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ONE “CLICK” TO SUICIDE: mechanistic understanding of fatal nitrite ingestion

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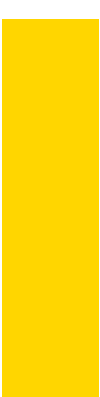
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ONE “CLICK” TO SUICIDE: mechanistic understanding of fatal nitrite ingestion

UM “CLIQUE” PARA O SUICÍDIO: entendimento mecanístico da intoxicação fatal com nitritos

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List of Abbreviations

AGS – Human gastric adenocarcinoma epithelial cells
DMEM – Dulbecco's modified Eagle's medium
DMSO – Dimethyl sulfoxide
ELISA – Enzyme-Linked Immunosorbent Assay
F – Female
FBS – Fetal bovine serum
FFUP – Faculty of Pharmacy, University of Porto
FiO₂ – Fraction of inspired oxygen
GC-MS – Gas Chromatography – Mass Spectrometry
G6PD – Glucose-6-phosphate dehydrogenase
Hb – Haemoglobin
HBSS (+/+) – Hanks' balanced salt solution with calcium and magnesium
HNO₂ – Nitrous acid
HS-GC-FID – Headspace Gas Chromatography with Flame Ionization Detection
IC – Inhibitory concentration
INMLCF – National Institute of Legal Medicine and Forensic Sciences, I.P.
LC-MS/MS – Liquid Chromatography with tandem Mass Spectrometry
M – Male
MetHb – Methaemoglobin
MTT – (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NA – Not available
NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NO – Nitric oxide
NOS – NO synthase enzymes
NR – 3-amino-7-dimethylamino-2-methylphenazine hydrochloride
PCO₂ – Partial pressure of carbon dioxide
PO₂ – Partial pressure of oxygen
ROS – Reactive oxygen species
SpO₂ – Oxygen saturation
THC – Tetrahydrocannabinol
THC-COOH – 11-Nor-9-carboxy- Δ^9 -tetrahydrocannabinol

Abstract

Sodium nitrite is a white, odourless and water-soluble powder, used in suicide attempts as increasingly reported, including in Portugal. It is described as a cheap, easily obtained, and pain-free lethal substance. Death is usually attributed to methaemoglobinemia, but additional toxicity mechanisms may be at play.

The purpose of this work was to characterize this type of intoxication and to investigate potential toxicity mechanisms. A toxicological analysis from a forensic autopsy of a suspected suicide that occurred in Portugal was carried out. Qualitative and quantitative analysis of nitrites in biological and non-biological samples were performed using the Griess reaction. Methaemoglobinemia was measured using a ferricyanide and potassium cyanide spectrophotometric technique. An *in vitro* investigation of sodium nitrite/nitrate toxicity was subsequently conducted to test for a possible direct toxic effect. H9c2, Caco-2, and SH-SY5Y cells were cultured and exposed to sodium nitrite/nitrate and cell viability assays were performed to obtain full concentration-toxicity curves. To study the possible conversions of sodium nitrite into other chemical species along the gastrointestinal tract, *in vitro* digestion assays were conducted. These tests mimicked the sequential physiological conditions during an oral, gastric, and intestinal phases, under conditions similar to a suicide by nitrite ingestion. Additionally, to understand the levels of nitrites/nitrates capable of inducing methaemoglobinemia, the ferricyanide and potassium cyanide spectrophotometric technique was applied using fresh human blood samples.

The values of nitrites found in the biological samples of the fatal case, as well as the high value of methaemoglobinemia, are consistent with death due to the ingestion of nitrites. The cell viability assays showed no significant toxicity at biologically relevant concentrations of sodium nitrite/nitrate for any of the tested cell lines. Digestion simulation tests revealed that nitrite could possibly convert to other nitrogen chemical species during the gastrointestinal tract, particularly in the gastric environment. *In vitro* exposure of sodium nitrite to human blood leads to methaemoglobin production, but nitrate does not.

The data obtained suggest that methaemoglobin formation is the major mechanism of toxicity in nitrite intoxications. Understanding the mechanisms involved is essential to better understand the course of these intoxications, so that prevention and treatment strategies can be improved.

Keywords: Nitrites; Methaemoglobinemia; Suicide; Poisoning; Forensic Sciences

Resumo

O nitrito de sódio é um pó branco, inodoro e solúvel em água, utilizado em tentativas de suicídio cada vez mais relatadas, inclusivamente em Portugal. É descrito como uma substância letal barata, de fácil obtenção e que causa uma morte sem sofrimento. A morte é geralmente atribuída à metahemoglobinemia, mas mecanismos de toxicidade adicionais podem estar envolvidos.

O objetivo deste trabalho foi caracterizar a intoxicação por nitritos e investigar possíveis mecanismos de toxicidade. Foi realizada uma análise toxicológica de uma autópsia forense de um caso suspeito de suicídio ocorrido em Portugal. Foram feitas análises qualitativas e quantitativas de nitritos em amostras biológicas e não biológicas utilizando a reação de Griess. A metahemoglobinemia foi determinada através de uma técnica espectrofotométrica com ferricianeto e cianeto de potássio. Uma investigação da toxicidade *in vitro* do nitrito/nitrato de sódio foi subsequentemente conduzida para estudar o possível efeito tóxico direto. As células H9c2, Caco-2 e SH-SY5Y foram cultivadas e expostas a nitrito/nitrato de sódio e realizados ensaios de viabilidade celular para obter curvas concentração-resposta. Para estudar possíveis conversões do nitrito em outras espécies químicas ao longo do trato gastrointestinal, foram conduzidos ensaios de digestão *in vitro*. Estes mimetizaram condições fisiológicas sequenciais das fases oral, gástrica e intestinal, sob condições semelhantes às verificadas num suicídio por ingestão de nitrito. Adicionalmente, para determinar os níveis de nitritos/nitratos capazes de induzir metahemoglobinemia, foi utilizada a técnica espectrofotométrica com ferricianeto e cianeto de potássio em amostras de sangue humano fresco.

Os valores de nitritos encontrados nas amostras biológicas do caso fatal, bem como o alto valor de metahemoglobinemia, são compatíveis com o diagnóstico de morte por ingestão de nitritos. Os ensaios de viabilidade celular não mostraram toxicidade significativa em concentrações biologicamente relevantes de nitrito/nitrato de sódio para nenhuma das linhas celulares testadas. Os ensaios de simulação de digestão mostraram que o nitrito se pode converter em outras espécies químicas de azoto no trato gastrointestinal, particularmente no ambiente gástrico. A exposição *in vitro* de nitrito de sódio ao sangue humano leva à produção de metahemoglobina, mas o nitrato não.

Os dados obtidos sugerem que a formação de metahemoglobina é o principal mecanismo de toxicidade nas intoxicações por nitrito. É essencial compreender melhor o curso destas intoxicações e os mecanismos envolvidos, para que possam ser melhoradas estratégias de prevenção e tratamento.

Palavras-chave: Nitritos; Metahemoglobinemia; Suicídio; Envenenamento; Ciências Forenses

Introduction

This dissertation fulfills “GOAL 3: Good Health and Well-Being” of the United Nations 2030 Agenda Goals. Nitrite poisonings are becoming increasingly popular as a suicidal method, so more studies are needed to better understand, prevent and treat such intoxications.

Nitrites are inorganic chemical compounds with a natural presence in the environment: in the air, soil, water and plants. Daily, humans are exposed from different sources, both endogenously and exogenously [1]. They were once considered inert substances, but their effects in small amounts on vital functions are now known, being recognized as a substantial source of nitric oxide (NO) [2].

Oral ingestion is the main route of exposure to nitrites, but it is not the only one. In food, the largest amounts are found in vegetables and meat or cured meat products [1,3]. Water ingestion is another possible source through faecal contamination or extensive agricultural activity associated with the spread of nitrogen fertilizers [4].

Sodium nitrite is a white, odourless water-soluble powder, like ordinary table salt [5]. It has multiple applications in daily life such as a food additive (E250) and preservative by inhibiting fat oxidation processes leading to rancidity, and the growth of pathogens such as *Clostridium botulinum*. It is also used as a food colouring agent, producing a pink colour in food products like meat and fish [6]. Nitrites have anti-corrosive and antifreeze properties and are therefore used in the construction industry [6,7]. In health, sodium nitrite is a valuable antidote for the treatment of cyanide intoxication [6]. Due to its vasodilating power, nitrites seem to be promising in the field of medicine (e.g., to treat pulmonary and systemic hypertension or delayed cerebral vasospasm after subarachnoid haemorrhage) and in sports (for performance enhancement) [3,8,9]. Organic nitrates, which include nitroglycerin or isosorbide dinitrate, are used in pharmacological therapy for the treatment of ischemic heart disease [6].

Some worrying effects of nitrites on human health have already been investigated. An acute intoxication with sodium nitrite leads to the development of methaemoglobinemia, decreasing the transport of oxygen in the blood and a consequent change in skin colour to blue. Known since the 1940's, the “Blue Baby Syndrome” is an example of an acute intoxication that occurs when babies are fed with drinking water containing high concentrations of nitrates, leading to nitrite intoxication [4]. The elevated pH in the intestine of infants may create a favorable environment for the growth of gram-negative organisms that can convert dietary nitrates to nitrites. These nitrites can act as

inducers of methaemoglobin (MetHb). Furthermore, erythrocytes of small infants exhibit lower levels of cytochrome-b5 reductase and fetal haemoglobin (Hb) is more prone to oxidation compared to adult-type Hb [10]. As a result of the consequent cyanosis, changes in skin colour are produced and accompanied by many other detrimental health consequences. For this reason, it is advisable to use bottled water in baby feeding. With regard to chronic exposures, nitrates and nitrites have been linked to digestive tract cancers through the formation of carcinogenic N-nitroso compounds like nitrosamines [4].

Unintentional poisonings with nitrites have been documented throughout history [7,11]. Lately, self-poisoning appears to be a new suicide trend. The choice of sodium nitrite as a killing agent seems to be related to several factors which include: i) similar properties to daily table salt (salty flavour, odourless and water-soluble); ii) easily acquired, including on the internet; iii) low cost; iv) easy, quick, peaceful and painless death [12]. The internet allows access to end-of-life social support forums, descriptive guides and the purchase of sodium nitrite, as well as “Suicide Kits” containing this salt and other drugs [11,12,13].

Death is usually attributed to methaemoglobinemia caused by the oxidation of Hb to MetHb. The oxidizing power of nitrites leads to a lack of oxygen transport in the blood, which at a certain point becomes incompatible with life [7,11]. However, information is lacking on additional toxicity mechanisms.

Post-mortem analysis shows some characteristic signs in victims, including a typical cyanotic appearance, a brownish colour of *livor mortis* and chocolate-brown coloured blood [5,9].

Since lethal poisonings involving nitrites are increasing, it is necessary to characterize this type of intoxication and to gather all mechanistic information that could help understand, prevent, and treat nitrite intoxications. With this purpose, this dissertation aims to (i) report a fatal case of suspected suicide by nitrite ingestion that occurred in Portugal, disclosing, among other information, the forensic toxicological analysis that were carried out, (ii) investigate the direct cytotoxic effect of sodium nitrite and sodium nitrate in three cell lines representative of target organs, to investigate other possible mechanistic pathways of toxicity in addition to methaemoglobinemia, (iii) investigate possible conversions of nitrite to other chemical species along the gastrointestinal tract after ingestion of a sodium nitrite solution, through *in vitro* digestion simulations and, lastly, (iv) assess the concentrations of sodium nitrite and sodium nitrate that are capable of inducing *in vitro* methaemoglobinemia.

PART I – Literature review

1. The role of nitrites in human health

Nitrites are ubiquitous compounds in daily life, with human exposure arising both from endogenous production and from external sources [14]. As previously mentioned, nitrites have been widely studied for their reactivity with amines, amides and other nitrosation precursors that lead to N-nitroso compounds formation, known for their possible carcinogenic effects [15]. Furthermore, nitrites are also a concern for human health due to their ability to oxidize Hb, inducing MetHb [10].

Although nitrites are usually associated with harmful effects, they also play an important role in physiological mechanisms as a stable physiological reservoir of NO [3]. NO serves as a crucial cell signaling molecule, playing significant roles in biological processes such as neural communication, blood vessel modulation and immune responses. However, excessive NO production can lead to pathological mechanisms, particularly in inflammatory conditions, being a critical cytotoxic mediator [16].

In the human body, nitrates and nitrites undergo continuous recycling in the blood and tissues [3]. Nitrite can be formed either by the oxidation of NO or by the reduction of nitrate. The auto-oxidation of NO, a non-enzymatic reaction of NO with oxygen, occurs slowly in tissues, but the oxidation catalyzed by multicopper oxidase ceruloplasmin (in plasma) or cytochrome c oxidase (in tissues) is fast [3,17]. In turn, NO can be formed physiologically by two pathways: (i) the L-arginine-NO synthase (NOS) pathway and (ii) the nitrate-nitrite-NO pathway. The L-arginine-NOS pathway, is dependent on oxygen, and the NOS enzymes use L-arginine and oxygen molecules to produce free radical gas •NO, serving as a crucial factor in regulating neurotransmission, vascular homeostasis, and host defence mechanisms. On the other hand, the nitrate-nitrite-NO pathway is activated in cases of oxygen deficiency and therefore, in blood and tissues, nitrites are reduced to NO through diverse enzymatic and non-enzymatic reactions (e.g., deoxyHb, myoglobin, xanthine oxidoreductase, ascorbate, polyphenols, protons). In this pathway, nitrite is used as an alternative electron acceptor to molecular oxygen and, under hypoxia and acidosis conditions, NO production is hugely increased. This nitrite-NO reduction plays a role in physiological hypoxic signaling, vasodilation, modulation of cellular respiration, including mitochondrial oxygen consumption, and cellular response to ischemic situations [3,9,18].

2. Sodium nitrite for suicide

Nitrite poisonings are almost entirely due to unintentional exposures [7]. These can happen by ingesting the wrong salt or consuming highly contaminated meats or vegetables [19]. Several cases of accidental ingestion have been reported over the years, with one of the first reported cases in 1936, when a family had a fatal outcome after using sodium nitrite as a food seasoning instead of sodium chloride. Other outbreaks have occurred throughout history, such as the episode involving 11 people in New York, in 1945, that were poisoned after having a meal in the same cafeteria [20].

Suicide represents a global public health problem and voluntary intoxication is a frequently chosen method to cause self-inflicted harm [21]. The choice of suicide method changes according to generational, cultural and social factors [18]. Self-poisoning from ingested nitrites is not a common form of suicide, however, numerous cases have already been reported in the literature in different countries, and some of them in Portugal [5,9,11]. The first reported case of suicide using this substance was in 1979. After that, other intentional cases were recorded in 1990, 1996, 2000 and 2010, but these numbers took other proportions from the year 2019 onwards [22]. An alert from the American Association of Poison Control Centers published in April 2022 shows that self-poisoning with nitrites and nitrates increased by 253% and also an 166% increase in deaths in 2021 compared to 2018 [23]. This form of deliberate self-harm, and its online purchase, has also been talked about in the celebrity world, after a popular news announcement of the fatal overdose of a famous person. This episode left the community concerned about how this mediatic episode might reach vulnerable populations and the impact that it could have on imitative suicide [24]. Although sodium nitrite is currently mostly related to suicide, cases of its use in homicides have also been reported. In China, a kindergarten teacher added this salt to the food of another teacher and 25 children, causing the death of one child [12].

Sodium nitrite can be seen as the ideal killing agent because it is cheap and relatively easy to obtain, especially through e-commerce [12]. The so-called “Suicide Kits” can be easily purchased, and they contain the materials needed: cups, co-adjuvant drugs (e.g., metoclopramide and ranitidine) and powdered sodium nitrite [9]. It is described that the taste of sodium nitrite dissolved in water is relatively similar to the taste of salty water [18]. Due to its high solubility in water, clinical symptoms appear in a very short period of time, contributing to a high mortality rate. The symptoms of intoxication include nausea, vomiting, diarrhoea, headache, dizziness, confusion, fatigue, bradycardia or tachycardia, hypotension, flushing, lethargy, and dyspnoea [9,25,26]. The most severe are convulsions, coma, respiratory failure, bradyasystolic cardiac arrest

and, at last, death [9,26]. After ingestion, a short time can precede death, which in some cases can occur within an hour of consumption [12,18,27]. The use of this salt for suicidal purposes seems to lead to an easy, quick, peaceful, and pain-free death, and all these factors may possibly contribute to its increasing popularity in suicidal attempts [9,28].

To mitigate the gastrointestinal side effects caused by nitrite ingestion and to increase its tolerance and absorption, other substances often appear at the scenes. Most of these are taken previously to the ingestion of sodium nitrite due to their antiemetic, gastric emptying and antacid properties. Most frequently used drugs found at the scene include metoclopramide, ondansetron (antiemetic and antivertigo), ranitidine, famotidine, cimetidine (antacid and antiulcer), bismuth subsalicylate (antacid and antidiarrheal) and olanzapine (antipsychotic with antiemetic properties) [5,11,12,13,25]. Anecdotal instructions are available online, advising nitrites dissolution in the organosulfur compound dimethyl sulfoxide (DMSO) to increase its absorption. It is mentioned that with DMSO, there is no need to take antiemetics since lethality occurs in a short period of time [11,29,30]. There are also reported cases of concomitant ingestion of drugs from other therapeutic classes such as analgesics (paracetamol, ibuprofen, tramadol, codeine), antipsychotics (quetiapine, olanzapine) and benzodiazepines (diazepam) [5,13].

Some victims are found accompanied by suicide letters while others sometimes share information on social networks or on forums for suicide support [5,11,18]. Victims have usually a psychiatric medical history (e.g., depression, schizophrenia), with previous suicide attempts or suicidal ideation and a history of searching online for suicide related information [9,22,31]. The choice of using nitrite as a suicide method seems to be a recent trend, and mostly associated with young population of both genders [32]. Social media and easy accessibility to websites are sources for (mis)information on this problematic topic [12]. Several online platforms provide access to sodium nitrite, called as the “secret powder”, and all the needed information explaining the procedure step-by-step is provided. The explanation for being considered a low-suffering method of ending one's life seems to be related to the action of reductase enzymes on nitrite and nitrate, with the subsequent production of NO, and its vasodilating potential. The phenomenon of vasodilation leads to hypotension and muscle relaxation and consequently to loss of consciousness [5]. Some of these effects are also harnessed for recreational purposes, through the inhalation of volatile alkyl nitrites, usually used by young people at parties due to its neurological effects, such as euphoria [5,33].

3. Nitrite toxicokinetics and mechanisms of toxicity

Oral intake is associated with an extremely high bioavailability of about 98% because this dissolved salt is quickly absorbed in the gastrointestinal tract (stomach and upper small intestine) [30]. The metabolism of nitrites takes place in various organs (with low hepatic first-pass metabolism) and in blood [30,34]. When nitrite reaches the stomach, a part is reduced to NO. The acidic environment promotes the protonation of nitrite, forming nitrous acid (HNO_2) that decomposes into NO and other nitrogen oxides. A large amount of the nitrite does not undergo conversion in the stomach, reaching systemic circulation. Nitrite has an effect on resistance vessels, originating NO, responsible for vasodilation and consequent hypotension. The existing NO can then be re-oxidized, forming nitrite. Subsequently, it is through the kidneys that excess nitrite or nitrate is eliminated from the body [16,35]. Because mammals lack nitrate reductase enzymes, if nitrates are ingested (instead of nitrites) they are reduced to nitrite by commensal bacteria present in the oral cavity as well as in the gastrointestinal tract [3].

Nitrite poses greater toxicological concerns compared to nitrate, primarily due to its complexity and distinctive chemical reactivity (specially the modification of the redox balance) [36]. Nitrites have a greater oxidative power compared to nitrates and are therefore more toxic [25]. They easily react with functional groups of both endogenous and food-related molecules [36]. Numerous biological effects caused by nitrite result from NO interactions with mitochondria as NO competes with oxygen by binding to cytochrome c oxidase, the terminal electron acceptor at the mitochondrial electron transport chain. The interaction of NO with cytochrome c oxidase initiates intracellular signaling processes, resulting in the redirection of oxygen towards non-respiratory substrates and the generation of reactive oxygen species (ROS), which may cause harmful effects [37].

In red blood cells, nitrites are oxidized, producing nitrate and MetHb [3]. However, it is important to note that methaemoglobinemia is not exclusively caused by nitrite. It can arise from different etiologies, such as genetic (e.g., cytochrome-b5 reductase deficiency or cytochrome-b5 deficiency, transmitted in an autosomal recessive pattern), idiopathic, dietary, or toxicological sources (drugs or chemical substances - Table 1) [10,38].

Table 1. Examples of drugs and chemical agents capable of inducing MetHb [10,38].

MetHb-inducing drugs			MetHb-inducing chemical agents	
Acetaminophen	Metoclopramide	Piperazine	Anilines	Nitrobenzene
Benzocaine	Nitrates	Prilocaine	Benzene	Nitrophenol
Chloroquine	Nitrites	Primaquine	Bivalent copper	Paraquat
Dapsone	Nitrofurantoin	Rifampicin	Dimethyl Sulfoxide	Sodium nitrate
Lidocaine	Nitroglycerine	Sulfonamides	Dinitrophenol	Sodium nitrite
Methylene Blue	Phenytoin	Valproic acid	Naphthalene	Trinitrotoluene

In blood, nitrites are rapidly oxidized into nitrates. Nitrates' half-life is 5-8 h whereas nitrites' half-life is only 110 s. In a normal situation, plasma levels of nitrites in the human body vary between 50 and 100 nM, but these concentrations can increase 4 to 5 times after the ingestion of nitrate-rich food, following its reduction into nitrite in blood and tissues [1]. Nitrites and nitrates seem to cross the blood-brain barrier [18,39,40].

The mechanism of nitrite toxicity involves several processes, with emphasis on MetHb and the vasodilator and hypotensive effect [25]. Sodium nitrite also induces haemolysis, contributing to the decreased oxygen supply to vital organs [5]. Haemolytic and oxidative anaemia caused by nitrites is well characterized in cases reported after the inhalation of so-called "poppers" (nitrite inhalants, like amyl nitrite). The recreational use of these volatile alkyl nitrites leads to the formation of MetHb, increased proteolytic susceptibility of Hb, leading to denaturation of the globin chain and formation of Heinz bodies. The red blood cells turn brownish and haemolysis occurs when the erythrocyte antioxidant defences are overloaded [5,41,42,43].

Nitrite intoxications are similar to carbon monoxide intoxications in the sense that there are changes in the conformation of Hb resulting in anaemic hypoxia. Ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) conversion leads to blockage of oxygen binding with the shift to the left of the oxygen-Hb dissociation curve [7,11,31]. The existence of one or more Fe^{3+} ions in the Hb tetramer leads to a greater affinity for oxygen by the remaining Fe^{2+} heme groups. In addition to the decrease in oxygen transport, the oxygen remaining in the unaffected heme groups cannot be released into the tissues, causing functional anaemia and lactic acidosis [11,30]. Myoglobin also has a heme subunit that can bind an oxygen molecule. If oxidized by nitrites, muscle hypoxia will occur, making the victims feel prostrated, weak and lethargic [12].

Under normal circumstances, the maintenance of a low percentage of MetHb in relation to total Hb is guaranteed by MetHb reductase enzymes in red blood cells,

protecting the erythrocytes from small amounts of oxidizing agents [12]. The maintenance of low physiological levels of MetHb depends on two mechanisms only: (i) the nicotinamide adenine dinucleotide (NADH)-dependent MetHb reductase (composed by cytochrome-b5 reductase and cytochrome-b5) that is responsible for 95-99% of daily MetHb reduction and the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent MetHb reductase for the remainder; and (ii), reduction by glutathione and ascorbate. With these mechanisms, low levels of MetHb are normally maintained between 0-3% but an imbalance can occur if the enzymatic activity of the MetHb reductases decreases or if oxidative stress increases [18,38]. If levels of MetHb increase to 10-20%, there is hypoxia and central/peripheral cyanosis, while MetHb levels greater than 30% lead to tachycardia, dysrhythmias and affected consciousness. MetHb levels above 55% result in loss of consciousness or coma and, at an extreme level, MetHb above 70% causes death [5].

Increased MetHb clinical manifestation is central and peripheral cyanosis and 70-90% oxygen saturation (SpO_2) values, that usually does not respond to oxygen administration [44]. The first body systems affected are those that need the greatest supply of oxygen, especially the central nervous system that controls the heart and respiratory centre [45]. If not treated quickly, systemic tissue hypoxia leads to death [11]. In cases of non-fatal poisoning, nitrites can produce delayed and persistent toxic effects such as the delayed post-hypoxic leukoencephalopathy. This demyelinating syndrome is the result of the lack of oxygen in brain over a period of time, resulting in mental sequelae, symptoms of the extrapyramidal system and motor disorders [26]. Another worrying effect of nitrites is vasodilation, explained by the relaxation of smooth muscles, leading to severe hypotension [45].

The lethal dose for sodium nitrite has been established at 2.6 g, or alternatively at 71 mg/kg [5,19]. Nevertheless, there are reports of survival after doses of 6 g or even 60 g and deaths with as much as 1 g [5,18]. In an acute intoxication, after the ingestion of 200-500 mg of nitrites, it takes about 1 h until the first symptoms appear, but it can vary between 20 min to 3 h [19]. Regarding the percentage of MetHb, as previously mentioned, levels above 70% can lead to death but there are fatal cases documented with MetHb levels of 9.87% as well as survival with 94% [9,18]. The harmful effects of nitrites become more worrying when victims have a history of poor health or previous illnesses, like anaemia, acidosis and a deficient cardiac and/or respiratory system [9].

In vitro and *in vivo* studies are limited with respect to acute exposure to nitrites. Understanding these effects could help explain several phenomena described in the reported intoxication cases. Ansari *et al.* (2016) studied the oxidizing effects of sodium nitrite (0.1-10 mM) directly on erythrocytes after an incubation of 30 min. Nitrite can enter

erythrocytes through the anion transporter and partly by diffusion, inducing membrane hyperpolarization and oxidation of Hb [46]. The hyperpolarization of the erythrocyte membrane appears to be related to changes in the electrochemical properties that occur due to changes in the activities of the membrane's ion pumps and/or to the accumulation of the nitrite ion [47]. The results of the study show that nitrite causes an enormous increase in ROS, a significant decrease in the antioxidant defenses, as well as inhibition of the plasma membrane redox system and ascorbate free radical reductase. These conditions lead to damage and senescence of red blood cells. A previous study [Ansari *et al.* (2015)], under the same conditions previously mentioned, had already revealed increased lipid and protein oxidation, membrane damage, osmotic fragility, and morphological disturbances in human erythrocytes [46,48].

To study cell proliferation and the cytotoxic effects of sodium nitrite, Sun *et al.* (2006) carried out *in vitro* work with human gastric epithelial cells. Human gastric adenocarcinoma epithelial (AGS) cells were exposed to increasing concentrations of sodium nitrite for 24, 48 and 72h. For cell proliferation assays, concentrations of 1.575-50 mM were tested and 6.25-25 mM concentrations for the cytokine expression assays. The correlation between cytotoxicity and the activity of inflammatory cytokines (IL-1 β , IL-6; IL-8 and TNF- α) was investigated. The authors noted that at low concentrations (0-6.25 mM), sodium nitrite promotes cell proliferation and at 50 mM cell proliferation was inhibited. At 6.25 mM, there was an increase in the release of IL-8 and at the highest concentration (25 mM) increases in IL-1 β , IL-6 and TNF- α were noted. In short, sodium nitrite induces the release of inflammatory cytokines and negatively affects the proliferation of AGS cells at high concentrations [49].

Ansari *et al.* (2017) studied *in vivo* the acute effect on the intestine of rats of a single oral dose of sodium nitrite (at 20, 40, 60 and 75 mg/kg body weight). In the intestinal cells of rats, nitrite caused dose-dependent intracellular redox reactions, with consequent production of NO, peroxynitrite, nitryl chloride and other ROS/reactive nitrogen species. In turn, there was a reduction in intracellular reductants (GSH/total SH) and in antioxidant enzymatic defence, DNA fragmentation, formation of DNA-protein crosslinks and oxidatively modified proteins, alteration of protein/enzyme function and lipid peroxidation [2]. The same authors reported very similar results in a parallel study targeting the rat kidney [50].

Petrova *et al.* (2020) studied the effect of acute sodium nitrite intoxication on the levels of essential metals (iron, calcium and zinc), that play an important role in antioxidant metalloenzymes, in mouse spleen. In this study, the animals were exposed to sodium nitrite through a single intraperitoneal injection of 120 mg/kg body weight. The

results revealed that acute intoxication leads to disturbances in the levels of the three essential metals, impairing the activity of antioxidant defence enzymes [51].

4. Forensic analysis

The forensic analysis of a suspected intoxication with nitrite should follow the steps [11]:

i) Meticulous examination of the scene with exhaustive description and documentation (e.g., by photography), paying special attention to elements that may indicate suicidal intent. Look for cups with liquid contents or traces, containers with a white crystalline powder and other drugs, including the co-adjuvants previously described. Searching for suicide notes, making phone calls to medical numbers, registering information on social media are examples;

ii) Examine the external habit, paying special attention to *livor mortis* colour;

ii) Examine the internal habit, paying special attention to the colour of the blood;

iii) Add to the analyses, the value of MetHb and search for auxiliary drugs.

To find the most relevant features of these intoxications, a literature search on the Pubmed database was conducted to examine the articles reported on intentional poisoning by nitrite ingestion. No time limits were placed on the research. After reading the obtained reports, the most important forensic aspects reported from cases involving nitrite intoxications were summarized in Tables I-III presented in the Annex I (Table I – Intentional fatal cases reported in Portugal; Table II – Intentional fatal cases reported in the literature in other countries; Table III – Intentional non-fatal cases reported in the literature in other countries). The following aspects were considered: year of publication, country, total number of cases, age, sex, clinical history, estimated dose of sodium nitrite ingested, scene findings, signs and symptoms, pathology findings, toxicological findings and, finally, the origin of nitrites. It is noteworthy that all listed articles in these tables were published in the year 2020 or after, except for 3 of them. These publications originated from countries on different continents. From the total number of cases (84), 69 were fatal (48 men and 21 women) and 15 were non-fatal (8 men and 7 women). From all cases, the lowest registered age was 16 and the highest 86; in most cases the victims were young adults under the age of 40. Often, a clinical history of depression, suicidal ideation, previous suicide attempts, bipolar disorder and schizophrenia was registered. The ingested amounts of sodium nitrite seemed to vary between 15-113 g in fatal cases (although Kim *et al.* (2022) reported one case in which the victim mentioned having ingested 1 kg of pickling salt dissolved in water [52]), and between 750 mg-60 g in non-

fatal cases. At the scenes, it was common to find cups and bags or flasks with sodium nitrite or its residues, as well as suicide letters; the signs and symptoms were varied, and it was common for victims to become unresponsive, asystolic, with cyanosis, hypotensive, with vomiting, among other symptoms. Regarding forensic pathology, blue-grey hypostasis, dark brown discoloration of the blood and cyanotic appearance were recurrent. The blood %MetHb reached the highest recorded level of 90.3%, in fatal cases, and of 88% in non-fatal cases; the origin of nitrites was mostly online acquisition; but in 2 cases there was access to the salt at the workplace.

5. Forensic Pathology

There are signs to be aware of in the *post-mortem* body examination that are indicative but not pathognomonic of nitrite intoxication. Regarding the external habit, sodium nitrite generally induces a typical cyanotic scenario with colour changes in different parts of the body such as face, fingers and toes [12]. The *livor mortis* may not be uniform and can be described as brown-grey-blue-red colour [9,12]. This colouring of the hypostases may be different in cases of low percentage of MetHb and according to the state of putrefaction of the body [18]. It may also be difficult to distinguish in individuals with dark skin [11].

In the examination of the internal habit during the autopsy, it is frequently possible to see massive scleral and polyvisceral congestion, pulmonary and cerebral edema, brown foam in trachea, bronchial aspiration of vomiting and some Tardieu petechiae [5,6,9,18]. The subcutaneous tissue and the oral mucosa may in turn acquire a red colour as in carbon monoxide poisoning [18]. Blood changes colour from red to brown (which normally appears at MetHb values higher than 15-20%) and the same can happen in some body organs, like in the kidney, heart and also in muscle tissue [5,12,53].

Briefly summarised, there are three characteristics that usually stand out in nitrite poisoning victims and that deserve the attention of forensic pathologists: (i) the asphyxia signs that are described as appearance of blue-grey colour mainly on the nails, facial skin and lips; (ii) brownish colour of *livor mortis* and (iii) chocolate-brown coloured blood [5,9,12].

6. Forensic Toxicology

Nitrite self-poisoning is a recent trend and because routine toxicological analysis usually contemplates only standard substances, it is important to be aware of the

features of suicidal nitrite ingestion. The analysis of the place of death can also provide key information in this regard [9].

For the analysis of nitrite and nitrate levels, several methodologies have been proposed, including colorimetry, fluorimetry, chemiluminescence, gas chromatography – mass spectrometry (GC-MS) and capillary electrophoresis. However, analysis on biological samples can be complicated by the interference from cadaveric phenomena, making the analysis matrix complex [6]. Due to the constant conversions of nitrite-nitrate and nitrate-nitrite in human blood, both chemicals should be investigated [18]. It is common to detect a higher concentration of nitrates than nitrites [9]. Therefore, detection of nitrates may confirm nitrite poisoning [18]. In fact, in a *post-mortem* setting, most nitrites in blood are converted to nitrates [27]. Nitrates remain stable in the *post-mortem* period and their concentrations do not change both in cardiac and peripheral blood. In a forensic toxicology analysis, Hwang *et al.* (2021) documented that it was possible to detect nitrates in the cerebrospinal fluid, but not nitrites. Analysis of cerebrospinal fluid may be useful in cases of putrefaction where blood analysis is compromised [18]. Surprisingly, some authors have investigated the presence of both nitrites and nitrates in the vitreous humour, but failed to detect both chemicals. This matrix, often useful in cases of putrefaction, does not seem to provide information in these cases, as it is thought that the blood-retinal barrier selectively regulates the absorption, secretion and diffusion of ions, water and proteins between blood, extracellular fluid and vitreous retina [6]. On the contrary, other authors have detected nitrite ions using the Griess method in both vitreous humour and costal cartilage samples. With regard to the costal cartilage, it was suggested that this matrix could be an alternative for cases in which, for example, the body is at an advanced state of putrefaction or missing limbs [54].

Regarding the laboratory analysis of nitrites/nitrates, this can be done through the Griess reaction, because nitrite ions react with N-(1-naphthyl)ethylenediamine dichloride and sulfanilamide which are the main constituents of the Griess reagent [54]. However, the interpretation becomes difficult because it is not known which concentrations of nitrite/nitrate are of toxicological importance, its stability, or *post-mortem* redistribution [11].

The absence of nitrite in gastric contents, body fluids or tissues, does not exclude the possibility of nitrite poisoning. Therefore, the determination of MetHb seems to be more relevant than the quantification of blood nitrite/nitrate levels [9,11,55]. However, there are aspects that have to be taken into account when interpreting the obtained MetHb values: on one hand, freeze-thaw cycles and/or the lack of preservatives in the sample can lead to falsely high values, possibly due to auto-oxidation during storage; on the other hand, *post-mortem* haemolysis (e.g., due to bacterial activity) and lack of

preservation (leading to intracellular enzymatic reduction of MetHb to Hb by the action of MetHb reductase, that could persist after death), can make the values falsely lower [11,56]. For these reasons, samples used for *post-mortem* determinations of MetHb values must be refrigerated (4-6 °C), contain EDTA as a preservative, ensure the smallest dead space in the tube and the analysis should be done as soon as possible [11,18]. If the sample is to be used within a short period of time, such as two weeks, the blood sample should contain EDTA as a preservative and be kept cold (4-6 °C) rather than frozen [18].

In conclusion, in a first approach to a fatal case without prior information, forensic experts may not have an easy job. Therefore, the importance of perinecropsy in all forensic cases is highlighted, and suicide with nitrites is no exception. All the data obtained will be able to help forward the pathological and toxicological analyses. Some typical forensic pathology signs (such as brown-grey-blue-red colour lividities and brown blood) may help to suspect this type of poisoning and guide toxicological research [9]. The laboratorial diagnosis consists of the determination of the MetHb percentage in blood. There have been some difficulties in the direct quantification of nitrites and nitrates in biological samples, which can lead to false negative results. The analysis should be carried out within the shortest time after death and the results interpretation with the greatest critical spirit, taking into account possible interferences such as decomposition or contamination. It would also be useful to determine the presence of other co-adjutant drugs often associated with these cases [11].

7. Nitrites poisoning treatment

The poisoning treatment involves reversing severe methaemoglobinemia by providing hyperoxic ventilation, exchange transfusion and administering reducing substances like thiazine dye methylene blue (methyl-thioninium chloride) [9,31]. Gastric lavage and the use of activated charcoal (1 g/kg) using an orogastric tube are also documented [57]. Vasopressors have also been used in some cases [44].

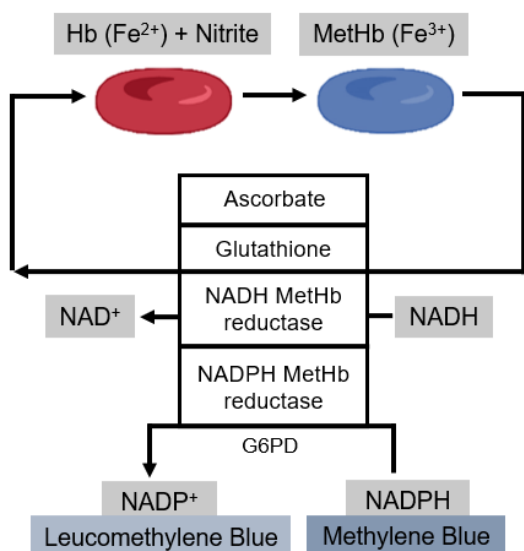


Figure 1. Hb-MetHb and MetHb-Hb conversion pathways in erythrocytes.

Methylene blue seems to act in two aspects of toxicity: at the hypoxic level (due to its effect on MetHb) and at the hemodynamic level (due to its effect on excess NO, which causes vasodilation) [58]. The primary pathway for the reduction of MetHb to Hb occurs through NADH MetHb reductase. The alternative pathway involves methylene blue as a cofactor in the presence of glucose-6-phosphate dehydrogenase (G6PD) (Figure 1) [53]. Methylene blue works as an antidote for nitrite poisoning by accelerating the enzymatic reduction from

Fe^{3+} to Fe^{2+} , due to its dose-dependent redox potential [7,31]. NADPH MetHb reductase originates a metabolic product, leucomethylene blue, transforming MetHb into Hb [7,12]. It is important to emphasize that this antidote is effective in intact erythrocytes and its effect is diminished in cases of installed haemolysis [31]. The use of this antidote should be considered in cases where the victim is symptomatic with MetHb>20% or has unfavourable factors such as anaemia or congestive heart failure [59]. Additionally, it can counteract the vasodilator effect by inhibiting NOS enzymes [30]. The usual dosage of methylene blue used in acute cases is 1-2 mg/kg from a 1% solution IV during 5 min. If symptoms and elevated MetHb rates (>30%) are still present, administration may be repeated within 30 to 60 min. A successful effect appears to occur in victims with intact circulation between 30 and 60 min after injection [31]. However, there are cases where methylene blue is not effective: patients with G6PD deficiency, NADPH MetHb reductase deficiency and sulphaemoglobinemia [7]. Methylene blue has a maximum daily dose of 7 mg/kg and it should not be used at much higher doses because the scenario can be quickly reversed, with the possibility of oxidizing Hb and causing haemolysis [30,60]. It is crucial for health professionals to know the toxicity of sodium nitrite and know how to act in an emergency situation [59]. In addition, health institutions should have methylene blue available, which is sometimes not the case, hindering an adequate treatment of the intoxication [30,58].

PART II – Experimental work

1. A suspected case of nitrite suicidal ingestion

At the end of 2021, the Laboratory of Toxicology of the Faculty of Pharmacy, University of Porto (FFUP) collaborated with the Forensic Chemistry and Toxicology Service of the National Institute of Legal Medicine and Forensic Sciences, I.P. (INMLCF) of the North Delegation in a toxicological analysis of a possible intentional intoxication from nitrite ingestion. It was intended to search for the presence of nitrites in biological and non-biological samples obtained from the victim's autopsy and at the crime scene. The disclosure of the reported case data was approved by the Ethics Committee and by the Department of Research, Training and Documentation of the INMLCF (CE-15/2023). This case was the starting point for continuing research work in this field. Suicide due to nitrite ingestion is an emergent issue, with some fatal cases already reported in Portugal but only few research studies related to it [5,9].

1.1. Fatal self-poisoning case report

A 20-year-old man with previous history of depression and suicide attempt, was found dead in his bedroom, along with a suicide note, a glass with residue inside and 3 small bottles containing a white powder, one transparent and one yellow liquid of unknown composition. According to the parents, the victim had received an online order package of sodium nitrite the day before.

1.2. Forensic Pathology

A medico-legal autopsy was performed, and the external *post-mortem* body examination revealed: (i) a purplish *livor mortis*, partially fixed, located in the posterior regions of the neck, chest, abdomen, and upper and lower limbs, (ii) foam with fine bubbles, whitish and pink, coming out of the oral cavity, (iii) facial, conjunctival and cervical congestion. The internal habit examination showed: (iv) vestigial subdural haemorrhage in the parieto-occipital region, (v) foam from small bubbles in the lumen of the trachea and bronchi, (vi) a marked congestion of the organs. Peripheral blood, gastric content, urine and vitreous humour were collected for toxicological analysis. Samples were taken for histological analysis but not processed.

1.3. Forensic Toxicology

The biological samples were sent to the Forensic Chemistry and Toxicology Service of the INMLCF of the North Delegation for the investigation of ethanol, drugs and drugs of abuse. Biological and non-biological samples (collected at the crime scene) were sent to the FFUP Toxicology Laboratory for nitrite investigation.

1.3.1. Material & Methods

1.3.1.1. Chemicals and materials

The Griess reagent (phosphoric acid 85%, sulfanilamide, N-(1-naphthyl)-ethylenediamine dihydrochloride and water) was provided by the Laboratory of Bromatology and Hydrology, Department of Chemical Sciences of the FFUP. Nitrite standards were prepared from a 1000 mg/L nitrite ion standard solution from Certipur® 1198990500 (Merck, Germany). Charcoal decolourising, zinc sulfate heptahydrate and Triton X-100 were obtained from Sigma-Aldrich, USA and potassium di-hydrogen phosphate from PanReac AppliChem, Spain. The following solutions were used: 2M and 0.5M NaOH; 0.5M phosphate buffer pH=7.2; 5% potassium hexacyanoferrate (III) solution (Fisher Scientific, UK), prepared monthly and stored in a dark bottle; 12% acetic acid solution (Honeywell, Germany); 5% potassium cyanide solution (Sigma-Aldrich, Germany), prepared monthly; ferricyanide-phosphate reagent, prepared daily (200 µL of 5% potassium ferricyanide, 2.5 mL of 0.5M phosphate buffer pH=7.2 and complete with water to 10 mL) and neutralized potassium cyanide solution, prepared at the time of use (1 mL of 5% potassium cyanide and 0.9 mL of 12% acetic acid).

1.3.1.2. Toxicological analysis

Qualitative and quantitative analysis of nitrites was performed in biological (whole blood, urine and gastric content) and non-biological (white powder, glass residues, transparent and yellow liquids) samples, using the Griess reaction and an UV/VIS spectrophotometric method. For the ethanol analysis, the blood sample was processed through Headspace Gas Chromatography with Flame Ionization Detection (HS-GC-FID) and, for the screening for drugs of abuse, blood and urine samples were analysed using an Enzyme-Linked Immunosorbent Assay (ELISA). To investigate other drugs, peripheral blood and urine were also analysed by Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) and glass residues and both liquids by GC-MS. Additionally,

methaemoglobinemia was measured in the whole blood sample using a ferricyanide and potassium cyanide spectrophotometric technique.

The Griess reaction, first reported in 1879 by Johann Peter Griess, is a simple, viable, inexpensive and specific analysis method for nitrite ion, widely used over the years for the analysis of nitrite in biological samples. This method has also been applied to the investigation of nitrates, with a previous chemical or enzymatic reduction of the nitrate to nitrite followed by the Griess reaction [61].

Briefly, this colorimetric method is based on the detection of nitrite ion after the formation of an azo dye originated by its reaction with the Griess reagent. In more detail, nitrite ion reacts with sulfanilamide under acidic conditions which is revealed after diazotization with N-(1-naphthyl)ethylenediamine. The azo chromophore compound produced (with red-violet colour) absorbs in the visible region, at a $\lambda_{\text{max}} \approx 540 \text{ nm}$ (Figure 2) [61,62].

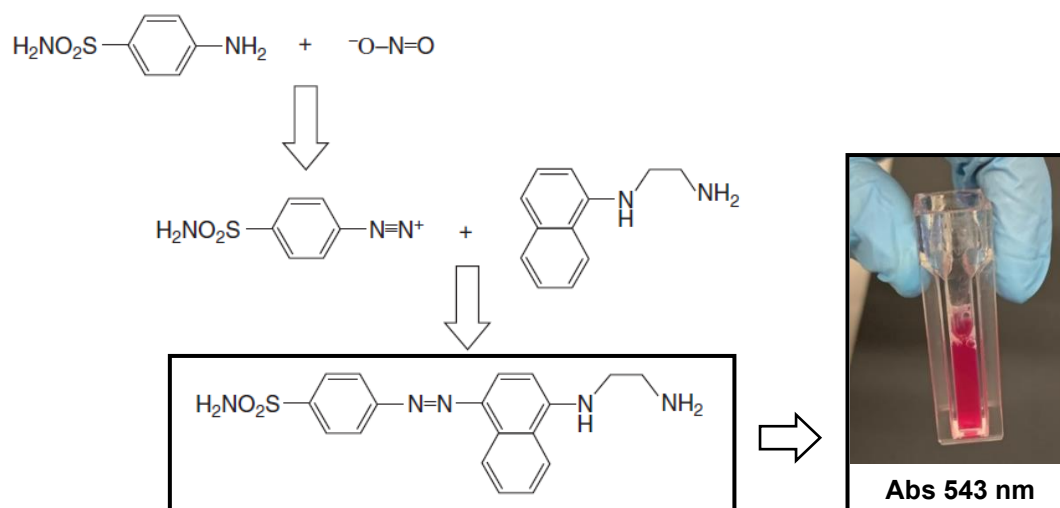


Figure 2. Scheme of the principle of the Griess reaction. Formation of the azo chromophore compound that forms a visible red-violet colour at an intensity that depends on the concentration of nitrite ion in the sample. Adapted from Giustarini *et al.* [62].

The samples were tested using the Griess reaction method and absorbance measurements were performed on an UV/VIS spectrophotometer (Shimadzu UV-1800 UV/VIS Scanning Spectrophotometer, U.S.A.). Before carrying out the reaction, the samples were pre-treated to eliminate possible interferences according to Zhao *et al.* [16]:

- *Urine sample*: to 10 mL of urine sample, 0.4 g of activated charcoal were added as decolouring agent. It was stirred for 30 s in a vortex and then filtered on filter paper (Figure 3). To obtain negative controls and standard samples of known nitrite concentrations, urine samples from a healthy donor were manipulated under the same conditions as those used in the case study sample. Due to the high absorption values obtained after spectrophotometer readings, it was necessary to further dilute the victim's samples.

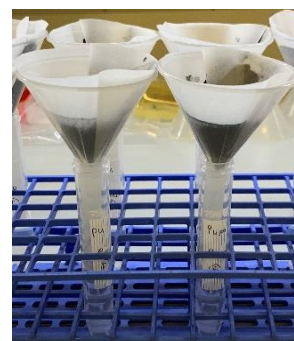


Figure 3. Treatment of urine samples using activated charcoal followed by filtration.

- *Whole blood and gastric content samples*: these samples were centrifuged in the Eppendorf Centrifuge (5810R, Germany) at 1100 g for 15 min at 4 °C; to 1 mL of supernatant 9 mL of distilled water were added; 1 mL of 2M NaOH and 2 g of zinc sulfate were used as a decolourizing agent and to precipitate proteins; mixed by vortex for 30 s; centrifuged at 4000 rpm for 10 min to aid protein precipitation, and the colourless and clear supernatant was used for the Griess reaction. To obtain negative controls and nitrite standard samples, distilled water blanks and blood samples from a healthy donor were manipulated under the same conditions as those used with the case study samples.

- *Non-biological samples*: transparent and yellow liquids were treated using the same protocol as for urine samples. Negative controls and nitrite standard solutions in water were submitted to the same treatment as the test samples. The white powder and the glass residues were dissolved in distilled water for analysis.

The Griess reaction method: due to sample scarcity, all samples were processed in duplicate for nitrite quantification, following the same protocol: (i) 50 μ L of Griess reagent to 1.25 mL of sample, (ii) 15 min incubation protected from light and (iii) UV/VIS absorbance readings at 543 nm in a 1 mL plastic cuvette.

The methaemoglobinemia evaluation technique was first developed in 1938 by Evelyn and Malloy, using cyanide derivatives [63]. Over the years, some authors have proposed modifications to this technique to increase its sensitivity [64,65]. Hb and MetHb in haemolyzed samples have maximum absorption at $\lambda \approx 630$ nm. To obtain the MetHb value, a reading is taken at 632 nm in the absence of cyanide and then a new reading is taken after the addition of a neutralized cyanide solution. The cyanide leads to the transformation of MetHb in the sample to cyanoMetHb, eliminating the contribution of MetHb at this λ . MetHb is determined by the change in absorbance from MetHb to cyanMetHb (reaction 1). To obtain the total Hb value, all forms of Hb are oxidized to MetHb by adding potassium ferricyanide and the reading is also taken at 632 nm

(reaction 2). Finally, a neutralized cyanide solution is added, followed by a new reading. The total Hb value is obtained by subtracting the two recorded values [66,67].

Reaction 1: MetHb + potassium cyanide = cyanMetHb

Reaction 2: Hb + potassium ferricyanide = MetHb

MetHb quantification: it was used the spectrophotometric technique of induction with ferricyanide and potassium cyanide adapted from the methods described by Evelyn & Malloy, Hegesh *et al.* and Camargo *et al.* [63,64,67]. The %MetHb determination method was applied in duplicate to the whole blood sample from the suspected case and to a whole blood sample from a healthy donor. Initially, distilled water (1.1 mL), phosphate buffer 0.5 M pH=7.2 (0.5 mL) and Triton X-100 (1 drop) were added to the whole blood sample (0.4 mL) to produce total haemolysis. From this, 1.2 mL were transferred to a plastic cuvette (A) and 0.1 mL to a cuvette (B) that contained 1.1 mL of ferricyanide-phosphate reagent. Readings were taken in an UV/VIS spectrophotometer at 632 nm, performing auto-zero against 1.2 mL of distilled water. Then, a neutralized potassium cyanide solution (50 µL) was added to cuvette A and B and readings were taken again at 632 nm, obtaining values C and D, respectively. MetHb is expressed as a percentage of total Hb using the following formula [67]:

$$\%MetHb = \frac{A - C}{(B - D) \times 12} \times 100$$

Due to the high absorption values obtained after spectrophotometer readings, it was necessary to perform 10 and 20x dilutions of the samples using phosphate buffer.

1.3.2. Results & Discussion

1.3.2.1. Toxicological findings

The results obtained are summarized in Table 2:

Table 2. Applied methods for the analysis of biological and non-biological samples of the suspected case of suicide by nitrite ingestion and respective results obtained.

Biological samples	Methods	Results
Blood	Griess reaction and UV/VIS spectrophotometry; Ferricyanide and potassium cyanide and UV/VIS spectrophotometry; HS-GC-FID; ELISA; LC-MS/MS	Extensive haemolysis; MetHb = 96.9%; Sertraline 456 ng/mL*; - ethanol and drugs of abuse*
Urine		[Nitrites] = 2.73 mg/L; Sertraline, risperidone and 9-hydroxyrisperidone*; - drugs of abuse*
Gastric content		[Nitrites] = 0.154 mg/L

Non-biological samples	Methods	Results
White powder	Griess reaction and UV/VIS spectrophotometry; GC-MS	[Nitrites] = 0.6 mg/mg
Glass residues		Camphor and lidocaine
Transparent liquid		
Yellow liquid		

*Data provided by the INMLCF (CE-15/2023); + positive result; - negative result.

Nitrites were present in all samples, excluding the glass residues and both liquids. Powder, blood, urine and gastric content samples tested positive in the Griess reaction, with the formation of a red-violet colour, but with different intensities. On the other hand, the glass residue and both liquids did not test positive in the reaction, with no formation of the characteristic coloration (Figure 4).

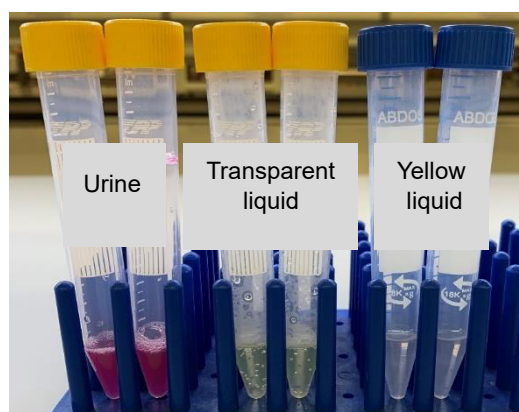


Figure 4. Griess reaction in urine sample and transparent and yellow liquids. In the urine tubes, it was possible to observe an intense red-violet colour after the addition of the Griess reagent. The same did not happen for the tubes of the liquid samples. The transparent liquid after the addition of the Griess reagent acquired a pale-yellow colour and the yellow liquid decreased its original colour with the addition of the reagent.

In the powder, urine and gastric content, the nitrite concentrations were 0.6 mg/mg, 2.73 mg/L, and 0.154 mg/L, respectively. Regarding the concentration of nitrites in the gastric content, the value obtained was much lower than those described in fatal cases in the literature: 1150 mg/L [68], 2200 mg/L [54], 8800 mg/L [5], 11220.1 mg/L [18], 16000 mg/L [9] and 9.2 mg/L (in a case that occurred in Portugal in 2021). On the other hand, in another case reported in 2021, a value <0.03 mg/L was measured in the gastric content of the victim, which is lower than the value found in this case study (Annex I – Table I). Regarding the value obtained in the urine sample, it was much lower than the 24.6 mg/L previously described [54]. The measurement of nitrites in urine and its interpretation has some limitations. For example, it is known that there are bacteria that can colonize the urinary tract producing nitrites, so the presence of nitrites in the urine can be an indication of urinary tract infection [69]. When toxicological analyses are carried out, the nitrite urinary concentration of the victim is usually unknown [22].

Overall, the interpretation of the values obtained from the biological samples becomes difficult due to the influence of multiple *ante-mortem* and *post-mortem* factors. In Forensic Toxicology routine, nitrites are not included in the list of standard substances searched and, therefore, the *post-mortem* concentrations of toxicological significance for either sodium nitrite or sodium nitrate are not established [11]. Although no further conclusions could be drawn, in the present case it was possible to conclude that the three biological samples tested positive for nitrites.

In the present toxicological analysis, a spectrophotometric technique was used for nitrite quantification. Currently, more precise methods and equipment can be used, such as Ion Chromatography with a Conductivity Detector, High-Performance Liquid Chromatography with Electrochemical Detection, and Capillary Ion Analysis with UV Detection [6,27,52].

The analysis requested by the INMLCF to the Laboratory of Toxicology of FFUP was focused on detecting nitrites in the samples. Due to the rapid conversion of nitrites to nitrates in the human body, it would have been interesting to have also analysed the nitrate content [9]. From nitrite, there is also a rapid and extensive formation of NO (leading to symptoms such as severe hypotension and muscle relaxation) which becomes immeasurable as a consequence of its half-life of milliseconds or less [5,70]. The *post-mortem* stability and redistribution of nitrites and nitrates are unknown [11]. The difficulties inherent to this determination in biological samples and the interpretation of the respective values, gives the determination of %MetHb a preponderant role in the diagnosis of the cause of death [9].

In blood, it was possible to detect, but not quantify nitrites. However, the methaemoglobinemia level was of 96.9%, adding evidence to probable nitrite poisoning.

The dark brown colour in the victim's blood sample is consistent with the MetHb value obtained. It is known that blood changes its normal red colour to brown for MetHb values above 15-20%, a characteristic finding usually called "chocolate-brown blood" (Figure 5) [38].



Figure 5. Comparison of the red coloration of the control blood sample with the "chocolate-brown" coloration of the victim's blood sample.

It is noteworthy in routine forensics, the determination of %MetHb is not common. The forensic pathology findings obtained during the autopsy together with the clues found at the scene lead to the investigation of this parameter [11]. MetHb values above 70% are considered lethal, although cases of survival with 94% MetHb and deaths with about 10% MetHb have been described [9,18,38]. Of the fatal cases reported in the literature (Annex I – Table I and II), the lowest MetHb value recorded was 8.5% and the highest was 90.3% [59,71]. The value obtained in this case was higher than those documented. The obtained MetHb value of 96.9%, and the absence of evidence for other toxicity mechanisms, explains the victim's fatal outcome. In addition to sodium nitrite, many other substances are capable of inducing methaemoglobinemia (Part I – Table 1) [38]. However, as it was possible to identify nitrites in the white powder and in the biological samples, these findings further supported the suspicion that sodium nitrite was the intoxicating agent. The victim's parents reported the arrival of an online order of sodium nitrite on the day before the death, an important and common aspect in this new suicide trend. It is worth mentioning the easy purchase of sodium nitrite in the online market specially by young individuals who easily use new technologies.

Analysis by LC-MS/MS, carried out at the Forensic Chemistry and Toxicology Service of INMLCF of the North Delegation, showed the presence of sertraline (456 ng/mL) in the femoral blood and sertraline, risperidone and its metabolite, 9-hydroxyrisperidone in the urine. The victim's clinical history of depression may explain the detection of these two drugs in the biological samples. It is known that the choice of nitrite as a method of suicide is usually made by victims with a psychiatric medical history, including depression [22]. Negativity was obtained for ethanol and drugs of abuse.

GC-MS results obtained from non-biological samples (excluding the white powder) showed the presence of camphor and lidocaine, indicating that other drugs were involved in this fatal intoxication and that may eventually be part of the “Suicide Kit”. Despite the fact that multiple substances are often involved in this type of suicide, these two substances have not been involved in the reported cases depicted in the Annex I. In those cases, it was found that drugs with antiemetic, antacid and gastric emptying properties, such as metoclopramide, ranitidine, famotidine, cimetidine and dimenhydrinate, are often associated with overdose by nitrite ingestion. The consumption of these drugs in this scenario can be understood as a way to counteract the gastrointestinal effects (like gastric reflux, nausea and vomiting) triggered by nitrite ingestion, thereby enhancing tolerance and absorption [5,11,12,13,25]. Interestingly, metoclopramide is classified as a MetHb-inducing drug (Part I – Table 1) [10,38]. Additionally, it was observed that drugs pertaining to other pharmacological categories, including antidepressants, antipsychotics, benzodiazepines and analgesics, are commonly detected [5,13]. However, it should be noted that these medications might be part of the victims' regular prescribed treatment. The victims' clinical history, often linked to depression and suicidal ideation, may grant them access to these medications [9,22,31].

In everyday life, camphor has several applications, being commonly used in medicine for its antipruritic, analgesic and anesthetic properties. Accidental poisoning by ingestion has been reported, and there is already a record in the literature of intentional use [72]. The neurotoxic and hepatotoxic effects caused by camphor are known. After its ingestion, convulsions can appear in a short time and death usually occurs due to respiratory arrest [72,73]. Lidocaine is used daily as a local anesthetic and as an antiarrhythmic agent. Suicide involving lidocaine poisoning is also documented in the literature. An overdose of this drug causes heart and central nervous system damage, leading to respiratory depression, convulsions, coma, ventricular fibrillation and cardiac arrest [74]. It is also known that, like sodium nitrite, lidocaine is an oxidizing agent also included in the list of compounds capable of inducing MetHb (Part I – Table 1) [10,75].

To conclude, this case study is one of the 9 known fatal cases of nitrite poisoning in Portugal (Annex I – Table I). Data obtained from these cases provided by INMLCF (CE-15-2023) showed that this method of suicide is a new trend in Portugal since 2020, often involving multiple drugs and victims at young ages.

2. Assessment of cell toxicity

The literature highlights nitrite-induced MetHb as the main mechanism of death involved in nitrite intoxications. However, other potential mechanisms of toxicity originated by the ingestion of nitrites remain to be explained. Using *in vitro* assays, the cytotoxic effects of sodium nitrite and sodium nitrate were studied in three cell models. The H9c2, Caco-2 and SH-SY5Y cells were considered representative of potential target organs of nitrite intoxication. Due to the toxicokinetics of nitrites, Caco-2 cells were chosen because the gastrointestinal tract is the first line of contact after ingestion. Another relevant aspect is related to toxicodynamics, and the appearance of the described side effects (e.g., nausea and vomits), which lead to the intake of other drugs, often contained in the "Suicide Kits". The studies with the SH-SY5Y and H9c2 cell lines were conducted since the brain and the heart are the main organs affected by the lack of oxygen caused by nitrites, expected by the MetHb effect. Lethargy, loss of consciousness, convulsions and coma are frequently reported neurologic symptoms, and cardiovascular outcomes are related to vasodilation and bradyasystolic arrest.

2.1. *In vitro* investigation of cytotoxicity

An *in vitro* investigation of sodium nitrite toxicity towards potential target organs (heart, intestine and brain) was conducted to test for a possible direct toxic effect. Since nitrites in the blood are quickly oxidized to nitrates, in addition to the study of sodium nitrite, sodium nitrate was also tested in parallel under the same conditions [3]. The cytotoxicity studies of both substances were performed using three cell lines: the rat derived cardiomyocyte H9c2 cell line, the human derived colorectal adenocarcinoma Caco-2 cell line and the human derived neuroblastoma SH-SY5Y cell line [76,77,78]. A screening of several time points was first performed to select the incubation time of sodium nitrite and sodium nitrate to be used in this *in vitro* research. Experiments with exposures lasting 2, 4, 6, 10, 24 and 48 h were carried out. The results obtained in these screenings, together with the toxicokinetic findings reported in the literature for nitrite intoxications, led to the choice of a 4 h incubation period for all performed tests.

2.2. Material & Methods

2.2.1. Chemicals and materials

Dulbecco's modified Eagle's medium (DMEM) high glucose, sodium bicarbonate, trypsin/EDTA solution, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride

(Neutral Red or NR), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Triton X-100, ethanol absolute and acetic acid glacial were obtained from Sigma-Aldrich (Germany). Antibiotic mixture (10.000 U/mL penicillin, 10.000 µg/mL streptomycin) was obtained from Biochrom (Germany). Heat inactivated fetal bovine serum (FBS), Hanks' balanced salt solution with calcium and magnesium [HBSS (+/+)] and non-essential amino acids were obtained from Gibco (United Kingdom). DMSO was obtained from Merck (Germany). Sodium nitrite and sodium nitrate (Sigma-Aldrich, Germany) stock solutions were prepared in sterile HBSS (+/+) and stored at 4-6 °C. All sterile plastic material was obtained from Corning Costar (United States of America). Immortalized human cells used in the research were previously purchased from Invitrogen within previous projects and are routinely used in the FFUP laboratory. Their use raises no ethical issues, as they fall outside of the legislation relevant to primary human tissue and require no contact with patients or primary human material.

2.2.2. Cell culture

2.2.2.1. H9c2 cells

The H9c2 cells were routinely cultured in 75 cm² flasks using complete DMEM high glucose medium, supplemented with 10% FBS and 1% antibiotic mixture, at 37 °C in a humidified 5%CO₂-95% air atmosphere. When cells reached 70–80% confluence, the cell culture medium was removed and a pre-warmed HBSS (+/+) wash was done. Then, the cells were trypsinized (trypsin/EDTA solution) and the cell suspension was counted before seeding into 96-well plates at 100 cells/µL density. The cells were incubated for 24 h and used undifferentiated in the cytotoxicity assays between passages 8 and 14 [79,80].

2.2.2.2. Caco-2 cells

The Caco-2 cells were routinely cultured in 75 cm² flasks using complete DMEM high glucose medium, supplemented with 10% FBS, 1% antibiotic mixture and 1% non-essential amino acids, at 37 °C in a humidified 5%CO₂-95% air atmosphere. Cultures were passaged by trypsinization (trypsin/EDTA solution) after a washout with HBSS (+/+). The cells were seeded in 96-well plates at a density of 128 cells/µL and used 3 days after seeding, when confluence was reached. For the cytotoxicity assays, Caco-2 cells were taken between passages 15 and 25 [77,81].

2.2.2.3. SH-SY5Y cells

The SH-SY5Y cells were routinely cultured in 75 cm² flasks using complete DMEM high glucose medium, supplemented with 10% FBS and 1% antibiotic mixture, at 37 °C in a humidified 5%CO₂-95% air atmosphere. Before reaching confluence, the cells were washed with pre-warmed HBSS (+/+) and trypsinized (trypsin/EDTA solution). The cell suspension was counted before seeding into 96-well plates at 250 cells/μL density. After a 24 h incubation, the cells were used undifferentiated in the cytotoxicity assays. To prevent phenotypic alterations, the SH-SY5Y cells were used between passages 21 and 25 [78,82,83].

2.2.3. Cytotoxicity assays

Cytotoxicity is the potentiality of a compound to induce cell death by interfering with structures or properties that are essential for cell function, proliferation or survival. Cytotoxicity assays are *in vitro* bioassays to predict the toxicity of substances to different tissues, by monitoring the response of the cells exposed to xenobiotics. The main types of assays that are used to monitor the response of cells to potentially toxic substances include: cell viability, cell membrane integrity, cell proliferation and metabolic activity. In this work, two assays were performed as a biological endpoint parameter for detecting cytotoxicity [84,85]. With the selected assays, lysosomal dysfunction and mitochondrial impairment were assessed, using the NR uptake and MTT reduction assays, respectively [78]. The culture media of the H9c2, Caco-2, and SH-SY5Y cells were removed and replaced with fresh complete DMEM medium. Then the cells were acutely exposed to sodium nitrite or sodium nitrate for 4 h at 37 °C, 5% CO₂. To avoid any effect of the solvent, the cells of the control wells were exposed to the same volume of HBSS (+/+) as that used as solvent for nitrite/nitrate-treated cells. A positive control (1% Triton X-100) was also tested in all cell culture experiments. The assays were performed at a wide concentration range (25–400 mM) and the cytotoxic effects were determined by testing full concentration-response cell death curves [78,82]. The Inhibitory Concentration (IC) levels were calculated at two different levels of cytotoxicity: (i) the IC₂₀ (concentration that causes 20% cell death), i.e., mildly, but significantly cytotoxic, and (ii) the IC₅₀ (concentration that causes 50% cell death), i.e., a greatly cytotoxic effect level. All assays were tested in at least six independent experiments performed at least in triplicate followed by analysis with the appropriate statistical tools.

2.2.3.1. Neutral Red uptake assay

The uptake and binding of NR within lysosomes only occurs in viable cells. This weak cationic dye penetrates cells by non-ionic diffusion and accumulates in lysosomes by interaction with anionic sites in the lysosomal matrix. Thus, the amount of dye absorbed allows inferring about lysosomal integrity and/or cell viability [80,86]. After the incubation period, the medium was replaced by fresh medium containing 33 µg/mL NR, and cells were incubated at 37 °C, 5% CO₂ for 1h30. The medium was then removed and cells were washed with a warmed HBSS (+/+) solution. A 50% ethanol/1% glacial acetic acid solution was used to promote the lysis of the incubated cells and dissolve the intracellular NR. The plates were stirred for 2 min and the absorbance was measured at 540 nm and 690 nm (the reference wavelength) in a multi-well plate reader (Biotek Instruments, United States of America) [82].

2.2.3.2. MTT reduction assay

To complement the cytotoxicity study, the MTT reduction assay was performed. The MTT colorimetric assay assesses cell viability by measuring the activity of reductases, being primarily an indicator of metabolically active mitochondria. Cellular enzymes can reduce yellow soluble MTT to purple insoluble formazan salts. This reaction can only occur if the enzymes are active, so this conversion allows the measurement of cell metabolism capacity and, consequently, cell viability [79,87]. After a 4 h exposure period, the medium of each well was carefully removed and replaced by fresh DMEM medium containing 500 µg/mL MTT. Then, the cells were incubated at 37 °C, 5% CO₂ for 1 h. The medium was removed and 100% DMSO was added. The plates were stirred for 2 min and then the amount of formazan formed was measured at 550 nm and 690 nm in a multi-well plate reader (Biotek Instruments, United States of America) [82].

2.2.3.3. Statistical analysis

All statistical calculations were performed with the GraphPad Prism 8.3.0 software and the IC₂₀ values were calculated using the online calculation tool available on the GraphPad website [88].

The t-test was used to see if there were significant differences ($p < 0.05$; confidence level 95%) between control wells with DMEM medium only and control wells with solvent. The solvent control was selected because there were no statistically significant differences between negative and solvent controls.

In the cytotoxicity assays, concentration–response curves were fitted using least squares as the fitting method and the comparisons between curves (IC₅₀, top, bottom, and hill slope) were made using the extra sum-of-squares F test. In each case, p-values lower than 0.05 were considered statistically significant and a significance level of 95% was accepted. All results are shown as mean and standard error of the mean from at least six independent experiments performed at least in triplicate.

The viability results were expressed as percentage of control cells, with mean values set at 100%, according to the following equation [78,89]:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \right) \times 100$$

2.2.4. Analysis of the cellular morphology by digital microscopy

The morphology of H9c2, Caco-2 and SH-SY5Y cells was evaluated 30 min before the end of the 4 h incubation with sodium nitrite/nitrate. Imaging of wells of all cell lines exposed to increasing concentrations was performed with a Lionheart FX Automated Microscope from BioTek (Vermont, USA). Image acquisition was performed using Gen5 Imager Software Version 3.04 with 4x PL ACHx setting, colour Bright Field and scale bar = 1000 µm.

2.3. Results & Discussion

2.3.1. Concentration-dependent cytotoxicity of sodium nitrite and sodium nitrate in H9c2 cells

A concentration-response analysis was carried out by incubating the H9c2 cells for 4 h with 50-400 mM of sodium nitrite and sodium nitrate in separate wells. Figure 6 presents the obtained concentration-response curves showing the NR (A) and MTT (B) assays. A summary of the calculated IC₅₀ values (representing the half-maximal inhibitory concentrations from the fitted curves), IC₂₀, top, bottom and hill slope values are presented in Table 3.

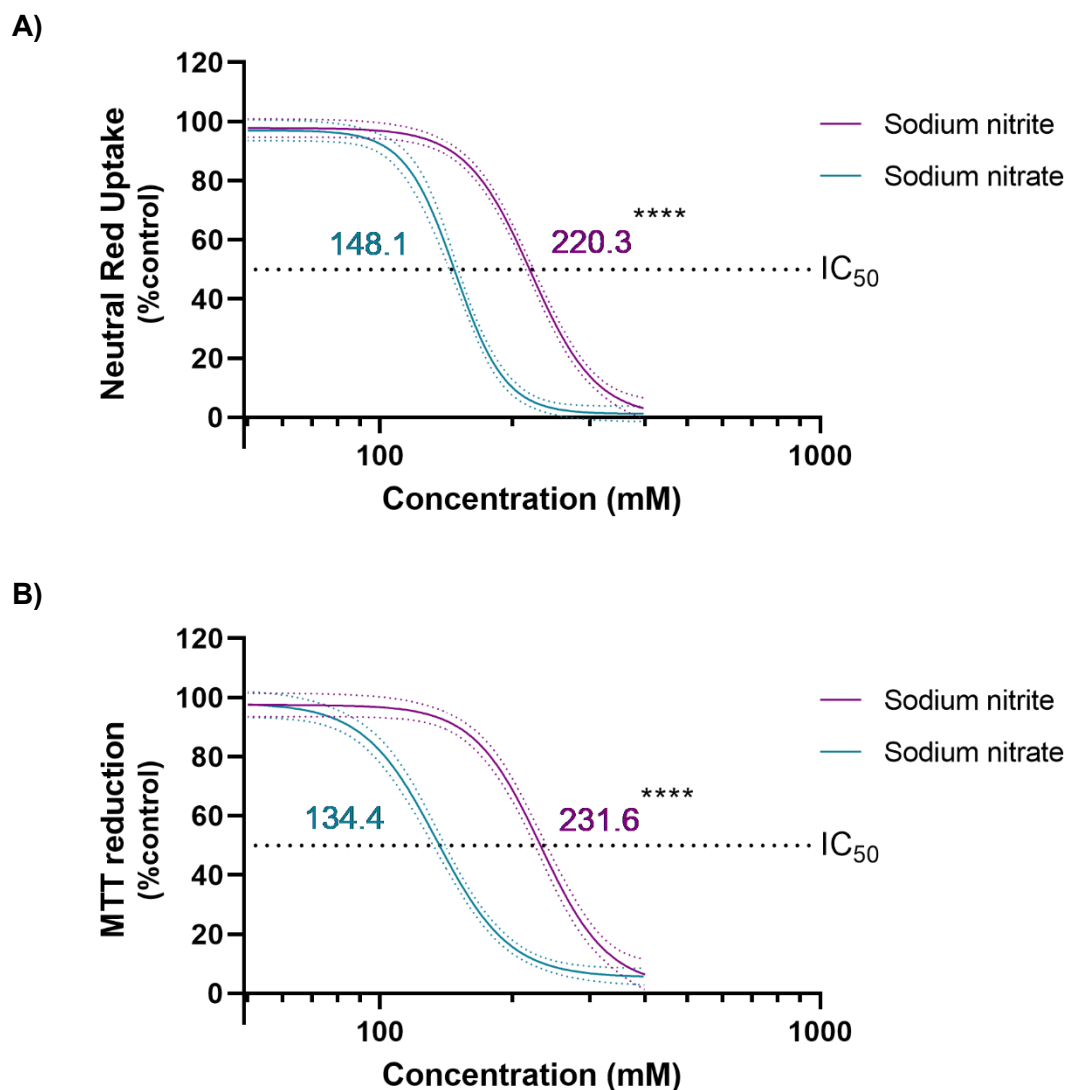


Figure 6. Concentration–response curves of sodium nitrite and sodium nitrate after 4 h incubation in H9c2 cells at 37 °C. Cell viability was evaluated by the NR uptake (A) and the MTT reduction (B) assays. Data are presented as percentage of NR uptake or MTT reduction $\pm$ 95% confidence interval relative to the respective negative controls. Curves were fitted using least squares as the fitting method and comparisons were made using extra sum-of-squares F test (**** $p < 0.0001$).

Table 3. IC₂₀, IC₅₀ (half-maximal inhibitory concentration), top (minimal inhibition), bottom (maximal inhibition) and hill slope values of the sodium nitrite and sodium nitrate concentration-response curves A (NR assay) and B (MTT assay) for H9c2 cells.

A) NR assay	Sodium nitrite	Sodium nitrate
IC ₂₀	173.78	123.38

IC ₅₀	220.3	148.1 ****
Top	97.83	97.04
Bottom	4.125e-006	1.117
Hill slope	-5.845	-7.591
Overall comparison	---	****

B) MTT assay	Sodium nitrite	Sodium nitrate
IC ₂₀	181.47	103.15

IC ₅₀	231.6	134.4 ****
Top	97.52	98.16
Bottom	2.259	5.356
Hill slope	-5.683	-5.238
Overall comparison	---	****

Concentration-response curves were fitted using least squares as the fitting method and the comparisons between sodium nitrite and sodium nitrate curves (IC₅₀, top, bottom and hill slope) were made using extra sum-of-squares F test (**** p<0.0001).

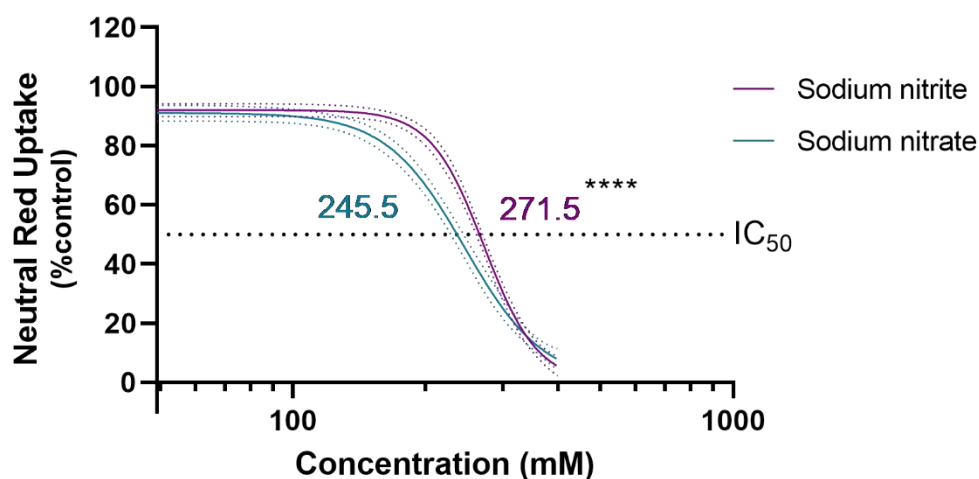
Significant differences were observed for the whole curves and the respective IC₅₀ values, but not for the other evaluated parameters. Based on this data, it was evident that under the experimental conditions mentioned, sodium nitrate (NR IC₅₀=148.1 mM; MTT IC₅₀=134.4 mM) is more cytotoxic than sodium nitrite (NR IC₅₀=220.3 mM; MTT IC₅₀=231.6 mM) for H9c2 cells.

2.3.2. Concentration-dependent cytotoxicity of sodium nitrite and sodium nitrate in Caco-2 cells

A concentration-response analysis was carried out by incubating the Caco-2 cells for 4 h with 25-400 mM of sodium nitrite and sodium nitrate in separate wells. Figure 7 presents the obtained concentration-response curves showing the NR (A) and MTT (B)

assays. A summary of the calculated IC_{50} values (representing the half-maximal inhibitory concentrations from the fitted curves), IC_{20} , top, bottom and hill slope values are presented in Table 4.

A)



B)

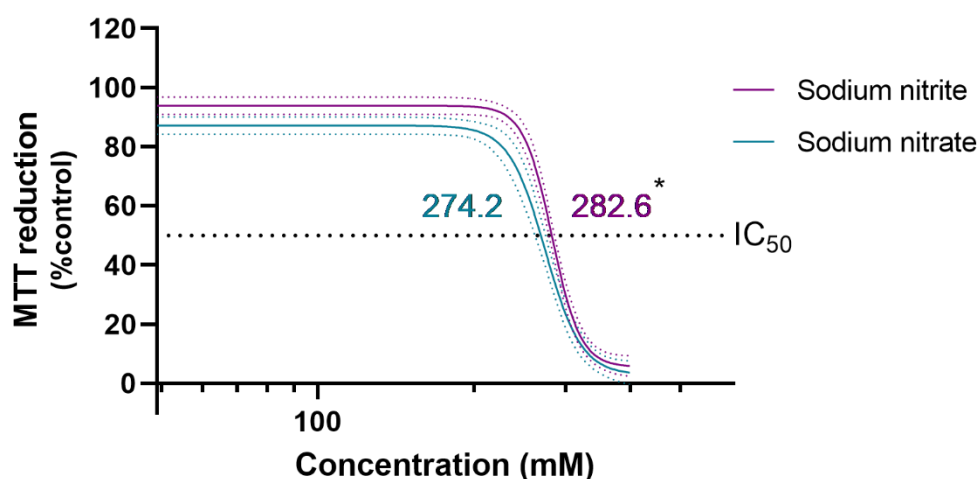


Figure 7. Concentration–response curves of sodium nitrite and sodium nitrate after 4 h incubation in Caco-2 cells at 37 °C. Cell viability was evaluated by the NR uptake (A) and the MTT reduction (B) assays. Data are presented as percentage of NR uptake or MTT reduction $\pm$ 95% confidence interval relative to the respective negative controls. Curves were fitted using least squares as the fitting method and comparisons were made using extra sum-of-squares F test (* $p < 0.05$; **** $p < 0.0001$).

Table 4. IC₂₀, IC₅₀ (half-maximal inhibitory concentration), top (minimal inhibition), bottom (maximal inhibition) and hill slope values of the sodium nitrite and sodium nitrate concentration-response curves A (NR assay) and B (MTT assay) for Caco-2 cells.

A) NR assay	Sodium nitrite	Sodium nitrate
IC ₂₀	223.77	184.75

IC ₅₀	271.5	245.5 ****
Top	91.99	91.04
Bottom	5.774e-008	5.951e-006
Hill slope	-7.170	-4.876 ****
Overall comparison	---	****

B) MTT assay	Sodium nitrite	Sodium nitrate
IC ₂₀	259.07	245.31

IC ₅₀	282.6	274.2 *
Top	93.84	87.13 **
Bottom	5.489	2.805
Hill slope	-15.95	-12.45
Overall comparison	---	****

Concentration-response curves were fitted using least squares as the fitting method and the comparisons between sodium nitrite and sodium nitrate curves (IC₅₀, top, bottom and hill slope) were made using extra sum-of-squares F test (* p<0.05; ** p<0.01; **** p<0.0001).

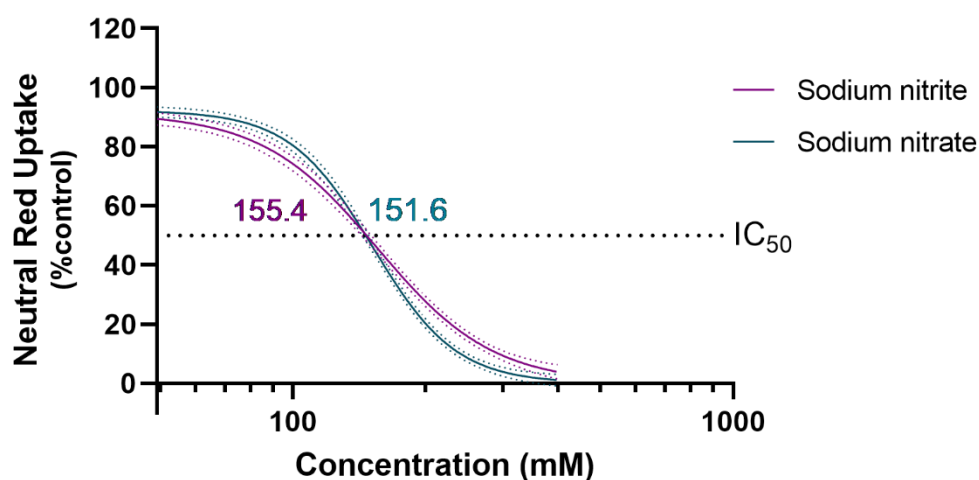
Significant differences were observed for the whole curves, their IC₅₀ values, the hill slope of the NR curve and for the top parameter of the MTT curve. Based on this data, it was evident that under these experimental conditions, sodium nitrate (NR IC₅₀=245.5 mM; MTT IC₅₀=274.2 mM) is more cytotoxic than sodium nitrite (NR IC₅₀=271.5 mM; MTT IC₅₀=282.6 mM) for Caco-2 cells.

2.3.3. Concentration-dependent cytotoxicity of sodium nitrite and sodium nitrate in SH-SY5Y cells

A concentration-response analysis was carried out by incubating the SH-SY5Y cells for 4 h with 25-400 mM of sodium nitrite and sodium nitrate in separate wells. Figure 8 presents the obtained concentration-response curves showing the NR (A) and MTT (B)

assays. A summary of the calculated IC_{50} values (representing the half-maximal inhibitory concentrations from the fitted curves), IC_{20} , top, bottom and hill slope values are presented in Table 5.

A)



B)

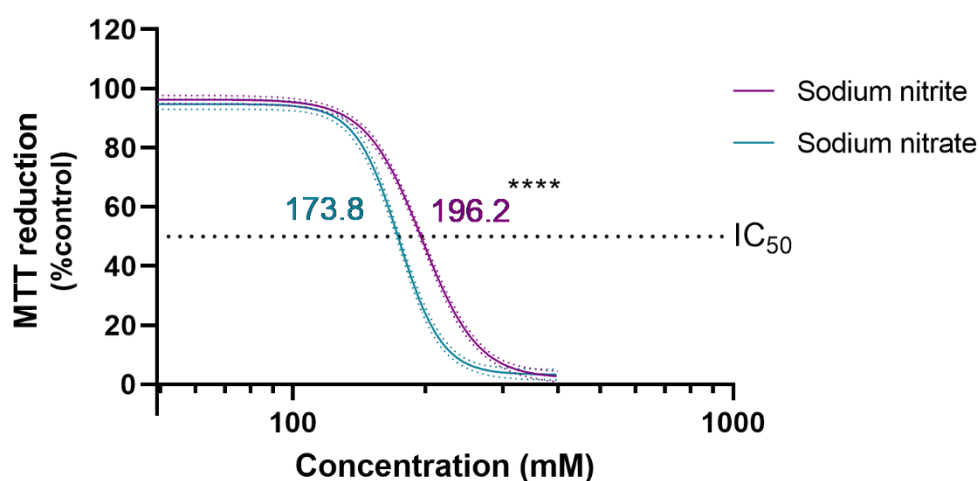


Figure 8. Concentration–response curves of sodium nitrite and sodium nitrate after 4 h incubation in SH-SY5Y cells at 37 °C. Cell viability was evaluated by the NR uptake (A) and the MTT reduction (B) assays. Data are presented as percentage of NR uptake or MTT reduction $\pm$ 95% confidence interval relative to the respective negative controls. Curves were fitted using least squares as the fitting method and comparisons were made using extra sum-of-squares F test (**** $p < 0.0001$).

Table 5. IC₂₀, IC₅₀ (half-maximal inhibitory concentration), top (minimal inhibition), bottom (maximal inhibition) and hill slope values of the sodium nitrite and sodium nitrate concentration-response curves A (NR assay) and B (MTT assay) for SH-SY5Y cells.

A) NR assay	Sodium nitrite	Sodium nitrate
IC ₂₀	102.21	112.10

IC ₅₀	155.4	151.6
Top	91.34	92.20 *
Bottom	9.099e-007	8.765e-007
Hill slope	-3.309	-4.593 *
Overall comparison	---	***

B) MTT assay	Sodium nitrite	Sodium nitrate
IC ₂₀	160.28	148.65

IC ₅₀	196.2	173.8 ****
Top	96.27	94.73
Bottom	1.948	3.227
Hill slope	-6.855	-8.869 ***
Overall comparison	---	****

Concentration-response curves were fitted using least squares as the fitting method and the comparisons between sodium nitrite and sodium nitrate curves (IC₅₀, top, bottom and hill slope) were made using extra sum-of-squares F test (* p<0.05; *** p<0.001; **** p<0.0001).

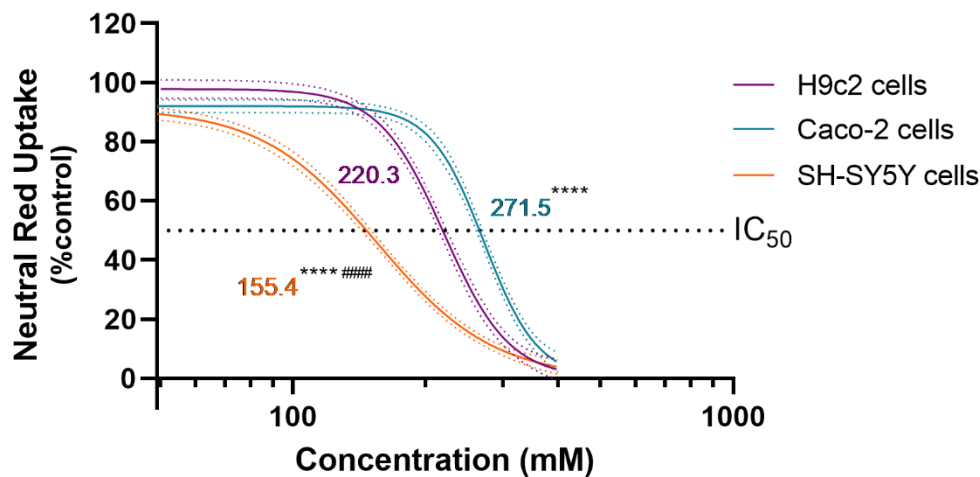
Significant differences were observed for the IC₅₀ values only in the MTT assay. The two curves showed statistically significant differences in the hill slope parameter and in the NR curve also in the top. Based on MTT data, sodium nitrate (NR IC₅₀=151.6 mM; MTT IC₅₀=173.8 mM) is more cytotoxic than sodium nitrite (NR IC₅₀=155.4 mM; MTT IC₅₀=196.2 mM) for SH-SY5Y cells.

2.3.4. Comparative study of cytotoxicity of sodium nitrite among H9c2, Caco-2 and SH-SY5Y cell lines

A comparative study of the cytotoxicity caused by sodium nitrite in the three proposed cell lines was carried out. Figure 9 presents the obtained concentration-response curves showing the NR (A) and MTT (B) assays. A summary of the calculated

IC₅₀ values (representing the half-maximal inhibitory concentrations from the fitted curves), IC₂₀, top, bottom and hill slope values are presented in Table 6.

A)



B)

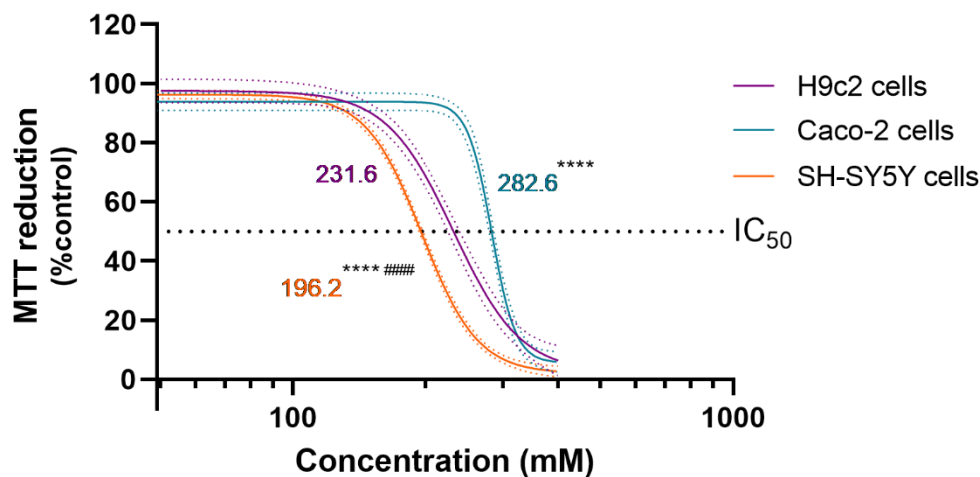


Figure 9. Concentration–response curves of sodium nitrite after 4 h incubation in H9c2, Caco-2 and SH-SY5Y cells at 37 °C. Cell viability was evaluated by the NR uptake (A) and the MTT reduction (B) assays. Data are presented as percentage of NR uptake and MTT reduction $\pm$ 95% confidence interval relative to the respective negative controls. Curves were fitted using least squares as the fitting method and comparisons were made using extra sum-of-squares F test (**** or #### p<0.0001). * Compares to the H9c2 cell line; # Compares to the Caco-2 cell line.

Table 6. IC₂₀, IC₅₀ (half-maximal inhibitory concentration), top (minimal inhibition), bottom (maximal inhibition) and hill slope values of the sodium nitrite concentration-response curves A (NR assay) and B (MTT assay) for H9c2, Caco-2 and SH-SY5Y cell lines.

A) NR assay	H9c2 cells	Caco-2 cells	SH-SY5Y cells
IC ₂₀	173.78	223.77	102.21

IC ₅₀	220.3	271.5 ****	155.4 **** ####
Top	97.83	91.99 ***	91.34 ****
Bottom	4.125e-006	5.774e-008	9.099e-007 ####
Hill slope	-5.845	-7.170 ****	-3.309 **** ####
Overall comparison	---	****	**** ####

B) MTT assay	H9c2 cells	Caco-2 cells	SH-SY5Y cells
IC ₂₀	181.47	259.07	160.28

IC ₅₀	231.6	282.6 ****	196.2 **** ####
Top	97.52	93.84	96.27
Bottom	2.259	5.489	1.948
Hill slope	-5.683	-15.95 ****	-6.855 ####
Overall comparison	---	****	**** ####

Concentration-response curves were fitted using least squares as the fitting method and the comparisons between H9c2, Caco-2 and SH-SY5Y curves (IC₅₀, top, bottom and hill slope) were made using extra sum-of-squares F test (** p<0.001; **** or #### p<0.0001). * Compares to the H9c2 cell line; # Compares to the Caco-2 cell line.

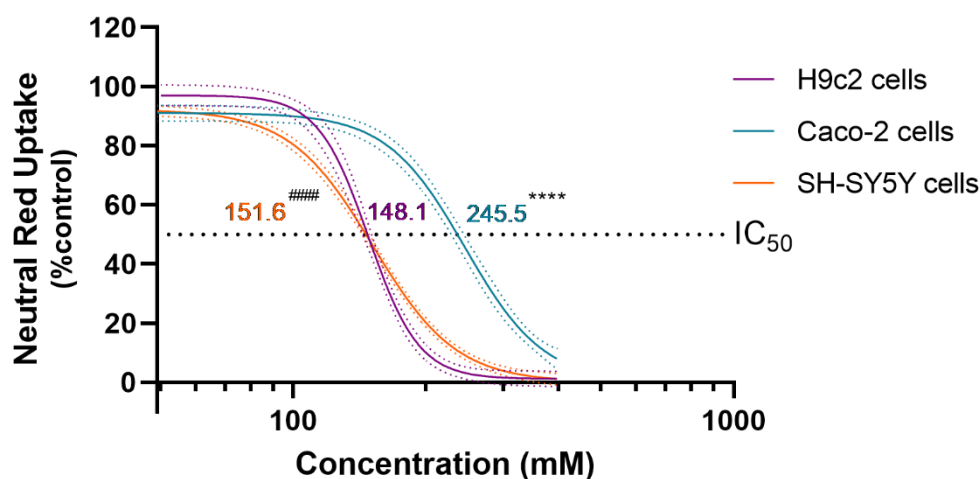
Significant differences were observed for the IC₅₀ values in both assays when comparing the three cell lines. The values obtained in the MTT assay agree with those obtained in the NR assay. For sodium nitrite, SH-SY5Y cells seem to be the most sensitive and Caco-2 cells the least sensitive.

2.3.5. Comparative study of cytotoxicity of sodium nitrate among H9c2, Caco-2 and SH-SY5Y cell lines

A comparative and simultaneous study of the cytotoxicity caused by sodium nitrate in the three proposed cell lines was carried out. Figure 10 presents the obtained

concentration-response curves showing the NR (A) and MTT (B) assays. A summary of the calculated IC_{50} values (representing the half-maximal inhibitory concentrations from the fitted curves), IC_{20} , top, bottom and hill slope values are presented in Table 7.

A)



B)

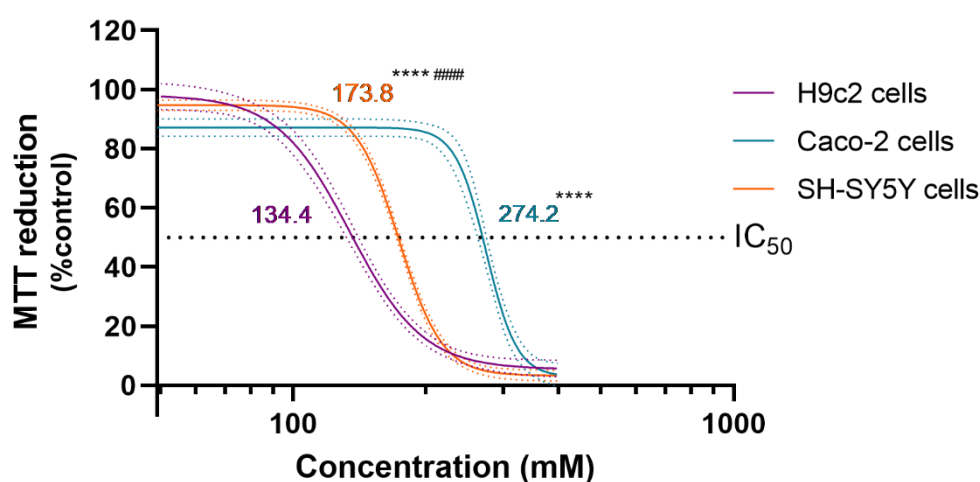


Figure 10. Concentration–response curves of sodium nitrate after 4 h incubation in H9c2, Caco-2 and SH-SY5Y cells at 37 °C. Cell viability was evaluated by the NR uptake (A) and the MTT reduction (B) assays. Data are presented as percentage of NR uptake and MTT reduction $\pm$ 95% confidence interval relative to the respective negative controls. Curves were fitted using least squares as the fitting method and comparisons were made using extra sum-of-squares F test (**** or #### $p < 0.0001$). * Compares to the H9c2 cell line; # Compares to the Caco-2 cell line.

Table 7. IC₂₀, IC₅₀ (half-maximal inhibitory concentration), top (minimal inhibition), bottom (maximal inhibition) and hill slope values of the sodium nitrate concentration-response curves A (NR assay) and B (MTT assay) for H9c2, Caco-2 and SH-SY5Y cell lines.

A) NR assay	H9c2 cells	Caco-2 cells	SH-SY5Y cells
IC ₂₀	123.38	184.75	112.10

IC ₅₀	148.1	245.5 ****	151.6 ####
Top	97.04	91.04 ***	92.20 **
Bottom	1.117	5.951e-006	8,765e-007
Hill slope	-7.591	-4.876	-4.593 ***
Overall comparison	---	****	**** ####

B) MTT assay	H9c2 cells	Caco-2 cells	SH-SY5Y cells
IC ₂₀	103.15	245.31	148.65

IC ₅₀	134.4	274.2 ****	173.8 **** ####
Top	98.16	87.13 ***	94.73 ###
Bottom	5.356	2.805	3.227
Hill slope	-5.238	-12.45 ****	-8.869 ****
Overall comparison	---	****	**** ####

Concentration-response curves were fitted using least squares as the fitting method and the comparisons between H9c2, Caco-2 and SH-SY5Y curves (IC₅₀, top, bottom and hill slope) were made using extra sum-of-squares F test (** p<0.01; *** or ### p<0.001; **** or #### p<0.0001). * Compares to the H9c2 cell line; # Compares to the Caco-2 cell line.

Significant differences were observed for the IC₅₀ values in both assays when comparing the three cell lines, except when comparing the IC₅₀ value of H9c2 cells (148.1 mM) with SH-SY5Y cells (151.6 mM) in the NR assay. The MTT assay shows that H9c2 cells appear to be the most sensitive to sodium nitrate. On the other hand, and similar to what was observed with sodium nitrite, the Caco-2 cells appear to be the least sensitive to sodium nitrate both in the NR and MTT assays.

2.3.6. Microscopic visualization of the cells

The effect of sodium nitrite and sodium nitrate on cell morphology and cell death was confirmed with cell imaging using the Lionheart FX Automated Microscope (Figures 11-13). The analysis conducted through microscope visualization, shortly before the end of incubation, showed that treatment with sodium nitrite and sodium nitrate led to morphologic changes with loss of normal cellular structure for all cell lines. In general, the analysis of the presented images was able to corroborate the data obtained from the NR and MTT assays and changes in cell morphology occur with increasing concentrations of the two compounds under study.

2.3.6.1. H9c2 cells

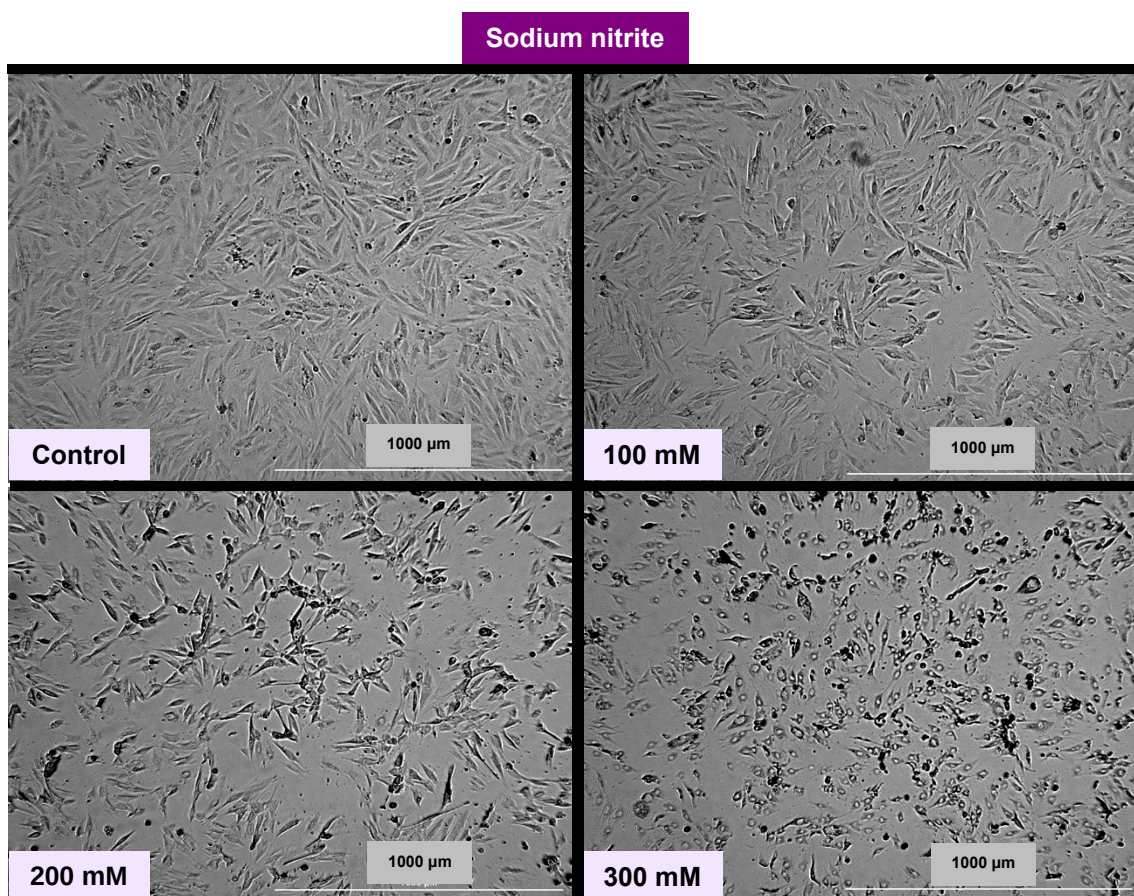


Figure 11a. Microscope cellular visualization through Lionheart FX of negative control wells and sodium nitrite treatment wells at concentrations of 100, 200 and 300 mM on H9c2 cells.

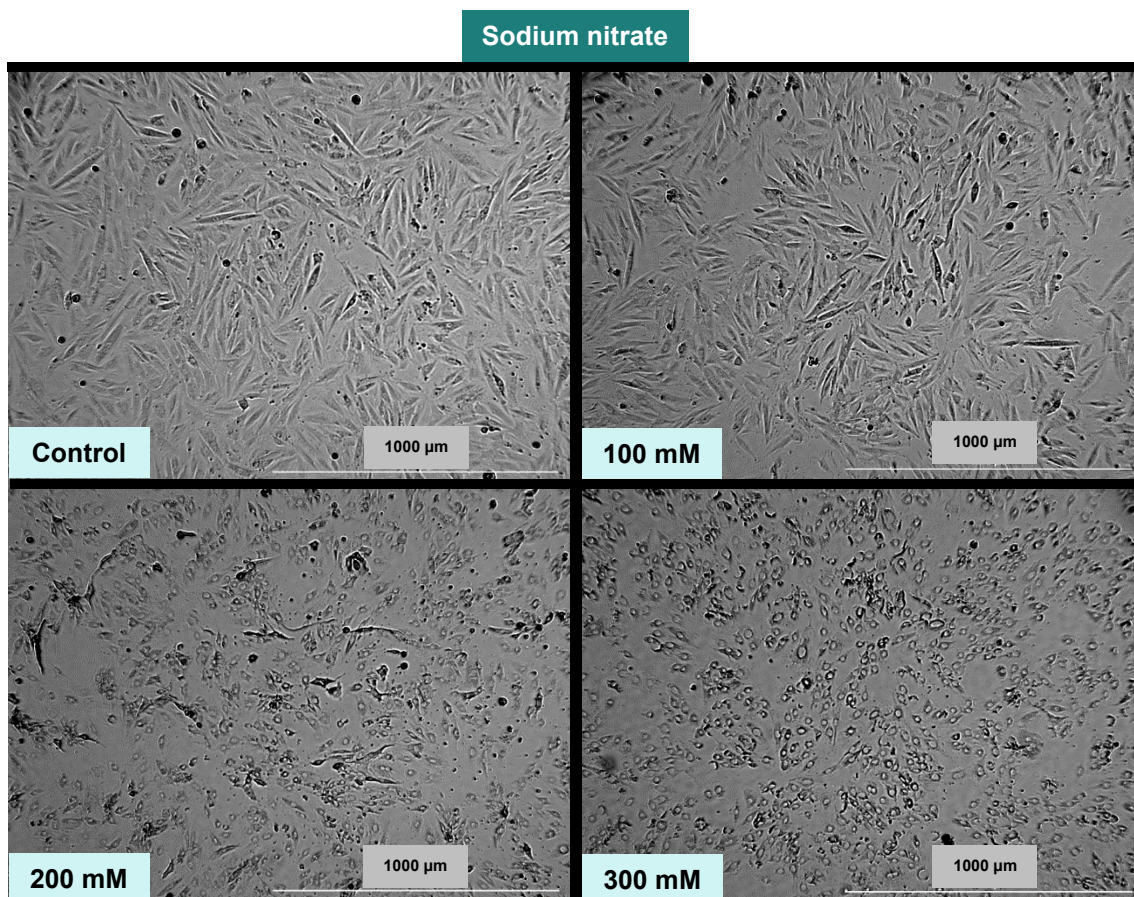


Figure 11b. Microscope cellular visualization through Lionheart FX of negative control wells and sodium nitrate treatment wells at concentrations of 100, 200 and 300 mM on H9c2 cells.

H9c2 cells are fusiform and normally have a parallel arrangement [90]. At a concentration of 100 mM, there is an increase in spaces without cells in the well, but those that remain adhered to the bottom show normal morphology. In the 200 mM sodium nitrite wells, there are many empty spaces, already some rounded cells and some dead cells floating in the culture medium. In the well of sodium nitrate, at this concentration, empty spaces can be observed, the cells are small and rounded, with a large amount of cells floating in the medium observed in other focus planes. So, in the image of sodium nitrate at 200 mM, a more drastic change in cell morphology can be seen as compared to the same concentration of sodium nitrite. At a concentration of 300 mM of both compounds, it is observed that almost all cells have a rounded morphology, completely different from that of the control well, and many dead cells. This is consistent with the values obtained in the NR and MTT assays, concluding that sodium nitrate is more cytotoxic for H9c2 cells than nitrite.

2.3.6.2. Caco-2 cells

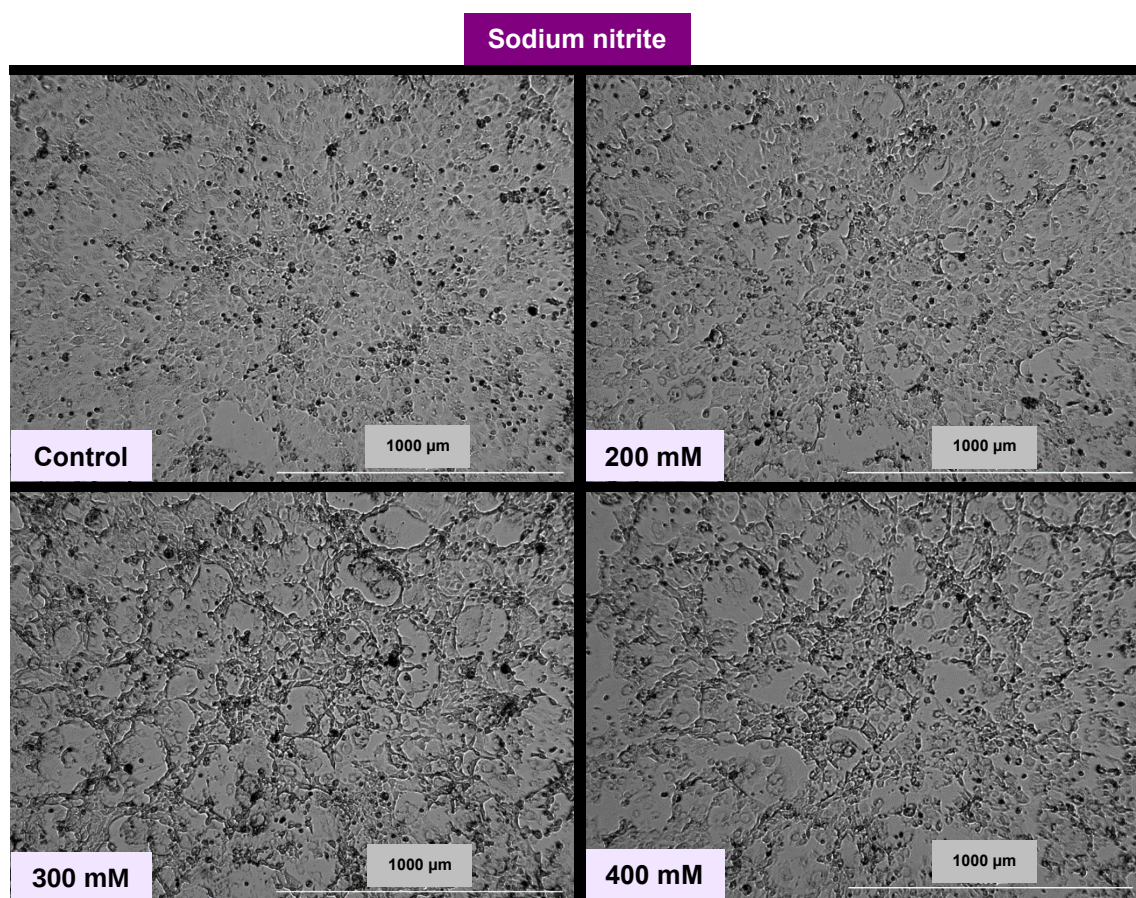


Figure 12a. Microscope cellular visualization through Lionheart FX of negative control wells and sodium nitrite treatment wells at concentrations of 200, 300 and 400 mM on Caco-2 cells.

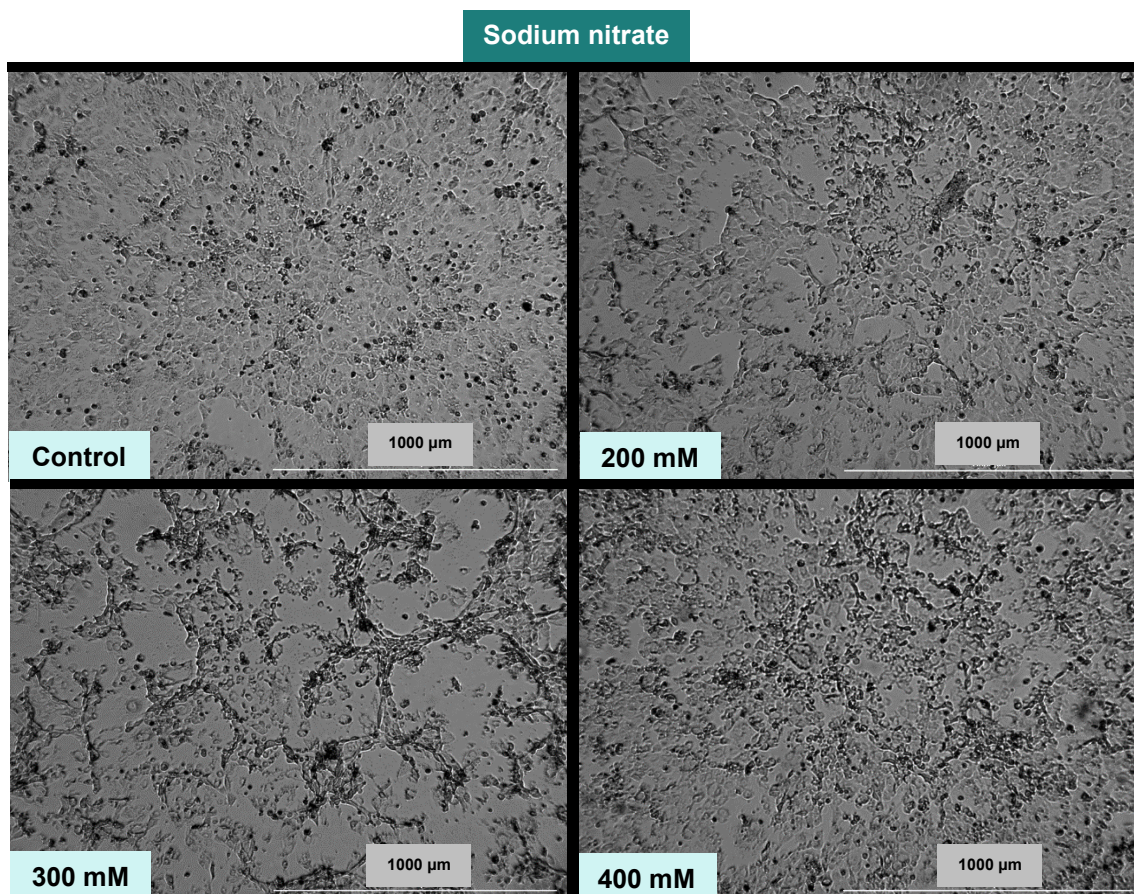


Figure 12b. Microscope cellular visualization through Lionheart FX of negative control wells and sodium nitrate treatment wells at concentrations of 200, 300 and 400 mM on Caco-2 cells.

Caco-2 cells grow in a cylindrical monolayer, possessing brush-border enzyme-secreting microvilli that develop on the apical side and form uniform tight unions with adjacent cells [91]. At the 200 mM concentration, a few empty spaces in the wells and some dead ones floating around can be observed. At 300 mM, similar findings were noted, but with extensive aggravation. A greater number of empty spaces is noticeable in the wells at 300 mM sodium nitrate compared to sodium nitrite at the same concentration. This is consistent with the results obtained in the NR and MTT assays, which demonstrate that nitrate is more toxic to Caco-2 cells than nitrite. At 400 mM, in addition to the existence of empty spaces, it is most notable the great amount of floating dead cells that are seen in several planes when the microscope is focused. Unlike H9c2 and SH-SY5Y cells, where a marked concentration-dependent change in cell morphology was noted, images of Caco-2 cells do not appear to show the same. Indeed, although Caco-2 cell imaging does not reveal significant change at higher concentrations of the compounds, data from the NR and MTT assays do. The explanation for this may be because the cells do not present morphological alterations, but only physiological

ones, since Caco-2 cells at high concentrations of nitrite and nitrate failed to incorporate NR dye into lysosomes or metabolize MTT.

2.3.6.3. SH-SY5Y cells

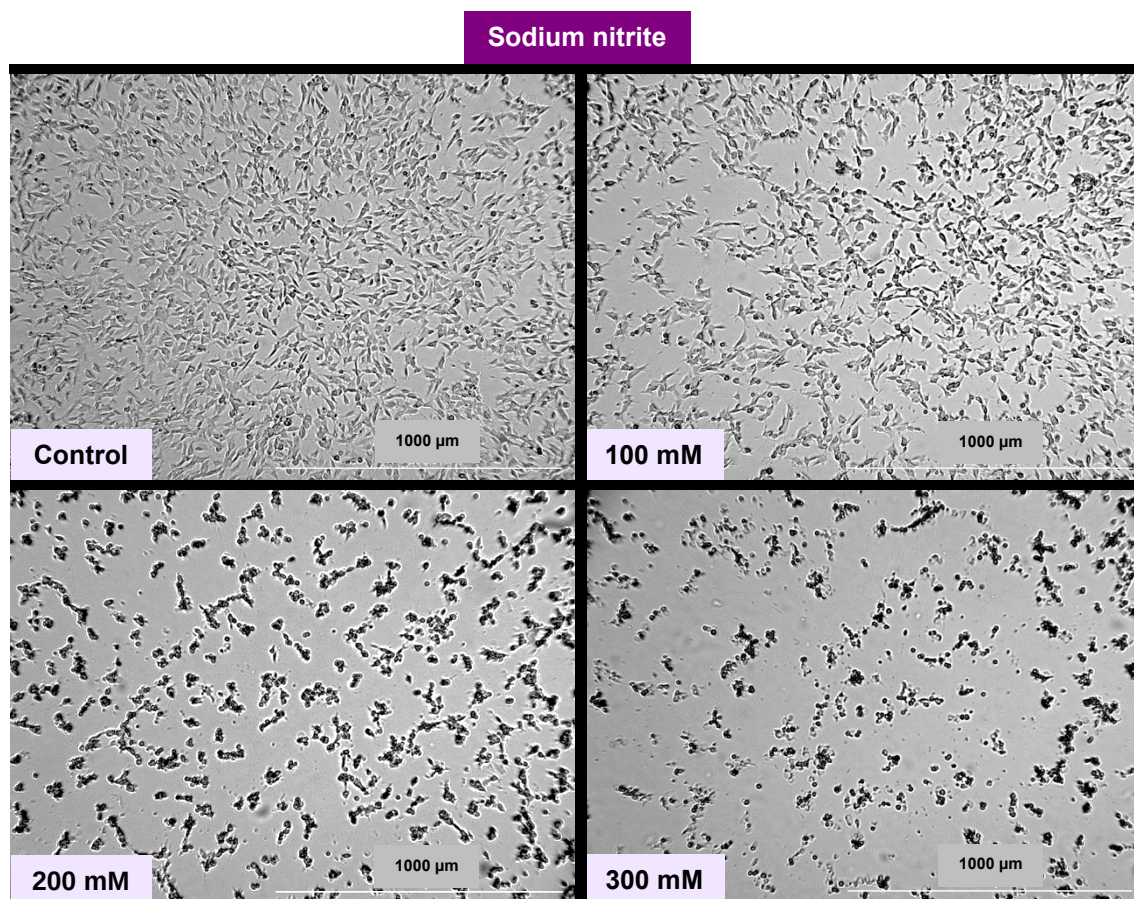


Figure 13a. Microscope cellular visualization through Lionheart FX of negative control wells and sodium nitrite treatment wells at concentrations of 100, 200 and 300 mM on SH-SY5Y cells.

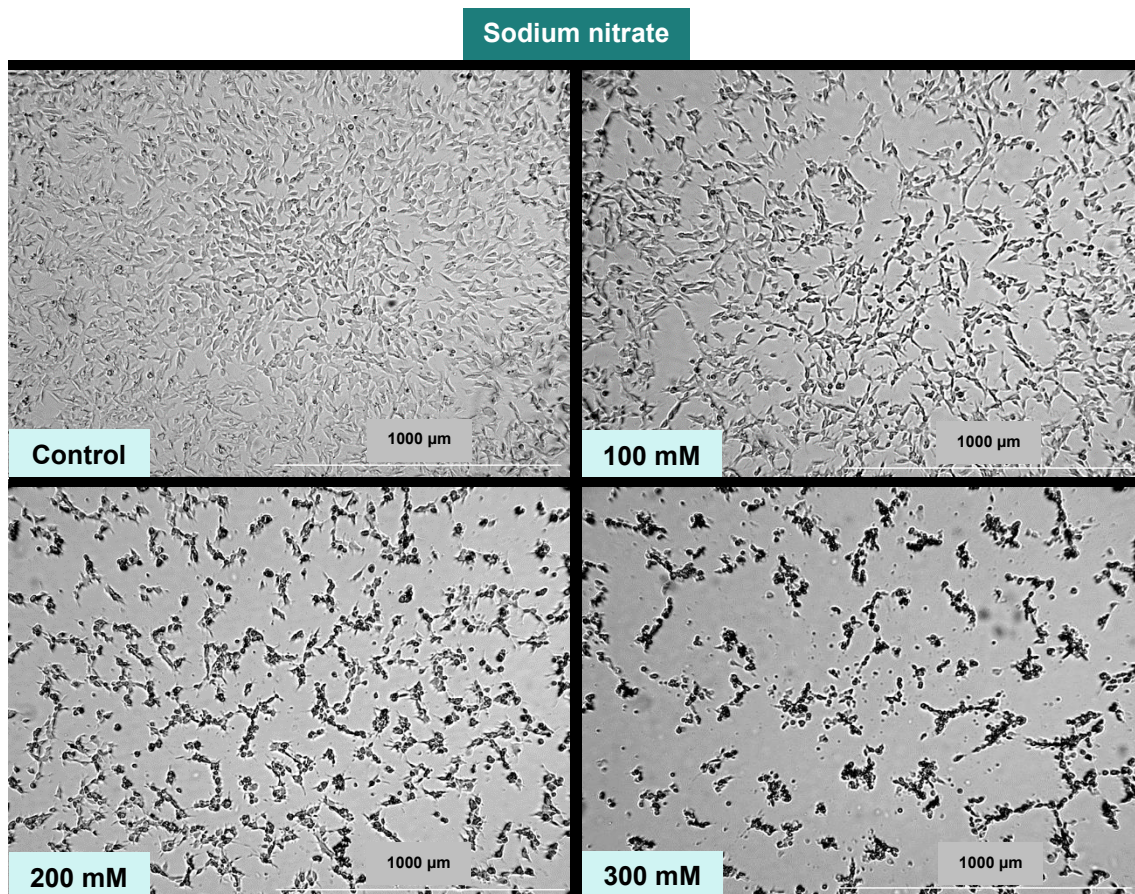


Figure 13b. Microscope cellular visualization through Lionheart FX of negative control wells and sodium nitrate treatment wells at concentrations of 100, 200 and 300 mM on SH-SY5Y cells.

Undifferentiated SH-SY5Y cells have morphological similarities to neuroblasts, with few truncated processes [92]. At a concentration of 100 mM, there are spaces with no cells, but those that remain adhered to the bottom of the well maintain their original morphology. At a concentration of 200 mM, numerous clusters and morphologically rounded cells are observed, completely different from those in the control wells. Furthermore, by focusing the microscope, an increase in the number of dead cells floating in the culture medium becomes perceptible in other planes of the image. At 300 mM, there are even fewer clumps of small, round cells and a large number of floating cells. In these images, sodium nitrite and sodium nitrate seem to show similar cytotoxicity for SH-SY5Y cells, which is consistent with the values obtained in the NR assay.

Overall, each studied cell line presents a specific toxicity profile. Both NR and MTT assays revealed that for the three cell lines, sodium nitrite and sodium nitrate induce cytotoxicity in a concentration-dependent manner. Despite that, it can be concluded that no significant cytotoxicity at biologically relevant concentrations of sodium nitrite and sodium nitrate were observed for any of the tested cell lines. It is important to note that nitrites and nitrates are not foreign substances to the human organism. In addition to daily exogenous exposure, these substances are endogenously produced [3]. In a physiological situation, a 50-100 nM concentration of nitrites in human plasma can be expected, so the IC₅₀ values obtained in the performed assays (154.4-282.6 mM) are comparatively high [1]. Even considering that at an acute intoxication scenario the attained concentrations largely exceed those that are expected at physiological levels, from the data depicted in the Annex I – Tables I and II, it can be concluded that the highest concentrations found in the blood of the intoxicated individuals (4.32 mM for nitrite and 6.5 mM for nitrate) are still lower than those corresponding to these calculated IC₅₀ values [6,52].

Nevertheless, these *in vitro* assays in cellular models have some limitations. An example of such limitations is the fact that SH-SY5Y and Caco-2 cells are from human lineage but H9c2 cells are derived from rat, and all three are immortalized cell lines, which may compromise their representativeness [76,77,78].

Based on these cytotoxicity data it seems unlikely that the direct cytotoxic effect of nitrite contributes significantly to the fatal outcome of intoxications or plays a major role in target-organ toxicity. The studies on nitrite and nitrate cytotoxicity available on the published literature are quite scarce. Therefore, it is difficult to compare the cytotoxic effect observed in our cell lines under our experimental conditions, or even with effects on other relevant cellular models. In fact, previously published studies mainly focus on the beneficial and cytoprotective effects of these substances, at concentrations that are obviously much lower than those involved in human intoxications [93,94,95]. Still, there are already some *in vitro* and *in vivo* studies on the harmful effects of these chemicals on some cell lines different from those studied in this work [49,96,97,98,99]. The integration of this information and the results presented above may contribute to deepen the knowledge on this topic and serve as a starting point for future investigations.

An *in vitro* study conducted by Vatasser *et al.* (2004) analysed the oxidative stress and the inhibition of oxidative phosphorylation in subcellular fractions of rat brain caused by nitrite. It was shown that this compound was able to inhibit mitochondrial oxidative phosphorylation (5 mM nitrite caused a 40% reduction in the rate of mitochondrial respiratory control). The mechanism by which nitrite appears to disrupt oxidative phosphorylation is through the modulation of NO levels, possibly causing a slight

uncoupling of mitochondria. The same study adds that nitrate would not exert these harmful actions unless it was first reduced to nitrite [97]. In another *in vitro* study, Jondeau *et al.* (2006) evaluated sublethal cytotoxicity in human hepatoma HepG2 cells using water contaminants, including sodium nitrate. For this, three cytotoxicity assays (RNA synthesis determination, alamar blue assay and ATP bioluminescence assay) were performed after exposing the cells to 11 substances: acrylamide, atrazine, B[a]P, BPA, 2,4-DAT, 17- α EE, H₂O₂, 4-OP, bromate, chlorate and nitrate (14.6-257 mM). For sodium nitrate, IC₅₀ values of 40.6 mM, 160 mM and 72.8 mM were obtained in the RNA, alamar blue and ATP assays, respectively. The authors concluded that sodium nitrate along with sodium chlorate were the compounds with the highest IC₅₀ values, therefore, considered the least toxic [100].

Regarding *in vivo* studies, El-Nabarawy *et al.* (2020) studied the effect of nitrite on hepatotoxicity and nephrotoxicity in adult rats by evaluating histopathological changes and activation of apoptosis. For this, 20, 40, 60, and 75 mg/kg body weight of sodium nitrite were administered in a single acute dose orally. In a dose-dependent manner, the researchers confirmed disruption in biochemical analysis and oxidative/antioxidative balance; methaemoglobinemia; histopathological changes; and activation of the Bax, Bcl2, and caspase 3 genes, associated with apoptosis in hepatic and renal tissues [96]. Other authors also demonstrated that sodium nitrite can trigger cytotoxicity through the formation of ROS, causing mitochondrial damage and the release of lysosomal enzymes, ultimately resulting in hepatocytes cell death [101].

In addition to the previously discussed studies, some reports of clinical cases related to nitrite intoxication can also elucidate on potential target-organ toxic effects. A reported case in Canada (2023) depicts a successful liver transplantation episode from a brain-dead donor after intentional ingestion of sodium nitrite. Opposite to the findings in the *in vitro* studies, this case reported no hepatic damage in human poisoning with sodium nitrite [102]. Another reported case of nitrite poisoning in China (2022), focused on brain damage, showing delayed post-hypoxic leukoencephalopathy, a condition associated to oxygen deprivation [26]. This case, together with our results in the SH-SY5Y cells, raise the hypothesis that the brain may possibly not be a target organ to the direct action of nitrite, but brain damage is certainly associated with the indirect hypoxic effects. Thus, the formation of MetHb seems to be the primary toxicity mechanism in the brain in cases of intoxication.

3. *In vitro* simulation of gastrointestinal digestion of a sodium nitrite solution

The *in vitro* digestion assays were conducted to study the possible conversions of sodium nitrite into other chemical species along the gastrointestinal tract after ingestion. This simulation involved mimicking the sequential physiological conditions during an oral, gastric and intestinal phases. A simplified model with representative electrolytes, enzymes and pH of each digestive phase was used. The experimental conditions were adapted to a context of suicide by nitrite ingestion and, therefore, 2.6 g of sodium nitrite were used, as this is documented lethal dose for adults [54]. Therefore, the oral intake of 2.6 g of sodium nitrite dissolved in a volume equivalent to a glass of water (250 mL) was simulated [9]. Samples were taken at each of the three stages of digestion and the respective nitrite ion contents were measured.

3.1. Material & Methods

3.1.1. Chemicals and materials

A solution of artificial saliva was prepared according to the British Standard Specification for Safety Harnesses (BS 6684): 450 mg sodium chloride (José Manuel dos Santos, LDA, Portugal), 30 mg potassium chloride (Chem-Lab, Germany), 30 mg sodium sulfate (Sigma-Aldrich, Germany), 40 mg ammonium chloride (Merck, Germany), 20 mg urea (Sigma-Aldrich, Germany) and 300 mg lactic acid (Sigma-Aldrich, Germany) dissolved in 100 mL distilled water adjusted to pH 4.5-5.0 with NaOH in water [103]. Sodium nitrite, zinc sulfate heptahydrate, 37% hydrochloric acid, α -amylase from *Aspergillus oryzae* ~1.5 U/mg, pepsin from porcine gastric mucosa $\geq$ 400 U/mg protein (P7125), pancreatin from porcine pancreas 4xUSP specifications (P1750) and bile salts (B8756) were acquired from Sigma-Aldrich, Germany. The Certificates of Analysis issued by Sigma-Aldrich were analysed to determine the exact values of U/mg and U/mg of protein of the used batches of α -amylase and pepsin, respectively. Calculations were made knowing that the α -amylase used contained 1.1 U/mg and that pepsin contained 1000 U/mg of protein and 11.6% protein. The protocol followed the experimental procedures reported by Ribeiro *et al.* (2020) [104]. 10M and 1M NaOH were used to adjust pH values. $\text{ZnSO}_4 + 2\text{M NaOH}$ were used to precipitate proteins and eliminate turbidity prior to the Griess reaction. Nitrite standards were prepared from a 1000 mg/L nitrite ion standard solution from Certipur® 1198990500 (Merck, Germany). The Griess reagent (phosphoric acid 85%, sulfanilamide, N-(1-naphthyl)-ethylenediamine

dihydrochloride and water) was provided by the Laboratory of Bromatology and Hydrology, Department of Chemical Sciences of the FFUP.

3.1.2. *In vitro* digestion method and nitrite ion content assessment

Before starting the digestion simulation tests, four calibration curves were plotted to guarantee that the sample pre-treatment with NaOH+ZnSO₄ and the pH adjustments did not interfere with the Griess reaction: (i) a curve in water with the addition of 300 µL of water and vortex-mixed 30 s; (ii) a curve in water (reflecting the oral phase conditions) with the addition of 300 µL of 2M NaOH and 0.6 g of ZnSO₄, vortex-mixed 30 s and centrifuged at 4000 rpm, 10 min; (iii) a curve in water at pH=2 (reflecting the gastric phase conditions) with the addition of 300 µL of 2M NaOH and 0.6 g of ZnSO₄, vortex-mixed 30 s and centrifuged at 4000 rpm, 10 min; (iv) a curve in water at pH=6 (reflecting the intestinal phase conditions) with the addition of 300 µL of 2M NaOH and 0.6 g of ZnSO₄, vortex-mixed 30 s and centrifuged at 4000 rpm, 10 min. Standard concentrations (0-7.246 mM) prepared from a certified standard stock solution of 1000 mg/L of nitrite ion were tested in duplicate.

As previously mentioned, the method used for simulating the *in vitro* digestion was based on the protocol described by Ribeiro *et al.* (2020), but small changes were made according to the INFOGEST protocol [104,105]. Mouth digestion was conducted with the addition of 0.6 mL of the artificial saliva solution with α-amylase (100 U/mL) to the initial 150.72 mM sodium nitrite solution. A pH value of 6.9 was recorded, which is compatible with the normal pH values described for this digestion stage (5.6-6.9) [106,107]. Incubation took place for 1 min, 200 rpm at 37 °C. For the gastric phase, pH=2 was adjusted using concentrated HCl and a freshly prepared pepsin solution was added at a rate of 0.05 mL/mL of sample, to ensure a final 2000 U/mL concentration. Incubation took place for 60 min, 130 rpm at 37 °C [106,107]. Lastly, intestinal digestion was monitored by the addition of 10M and 1M NaOH solutions to adjust the pH to 6. Pancreatin and bile salts powders were added at a concentration of 2 g/L and 12 g/L, respectively, in a ratio of 0.25 mL/mL of sample. Incubation took place for 60 min, 45 rpm at 37 °C [106,107]. The assays were performed under controlled temperature to simulate normal body temperature (≈37 °C). Light protection was achieved using aluminium foil.

Aliquots of digests (10 mL) were collected at the end of the oral (sampling time=1 min), gastric (sampling time=61 min) and intestinal phases (sampling time=121 min), immediately treated and analysed for their nitrite content. Before starting the digestion process, an aliquot of the original solution of sodium nitrite, was submitted to the same

procedure of sample pre-treatment to accurately measure the initial amount of nitrite. To each 3 mL of sample, 300 μ L of 2M NaOH and 0.6 g of ZnSO₄ were added as a decolouring reagent and to precipitate proteins. It was vortexed for 30 s and centrifuged at 4000 rpm for 10 min to accelerate the protein precipitation process (Figure 14) [16]. Serial dilutions were performed from the obtained supernatant. To evaluate the nitrite ion content, the samples were tested using the Griess reaction method and absorbance measurements performed on an UV/VIS spectrophotometer, as described in 1.3.1.2. To 2 mL of sample, 80 μ L of Griess reagent were added, incubated 15 min in the dark and, finally, absorbance measurements were made at 543 nm. All assays were tested in five independent experiments performed in duplicate and the appropriate statistical tools were applied to the obtained data.

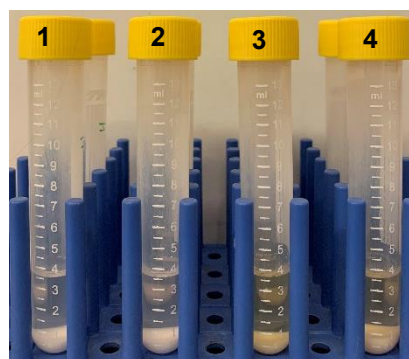


Figure 14. Aliquots of digests samples after treatment with NaOH, ZnSO₄ and centrifugation. A clear supernatant was obtained. 1-Initial sodium nitrite solution; 2-Mouth digestion sample; 3-Gastric digestion sample; 4-Intestinal digestion sample.

3.1.3. Statistical analysis

All graphics and statistical calculations were performed with the GraphPad Prism 8.3.0 software. The linear regression statistical analysis between curves was done to test if slopes and intercepts were significantly different as compared to each other ($p < 0.05$), following the methodology proposed by Jerrold H. Zar [108]. This approach is analogous to the analysis of covariance (ANCOVA) [109]. Standards were tested in duplicate.

The normality of the data distribution was assessed by four different tests: Anderson-Darling test, D'Agostino & Pearson omnibus normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lillie for P value. For data with parametric distribution, statistical comparisons were estimated using the parametric method of Holm-Sidak's (one-way ANOVA) ($p < 0.05$). Results are shown as mean $\pm$ SD from five independent experiments performed in duplicate.

3.2. Results & Discussion

The four calibration curves were plotted (Figure 15) and all R^2 values obtained were > 0.999 .

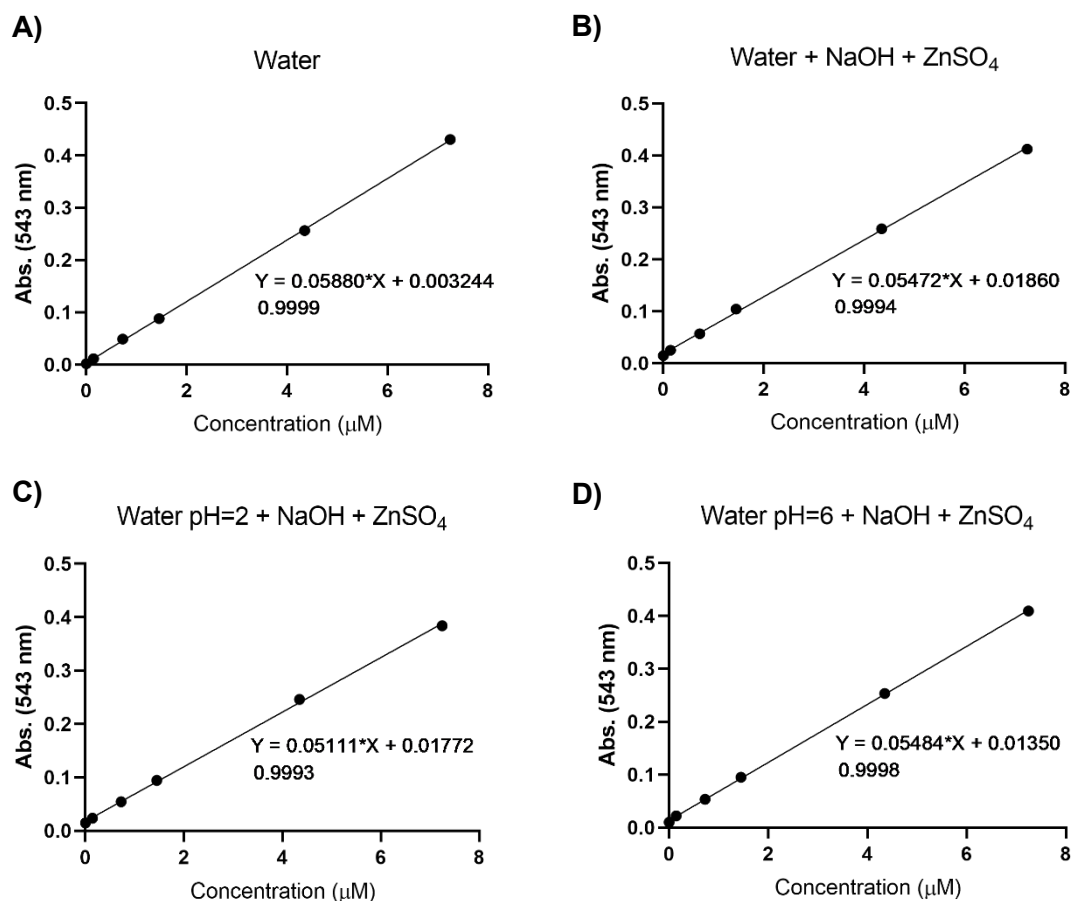


Figure 15. Nitrite quantification in the four calibration curves performed using the Griess reaction.

A) Calibration curve in water with the addition of 300 μL of water and vortex-mixed 30 s. **B)** Calibration curve in water with the addition of 300 μL of 2M NaOH and 0.6 g of ZnSO_4 , vortex-mixed 30 s and centrifuged at 4000 rpm, 10 min. **C)** Calibration curve in water at pH=2 with the addition of 300 μL of 2M NaOH and 0.6 g of ZnSO_4 , vortex-mixed 30 s and centrifuged at 4000 rpm, 10 min. **D)** Calibration curve in water at pH=6 with the addition of 300 μL of 2M NaOH and 0.6 g of ZnSO_4 , vortex-mixed 30 s and centrifuged at 4000 rpm, 10 min. Standards were tested in duplicate.

The four curves obtained were plotted on the same graph (Figure 16) and submitted to statistical analysis. The calibration curves data obtained are summarized in Table 8. As the slopes were significantly different for all curves ($p < 0.0001$), the

comparison between intercepts was not done. Due to these statistical differences, for further calculations, each curve was used for the corresponding phase of the gastrointestinal simulation test.

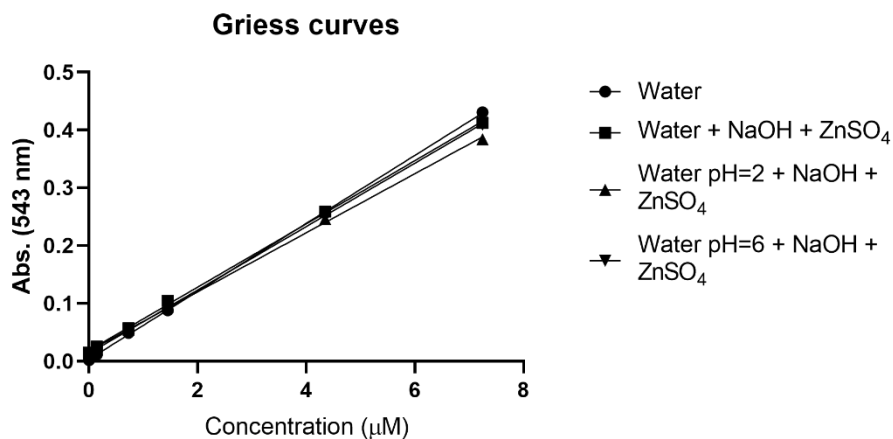


Figure 16. Simultaneous comparison of the four calibration curves obtained in Figure 15.

Table 8. Data summary of the calibration curves performed using the Griess reaction.

Curve	Equation*	R square	95% confidence intervals		
			Slope	Y-intercept	X-intercept
Water	$Y = 0,05880 \cdot X + 0,003244$	0,9999	0,05831 to 0,05928 ****	0,001551 to 0,004937	-0,08442 to -0,02623
Water + NaOH + ZnSO ₄	$Y = 0,05472 \cdot X + 0,01860$	0,9994	0,05374 to 0,05571 ****	0,01516 to 0,02205	-0,4080 to -0,2736
Water pH=2 + NaOH + ZnSO ₄	$Y = 0,05111 \cdot X + 0,01772$	0,9993	0,05016 to 0,05207 ****	0,01438 to 0,02107	-0,4176 to -0,2777
Water pH=6 + NaOH + ZnSO ₄	$Y = 0,05484 \cdot X + 0,01350$	0,9998	0,05425 to 0,05544 ****	0,01140 to 0,01559	-0,2864 to -0,2064

*Y = bX+a; Standards were tested in duplicate; **** p<0.0001.

The previously obtained samples of each digestion phase were then submitted to the Griess reaction, obtaining a red-violet coloration in all cases, but with different intensities. It can be concluded that all samples tested positive for nitrite ion, with a greater intensity of coloration being observed in tubes with samples of the initial solution and oral phase and lower in tubes with samples of gastric and intestinal phases. The colour of the tube of the initial solution is visually similar to that of the tube of the oral phase, so a similar concentration of nitrite ion would be expected, and the same happens for the tubes of the gastric and intestinal phases (Figure 17).

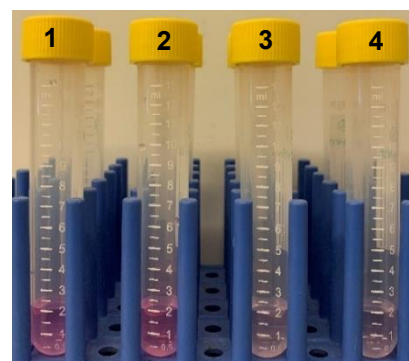


Figure 17. Samples of each digestion phase after the Griess reaction. 1-Initial sodium nitrite solution; 2-Mouth digestion sample; 3-Gastric digestion sample; 4-Intestinal digestion sample.

For each sample, and using the respective calibration curve, the nitrite concentration was calculated, taking into account all the previous dilutions, and the statistical analysis was carried out (Figure 18). After calculating the average value of each phase (from five independent assays), the following results were obtained: 144.37 mM for the initial solution, 143.73 mM for the oral phase, 40.61 mM for the gastric phase and 37.45 mM for the intestinal phase. The 37.45 mM value from the last phase was theoretically considered as the amount that would be available to be absorbed (considering the high bioavailability of nitrite > 98%) and, in turn, reach the bloodstream. The results show that the sample of the initial solution and the sample of the oral phase do not have statistically significant differences ($p=0.5542$), so in the oral phase (artificial saliva and α -amylase) there is no significant change in the nitrite content. Both the initial solution sample and the oral phase sample showed statistically significant differences compared to both the gastric and intestinal phases ($p<0.0001$). The gastric phase also presents statistically significant differences in relation to the intestinal phase ($p<0.01$). It should be noted that from the gastric phase onwards there is a loss of nitrite content greater than 1/3 of the initial concentration.

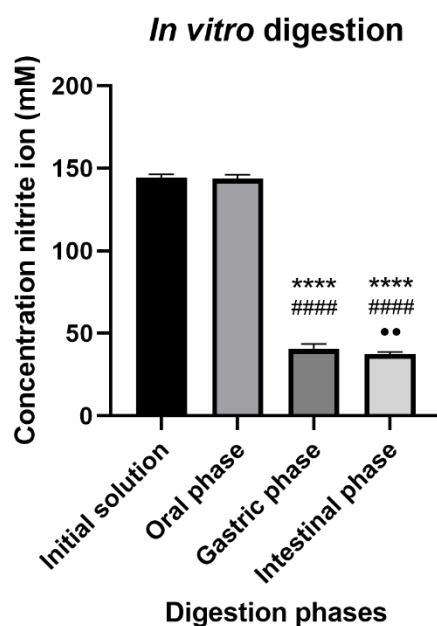


Figure 18. Nitrite concentrations (mM) obtained in the four analysed samples obtained in the gastrointestinal digestion assay. Statistical comparisons were estimated using the parametric method of Holm-Sidak's (one-way ANOVA) ($p < 0.05$). Results are shown as mean $\pm$ SD from five independent experiments performed in duplicate. * Compares to the initial solution; # Compares to the oral phase; • Compares to the gastric phase. •• $p < 0.01$; **** or ##### $p < 0.0001$.

The decrease in nitrite concentration in the gastric phase may be explained by the acidic environment of the stomach that can cause a notable instability for nitrites [36]. Under such conditions, nitrites are protonated to HNO_2 , giving rise to NO and other nitrogen oxides [3,110]. Accordingly, Björne *et al.* (2004) observed that NO gas was swiftly produced when nitrite-rich human saliva was exposed to the gastric mucosa of rats [3,111]. It is known that the NO produced in the gastric environment plays a vital role in defending against enteropathogens. While the acidic pH alone might not be sufficient to cause pathogens' death, the combination of acid pH with nitrite seems to be effective. The NO and other reactive nitrogen oxides, generated from acidified nitrite, can target multiple bacterial elements, including proteins, DNA, and cell wall components [3]. Kim *et al.* (2017) performed an *in vitro* human digestion study to determine the structural changes in nitrite along the gastrointestinal tract, using enterobacteria *Escherichia coli* and/or *Lactobacillus casei* in the large intestine phase. At the beginning, the sodium nitrite concentration was 150 ppm and after mouth digestion it decreased to 127.1 ppm. In the gastric digestion phase, the concentrations decreased again to 60.7 ppm, but in the small intestine, the value obtained was 63.2 ppm. During the large intestine digestion, sodium nitrite reacted with *Escherichia coli* and *Lactobacillus casei* and it was found that

the sodium nitrite concentration was reduced in the presence of both bacteria, but with greater impact notes for *Escherichia coli*. The reduction in sodium nitrite concentration by the action of these two bacteria suggests that enterobacteria interact with nitrite in the large intestine. *Escherichia coli* possibly converts nitrite to NO using cytochrome c nitrite reductase and *Lactobacillus casei* secretes nitrite reductase and lactic acid. The authors concluded that the exact mechanisms for the chemical changes in sodium nitrite in the human body during digestion are not totally clear. However, they assume that changes in pH (especially low pH), digestive enzymes, ionic strength or enterobacteria can affect the chemical structure, and the reactivity of this compound [112].

It is important to mention that the *in vitro* digestion method that was carried out has limitations. A static digestion method was employed to simulate the *in vitro* gastrointestinal digestion, making a sequential exposure in order to mimic digestion in different compartments (mouth, stomach and intestine) [113]. However, the INFOGEST guidelines highlight its disadvantages compared to a dynamic model. The static model does not simulate the complex dynamics present during the digestion process. For instance, during the test gastric phase, the pH remains constant throughout the entire incubation period, lacking the gradual addition of gastric fluid (acid, minerals, and pepsin) or gastric emptying. Moreover, the intestinal phase is represented as one, disregarding the distinct conditions of the sequential duodenal, jejunal, and ileal phases, which exhibit different conditions such as the mineral content, pH, enzymatic activities, and bacterial flora. Therefore, static models are simple to perform and allow understanding trends in certain test conditions, whereas the dynamic models are more representative of natural human digestion by reproducing the gradual transit through the gastrointestinal tract with a multi-compartment system controlled by computer [105,113]. This last model is capable of providing more reproducible and reliable results but has the disadvantage of being complex and expensive because it requires specific hardware and software [113].

In addition, in the assays performed the commensal bacterial flora of the intestine is lacking, which assumes great relevance in the conversions of nitrite into other chemical species [112]. It would also be equally pertinent to represent, in addition to enterobacteria, the oral microflora. These microorganisms use nitrate and nitrite as electron acceptors in respiration, generating nitrite and NO [114]. Interestingly, one study revealed that the use of an antiseptic mouthwash treatment resulted in a 90% decrease in oral nitrite production, demonstrating that the microbial oral flora plays major influence in these nitrogen species [1,115]. Ultimately, as a future perspective, to obtain closer results of the reactions that occur *in vivo*, it would be pertinent to carry out the *in vitro* digestion tests using a dynamic model and with commensal bacteria in each digestion phase. Despite the difficulty in quantifying NO due to its short half-life, it would also be

interesting to detect this species, as well as other nitrogen oxides, and if possible, to quantify them [62].

After simulating the gastrointestinal digestion of a sodium nitrite solution and obtaining the amount that would theoretically be available for absorption, the effect on the MetHb formation in the blood remained to be determined. For this purpose, sodium nitrite exposure test (and also sodium nitrate, assuming that conversion into this stable species could occur during gastrointestinal digestion) were conducted to determine the relation between nitrite and nitrate concentration and the extension of MetHb formation in human blood. Additionally, it was intended to demonstrate if the 37.45 mM nitrite concentration obtained after the ingestion of a lethal 2.6 g dose was in fact capable of producing a theoretically lethal %MetHb.

4. *In vitro* exposure of human blood to sodium nitrite and sodium nitrate for MetHb determination

The determination of methaemoglobinemia plays a crucial role in the diagnosis of poisoning by oxidizing agents, including sodium nitrite [65]. Some authors refer that quantifying MetHb levels in a toxicological analysis in a context of nitrite overdose may be more important than quantifying nitrite and nitrate levels [9]. To understand the levels of nitrites and nitrates that are capable of inducing methaemoglobinemia and, more specifically, to estimate a theoretically lethal %MetHb, a ferricyanide and potassium cyanide spectrophotometric technique using small volumes of blood samples was applied.

4.1. Material & Methods

4.1.1. Chemicals and materials

Sodium nitrite and sodium nitrate (Sigma-Aldrich, Germany) stock solutions were prepared in distilled water and stored at 4-6 °C. Potassium di-hydrogen phosphate was obtained from PanReac AppliChem, Spain and Triton X-100 from Sigma-Aldrich, USA. The following solutions were used: 2M and 0.5M NaOH; 0.5M phosphate buffer pH=7.2; 5% potassium hexacyanoferrate (III) solution (Fisher Scientific, UK), prepared monthly and stored in a dark bottle; 12% acetic acid solution (Honeywell, Germany); 5% potassium cyanide solution (Sigma-Aldrich, Germany), prepared monthly; ferricyanide-phosphate reagent, prepared daily (200 µL of 5% potassium ferricyanide, 2.5 mL of 0.5M phosphate buffer pH=7.2 and complete with water to 10 mL) and neutralized potassium cyanide solution, prepared at the time of use (1 mL of 5% potassium cyanide and 0.9 mL of 12% acetic acid).

To carry out these experiments, all procedures and protocols concerning the patients were conducted following the Declaration of Helsinki and received approval from the Ethics Committee of the Centro Hospitalar do Porto. After obtaining informed consent, venous blood was collected from each healthy human volunteer, males and nonpregnant females aged 18–65 years, via antecubital venipuncture into vacuum tubes containing heparin. No other information was collected regarding the donor, so anonymity was always guaranteed.

4.1.2. Analytical method for MetHb determination in human blood

To study the toxic effect of sodium nitrite/nitrate in human blood through MetHb production, solutions with increasing concentrations of sodium nitrite and sodium nitrate were added to fresh human blood and the %MetHb was determined. First, different exposure times (15, 20, 30, 40 min and 1 h) of the fresh blood to sodium nitrite and sodium nitrate were tested. Afterwards, the exposure time of 20 min was selected and applied in all experiments, as no further increases in %MetHb were noted after that time point.

For MetHb determination, as in 1.3.1.2., the ferricyanide and potassium cyanide spectrophotometric method was applied using small volumes of samples. Whole blood was used as recommended [63,64,67].

Whole blood (72 μ L) was exposed for 20 min to sodium nitrite and sodium nitrate solutions (8 μ L) to obtain final concentrations between 1-500 mM. Sodium nitrite and sodium nitrate solutions 10 times more concentrated were prepared by serially diluting 5M stock solutions. Negative controls with and without solvent (distilled water) were also tested. Distilled water (220 μ L) and Triton X-100 (5 μ L) were added to produce total haemolysis, followed by the addition of 0.5M phosphate buffer pH=7.2 (100 μ L). Two test conditions were applied: (A) into a 96-well plate, the previous preparation was diluted 1:4 to a final volume of 240 μ L; (B) a buffered ferricyanide solution (220 μ L) was placed and the original preparation was 1:4 diluted to a final volume of 20 μ L. Readings were taken in an UV/VIS spectrophotometer at 632 nm, performing auto-zero against 240 μ L of distilled water. Then, a neutralized potassium cyanide solution (10 μ L) was added to all wells (A and B) and readings were taken again at 632 nm, obtaining values C and D, respectively. The %MetHb was calculated using the formula mentioned in 1.3.1.2. [67].

4.1.3. Statistical analysis

All graphics and statistical calculations were performed with the GraphPad Prism 8.3.0 software. The t-test was used to check for significant differences ($p < 0.05$; confidence level 95%) between control wells with and without solvent (distilled water). The solvent control was selected for further statistical comparisons, but no statistically significant differences between negative and solvent controls.

The linear regression statistical analysis between curves was done to test if slopes and intercepts were significantly different as compared to each other ($p < 0.05$), following the methodology proposed by Jerrold H. Zar [108]. This approach is analogous

to the analysis of covariance (ANCOVA) [109]. Experiments were done in at least triplicate in at least three independent assays.

The normality of the data distribution was assessed by four different tests: Anderson-Darling test, D'Agostino & Pearson omnibus normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lillie for P value. For data with parametric distribution, statistical comparisons were estimated using the parametric method one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test ($p < 0.05$). Results are shown as mean $\pm$ SD from three independent experiments performed in triplicate.

4.2. Results & Discussion

4.2.1. Sodium nitrite exposure

The conversion of Hb to MetHb followed an increasing and linear trend between 1-20 mM but, at higher concentrations, this was not observed (Figure 19A). The average %MetHb of the controls was calculated and a mean value of 0.45% was obtained, which is consistent with the normal expected physiological levels (0-3%) [18]. The curve of %MetHb *versus* sodium nitrite concentration (1-20 mM) yielded an R^2 of 0.932 (Figure 19B). The obtained R^2 value can be explained by the variability of blood donors. In fact, only one test was performed per day, in triplicate, so for each experiment the blood used was originated from a different donor. As previously mentioned, no information was available about the donors.

It has been described that MetHb reductase activities exhibit considerable variation across different age groups and also species [116]. There are individuals who naturally have moderately high MetHb levels. As previously mentioned, this could be due to cytochrome-b5 reductase deficiency or cytochrome-b5 deficiency, transmitted in an autosomal recessive pattern, leading to MetHb of genetic origin. This interindividual variability in metabolism means that distinct levels of MetHb can be expected from exposure to fairly similar amounts of oxidizing agents [10]. Therefore, it is expected that the deviations found from assay to assay can be explained by the variability between donor individuals.

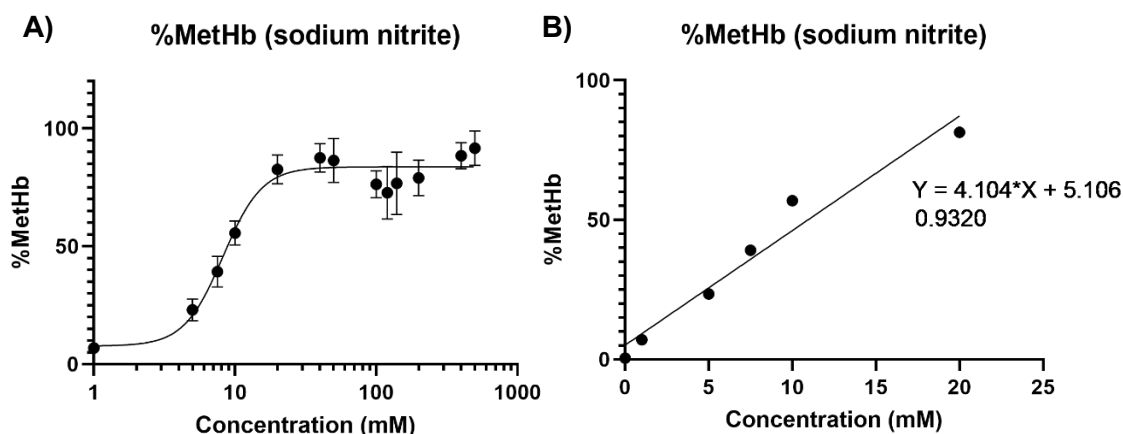


Figure 19. %MetHb curves obtained after sodium nitrite exposure to different donor blood samples. **A)** Dose-response curve obtained after 20 min of exposure to 1-500 mM sodium nitrite concentrations in fresh human blood from different donors. The curve was fitted using least squares as the fitting method. Results are shown as %MetHb $\pm$ 95% confidence interval from at least nine independent experiments performed in triplicate. **B)** Curve (linear portion) of 1-20 mM of sodium nitrite exposure in fresh human blood from different donors from at least nine independent experiments performed in triplicate.

Fresh human blood samples from different donors were exposed to 37.45 mM sodium nitrite (i.e., the concentration obtained in the *in vitro* gastrointestinal assays), in three independent tests run in triplicate, to determine the %MetHb at that nitrite concentration. The mean value of these determinations was 95.36% (a theoretically lethal value), which would be expected according to the data shown in Figure 19A.

Due to the variability of results obtained using blood from different donors, three independent assays ($n=3$) with six replicates each, were performed using blood samples from the same donor. The graph of the three curves is represented in Figure 20A and the calibration curves' data obtained are summarized in Table 9. A statistical comparison between slopes and intercepts was made to test if there were statistically significant differences ($p < 0.05$). It was verified that there were no statistically significant differences in slopes ($p = 0.2327$) and in Y intercepts ($p = 0.7595$). Therefore, with the values of the three independent assays, a single graph was constructed and it presents a $R^2 = 0.961$ (Figure 20B). The R^2 value increased when compared with the results using blood samples from different donors, so it was confirmed that the use of the same blood sample decreased the variability of the results.

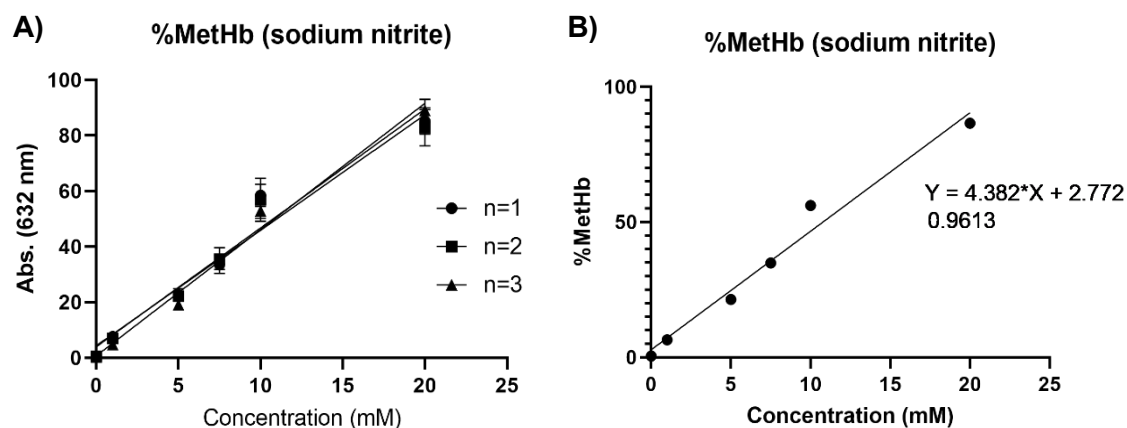


Figure 20. %MetHb curves obtained after sodium nitrite exposure to same donor blood samples.

A) Simultaneous comparison of calibration curves (three independent assays with six replicates) using blood from the same donor exposed to 1-20 mM of sodium nitrite. **B)** Curve (linear portion) of 1-20 mM of sodium nitrite exposure in fresh human blood from the same donor from three independent experiments with six replicates.

Table 9. Data summary of the calibration curves performed using human blood from the same donor.

Curve	Equation*	R square	95% confidence intervals		
			Slope	Y-intercept	X-intercept
n=1	$Y = 4,277 \cdot X + 3,959$	0,9513	3,923 to 4,631	0,3130 to 7,604	-1,904 to -0,06880
n=2	$Y = 4,156 \cdot X + 4,339$	0,9427	3,781 to 4,532	0,4751 to 8,202	-2,128 to -0,1069
n=3	$Y = 4,542 \cdot X + 0,7243$	0,9796	4,298 to 4,787	-1,809 to 3,258	-0,7486 to 0,3828

* $Y = bX + a$; Results from three independent assays with six replicates. Slopes ($p=0.2327$) and in Y intercepts ($p=0.7595$).

The applied method allowed evaluating which concentrations of sodium nitrite are capable of inducing methaemoglobinemia. It was possible to draw a calibration curve, having calculated that an exposure to a concentration >15.81 mM of sodium nitrite in human blood can be theoretically lethal (MetHb>70%). The use of blood samples from different donors for this calibration curve resulted in a lower than expected R^2 value, but this value improved in the assays using blood from the same donor.

During the experimental work, before the addition of neutralized cyanide, it was evident a change in blood colour to brown in the wells exposed to sodium nitrite.

Increasing nitrite concentration leads to a gradual increase in brown colour intensity (Figure 21).

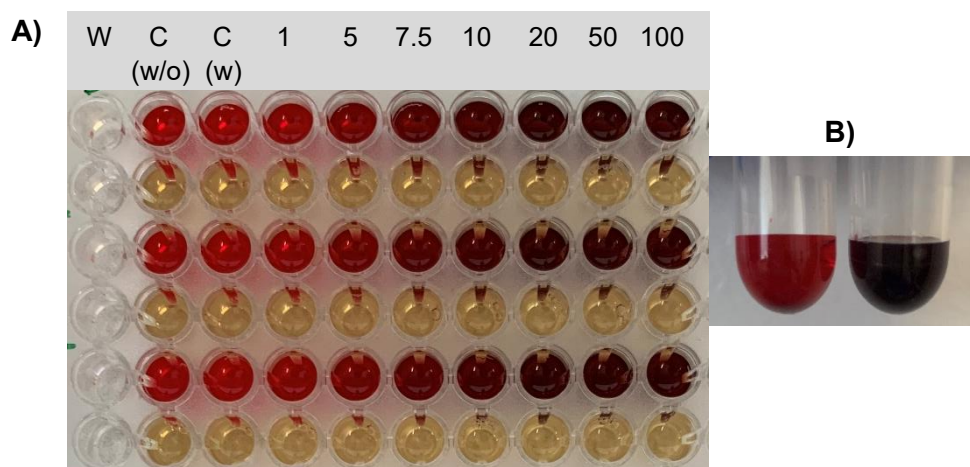


Figure 21. Blood sample colour analysis when exposed to increasing concentrations of sodium nitrite. **A)** Comparison of the red colour of blood control wells versus the brown colour seen in blood wells exposed to 1, 5, 7.5, 10, 20, 50 and 100 mM of sodium nitrite (before the addition of neutralized cyanide). W – water; C(w/o) – control without solvent; C(w) – control with solvent. Experiments performed in triplicate. **B)** Control blood (red) on the left and blood exposed to 50 mM sodium nitrite (brown) on the right.

Interestingly, it was found that after the addition of neutralized cyanide there is a reversal of blood colour. This again turns the colour from brown to red. This phenomenon is explained by the formation of cyanoMetHb, which is bright red, in contrast to the brownish colour characteristic of MetHb [10]. This mechanism helps to understand why sodium nitrite can be used as an antidote for cyanide poisoning. In that type of intoxication, sodium nitrite is administered to convert Hb into MetHb, which reacts with cyanide to form cyanoMetHb, preventing the lethal cyanide binding to the mitochondrial electron transport chain [117].

4.2.2. Sodium nitrate exposure

The exposure of human blood to sodium nitrate occurred under the same experimental conditions as those described for sodium nitrite. However, there were no statistically significant differences in %MetHb relative to controls. Normal physiological levels of MetHb in the blood are between 0 and 3% [18]. In these assays, the %MetHb was always below 1% for all tested concentrations (1-500 mM) of sodium nitrate (Figure 22). With the present data it is concluded that MetHb can be induced *in vitro* by sodium nitrite but not by sodium nitrate.

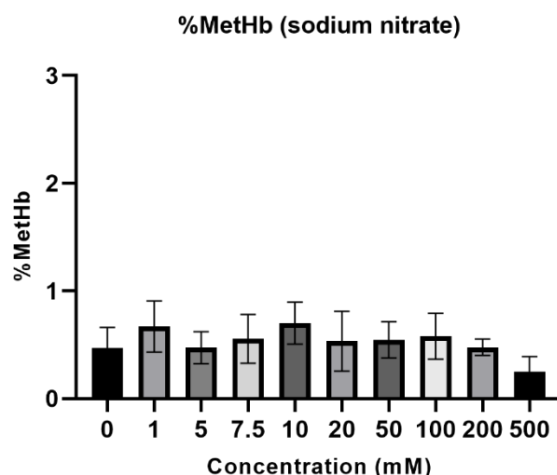


Figure 22. Exposure of fresh human blood to increasing concentrations (0-500 mM) of sodium nitrate and determination of the respective %MetHb. Scale from %MetHb up to 3%, as this is the upper limit that is considered normal. Statistical comparisons were estimated using the parametric test one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test ($p < 0.05$). Results are shown as mean $\pm$ SD from three independent experiments performed in triplicate.

No change in colour of the blood sample was observed after the exposure period, contrary to what was observed in the blood samples exposed to sodium nitrite (Figure 23). These findings are consistent with the values obtained for %MetHb. When neutralized cyanide is added to wells previously exposed with sodium nitrate, the reddish colour remains. As MetHb is not formed, no brown colour appears and therefore cyanoMetHb is not formed after the addition of neutralized cyanide.

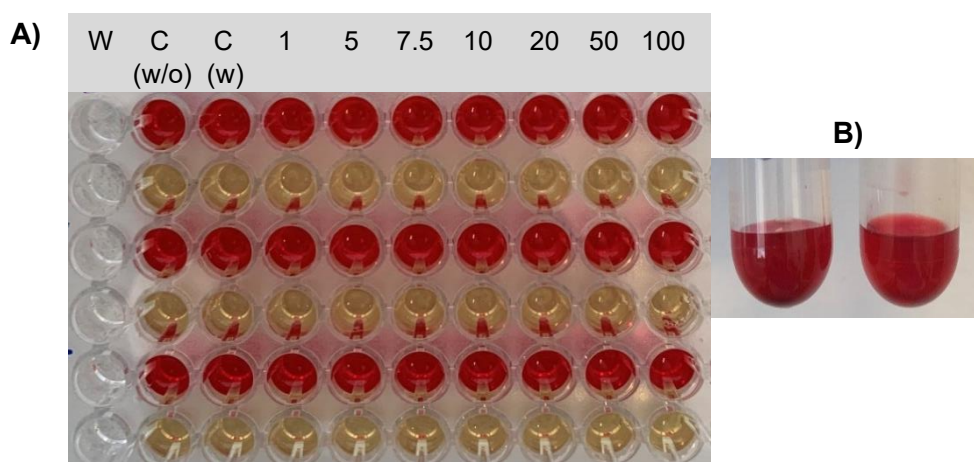


Figure 23. Blood sample colour analysis when exposed to increasing concentrations of sodium nitrate. **A)** Observed red colour in all blood samples, including control wells and wells exposed to 1, 5, 7.5, 10, 20, 50 and 100 mM of sodium nitrate. W – water; C(w/o) – control without solvent;

C(w) – control with solvent. Experiments performed in triplicate. **B)** Control blood (red) on the left and blood exposed to 50 mM sodium nitrate (also red) on the right.

It is known that nitrite easily permeates across the erythrocyte membrane through anion exchanger 1 and HNO_2 diffusion. Fens *et al.* (2014) studied the ability of red blood cells to convert nitrite to NO under hypoxic environment. Under normal conditions, red blood cells are capable of producing NO by endothelial NOS enzymes present in the membrane. However, in anoxia, these enzymes are inactivated and the formation of NO in the erythrocyte occurs by another route, through nitrite. In these cases, for the nitrite-NO conversion, xanthine oxidoreductase seems to be involved. The study carried out by these authors shows that a concentration of 5 mM nitrite in red blood cell samples leads to the release of NO at a lower limit of detection. Furthermore, they confirmed that the presence of oxygen leads to a decrease in NO release and that, on the contrary, under hypoxic conditions the pH decrease leads to its release. In particular, the authors verified that with the addition of 5 mM of sodium nitrate, under hypoxic conditions, there was no influx of nitrate into red blood cells, nor any release of NO. This demonstrates that nitrate is not absorbed by red blood cells, which may explain the results obtained in the determination of %MetHb in the present assays [118].

Still, the results obtained with this experiments should be interpreted with caution because nitrate does not cause *in vitro* MetHb but *in vivo* they are converted into nitrites by commensal bacterial flora [10]. Therefore, the toxicity of the nitrate ion is probably due to its bioconversion into nitrite [3].

It is crucial to contemplate the significance of erythrocytes as a distinctive target of nitrite toxicity. These cells are particularly vulnerable to oxidative stress. Their role in transporting substantial amounts of oxygen makes them constantly exposed to elevated concentrations of oxygen free radicals. To counteract these effects, various enzyme systems work to safeguard red blood cells from oxidation reactions. Nevertheless, unlike other cells, erythrocytes lack a nucleus and, as a result, cannot synthesize new proteins. Over time, the enzymes that typically detoxify oxidizing agents undergo degradation. Additionally, due to the absence of mitochondria in red blood cells, they exhibit lower efficiency in generating energy and cofactors for chemical detoxification when compared to other cells. Therefore, the oxidative toxicity caused by nitrites in the blood is noteworthy, manifesting itself by triggering haemolysis and methaemoglobinemia [10].

In addition to the effects studied *in vitro* on erythrocytes, Miasoedova *et al.* (2004) simulated *in vivo* an acute intoxication by sodium nitrite in 30 mature white female rats, injecting 10 and 50 mg/kg subcutaneously. One hour later, they verified the appearance of haemolytic anemia, MetHb, leukocytolysis and depression of macrophages. Thus, the

authors concluded that sodium nitrite unbalances the erythron and leukocyte system, in a dose-dependent manner, showing that the harmful effects of nitrite in blood are not exclusively directed at erythrocytes, but also at other blood cells [119].

Finally, the protocol used allows evaluating %MetHb in relation to total Hb. However, the Hb concentration was not determined for each sample and this may be a limitation. Other factors that were also not considered because no information about the donors was available, and that may have influenced the results, include age, gender, illnesses or medication intake. Regarding the ferricyanide and potassium cyanide spectrophotometric technique it is important to note that this is a laborious and time-consuming method and implies handling cyanides, known for its toxicity. A possible alternative to this technique is the "Blood Gas Analyzer" equipment (for example, ABL800 FLEX Blood Gas Analyzer), a simpler and automated method, which is able to measure six oximetry parameters, including MetHb. In hospital practice, this equipment allows the determination of several analytical parameters simultaneously using the same blood sample [18].

Conclusions

While in the past nitrite poisonings were mostly accidental, we are currently witnessing an increase in this type of intentional intoxication. The use of nitrites seems to be a current trend more related to young adults, which can be explained by the fact that they are more prone to surf the web. Unfortunately, insufficient control over the accessibility of this salt has led to many deaths in several countries. This worrying trend also reached Portugal, with fatal cases already recorded in the literature. In this work, it was possible to report one more case, in addition to the eight registered since 2020. All findings obtained from perinecropsy, necropsy, as well as those from complementary exams (including forensic toxicology), are consistent with a scenario of suicide by nitrite ingestion. The toxicological analyses carried out revealed a high value of MetHb in the blood and positivity for nitrites in all biological samples and in the powder found near the victim, which helped to characterize the cause of death. It was also possible to detect camphor and lidocaine in the liquids, two substances that had not been reported so far in the cases analysed in the literature, leading to the hypothesis that they could be part of new "Suicide Kits". As the victim received an online order with sodium nitrite, it seems that the online purchase of these kits is facilitated. Indeed, nitrites have a wide applicability in everyday life, which may partly explain their easy acquisition. There are websites and books that provide access to a guide to pain-free suicide, promising and supporting it. These claim that the choice of this method of ending life is peaceful, non-violent, easy to acquire, prepare and store, undetectable, fast and at an affordable cost. Therefore, it would be essential to better control the access to this substance, especially its easy purchase in bulk. Accordingly, authorities must be alert and take appropriate measures to restrict nitrites misuse. With regard to health professionals, they should be aware of the clinical aspects related to nitrite or nitrate poisoning (e.g., hypotension, desaturation, cyanosis). It would be important to know how to act appropriately and for health units to have the treatments available, including methylene blue. In terms of forensic pathology, blue-grey colour in different parts of the body, brownish colour of *livor mortis* and brown blood are common findings. As for forensic toxicology, it is usual to analyse nitrites and nitrates in different biological samples and also to quantify MetHb. It is advisable to search for other co-adjuvant drugs, as these are frequently part of the "Suicide Kits".

The studied forensic case was, in fact, the starting point for the investigation of toxicity mechanisms involved in cases of poisoning by nitrite ingestion. It became pertinent to study the direct cytotoxicity of nitrites, which is why *in vitro* studies were carried out on cell lines representative of three possible target organs. The high IC₅₀

values obtained through the assays performed on H9c2, Caco-2 and SH-SY5Y cells lead to the conclusion that these are not physiologically relevant concentrations, even at intoxication scenarios. For this reason, the research work was continued by simulating *in vitro* gastrointestinal ingestion of a supposed fatal ingestion of a sodium nitrite solution. These tests demonstrated that nitrite can be converted into different nitrogen species along the gastrointestinal tract, with greater emphasis in the gastric phase due to acidic conditions.

The formation of MetHb in blood was also studied. Exposure studies of sodium nitrite and sodium nitrate in fresh human blood have led to the conclusion that nitrite is indeed a direct agent for inducing MetHb and, on the contrary, nitrate is not. Despite being usually listed in the substances capable of inducing MetHb, sodium nitrate seems to exert its effect indirectly, requiring previous conversion into nitrite.

To conclude, other mechanisms involved in this type of intoxications are yet to be explored. Further *in vivo* and *in vitro* investigation are needed to complement the results obtained in all the experimental work carried out. As future perspectives, it would be pertinent to perform, in addition to those previously mentioned: (i) studies in neuronal models to assess permanent neuronal damage and possible treatments; (ii) investigation on possible drug interactions involved in these cases and their role in toxicity and (iii) researching on the impact of antioxidant substances on the prevention of erythrocytes damage, in order to improve existing treatment options. Investigating other mechanisms could help to better understand these intoxications and find more efficient treatments.

Acknowledgments

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Annex I – Tables

Table I – Intentional fatal cases reported in Portugal

Year of publication and reference	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Pathology findings	Toxicology findings	Origin of nitrites
2020*	1	20 F	NA	NA	1 packet of powder and 2 packets of Vomidrine® (dimenhydrinate)	NA	+ powder for nitrites; + blood for nitrites; - drugs; - ethanol; - drugs of abuse	NA
2020 [9]	1	37 M	Depression, schizophrenia, previously attempted suicide, consulted suicide support websites	NA	A box with 2 glass cups, metoclopramide and ranitidine; 35 g package of sodium nitrite	Signs of asphyxia (intense scleral congestion); cyanosis of the extremities (hands and lips); brown-grey-blue-red <i>livor mortis</i> ; Tardieu petechiae; intense polyvisceral congestion; chocolate brown blood colour; histological examination: nonspecific changes (pulmonary edema and coronary artery disease without major expression)	Femoral blood: - Desmethylcitalopram (90 ng/mL); - Citalopram (252 ng/mL); - Trazodone (280 ng/mL); Nitrites: - 16 g/L of nitrite ion and 24 g/L of sodium nitrite (gastric content) - 30 ng/mL of nitrite ion (blood)	Online
2020 [5]	1	37 F	NA	NA	500 g plastic bottle containing sodium nitrite; suicide note; a bag containing different medicines (tramadol+paracetamol, quetiapine, ondansetron, diazepam, olanzapine, codeine+paracetamol)	General signs of asphyxia on lips and facial cyanosis; greyish <i>livor mortis</i> ; trachea with brown foam and bronchial aspiration of vomiting; lung edema and a dark violet and congested cut surface; histological examination: nonspecific changes (pulmonar congestion and alveolar edema); heart congestion and areas of cicatricial fibrosis in the interventricular region of the septum	Femoral blood: - Codeine (194 ng/mL); - Quetiapine (1755 ng/mL); - Norquetiapine (418 ng/mL); - Tramadol (5808 ng/mL); - O-desmethyl-tramadol (254 ng/mL); - N-desmethyl-tramadol (180 ng/mL); - Topiramate (4838 ng/mL); - Diazepam (44 ng/mL); - Nordiazepam (24 ng/mL); - Olanzapine (157 ng/mL); Nitrites: - 8.8 g/L for nitrite ion and 13 g/L for sodium nitrite (gastric contents) - 35 mg/L for nitrite ion (cardiac blood)	Online
2021*	1	59 M	NA	NA	NA	NA	+ cardiac blood for nitrites; gastric content: nitrite ion 9.2 mg/L and sodium nitrite 14 mg/L; metoclopramide 18200 ng/mL; paracetamol 18200 ng/mL; - ethanol; - drugs of abuse	NA
2021*	1	17 M	NA	NA	NA	NA	+ cardiac blood for nitrites; gastric content: nitrite ion <0.03 mg/L and sodium nitrite <0.02 mg/L; - drugs; - ethanol; - drugs of abuse	NA
2023*	1	27 M	NA	NA	NA	NA	- cardiac blood for nitrites; - urine for nitrites; + glass with yellow liquid for nitrites; metoclopramide 222 ng/mL; paracetamol 9500 ng/mL; - ethanol; THC 1.9 ng/mL; THC-COOH 13 ng/mL	NA
2023*	1	18 M	NA	NA	NA	NA	+ gastric content for nitrites; + glass residues for nitrites; venlafaxine; mirtazapine; doxylamine; ethanol <0.5 g/L; - drugs of abuse	NA
2023*	1	30 M	NA	NA	NA	NA	+ gastric content for nitrites; - blood for nitrites; citalopram; ibuprofen; - ethanol; - drugs of abuse	NA

*Data provided by the INMLCF (CE-15/2023); F – female; M – male; NA – not available; THC – tetrahydrocannabinol; THC-COOH – 11-Nor-9-carboxy- Δ^9 -tetrahydrocannabinol; + positive result; - negative result.

Table II – Intentional fatal cases reported in the literature in other countries

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
2023 [68]	Belgium	1	18 F	Psychiatric issues and attempted suicide (auto-intoxication) a few months prior	NA	A bag ($\approx$ 63 g) containing white-yellowish crystal powder with the label "Nitryt do mięsa 100 g Azotyn Sodu E250", a measuring cup, a kitchen scale, 2 teaspoons with powder remnants, an opened water bottle, and a cup containing residual viscous liquid	Unresponsive	Healing self-harm injuries on the forearms; grey-brownish <i>livor mortis</i> ; cyanotic changes of the lips and fingernails; pulmonary edema with prominent brownish foam in the airways; signs of mild cerebral edema; congestions of the visceral organs; brown discoloration of the blood, internal blood vessel walls, organ and of the bodily membranes such as the perist; increased bleeding tendency; stomach wall dark brown with small, adherent white granulations; histological examination: nonspecific changes (pulmonary edema, intra-alveolar; haemorrhages, and generalized congestion of the organs, including the stomach); moderate liver steatosis	MethHb 35%; nitrite in serum (38 μ g/mL) and in stomach content (1.15 g/L); caffeine and theophylline	Probably online
2023 [71]	USA	5	1) 19 F 2) 43 F 3) 61 F 4) 16 F 5) 41 F	1) Major depressive disorder, post-traumatic stress disorder, attention-deficit hyperactivity disorder, borderline personality disorder and obesity; 2) Anxiety, panic attacks, headaches,	NA	1) A bottle of sodium nitrite containing a yellowish substance, which was also found in a water bottle; 2) An open, partially filled pouch of sodium nitrite was found on the desk with a	1) NA 2) Unresponsive; 3) Unresponsive; 4) Unresponsive; 5) Unresponsive with shallow breaths, a weak radial pulse and pale cyanotic skin; supplemental oxygen	NA	1) Serum and vitreous humour tested positive for nitrites; 2) Vitreous humour tested positive for nitrites; 3) Urine and vitreous humour tested positive for nitrites;	NA

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
				major depressive disorder and previous suicide attempts; 3) Depression due to the loss of her husband for 9 years 4) 2 suicide attempts by overdose ingesting acetaminophen the 1 st time and quetiapine and ethanol the 2 nd time; medical history of depression and anxiety; 5) Suicidal ideations		handwritten note "CAUTION! SODIUM NITRITE POISONOUS" 3) Numerous prescription medications, over-the-counter medications, and unidentified liquids found at the scene; 4) A bottle of opened sodium nitrite	causing improvement in her colour; i.v. naloxone with no improvement and ketamine; cardiac arrest after receiving ketamine; 50 mg of methylene blue without improvement, and 200 mL of brown bloody fluid was collected from her gastric tube; <i>ante-mortem</i> MetHb 58%		4) MetHb 8.5%; Serum, urine and vitreous humour tested positive for nitrite; 5) Serum and vitreous humour tested positive for nitrite	
2022 [52]	Republic of Korea	10	1) 29 M 2) 35 M 3) 33 F 4) 28 M 5) 22 M 6) 19 M 7) 28 M 8) 25 M 9) 26 M 10) 21 F	NA	7) 1 kg of pickling salt dissolved in water according to his text messages	1) Victim reported taking sodium nitrite; found dead in his car parked in rural area; 2) Victim found dead with a suicide note in a motel room; 3) Victim found dead with a suicide note in a motel room; 5) Suicide note found in his laptop; 6) Victim found dead with empty bottle labelled as "sodium nitrite"; 8) Victim found dead in a motel room with sodium nitrite in his bag; 9) His suicide mate who changed her mind reported that he drank sodium nitrite with green tea; 10) Her family suspected adverse effect of vaccine which she took 6 days before her death	4) Unconscious 8) Unconscious	NA	1) Nitrate 292 mg/L (cardiac blood); peripheral blood tested positive for nitrate; 2) Nitrite 8.1 mg/L (peripheral blood), 3.5 mg/L (cardiac blood) and gastric contents tested positive for nitrite; Nitrate 236.9 mg/L (peripheral blood), 131.5 mg/L (cardiac blood); 3) Nitrite 1.2 mg/L (peripheral blood), 1.3 mg/L (cardiac blood) and gastric contents tested positive for nitrite; Nitrate 363 mg/L (peripheral blood), 201.6 mg/L (cardiac blood); 4) Nitrite 298 mg/L (peripheral blood) and gastric contents tested positive for nitrite; Nitrate 319 mg/L (peripheral blood); 5) Nitrite 9.4 mg/L (peripheral blood), 8.4 mg/L (cardiac blood) and gastric contents tested positive for nitrite; Nitrate 476.4 mg/L (peripheral blood), 524.8 mg/L (cardiac blood); 6) Nitrite 7.0 mg/L (peripheral blood), 170.0 mg/L (cardiac blood) and gastric contents tested positive for nitrite; Nitrate 340.0 mg/L (peripheral blood), 277.0 mg/L (cardiac blood);	NA

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
									7) Gastric contents tested positive for nitrite; Nitrate 220.0 mg/L (peripheral blood), 218.5 mg/L (cardiac blood); 8) Gastric contents tested positive for nitrite; Nitrate 237.0 mg/L (peripheral blood), 212.0 mg/L (cardiac blood); 9) Gastric contents tested positive for nitrite; Nitrate 282.0 mg/L (peripheral blood), 369.0 mg/L (cardiac blood); 10) Gastric contents tested positive for nitrite; Nitrate 213.8 mg/L (peripheral blood), 172.7 mg/L (cardiac blood)	
2022 [59]	Korea	1	26 M	NA	18 g	NA	Unconscious; asystole rhythm; cardiac arrest	NA	MetHb 90.3%	NA
2022 [58]	France	1	Adult F	NA	25 g	NA	Found deceased	NA	NA	Workplace (curing meat)
2022 [53]	USA	1	Mid-20s M	NA	25 g	A scale and a white powder	At 1 st somnolent; cyanosis; diaphoresis; systolic blood pressure 110 mmHg; heart rate 130 bpm; SpO ₂ 79%; tonic-clonic seizures; ventricular fibrillation. Later: asystole; end-tidal CO ₂ 12 mmHg; skin blue-grey discoloration; brown-black blood with pH 7.03; PCO ₂ 87 mmHg, bicarbonate 22 mmol/L, lactate 30 mmol/L; Hb 12.4 g/dL; MetHb 28.8%; intubated; defibrillated; intravenous medications: 2.5 mg epinephrine 1:10000, 1 g calcium chloride, 100mEq sodium bicarbonate 8.4%, 5 g hydroxocobalamin, 100 mg methylene blue	Brown discoloration of muscle tissue	<i>Postmortem</i> -combined nitrate and nitrite: 5300 µmol/L	NA

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
2022 [22]	Australia	10	8 male (22-74 years); 2 female (23-29 years)	History of depression (7 victims); 4 expressed suicidal ideation; 1 with complex chronic medical history and expressed wish to be placed in palliative care; 1 decedent with no recorded history of depression; medical histories of the 2 remaining decedents were NA; 3 who had previously attempted suicide including one previous attempt using sodium nitrite	NA	In all cases, evidence of sodium nitrite ingestion found at the death scene (e.g., labelled NaNO ₂ packaging, drinking glasses and white powder residue); suicide note(s) in all but 2 cases, with a verbal expression of intent to commit suicide in one additional case	NA	Blue-grey hypostasis; dark brown blood and internal organs in all but one case	MetHb 87.5% in one case	Foodgrade sodium nitrite sourced from a homebrew supply in 2 cases and from a food store in another. In 1 case, a sodium nitrite suicide kit sourced via the post. The source of sodium nitrite in the remaining cases was NA
2021 [11]	Canada	16	1) 17 M 2) 68 M 3) 20 F 4) 86 M 5) 17 M 6) 22 F 7) 45 M 8) 22 M 9) 20 M 10) 42 M 11) 21 M 12) 23 M 13) 34 F 14) 23 M 15) 20 M 16) 20 M	NA	NA	NA	NA	1) Greyish-brown <i>livor mortis</i> 2) Brown purple <i>livor mortis</i> 3) Red-purple-brown <i>livor mortis</i> 4) Unremarkable <i>livor mortis</i> 5) Dark-purple/blue <i>livor mortis</i> 6) Unremarkable <i>livor mortis</i> 7) Purple-grey <i>livor mortis</i> 8) Grey-blue/purple <i>livor mortis</i> 9) Purple-grey <i>livor mortis</i> 10) Grey <i>livor mortis</i> 11) Unremarkable <i>livor mortis</i> 12) Maroon <i>livor mortis</i> ; brown blood 13) Unremarkable <i>livor mortis</i> 14) Unremarkable <i>livor mortis</i> 15) Ash-coloured <i>livor mortis</i> 16) Blue-grey <i>livor mortis</i>	2) MetHb 50% 3) MetHb 17%; diphenhydramine, citalopram, trazodone, bupropion, clonazepam 4) MetHb 10%; ethanol 5) MetHb 6% 6) MetHb 39%; diphenhydramine, quinine, quinidine, 7) MetHb 61%; venlafaxine, bupropion 8) MetHb 49%; diphenhydramine 9) MetHb 22% 10) MetHb 65%; diphenhydramine 11) diphenhydramine 13) MetHb 35%; amlodipine 15) MetHb >80% 16) MetHb 79%	NA

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
2021 [13]	USA	3	1) 17 F 2) 35 M 3) 22 M	1) NA 2) NA 3) NA	1) "Sodium something" pills 2) NA 3) NA	1) NA 2) NA 3) A white powder in the residence	1) Unresponsive; cyanotic; SpO ₂ post intubation 79%; "chocolate brown" blood; 2) Initial blood pressure 90/70 mmHg; initial heart rate 40-59 beats/min; initial SpO ₂ 80%; cardiac arrest; alert but in respiratory distress; profoundly cyanotic; unresponsive and asystolic; blood "very dark"; methylene blue 1 mg/kg (75 mg) i.v. twice + 50mg i.v. 3) Minimally responsive, hypotensive; cardiac arrest; initial blood pressure 67/36 mmHg; initial heart rate 102 beats/min; initial SpO ₂ 80%; Hb 7.4 g/dL; methylene blue 2 mg/kg i.v.	1) NA 2) NA 3) NA	1) MetHb 84% before cardiac arrest 2) NA 3) MetHb 30% after 1 st dose of methylene blue	1) Online 2) NA 3) NA
2021 [18]	Republic of Korea	1	28 M	NA	84 g	Victim found to be logged-in and chatting about suicide; posted on a social network service detailing the attempt at suicide	NA	Face dark brown colour, oral mucosa bright red colour; reddish-purple <i>livor mortis</i> ; cyanotic changes of the nail; oral mucosa and subcutaneous tissue of the trunk bright red; congestion of the internal organs and pulmonary edema	MetHb 33% (peripheral blood during <i>post-mortem</i> inspection); MetHb 26% (peripheral blood during the autopsy); Nitrate levels: - 220.6 mg/L (peripheral blood during <i>post-mortem</i> inspection) - 220.0 mg/L (peripheral blood during the autopsy); - 218.5 mg/L (cardiac blood); - 91.7 mg/L (pericardial fluid) - 137.8 mg/L (gastric content) - 50.5 mg/L (cerebrospinal fluid) Nitrite levels: - 181.0 mg/L (pericardial fluid) - 11,220.1 mg/L (gastric content)	Online

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
2021 [6]	Italy	2	1) 28 M 2) 33 M	1) Mild depression 2) Severe depression	1) NA 1) NA	1) Evidence of vomit; small school bag containing 700 g of a white powder 2) Two 250 g plastic bag labelled as "Anglia NaNO ₂ 99/95%", one full and one empty	1) Unresponsive 2) NA	1) Cyanosis of hands, lips and fingernail beds; brown-red <i>livor mortis</i> ; multi-organ congestion, cerebral, pulmonary edema 2) Cyanosis of the extremities; grey-blue-red <i>livor mortis</i> ; fingernail beds dark blue	1) Blood alcohol concentration 0.6 g/L; nitrates 6.5 mmol/L (blood) 2) Nitrates 4.4 mmol/L (blood)	1) NA 2) NA
2021 [54]	Poland	1	23 M	NA	NA	A wine glass and another glass filled with translucent fluid; sodium nitrite container stored in the under washbasin cabinet; a farewell letter	NA	Labial red of the upper and lower lips dark purple; cyanosis in fingers of both hands; mucous membrane of the trachea grey-yellowish-brown colour; stomach and intestines walls bloodshot; dark purple intestines wall from the outsider and mucous membrane of duodenum; small intestine filled with bloody content; large intestine's content uncharacteristic; stomach lumen filled with ≈ 50mL of thick brownish mucous content	Nitrite levels: - 0.2 µg/mL (blood) - 24.6 µg/mL (urine) - 57.7 µg/mL (vitreous humour) - 2200 µg/mL (gastric content) - 0.3 µg/g (liver tissue) - 3.6 µg/g (kidney tissue) - 3.4 µg/g (costal cartilage)	NA
2021 [7]	USA	1	17 F	Bipolar disorder; substance use disorder; previous self-harm attempt; depressed recently	1 tablespoon	Online purchase 100 g sodium nitrite 2 weeks before the suicide	Vomiting; tired and laid down; unresponsive; foam coming from mouth; perioral cyanosis; heart rate 69 beats/min; blood pressure 82/33 mmHg, respiratory rate 15/min, SpO ₂ 74%; end-tidal CO ₂ 19 mmHg; capillary blood glucose 184 mg/dL; became bradycardic and pulseless; cyanotic	Blood samples brown	MetHb 48%; qualitative presence of trazodone and aripiprazole (both prescribed medications), in addition to midazolam	Online

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
							with a slightly green hue; blood chocolate brown; treatment i.v.: 1L 0.9% normal saline, epinephrine, sodium bicarbonate and calcium chloride; 3 doses methylene blue (2 mg/kg); 2 units of packed red blood cells transfused			
2021 [12]	USA	3	1) 24 F 2) 22 M 3) 39 M	1) NA 2) NA 3) Suicidal for years and recently developed paranoia	1) NA 2) NA 3) NA	1) Open boxes of famotidine and cimetidine; white powder within a reagent grade bottle "NaNO ₂ "; IP-6; inositol; acetaminophen and a 2 nd unopened reagent bottle of sodium nitrite; 2) A four-page suicide note; a warning note, stating "Danger! Lethal dosage of NaNO ₂ Call 911 immediately if drank!" (sic); a bottle labelled "NaNO ₂ "; a glass with white residue near the notes. 3) A digital scale; a white powder; several baggies with white powdery residue; a spoon with white residue	1) NA 2) Vomiting; unresponsive; discoloured; agonal breathing; pulse 40 beats/min; naloxone i.v. 3) Unresponsive	1) Fingertips and nail beds dark; mottled purple–grey <i>livor mortis</i> across the right side of the face; white foam along the length of tracheobronchial tree 2) Face gunmetal grey (blue–grey) discoloration; some of the skin on the fingers grey; nailbeds dusky; brown blood; linear haemorrhages in the mucosa of the body of the stomach on the peaks of the rugal folds; similar haemorrhages in the distal portion of the small intestine and the proximal portion of the large intestine; gastric content composed of brown liquid; kidneys brown, especially the medullae; multiple purpural haemorrhages beneath the scalp 3) A plume of white and red-tinged white foam coming from the nose; gunmetal grey discoloration	1) MetHb 62%; 2) MetHb 33%; vitreous electrolyte panel: elevated levels of sodium (148 mmol/L) and chloride (124 mmol/L). 3) MetHb 44%.	1) NA (reagent bottle of sodium nitrite can be easily purchased on a well-known online product) 2) NA (jar of product available at a local retail store for about \$20) 3) NA

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
								of the facial skin and the lips; <i>livor mortis</i> red–purple and grey; fingernail beds blue–grey; toenail beds pale, but slightly grey-tinged; brown blood; internal organs, especially the liver, the kidneys and the heart brown; lungs and brain edematous; haemorrhagic gastritis; acute passive congestion of the liver and the spleen		
2020 [30]	USA	3	1) 27 M 2) 16 F 3) 25 M	1) NA 2) NA 3) NA	1) 15 g 2) NA 3) 113 g	1) NA 2) NA 3) NA	1) Unresponsive; agonal respirations; cyanosis; initial SpO ₂ 68%; cardiopulmonary arrest; initial blood pressure 116/68 mmHg; initial heart rate 72 beats/min; methylene blue 2 mg/kg i.v. 2) Unresponsive; cyanotic; 2 doses methylene blue 1 mg/kg i.v. 3) Unresponsive; initial blood pressure 98/64 mmHg; initial heart rate 95 beats/min; initial SpO ₂ 80%; “chocolate brown blood”; cardiopulmonary arrest; methylene blue 1 mg/kg i.v.	1) NA 2) NA 3) NA	1) MetHb>32.4% 2) MetHb>30% 3) MetHb 29%	1) NA 2) Online 3) NA
2010 [31]	New Zealand	1	76 M	Combined right hemicolectomy/radical nephrectomy for locally advanced renal cell carcinoma and subsequent chronic pain syndrome, depression and previous attempted suicide by overdose	NA	NA	Agitated; collapsed; brady-asystolic cardiac arrest; heart rate 110/min; blood pressure 107/56 mmHg; SpO ₂ 85%; skin deeply cyanosed with a generalized bluish hue; blood ‘chocolate brown’;	NA	MetHb 82.6%	NA

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs, symptoms and treatment	Pathology findings	Toxicology findings	Origin of nitrites
							arterial blood gas pH 7.33, sodium 146 mmol/L and potassium 5.4 mmol/L; lactate 9.6 mmol/L; MetHb 82.6%; Hb 110 g/L; ventilated 100% oxygen; adrenaline 1 mg i.v. and repeated at 5 min intervals; atropine total dose 1.8 mg; 250 mg methylene blue i.v. ($\approx$ 5 mg/kg) in divided aliquots 50 mg over 4 min each; 1500 mL 0.9% saline solution			
1990 [55]	UK	1	17 F	No history obtained from her; her boyfriend reported normal behaviour earlier in the day and no evidence of drug ingestion at her house	1 g tablet	NA	Hysterical condition and bright blue; central cyanosis, tachycardia and tachypnoea; chest clear; systolic blood pressure of 90 mmHg; vomited; suffered a respiratory arrest; arterial blood gases normal; venous blood sample chocolate brown in colour; cardiac arrhythmias; aspirated, intubated and ventilated; methylene blue i.v. 2 mg/kg over 10 min	NA	MetHb 35%; serum nitrite ion 13 mg/L	Workplace (dental nurse)

F – female; Hb – haemoglobin; M – male; MetHb – methaemoglobin; NA – not available; PCO₂ – partial pressure of carbon dioxide; PO₂ – partial pressure of oxygen; SpO₂ – oxygen saturation.

Table III – Intentional non-fatal cases reported in the literature in other countries

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs and symptoms	Toxicology findings	Treatment	Origin of nitrites
2022 [57]	Canada	1	20 M	NA	21 g	“Suicide kit”: a measurement scale, a flask for dissolution in water and antiemetics (metoclopramide and famotidine)	Comatose; centrally cyanotic; heart rate 60 beats/min; systolic blood pressure 80 mmHg; body temperature 36.5 °C; SpO ₂ 85%; conjunctival, nail bed and palmar pallor; arterial blood dark and chocolate coloured	MetHb>29%	Fluid resuscitation; vasopressor support; intubation; gastric lavage and activated charcoal (1 g/kg); 1 st methylene blue (1 mg/kg) i.v., in a total of 4 mg/kg	Online
2022 [120]	USA	1	3 rd decade of life F	NA	NA	Victim consulted a website indicating the use of nitrites to induce fatal methaemoglobinemia as a suicide method	Vomiting; decompensated; ashen in colour; SpO ₂ 83%; hypotension (60/30 mmHg); chocolate-brown blood	MetHb>30%	Intubated; crystalloid; methylene blue; ascorbic acid; vasopressors	Probably online
2022 [58]	France	3	1) 17 F 2) 31 M 3) 17 M	NA	1) 25-30 g 2) 20 g 3) 1 g	NA	1) Hypotension; tachycardia; cyanosis; agitation; coma 2) Vomiting; hypotension; tachycardia; cyanosis; coma 3) Vomiting; tachycardia	1) MetHb 88% 1h30 after ingestion 2) MetHb>30% after the first methylene blue injection 3) MetHb 8.5%	1) Intubation; mechanical ventilation; 2 injections methylene blue (2 mg/kg+1mg/kg) 2) Intubation; mechanical ventilation; norepinephrine; 2 injections methylene blue (100 mg+100 mg) 3) Normobaric oxygen	1) Online 2) Online 3) Online
2022 [60]	Republic of Korea	2	1) 24 F 2) 20 M	1) Healthy; depressed; unable to work; trouble sleeping; distress 2) Major depressive disorder (unmedicated); under stressful conditions; repeatedly thought of death	1) 10 g 2) 750 mg	1) Instructions obtained from a suicide blog 2) Obtained information on how to make the suicide pill from an online suicide forum	1) Acute dyspnoea; cyanosis; blood pressure 117/54 mmHg; pulse 104 beats/min; respiratory rate 24 breaths/min; SpO ₂ 93% 2) Mild nausea; no signs of central or peripheral cyanosis; hemodynamically stable	1) MetHb 40.9% 2) MetHb 4.8%	1) 1 mg/kg methylene blue; neuropsychiatric evaluation; treatment for mental illness, suicidal ideation and suicide attempts 2) Conservative supportive treatment	1) Online 2) Online
2022 [24]	USA	1	23 M	Previous suicide attempts; polysubstance use disorder; in remission from alcohol and benzodiazepine use; nicotine dependence; current marijuana use;	2 teaspoons	Marijuana use that day	Losing consciousness; confused; nausea, vomiting; bowel incontinence; severe diffuse abdominal pain; appearing ill, ashen, discoloured; in distress; tachycardic to 108; respiratory rate at 22; blood pressure in the 110 s systolic; SpO ₂ 84%; skin grey; perioral cyanosis; cardiopulmonary examination	MetHb 26.3%; pH 7.39, PCO ₂ 33 mmHg; PO ₂ 38 mmHg; platelet 329; leukocytosis 23.3 with strong left shift of 18.75; a mild decrease in bicarbonate at 19 and increased anion gap 16;	1 mg/kg methylene blue; further psychiatric care	Online

Year of publication and reference	Country	Total cases	Age and sex	Clinical history	Estimated amount ingested of sodium nitrite	Scene findings	Signs and symptoms	Toxicology findings	Treatment	Origin of nitrites
				bipolar disorder; post-traumatic stress disorder; irritable bowel syndrome			demonstrated mild tachycardia	urine drug screen was positive for THC and amphetamines; denied use of amphetamines, which may be a false positive from bupropion		
2022 [8]	Nepal	1	37 M	NA	NA	NA	Altered mental state; cyanosed; SpO ₂ 68%; blood pressure 107/65 mmHg; pulse 105 beats/min; respiration 15 breaths/min	oxyHb 25.1%; carboxyHb 10.4%; MetHb>10.0%	Intubated; mechanical ventilation with FiO ₂ of 100% with intensive care; methylene blue infusion; transferred to a psychiatric unit; enrolled in cognitive behavioural and antidepressant therapy for underlying depression and suicidal behaviour	NA
2022 [59]	Korea	1	20 F	NA	12.5 g	NA	Vomited; dyspneic; cyanotic; SpO ₂ 86%	Before methylene blue: MetHb 54.6%; 3 h after methylene blue: MetHb 1.2%	1 mg/kg methylene blue; psychiatric interview	NA
2021 [13]	USA	2	1) 17 F 2) 24 M	1) NA 2) Depression	1) 21 g 2) "1 cup"	1) Also ingested 600 mg ibuprofen and "half of a bottle" of bismuth subsalicylate	1) Pale; cyanotic; initial blood pressure 115/74 mmHg; initial heart rate 130-140 beats/min; initial SpO ₂ 84%; Hb 13.3 g/dL 2) Fingertip cyanosis; hypotension; initial blood pressure 116/54 mmHg; initial heart rate 140 beats/min; initial SpO ₂ 87%; Hb 15.3 g/dL	1) MetHb>28% before antidote; MetHb 5% 2 h after treatment 2) MetHb 69%; MetHb 10.5% 1 h after methylene blue	1) Methylene blue 1 mg/kg i.v. 2) Methylene blue 2 mg/kg i.v.	1) Online 2) Online
2020 [30]	USA	2	1) 18 M 2) 16 F	NA	1) 15 g + 100 mg of DMSO 2) 60 g	1) Followed instructions from a suicide blog 2) Learned about the suicide method from an online suicide forum	1) Confusion; cyanosis; initial blood pressure 109/58 mmHg; initial heart rate 95 beats/min; initial SpO ₂ 90% (post-methylene blue) 2) Somnolent; cyanotic; hypoxic; initial blood pressure 101/59 mmHg; initial heart rate 128 beats/min; SpO ₂ 76%	1) Peak MetHb level 70.8% 2) Peak MetHb level 73%	1) Methylene blue (1.5 mg/kg) 2) Methylene blue (6 mg/kg), 2 units packed red blood cells; intubated, placed on 100% FiO ₂ , and started on multiple vasopressors; red blood cell transfusion	1) NA 2) Online
2016 [27]	China	1	30 F	NA	NA	NA	Hypoxemia; disorder of consciousness; cyanotic lips and extremities	Blood nitrite ion 44 µmol/L (i.e., 2 µg/mL)	Proper therapy with specific antidote	NA

F – female; FiO₂ – fraction of inspired oxygen; Hb – haemoglobin; M – male; MetHb – methaemoglobin; NA – not available; PCO₂ – partial pressure of carbon dioxide; PO₂ – partial pressure of oxygen; SpO₂ – oxygen saturation; THC – tetrahydrocannabinol.

Annex II – Other activities

▪ Scientific communications:

During the academic year 2022/2023, the scientific work carried out within the scope of the dissertation was presented at some scientific events (Table 10).

Table 10. Oral and poster communications at scientific events in the academic year 2022/2023.

Event name	Date	Local	Title	Authors	Type of presentation	Publication of abstract
International Congress APCF-TOXRUN	08.04.2022	Porto	"Nitrites: one "click" to suicide"	S. Pinheiro; F. Carvalho; H. Carmo	Poster	RevSALUS - Revista Científica Internacional Da Rede Académica Das Ciências Da Saúde Da Lusofonia, 4(Sup), 153. https://doi.org/10.51126/revsalus.v4iSup.384
International Congress APCF-TOXRUN	08.04.2022	Porto	"Nitrite ingestion: a pain free lethal poison? Report of a fatal intoxication"	S. Pinheiro; H. Carmo; S. Costa; L. Coelho; R. Rangel; F. Carvalho	Poster	RevSALUS - Revista Científica Internacional Da Rede Académica Das Ciências Da Saúde Da Lusofonia, 4(Sup), 122-123. https://doi.org/10.51126/revsalus.v4iSup.352
SPF Meeting	03.02.2023	Coimbra	"Self-poisoning with nitrite. Toxicological analysis from a forensic autopsy and <i>in vitro</i> investigation of cytotoxicity"	S. Pinheiro; H. Carmo; S. Costa; L. Coelho; R. Rangel; F. Carvalho.	Oral	-----
IJUP	12.05.2023	Porto	"Nitrite ingestion as an emerging trend in suicide: investigating <i>in vitro</i> cytotoxicity"	S. Pinheiro; F. Carvalho; H. Carmo	Oral	-----

▪ Internship at the INMLCF of the Centre Delegation:

An observational internship was carried out at the Forensic Chemistry and Toxicology Service of the INMLCF of the Centre Delegation, between the 26th and the 30th of June, 2023.

References

- [1] Ma L, Hu L, Feng X, Wang S. Nitrate and Nitrite in Health and Disease. *Aging Dis*, **9**: 938-945, 2018
- [2] Ansari FA, Ali SN, Arif H, Khan AA, Mahmood R. Acute oral dose of sodium nitrite induces redox imbalance, DNA damage, metabolic and histological changes in rat intestine. *PLoS One*, **12**: e0175196, 2017
- [3] Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*, **7**: 156-167, 2008
- [4] Powlson DS, Addiscott TM, Benjamin N, Cassman KG, de Kok TM, van Grinsven H, L'Hirondel JL, Avery AA, van Kessel C. When does nitrate become a risk for humans? *J Environ Qual*, **37**: 291-295, 2008
- [5] Durão C, Pedrosa F, Dinis-Oliveira RJ. Another suicide by sodium nitrite and multiple drugs: an alarming trend for "exit"? *Forensic Sci Med Pathol*, **17**: 362-366, 2021
- [6] Taus F, Pigaiani N, Bortolotti F, Mazzoleni G, Brevi M, Tagliaro F, Gottardo R. Direct and specific analysis of nitrite and nitrate in biological and non-biological samples by capillary ion analysis for the rapid identification of fatal intoxications with sodium nitrite. *Forensic Sci Int*, **325**: 110855, 2021
- [7] Neth MR, Love JS, Horowitz BZ, Shertz MD, Sahni R, Daya MR. Fatal Sodium Nitrite Poisoning: Key Considerations for Prehospital Providers. *Prehosp Emerg Care*, **25**: 844-850, 2021
- [8] Sedhai YR, Atreya A, Basnyat S, Phuyal P, Pokhrel S. The use of sodium nitrite for deliberate self-harm, and the online suicide market: Should we care? *Med Leg J*, **90**: 79-80, 2022
- [9] Durão C, Pedrosa F, Dinis-Oliveira RJ. A fatal case by a suicide kit containing sodium nitrite ordered on the internet. *J Forensic Leg Med*, **73**: 101989, 2020
- [10] Wright RO, Lewander WJ, Woolf AD. Methemoglobinemia: Etiology, Pharmacology, and Clinical Management. *Annals of Emergency Medicine*, **34**: 646-656, 1999
- [11] Hickey TBM, MacNeil JA, Hansmeyer C, Pickup MJ. Fatal methemoglobinemia: A case series highlighting a new trend in intentional sodium nitrite or sodium nitrate ingestion as a method of suicide. *Forensic Sci Int*, **326**: 110907, 2021
- [12] Dean DE, Looman KB, Topmiller RG. Fatal methemoglobinemia in three suicidal sodium nitrite poisonings. *J Forensic Sci*, **66**: 1570-1576, 2021
- [13] McCann SD, Kennedy JM, Tweet MS, Bryant SM. Sodium Nitrite Ingestion: an Emerging Trend in Suicide Attempts Shared via Online Communities. *J Emerg Med*, **60**: 409-412, 2021
- [14] Karwowska M, Kononiuk A. Nitrates/Nitrites in Food-Risk for Nitrosative Stress and Benefits. *Antioxidants (Basel)*, **9**, 2020
- [15] Dellavalle CT, Xiao Q, Yang G, Shu XO, Aschebrook-Kilfoy B, Zheng W, Lan Li H, Ji BT, Rothman N, Chow WH, Gao YT, Ward MH. Dietary nitrate and nitrite intake and risk of colorectal cancer in the Shanghai Women's Health Study. *Int J Cancer*, **134**: 2917-2926, 2014
- [16] Zhao J, Wang J, Yang Y, Lu Y. The Determination of Nitrate and Nitrite in Human Urine and Blood by High-Performance Liquid Chromatography and Cloud-Point Extraction. *J Chromatogr Sci*, **53**: 1169-1177, 2015
- [17] Shiva S. Nitrite: A Physiological Store of Nitric Oxide and Modulator of Mitochondrial Function. *Redox Biol*, **1**: 40-44, 2013
- [18] Hwang C, Yeon SH, Jung J, Na JY. An autopsy case of sodium nitrite-induced methemoglobinemia with various post-mortem analyses. *Forensic Sci Med Pathol*, **17**: 475-480, 2021
- [19] Wang R, Teng C, Zhang N, Zhang J, Conway G. A Family Clustered Nitrite Intoxication Investigation in Gaoxin District, Suzhou, China, 2013. *Iran J Public Health*, **42**: 1465-1471, 2013

- [20] Cvetković D, Živković V, Lukić V, Nikolić S. Sodium nitrite food poisoning in one family. *Forensic Sci Med Pathol*, **15**: 102-105, 2019
- [21] Andelhofs D, Van Den Bogaert W, Lepla B, Croes K, Van de Voorde W. Suicidal sodium nitrite intoxication: a case report focusing on the postmortem findings and toxicological analyses-review of the literature. *Forensic Sci Med Pathol*, 2023
- [22] Stephenson L, Wills S, van den Heuvel C, Humphries M, Byard RW. Increasing use of sodium nitrite in suicides—an emerging trend. *Forensic Science, Medicine and Pathology*, 2022
- [23] AAPCC. American Association of Poison Control Centers. <https://poisoncenters.org/news-alerts/12760202>. [Accessed 26 November 2022]. 2022
- [24] Matin AM, Boie ET, Moore GP. Survival after self-poisoning with sodium nitrite: A case report. *J Am Coll Emerg Physicians Open*, **3**: e12702, 2022
- [25] Joosen D, Stolk L, Henry R. A non-fatal intoxication with a high-dose sodium nitrate. *BMJ Case Rep*, **2014**, 2014
- [26] Chen Y, Liu Q, Wang J, Li H, Zhang Y, Sun L, Liu J. Delayed Post-Hypoxic Leukoencephalopathy Following Nitrite Poisoning: A Case Report and Review of the Literature. *Front Neurol*, **13**: 836844, 2022
- [27] Yan H, Zhuo X, Shen B, Xiang P, Shen M. Determination of Nitrite in Whole Blood by High-Performance Liquid Chromatography with Electrochemical Detection and a Case of Nitrite Poisoning. *J Forensic Sci*, **61**: 254-258, 2016
- [28] Sedhai YR, Atreya A, Basnyat S, Phuyal P, Pokhrel S. The use of sodium nitrite for deliberate self-harm, and the online suicide market: Should we care? *Med Leg J*: 25817221998119, 2021
- [29] SanctionedSuicide. Suicide Discussion Forum. <https://sanctioned-suicide.org/threads/dmso-dimethyl-sulfoxide.10427/>. [Accessed 31 December 2022]. 2022
- [30] Mudan A, Repplinger D, Lebin J, Lewis J, Vohra R, Smollin C. Severe Methemoglobinemia and Death From Intentional Sodium Nitrite Ingestions. *J Emerg Med*, **59**: e85-e88, 2020
- [31] Harvey M, Cave G, Chanwai G. Fatal methaemoglobinaemia induced by self-poisoning with sodium nitrite. *Emerg Med Australas*, **22**: 463-465, 2010
- [32] Padovano M, Aromatario M, D'Errico S, Concato M, Manetti F, David MC, Scopetti M, Frati P, Fineschi V. Sodium Nitrite Intoxication and Death: Summarizing Evidence to Facilitate Diagnosis. *Int J Environ Res Public Health*, **19**, 2022
- [33] Barrangou-Pouey-Darlas M, Gerardin M, Deheul S, Istvan M, Guerlais M, Fan, Jolliet P, Dejoie T, Victorri-Vigneau C. Poppers Use and High Methaemoglobinaemia: 'Dangerous Liaisons'. *Pharmaceuticals (Basel)*, **14**, 2021
- [34] Blood AB, Power GG. Nitrite: on the journey from toxin to therapy. *Clin Pharmacokinet*, **54**: 221-223, 2015
- [35] Castiglione N, Rinaldo S, Giardina G, Stelitano V, Cutruzzolà F. Nitrite and nitrite reductases: from molecular mechanisms to significance in human health and disease. *Antioxid Redox Signal*, **17**: 684-716, 2012
- [36] de Saint-Blanquat G. [Toxicologic and nutritional aspects of nitrates and nitrites]. *Ann Nutr Aliment*, **34**: 827-864, 1980
- [37] Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR, Jr., Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S, Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E. Nitrate and nitrite in biology, nutrition and therapeutics. *Nat Chem Biol*, **5**: 865-869, 2009
- [38] do Nascimento TS, Pereira RO, de Mello HL, Costa J. Methemoglobinemia: from diagnosis to treatment. *Rev Bras Anesthesiol*, **58**: 651-664, 2008

- [39] Jung KH, Chu K, Ko SY, Lee ST, Sinn DI, Park DK, Kim JM, Song EC, Kim M, Roh JK. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke*, **37**: 2744-2750, 2006
- [40] Presley TD, Morgan AR, Bechtold E, Clodfelter W, Dove RW, Jennings JM, Kraft RA, King SB, Laurienti PJ, Rejeski WJ, Burdette JH, Kim-Shapiro DB, Miller GD. Acute effect of a high nitrate diet on brain perfusion in older adults. *Nitric Oxide*, **24**: 34-42, 2011
- [41] Shortt J, Polizzotto MN, Opat SS, Cole-Sinclair MF. Oxidative haemolysis due to 'poppers'. *Br J Haematol*, **142**: 328, 2008
- [42] Elshikh AH, Kango G, Baalbaki M, Lankowsky J, Bawa A. Amyl Nitrite-Induced Hemolytic Anemia: Acute Therapy and Prevention. *Cureus*, **13**: e16099, 2021
- [43] Atyabi N, Yasini SP, Jalali SM, Shaygan H. Antioxidant effect of different vitamins on methemoglobin production: An in vitro study. *Vet Res Forum*, **3**: 97-101, 2012
- [44] McCann SD, Tweet MS, Wahl MS. Rising incidence and high mortality in intentional sodium nitrite exposures reported to US poison centers. *Clin Toxicol (Phila)*, **59**: 1264-1269, 2021
- [45] Gutiérrez AJ, Rubio C, Caballero JM, Hardisson A. Nitrites. In: Wexler P, editor. *Encyclopedia of Toxicology (Third Edition)*. Oxford: Academic Press, pp. 532-535, 2014
- [46] Ansari FA, Mahmood R. Sodium nitrite enhances generation of reactive oxygen species that decrease antioxidant power and inhibit plasma membrane redox system of human erythrocytes. *Cell Biol Int*, **40**: 887-894, 2016
- [47] Zavodnik IB, Lapshina EA, Rekawiecka K, Zavodnik LB, Bartosz G, Bryszewska M. Membrane effects of nitrite-induced oxidation of human red blood cells. *Biochim Biophys Acta*, **1421**: 306-316, 1999
- [48] Ansari FA, Ali SN, Mahmood R. Sodium nitrite-induced oxidative stress causes membrane damage, protein oxidation, lipid peroxidation and alters major metabolic pathways in human erythrocytes. *Toxicol In Vitro*, **29**: 1878-1886, 2015
- [49] Sun J, Aoki K, Wang W, Guo A, Misumi J. Sodium nitrite-induced cytotoxicity in cultured human gastric epithelial cells. *Toxicol In Vitro*, **20**: 1133-1138, 2006
- [50] Ansari FA, Ali SN, Khan AA, Mahmood R. Acute oral dose of sodium nitrite causes redox imbalance and DNA damage in rat kidney. *J Cell Biochem*, **119**: 3744-3754, 2018
- [51] Petrova E, Gluhcheva Y, Pavlova E, Vladov I, Voyslavov T, Ivanova J. Effect of acute sodium nitrite intoxication on some essential biometals in mouse spleen. *J Trace Elem Med Biol*, **58**: 126431, 2020
- [52] Kim M, Kim S, Yang W, Sim J. Determination of nitrite and nitrate in postmortem whole blood samples of 10 sodium nitrite poisoning cases: The importance of nitrate in determining nitrite poisoning. *Forensic Science International*, **335**: 111279, 2022
- [53] Wettstein ZS, Yarid NA, Shah S. Fatal methaemoglobinemia due to intentional sodium nitrite ingestion. *BMJ Case Rep*, **15**, 2022
- [54] Tomsia M, Głaz M, Nowicka J, Szczepański M. Sodium nitrite detection in costal cartilage and vitreous humor - Case report of fatal poisoning with sodium nitrite. *J Forensic Leg Med*, **81**: 102186, 2021
- [55] Gowans WJ. Fatal methaemoglobinaemia in a dental nurse. A case of sodium nitrite poisoning. *Br J Gen Pract*, **40**: 470-471, 1990
- [56] Varlet V, Ryser E, Augsburg M, Palmiere C. Stability of postmortem methemoglobin: Artifactual changes caused by storage conditions. *Forensic Sci Int*, **283**: 21-28, 2018
- [57] Saleh D, Lucyk S, McGillis E. Methemoglobinemia caused by sodium nitrite overdose. *Cmaj*, **194**: E1066-e1067, 2022
- [58] Vodovar D, Tournoud C, Boltz P, Paradis C, Puskarczyk E. Severe intentional sodium nitrite poisoning is also being seen in France. *Clin Toxicol (Phila)*, **60**: 272-274, 2022

- [59] Mun SH, Park GJ, Lee JH, Kim YM, Chai HS, Kim SC. Two cases of fatal methemoglobinemia caused by self-poisoning with sodium nitrite: A case report. *Medicine (Baltimore)*, **101**: e28810, 2022
- [60] Yoon JC, Kim SE. Suicide attempt using sodium nitrite ordered on the internet: Two case reports. *Medicine (Baltimore)*, **101**: e29355, 2022
- [61] Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J Chromatogr B Analyt Technol Biomed Life Sci*, **851**: 51-70, 2007
- [62] Giustarini D, Rossi R, Milzani A, Dalle-Donne I. Nitrite and nitrate measurement by Griess reagent in human plasma: evaluation of interferences and standardization. *Methods Enzymol*, **440**: 361-380, 2008
- [63] Evelyn KA, Malloy HT. MICRODETERMINATION OF OXYHEMOGLOBIN, METHEMOGLOBIN, AND SULFHEMOGLOBIN IN A SINGLE SAMPLE OF BLOOD. *Journal of Biological Chemistry*, **126**: 655-662, 1938
- [64] Hegesh E, Gruener N, Cohen S, Bochkovsky R, Shuval HI. A sensitive micromethod for the determination of methemoglobin in blood. *Clin Chim Acta*, **30**: 679-682, 1970
- [65] Sato K. Methemoglobin. In: Suzuki O, Watanabe K, editors. *Drugs and Poisons in Humans: A Handbook of Practical Analysis*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 655-657, 2005
- [66] Arnaud F, Higgins A, McCarron R, Moon-Massat PF. Determination of methemoglobin and hemoglobin levels in small volume samples. *Artif Cells Nanomed Biotechnol*, **45**: 58-62, 2017
- [67] Camargo TM, Alves MIF, Oliveira SJ, Shitara ES, Oshima-Franco Y. Comparative study between two techniques of methemoglobin (MHb) determination. *Revista Brasileira de Análises Clínicas*, **39**: 95-98, 2007
- [68] Andelhofs D, Van Den Bogaert W, Lepla B, Croes K, Van de Voorde W. Suicidal sodium nitrite intoxication: a case report focusing on the postmortem findings and toxicological analyses—review of the literature. *Forensic Science, Medicine and Pathology*, 2023
- [69] Tsikas D, Suchy MT, Mitschke A, Beckmann B, Gutzki FM. Measurement of nitrite in urine by gas chromatography-mass spectrometry. *Methods Mol Biol*, **844**: 277-293, 2012
- [70] Giustarini D, Rossi R, Milzani A, Dalle-Donne I. Nitrite and Nitrate Measurement by Griess Reagent in Human Plasma: Evaluation of Interferences and Standardization. *Methods in Enzymology*: Academic Press, pp. 361-380, 2008
- [71] Zhang M, Truver MT, Hoyer JL, Chronister CW, Goldberger BA. Presumptive Identification of Nitrite by Griess Reagent Test Strips - Case reports of fatal poisoning with sodium nitrite. *J Anal Toxicol*, 2023
- [72] Lahoud CA, March JA, Proctor DD. Campho-Phenique ingestion: an intentional overdose. *South Med J*, **90**: 647-648, 1997
- [73] Tekin HG, Gökben S, Serdaroğlu G. Seizures due to high dose camphor ingestion. *Turk Pediatri Ars*, **50**: 248-250, 2015
- [74] Centini F, Fiore C, Riezzo I, Rossi G, Fineschi V. Suicide due to oral ingestion of lidocaine: a case report and review of the literature. *Forensic Sci Int*, **171**: 57-62, 2007
- [75] Barash M, Reich KA, Rademaker D. Lidocaine-induced methemoglobinemia: a clinical reminder. *J Am Osteopath Assoc*, **115**: 94-98, 2015
- [76] Pereira-Oliveira M, Reis-Mendes A, Carvalho F, Remião F, Bastos ML, Costa VM. Doxorubicin Is Key for the Cardiotoxicity of FAC (5-Fluorouracil + Adriamycin + Cyclophosphamide) Combination in Differentiated H9c2 Cells. *Biomolecules*, **9**, 2019
- [77] Silva R, Carmo H, Vilas-Boas V, Barbosa DJ, Palmeira A, Sousa E, Carvalho F, Bastos MdL, Remião F. Colchicine effect on P-glycoprotein expression and

- activity: In silico and in vitro studies. *Chemico-Biological Interactions*, **218**: 50-62, 2014
- [78] Soares J, Costa VM, Gaspar H, Santos S, Bastos ML, Carvalho F, Capela JP. Adverse outcome pathways induced by 3,4-dimethylmethcathinone and 4-methylmethcathinone in differentiated human SH-SY5Y neuronal cells. *Arch Toxicol*, **94**: 2481-2503, 2020
- [79] Martins MJ, Roque Bravo R, Enea M, Carmo H, Carvalho F, Bastos ML, Dinis-Oliveira RJ, Dias da Silva D. Ethanol additively enhances the in vitro cardiotoxicity of cocaine through oxidative damage, energetic deregulation, and apoptosis. *Arch Toxicol*, **92**: 2311-2325, 2018
- [80] Arbo MD, Silva R, Barbosa DJ, da Silva DD, Rossato LG, Bastos Mde L, Carmo H. Piperazine designer drugs induce toxicity in cardiomyoblast h9c2 cells through mitochondrial impairment. *Toxicol Lett*, **229**: 178-189, 2014
- [81] Silva R, Palmeira A, Carmo H, Barbosa DJ, Gameiro M, Gomes A, Paiva AM, Sousa E, Pinto M, Bastos Mde L, Remião F. P-glycoprotein induction in Caco-2 cells by newly synthesized thioxanthenes prevents paraquat cytotoxicity. *Arch Toxicol*, **89**: 1783-1800, 2015
- [82] Soares J, Costa VM, Gaspar H, Santos S, de Lourdes Bastos M, Carvalho F, Capela JP. Structure-cytotoxicity relationship profile of 13 synthetic cathinones in differentiated human SH-SY5Y neuronal cells. *Neurotoxicology*, **75**: 158-173, 2019
- [83] Lv M, Zhang Y, Liang L, Wei M, Hu W, Li X, Huang Q. Effect of graphene oxide on undifferentiated and retinoic acid-differentiated SH-SY5Y cells line. *Nanoscale*, **4**: 3861-3866, 2012
- [84] Sahu SC, Zheng J, Graham L, Chen L, Ihrie J, Yourick JJ, Sprando RL. Comparative cytotoxicity of nanosilver in human liver HepG2 and colon Caco2 cells in culture. *J Appl Toxicol*, **34**: 1155-1166, 2014
- [85] Tolosa L, Donato MT, Gómez-Lechón MJ. General Cytotoxicity Assessment by Means of the MTT Assay. *Methods Mol Biol*, **1250**: 333-348, 2015
- [86] Enea M, Pereira E, Silva DD, Costa J, Soares ME, de Lourdes Bastos M, Carmo H. Study of the intestinal uptake and permeability of gold nanoparticles using both in vitro and in vivo approaches. *Nanotechnology*, **31**: 195102, 2020
- [87] Roque Bravo R, Carmo H, Valente MJ, Silva JP, Carvalho F, Bastos ML, Dias da Silva D. From street to lab: in vitro hepatotoxicity of buphedrone, butylone and 3,4-DMMC. *Arch Toxicol*, **95**: 1443-1462, 2021
- [88] GraphPad. Free web calculators. <https://www.graphpad.com/quickcalcs/Ecanything1.cfm>. [Accessed 8 May 2023]. 2023
- [89] Eiras F, Amaral MH, Silva R, Martins E, Lobo JMS, Silva AC. Characterization and biocompatibility evaluation of cutaneous formulations containing lipid nanoparticles. *International Journal of Pharmaceutics*, **519**: 373-380, 2017
- [90] Kobuszewska A, Tomecka E, Zukowski K, Jastrzebska E, Chudy M, Dybko A, Renaud P, Brzozka Z. Heart-on-a-Chip: An Investigation of the Influence of Static and Perfusion Conditions on Cardiac (H9C2) Cell Proliferation, Morphology, and Alignment. *SLAS TECHNOLOGY: Translating Life Sciences Innovation*, **22**: 536-546, 2017
- [91] Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stamatii A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol*, **21**: 1-26, 2005
- [92] Kovalevich J, Langford D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods Mol Biol*, **1078**: 9-21, 2013
- [93] Dezfulian C, Kenny E, Lamade A, Misse A, Krehel N, St Croix C, Kelley EE, Jackson TC, Uray T, Rackley J, Kochanek PM, Clark RS, Bayir H. Mechanistic characterization of nitrite-mediated neuroprotection after experimental cardiac arrest. *J Neurochem*, **139**: 419-431, 2016

- [94] Liu Y, Croft KD, Hodgson JM, Mori T, Ward NC. Mechanisms of the protective effects of nitrate and nitrite in cardiovascular and metabolic diseases. *Nitric Oxide*, **96**: 35-43, 2020
- [95] Lu N, Ding Y, Tian R, Peng YY. Inhibition of myeloperoxidase-mediated oxidative damage by nitrite in SH-SY5Y cells: Relevance to neuroprotection in neurodegenerative diseases. *Eur J Pharmacol*, **780**: 142-147, 2016
- [96] El-Nabarawy NA, Gouda AS, Khattab MA, Rashed LA. Effects of nitrite graded doses on hepatotoxicity and nephrotoxicity, histopathological alterations, and activation of apoptosis in adult rats. *Environ Sci Pollut Res Int*, **27**: 14019-14032, 2020
- [97] Vatassery GT, SantaCruz KS, DeMaster EG, Quach HT, Smith WE. Oxidative stress and inhibition of oxidative phosphorylation induced by peroxynitrite and nitrite in rat brain subcellular fractions. *Neurochem Int*, **45**: 963-970, 2004
- [98] Shumilova TE, Shereshkov VI, Nozdrachev AD. Cardiovascular manifestations of acute nitrite intoxication in laboratory rats. *Journal of Evolutionary Biochemistry and Physiology*, **47**: 464-473, 2011
- [99] May JM, Qu ZC, Li X. Nitrite generates an oxidant stress and increases nitric oxide in EA.hy926 endothelial cells. *Free Radic Res*, **38**: 581-589, 2004
- [100] Jondeau A, Dahbi L, Bani-Estivals MH, Chagnon MC. Evaluation of the sensitivity of three sublethal cytotoxicity assays in human HepG2 cell line using water contaminants. *Toxicology*, **226**: 218-228, 2006
- [101] Kiani A, Yousefsani BS, Doroudian P, Seydi E, Pourahmad J. The mechanism of hepatotoxic effects of sodium nitrite on isolated rat hepatocytes. *Toxicology and Environmental Health Sciences*, **9**: 244-250, 2017
- [102] Mathuram Thiagarajan U, James Shapiro AM. Successful Liver Transplantation After Neurologically Determined Death Donation Following Sodium Nitrite Poisoning. *Cureus*, **15**: e33278, 2023
- [103] Rajbux C, Pereira J, Selbourne MdC, Costa-Pinto AR, Poças F. Assessment of baby Bibs. GC-MS screening, migration into saliva and insight of toxicity with QSAR tools. *Food Control*, **109**: 106951, 2020
- [104] Ribeiro TB, Oliveira A, Campos D, Nunes J, Vicente AA, Pintado M. Simulated digestion of an olive pomace water-soluble ingredient: relationship between the bioaccessibility of compounds and their potential health benefits. *Food & Function*, **11**: 2238-2254, 2020
- [105] Brodkorb A, Egger L, Alminger M, Alvito P, Assunção R, Ballance S, Bohn T, Bourlieu-Lacanal C, Boutrou R, Carrière F, Clemente A, Corredig M, Dupont D, Dufour C, Edwards C, Golding M, Karakaya S, Kirkhus B, Le Feunteun S, Lesmes U, Macierzanka A, Mackie AR, Martins C, Marze S, McClements DJ, Ménard O, Minekus M, Portmann R, Santos CN, Souchon I, Singh RP, Vegarud GE, Wickham MSJ, Weitschies W, Recio I. INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, **14**: 991-1014, 2019
- [106] Costa JR, Amorim M, Vilas-Boas A, Tonon RV, Cabral LMC, Pastrana L, Pintado M. Impact of in vitro gastrointestinal digestion on the chemical composition, bioactive properties, and cytotoxicity of Vitis vinifera L. cv. Syrah grape pomace extract. *Food Funct*, **10**: 1856-1869, 2019
- [107] Sánchez-Gutiérrez M, Gómez-García R, Carrasco E, Bascón-Villegas I, Rodríguez A, Pintado M. Quercus ilex leaf as a functional ingredient: Polyphenolic profile and antioxidant activity throughout simulated gastrointestinal digestion and antimicrobial activity. *Journal of Functional Foods*, **91**: 105025, 2022
- [108] Zar JH. Biostatistical analysis. Englewood Cliffs, N.J.: Prentice-Hall Englewood Cliffs, N.J., 1984
- [109] GraphPad. Comparing slopes and intercepts. https://www.graphpad.com/guides/prism/latest/curve-fitting/reg_comparingslopesandintercepts.htm. [Accessed 20 July 2023]. 2023

- [110] Petersson J, Phillipson M, Jansson EA, Patzak A, Lundberg JO, Holm L. Dietary nitrate increases gastric mucosal blood flow and mucosal defense. *Am J Physiol Gastrointest Liver Physiol*, **292**: G718-724, 2007
- [111] Björne HH, Petersson J, Phillipson M, Weitzberg E, Holm L, Lundberg JO. Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. *J Clin Invest*, **113**: 106-114, 2004
- [112] Kim HS, Hur SJ. Changes of sodium nitrate, nitrite, and N-nitrosodiethylamine during in vitro human digestion. *Food Chem*, **225**: 197-201, 2017
- [113] Lee EH, Cha KH, Vuong TT, Kim SM, Pan C-H. Comparison of static and dynamic in vitro digestion models to estimate the bioaccessibility of lutein in lutein-rich foods. *Applied Biological Chemistry*, **61**: 441-447, 2018
- [114] González-Soltero R, Bailén M, de Lucas B, Ramírez-Goercke MI, Pareja-Galeano H, Larrosa M. Role of Oral and Gut Microbiota in Dietary Nitrate Metabolism and Its Impact on Sports Performance. *Nutrients*, **12**, 2020
- [115] Kapil V, Haydar SM, Pearl V, Lundberg JO, Weitzberg E, Ahluwalia A. Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic Biol Med*, **55**: 93-100, 2013
- [116] Power GG, Bragg SL, Oshiro BT, Dejam A, Hunter CJ, Blood AB. A novel method of measuring reduction of nitrite-induced methemoglobin applied to fetal and adult blood of humans and sheep. *J Appl Physiol (1985)*, **103**: 1359-1365, 2007
- [117] Holland MA, Kozlowski LM. Clinical features and management of cyanide poisoning. *Clin Pharm*, **5**: 737-741, 1986
- [118] Fens MH, Larkin SK, Oronsky B, Scicinski J, Morris CR, Kuypers FA. The capacity of red blood cells to reduce nitrite determines nitric oxide generation under hypoxic conditions. *PLoS One*, **9**: e101626, 2014
- [119] Miasoedova EE, Nazarov SB. [The response of erythrocytic system of mature rats to acute nitrite intoxication]. *Patol Fiziol Eksp Ter*: 16-18, 2004
- [120] Pires KD, Hart K, Tomassoni AJ. Internet-assisted suicide by nitrite poisoning - a case report and increase in reported intentional nitrite/nitrate exposures in U.S. Poison Center data. *Clin Toxicol (Phila)*, **60**: 271-272, 2022