol and tapentadol overdose (Kemp et al., 2013).

The brain is particularly susceptible to oxidative damage, because of its high levels of oxygen consumption; however, the analysis of oxidative damage in the brain did not detect increased damage relative to the control, under our experimental conditions, with these results being consistent with our previous *in vitro* results, in a neuronal cell model, which deemed that LPO is not an implied mechanism of acute toxicity (Faria et al., 2016). Likewise, Kose et al. also did not detect statistically significant differences regarding brain LPO levels after tramadol administration, by the intracisternal route, in rat cerebrospinal fluid (Kose et al., 2014). However, our results also evidenced that oxidative damage to other organs is dose-dependent. Therefore, the possibility of LPO should be considered for a prolonged exposure to high doses, as a consequence of oxidative damage, as suggested by several authors, concerning different opioids (Atici et al., 2005; Awadalla and Salah-Eldin 2016; Ragab and Mohamed 2016; Zhang et al., 2004).

The histopathological analysis of rat brain cortex after tramadol and tapentadol exposure showed damage in all tested conditions. In this sense, in the specific case of tramadol, histological abnormalities have been described in the brain, after prolonged administration, by Ghoneim et al., including darkly stained irregular pyramidal cells, marked vacuolization, neuronal cell disorganization and hypercellularity, as well as inflammatory cell infiltration (Ghoneim et al., 2014). Awadalla and Salah-Eldin also observed several histological alterations after chronic treatment with tramadol in brain cortex and hippocampus, such as disorganization of cortical layers, degeneration and congestion (Awadalla and Salah-Eldin, 2016). Similarly, El Fatoh et al. (2014) also detected brain changes, such as neuronal degeneration, after prolonged exposure to tramadol. In turn, Ragab and Mohamed reported several brain alterations after exposure for several days, such as neuronal cell disorganization, intercellular edema, apoptotic cells, degenerative vacuolization and dark neurons with hyperbasophilia (Ragab and Mohamed, 2016). In the same way, Nafea et al. observed neurotoxic effects, through biochemical and histopathological analyses, in rats upon exposure to tramadol, or tramadol in association with cannabis (Nafea et al., 2016). Consistently with our results, 24 h after a single 300 mg/kg tramadol dose, by oral gavage, brain alterations were observed, such as brain congestion and inflammatory cell infiltrates (Samaka et al., 2012). Regarding the exposure to tapentadol, histological alterations were similar, but more pronounced, to those observed for tramadol, which is expectable given the structural and mechanistic similarity of both drugs (Barbosa et al., 2016). *In vivo* studies showed that the combined mechanism of action of tapentadol results in a lower inhibition of hippocampal neurogenesis than that induced by morphine, as well as other classical opioids, which make tapentadol a good alternative in neuropathic pain, offering a lower risk for cognitive impairment (Meneghini et al., 2014). Nevertheless, our

results support that tramadol and tapentadol cause toxicity to brain tissues. Then, it is important to focus the attention on the toxic potential of tapentadol, which has been underexplored. Different authors suggest that the neuronal degeneration found in rat brains exposed to high doses of morphine or tramadol probably contribute to brain dysfunction (Atici et al., 2004; Ragab and Mohamed 2016). According to our results, this also happens after tapentadol exposure. Previously, it was suggested that brain histological changes and neuronal degeneration result from the effect of tramadol on neuronal glucose metabolism and the insulin signaling pathway (Ghoneim et al., 2014). Thus, the increase in lactate levels detected in the brain, after exposure to tramadol and tapentadol at higher doses, may contribute to neuronal degeneration. Accordingly, our group has demonstrated, in cellular models, that tramadol, as well as tapentadol, cause a bioenergetic crisis (Faria et al., 2016). Consequently, metabolic changes may be responsible for some of the neuronal damage detected.

In order to search for apoptosis evidence, caspase 3 activity was assessed, but no increases were detected. Likewise, other *in vivo* studies showed no apoptosis induction after tramadol exposure in cerebrospinal fluid obtained from patients (Bonelli et al., 2005), and *in vitro* studies show that necrosis is the main mechanism for cell death after tramadol and tapentadol exposure (Faria et al., 2016). In lung samples, analyses after chronic exposure to tramadol also did not detect a significant increase of caspase 3 activity (Awadalla and Salah-Eldin, 2016). However, chronic treatment with tramadol was associated with apoptotic changes in brain tissues (Awadalla and Salah-Eldin, 2016); therefore, under chronic exposure, a possible apoptotic effect should be considered. In this sense, a single tramadol dose provides some protective effects, such as the decrease in brain TBARS or caspase 3 lung activity, for which tramadol may represent a better alternative in acute treatment situations. The difference observed between tramadol and tapentadol, in these aspects, might be due to mechanistic differences between both opioids. However, as previously discussed, prolonged exposure should be pondered for both compounds.

It must be added that, although the same doses were used for tramadol and tapentadol, these are not pharmacologically equivalent, given the distinct bioavailability of both compounds. Indeed, although they are both rapidly absorbed after oral administration, tramadol bioavailability is reported as approximately 68–84%, while that of tapentadol is only 32% (Barbosa et al., 2016; Grond and Sablotzki 2004). In this study drugs were administered ip, and although it is considered a parenteral administration since it bypasses the intestine, the primary route of absorption is into the mesenteric vessels, which drain into the portal vein and pass through the liver (Lukas et al., 1971). Therefore, substances also undergo hepatic metabolism upon intraperitoneal administration, before reaching the systemic circulation (Lukas et al., 1971). Accordingly, in order to obtain pharmacologically equivalent doses, tapentadol doses could actually be increased, to compensate its lower bioavailability. However, even at the administered doses, tapentadol was shown to induce more toxic effects; it therefore may be anticipated that these results would be exacerbated for higher doses. It should be also mentioned that, since a greater toxicity was registered in tapentadol-exposed rats, differences between tramadol and tapentadol regarding their affinity for receptors and transporters and the potential pharmacodynamics of their metabolites, should be considered as possible causes for the observed differences.

Lastly, it is also important to highlight that toxic changes have also been described, in the case of tramadol, in other organs, such as liver and kidney (Atici et al., 2005; Awadalla and Salah-Eldin 2015; Ezzeldin et al., 2014; Saleem et al., 2014; Samaka et al., 2012), and a multiple organ dysfunction syndrome was described in a situation of tramadol intoxication alone (Wang et al., 2009).

5. Conclusions

The results of the present study demonstrate that tramadol and

tapentadol cause brain, pulmonary and cardiac toxic damage after a single exposure, in typical effective doses, intermediate doses and maximum recommended daily doses. From the histological and serum biochemical perspective, the treatment with tapentadol seems to be more toxic. Exposure to tramadol and tapentadol causes brain injury, evidenced by the increased lactate levels, and histological damage, namely swollen neurons, poorly defined nuclear membranes and neuronal death. Pulmonary alterations, such as intra-alveolar hemorrhage and alveolar collapse and destruction, were also detected after tramadol and tapentadol exposure. In addition, both drugs cause an increase in AST, LDH and CK activities, which, together with the histological damage to the heart, indicate that these drugs cause heart damage. Further studies are required to evaluate the toxicity in other organs, such as liver and kidney, as well as to perform chronic exposure experimental designs. Indeed, it should be reminded that these opioids are frequently prescribed on a chronic basis, for which prolonged exposure assays would mimic clinical reality. In addition, since opioids are often co-administered with other drugs, such as selective serotonin re-uptake inhibitors, tricyclic antidepressants and monoamine oxidase inhibitors, *in vivo* combined exposure assays would also be relevant.

The present study highlights *in vivo* toxic effects triggered by tramadol and tapentadol even in acute exposure situations and considering lower, effective doses, emphasizing that these modifications (although minor) with therapeutic dosage occur with just one administration. Our study underlined the need for a careful selection and prescription of both opioids, since prolonged daily therapy may lead to damage accumulation.

Acknowledgements

This work was supported by grants from CESPU (TETT-CESPU-2014, TramTap-CESPU-2016 and ChronicTramTap-CESPU-2017); and project NORTE-01-0145-FEDER-000024, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). Juliana Faria is a PhD fellowship holder from FCT (SFRH/BD/104795/2014). Ricardo Dinis-Oliveira acknowledges Fundação para a Ciência e a Tecnologia (FCT) for his Investigator Grant (IF/01147/2013).

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PART III

1. INTEGRATED OVERVIEW OF THE PERFORMED STUDIES

The increase in the consumption and abuse of opioid pain medications is a worldwide problem. Likewise, the mortality due to opioid overdose is a major issue in public health. Accordingly to the Portugal Country Drug Report concerning year 2015, produced by the European Monitoring Centre for Drugs and Drugs Addiction, 31858 high-risk opioid users (4.9 per 1000), and 17011 people in opioid substitution treatment were reported (EMCDDA 2017). Another important information reported was the detection of opioids in 73% of all cases where toxicological analysis was performed, with heroin being the most frequently detected opioid (EMCDDA 2017). In addition, since 2013 there has been an increasing trend in the number of deaths (EMCDDA 2017). Furthermore, a retrospective analysis of fatal intoxication cases by xenobiotics, between 2001 and 2013, autopsied in northern Portugal, reported the highest incidences for medicines (22.9%), with opioids being involved in 5.75% of cases, thereby corresponding to the fifth place on xenobiotic detection in such *post-mortem* analysis (Alves et al., 2017).

In this regard, and considering the risk and the high number of opioid users, it is important to be alert for the easy access to prescription opioids, which may be an alternative, more accessible and cheaper, for illicit opioid users. For instance, in Iran, tramadol poisoning has been one of the most frequent causes of drug intoxication (Shadnia et al., 2008). Therefore, opioid therapy should be considered only when non-opioid therapies are inefficient, and the patients should be maintained under medical supervision (O'Brien et al., 2017). In addition, and considering the high prescription of opioids, it is important to understand the possible toxicity triggered by them. In fact, in the Portuguese list of “Top 100 Active Substances with Highest Number of Packages in the NHS” by the “Medicine and healthcare products statistics 2014”, tramadol occupies the 80th position, but the formulation that combines tramadol and paracetamol is in the 20th position (INFARMED 2014). In turn, tapentadol is a more recent drug, being included in the table of narcotic drugs and psychotropic substances, amended by the decree-law 15/93 of 22 January in 26 March 2012 (law number 13/2012 of 26 March), which approved the legal regime applicable to the trafficking and consumption of narcotic drugs and psychotropic substances.

As previously described, tramadol and tapentadol are synthetic analgesic opioids with a dual and synergic mechanism of action, which increases their analgesic efficiency and decreases the number of side effects. Both are intensively prescribed in

acute and chronic contexts, for different types of pain (such as postoperative and cancer pain), for which their consumption in clinical settings is also increasing nowadays. Despite their lower incidence of side effects, as described, cases of abuse and toxicity were reported, and their safety should be carefully analysed. For instance, in some countries, such as Iran, Sweden, Ukraine, Egypt, Brazil, China, the United Kingdom and the United States, increased tramadol use and abuse is a problematic concern. In contrast, several reports suggest that tapentadol abuse is infrequent (Butler et al., 2015; Pergolizzi et al., 2017), which may be due to its lower MOR affinity (Tzschentke et al., 2007). However, more studies are needed to evaluate tapentadol potential abuse, and to support the comparison between tramadol and tapentadol pharmacological and toxicological profiles. Regarding toxic effects, in addition to the more common adverse effects (such as dizziness, vomiting, constipation and sweating), fatal intoxications have been reported for both opioids (Cantrell et al., 2016; Clarkson et al., 2004; Franco et al., 2014; Kemp et al., 2013; Larson et al., 2012; Pilgrim et al., 2011; Shadnia et al., 2008). Although some studies have been performed in order to analyse tramadol and tapentadol toxic damage in different organs, and to explain their toxicity, the corresponding mechanisms are not completely understood yet. Concerning tapentadol in particular, few studies had been accomplished to evaluate its toxicity, and little is known about its potential toxicity and underlying mechanisms.

Accordingly, the work presented in this thesis was developed with the global objective of comparing the acute toxicity of tramadol and tapentadol, with the purpose of increasing the knowledge about the toxic effects of opioid therapy in target organs, and thus to alert the health professionals to carefully and reasonably balance opioid prescription.

The work presented in CHAPTER I contributed with some advances in the characterization of the mechanisms of tramadol and tapentadol toxicity, and showed a comparative approach to the potential toxic effects of both opioids. A neuronal cell model (the SH-SY5Y cell line) was exposed to both opioids, at the same doses and time periods, to assess putative damage. After exposure, a decrease in cell viability was observed, showing that both compounds cause toxicity in this cell model, with tapentadol causing more toxic damage. *In vitro* results also showed that necrosis is the main mechanism of cell death, after tramadol and tapentadol treatment, with no significant increase in oxidative damage or in cytochrome *c* release. The toxicity

observed was a consequence of a bioenergetic crisis, characterized by a decrease in ATP intracellular levels, as well as an increase in glucose and lactate intracellular levels. Our results showed that the alteration in the expression of genes involved in energetic metabolism is an effect of tramadol and tapentadol exposure, and might be the cause of the bioenergetic crisis. These results are in agreement with proteomic studies that suggested that tramadol causes several alterations in energetic metabolism enzymes (Zhuo et al., 2012). Nevertheless, at least to our knowledge, we are presenting the first report concerning tapentadol exposure. Gene expression alterations, as a result of opioid exposure, were previously associated with dependence and memory deficit, as well as with neurodegeneration (Chen et al., 2007; Zhuo et al., 2012), so we should be alert to the possibility that tramadol and tapentadol may also cause such damage even after an acute exposure, and that it may increase when they are prescribed in high doses or within a chronic treatment. In addition, it is also important to emphasize that hypoglycemic events have been observed in real situations of tramadol medication (Choi et al., 2005). Nowadays, tapentadol is frequently suggested as a safer option than tramadol, for instance after cardiac surgery (Iyer et al., 2015). However, and considering our results, where tapentadol caused higher alterations in the parameters studied, we recommend careful and short-term prescription of both opioids, as well as the patient's follow-up throughout the entire treatment period.

Aiming at a more comprehensive analysis of the toxicity of tramadol and tapentadol, *in vivo* assays were performed, using male Wistar rats, as described in CHAPTER II. 24 hours after intraperitoneal administration of doses corresponding to a typical analgesic dose, an intermediate dose and the maximum recommended daily dose, the toxicological effects were assessed. The analysis of serum biochemical biomarkers showed that both drugs cause an increase in the AST/ALT ratio and in the activity of LDH, CK and CK-MB enzymes. These results suggested muscle damage, probably cardiac injury, as a result of the exposure to both opioids. In this context, the increase in the AST/ALT ratio and in CK and CK-MB activities had already been reported in a case of tramadol overdose; and accordingly to that, myocardial damage had been described in an autopsy finding, in a case of death associated to tramadol consumption (Wang et al., 2009). Accordingly, the increase in LDH activity suggests cell lysis, and the increase in CK-MB – a specific cardiac marker –, also corroborates the hypothesis of cardiac injury. In addition to serum analysis, histological assays were

performed and oxidative stress parameters were studied in brain, lung and cardiac tissues. Oxidative damage, through an increase in protein oxidation, was detected in lung and heart samples. In turn, the histological approach evidenced alterations in all the tissues under analysis (brain, lung and heart). Histological changes promoted by tramadol had previously been described by other authors, particularly in a chronic context (Awadalla and Salah-Eldin 2016; Samaka et al., 2012); however, we did also observe these alterations in an acute exposure and after exposure to tapentadol, with this being more toxic than tramadol. These findings, together with the *in vitro* results, support that tramadol and tapentadol cause brain toxicity, and underline the need for further assays concerning tapentadol toxicity. In addition, *in vivo* results showed an increase in lactate levels in brain tissue, which may reflect the bioenergetic crisis triggered by tramadol and tapentadol, as we had suggested in our *in vitro* assays (see CHAPTER I). Figure 1 lists the main toxic effects detected in brain, lung and heart specimens, after tramadol or tapentadol acute exposure, as assessed through biochemical and histological approaches.

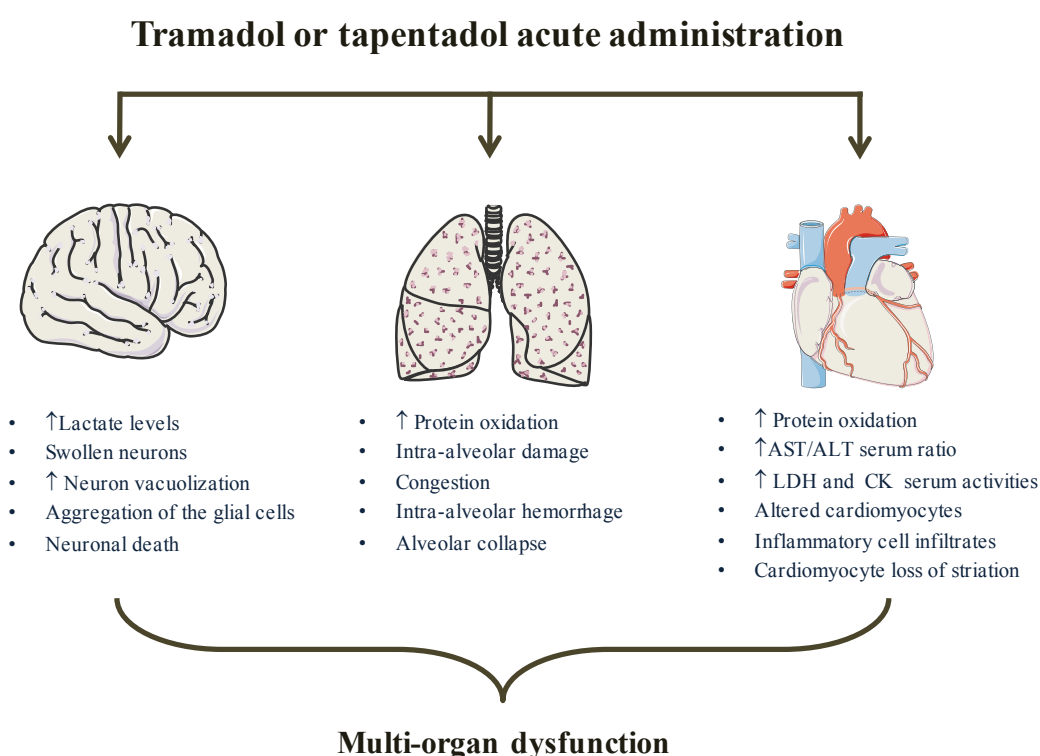


Figure 1: Main brain, lung and heart alterations detected after acute administration of effective analgesic doses of tramadol or tapentadol to Wistar rats. AST/ALT: aspartate aminotransferase/alanine aminotransferase; LDH: lactate dehydrogenase; CK: creatine kinase.

Although, in CHAPTER II, we present brain, lung and heart specific analysis, the multiple organ dysfunction syndrome, encompassing other organs, was previously described for tramadol intoxication (Wang et al., 2009). In this sense, additional studies by our group, reported in a paper by Barbosa *et al.* (2017), showed that tramadol and tapentadol acute administration causes hepatic and renal impairment, and once again tapentadol was comparatively more toxic than tramadol. For instance, an increase in the activity of ALT and a decrease in urea levels were observed, which, together with histological observations, such as sinusoidal dilatation, microsteatosis and cell degeneration, showed that tramadol and tapentadol cause liver damage, particularly at the highest dose tested (Barbosa et al., 2017). In this paper, through periodic acid-Schiff (PAS) staining, glycogen depletion was observed, after exposure to tramadol and tapentadol (Barbosa et al., 2017), in agreement with the metabolic and energetic changes we suggest in CHAPTER I and II, as a result of the exposure to tramadol and tapentadol. In the same context, changes in renal function, including proteinuria and a decrease in the glomerular filtration rate (GFR), were observed, while histological examination evidenced signs of tubular disorganization, inflammatory infiltrates and cell vacuolization. These results corroborate those presented in this thesis, showing that tramadol and tapentadol cause multi-organ toxic effects, even in an acute exposure and in a low-dose range.

We highlight that the results obtained for tapentadol are particularly important, since, to our knowledge, there were no *in vivo* studies with animal models on their toxicity in different tissues. In addition, it is important to be alert to the toxicological potential, as well as to the increased risk of their abuse, when these opioids are used in a chronic context. Thus, it is crucial to evaluate the effects of tramadol or tapentadol chronic exposure. In this context, chronic tramadol administration was associated with memory impairment, as a consequence of increased reactive oxygen species (ROS), mitochondrial membrane potential (MMP) collapse, and increased cytochrome *c* release, leading to brain mitochondrial dysfunction (Mehdizadeh et al., 2017). Therefore, we may expect tapentadol to cause the same alterations. In fact, in order to minimize the potential consequences of tramadol and tapentadol therapy, in cases of chronic treatment, a prolonged dose interval or a dose reduction are recommended during the treatment (O'Brien et al., 2017).

Our results are also a starting point for the study of the potential increase in toxicity resulting from the simultaneous consumption and misuse of opioids with other drugs. In accordance, the concurrent use of drugs is now a trend and a worldwide problem. For instance, in a study that analysed a sample of 2000 Iranian adolescents, the authors concluded that tramadol could be a related factor or co-factor for alcohol, *Cannabis* and MDMA (3,4-methylenedioxymethamphetamine; “ecstasy”) abuse in the adolescent population (Nazarzadeh et al., 2014). In Portugal, *Cannabis* is the most frequently used illicit drug among young adults (15-24 years), while MDMA is the second most reported drug (EMCDDA 2017).

Despite the higher incidence of simultaneous consumption of multiple drugs, the underlying toxic effects and corresponding cellular mechanisms are not completely understood yet. For instance, tramadol and MDMA co-administration may increase the risk of serotonergic syndrome (SS), given the increased risk of 5-HT accumulation in the central nervous system (CNS). Therefore, more studies should be performed in order to evaluate the effects of tramadol and MDMA combination. Regarding tapentadol, Franco *et al.* (2014) reported a fatal intoxication by this opioid, suggesting respiratory depression, CNS depression and SS as possible mechanisms of death. Additionally, and although tapentadol serotonergic activity is much more limited than its norepinephrinergic action (Raffa et al., 2012; Tzschentke et al., 2007), Walczyk *et al.* (2016) also reported a case of probable SS after tapentadol overdose, in combination with other medications, in particular duloxetine and amitriptyline. These authors contacted Janssen Pharmaceuticals, tapentadol manufacturer, by telephone, in order to inquire if SS had been implied as a consequence of tapentadol intake (Walczyk et al., 2016). The manufacturer’s senior therapeutic specialist replied that no cases were reported in phase 2 and 3 clinical trials; nonetheless, in post-marketing, SS had been reported in patients using tapentadol in combination with other serotonergic drugs (Walczyk et al., 2016). In this sense, further studies are needed to assess whether tapentadol, combined with other drugs, may cause SS.

The possibility of drug-drug interactions should also be emphasized for all drug mixtures. Tramadol is metabolized by cytochrome P450 enzymes, for which pharmacogenetic variations may be responsible for variability in their pharmacokinetics, accounting for their toxicity and analgesic efficiency. In this context, the CYP2D6 ultra-rapid metabolizer (UM) phenotype has been associated with toxicity

cases, for which a 30%-decrease in tramadol dose is recommended (Barbosa et al., 2016; Lassen et al., 2015). On the other hand, tramadol analgesic efficacy is lower in CYP2D6 poor metabolizers (PMs), who show increased tramadol half-life and decreased M1 concentrations. In these cases, an alternative drug is recommended (Barbosa et al., 2016; Lassen et al., 2015). In addition, concurrent consumption of other drugs may increase tramadol toxicity (Barbosa et al., 2016; Pilgrim et al., 2010; 2011). Concerning tapentadol, since it has a simpler metabolism and does not require metabolic activation, it is less susceptible to cases of metabolic interaction. In this context, and again using the MDMA example, Jamali *et al.* (2017) concluded, in a rat model, that MDMA affects tramadol absorption, distribution and metabolism. Indeed, *in vitro* studies suggest that MDMA may inhibit the CYP2D6 isoenzyme by competitive interaction and/or the formation of a metabolic intermediate (Capela et al., 2009), thereby decreasing the metabolism of tramadol when taken together. Accordingly, it is important to better understand the effects of drug mixtures in order to recognize and prevent their adverse reactions.

The results obtained within the scope of this thesis suggest that tramadol and tapentadol single exposure cause toxicity in an *in vitro* neuronal model, as well as in different organs in an *in vivo* model. Therefore, we suggest increased education and awareness about opioid prescription, and that the doses are carefully adjusted to each patient, in ways that maximise benefit and minimise harm. Lastly, we underline the recommendation of other authors for the reduction of the period of opioid prescription, in order to decrease the risk of opioid toxicity, misuse, addiction and overdose.

PART III

2. CONCLUSIONS

This thesis evidenced several alterations in *in vitro* and *in vivo* models, as a result of tramadol and tapentadol exposure. The major conclusions are summarized below:

1. Tramadol and tapentadol caused neurotoxic effects in an *in vitro* model, the SH-SY5Y cell line;
2. Tapentadol showed to trigger much more prominent toxic effects than tramadol, for the same concentrations and time exposure period, causing a higher decrease in cell viability, which is reflected in its lower IC₅₀ value (200 μ M and > 600 μ M, respectively);
3. The main mechanism of cell death, after exposure to both opioids, is necrosis, although apoptotic cells were observed after tapentadol exposure, as well as a slight increase in caspase 3 and 9 activities;
4. Oxidative stress does not have a major contribution to tramadol and tapentadol toxic effects, given that no significant oxidative alterations in lipids and proteins were detected;
5. Both opioids cause bioenergetic alterations, through an increase in intracellular glucose and lactate levels, and a decrease in ATP levels;
6. Both drugs lead to alterations in the expression of genes involved in energetic metabolism (they decrease the expression of LDH, NDUFS1 and CKB, and increase the expression of PDK3 and ALDOC), leading to a bioenergetic crisis;
7. *In vivo* assays, after exposure to typical effective doses, intermediate doses and maximum recommended daily doses, showed that tramadol and tapentadol cause brain, lung and heart toxicity, with tapentadol being more toxic;
8. The AST/ALT ratio, LDH, CK and CK-MB activities and lactate levels increase in rat serum samples as a consequence of the treatment with tramadol and tapentadol;
9. Tramadol and tapentadol acute treatment causes oxidative damage, at the protein level, in lung and heart rat tissues;
10. Brain, pulmonary and cardiac rat tissues are affected by tramadol and tapentadol, evidencing signs of inflammation, cell death and cell morphology alterations after histological analysis.

PART III

3. DIRECTIONS FOR FUTURE RESEARCH

Future studies are required in order to better understand the results obtained, as well as to continue with the study of tramadol and tapentadol toxicity.

Concerning *in vitro* experiments, and the metabolic alterations reported, it would be important to determine the expression of other energetic metabolism enzymes, such as PDH and ATP synthase beta-chain. Likewise, it will also be important to study the toxic effects in other cell line models, such as hepatic and renal cells, in order to analyse the effects in metabolizing organs.

Regarding *in vivo* studies, tramadol, tapentadol and their metabolites should be quantified in different organs, in order to evaluate their distribution and accumulation. In the same way, the use of a wider dose range is essential in further studies, to understand overdose cases. In addition, tramadol and tapentadol are often used in chronic treatments, and data on toxic effects resulting from chronic exposure are lacking, for which *in vivo* chronic assays should be performed. In chronic assays, alterations in several organs, such as metabolic and target organs, should be researched and correlated. Furthermore, although the simultaneous consumption of opioids with other drugs is an increasing trend, the underlying toxic effects and corresponding cellular mechanisms are not completely understood yet. Thus, *in vivo* combined exposure assays, such as with selective serotonin reuptake inhibitors, monoamine oxidase inhibitors and tricyclic antidepressants, would be relevant to evaluate possible interactions among different compounds, and the potential toxicity increase as a consequence of drug–drug interactions. In this context, we highlight the simultaneous consumption of opioids and MDMA, given that some MDMA consumers report opioid use in order to “come-down from ecstasy” or to minimize dysphoria following administration, which may increase the risk of serotonergic syndrome, as well as the risk of metabolic impairment.

Finally, the aim would be to contribute to the improvement of the patients’ quality of life, by individually selecting the best opioid and dose, as well as to alert the medical community to the potential opioid toxicity and abuse.

PART IV

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