

## Chapter

# Interaction between Pyridostigmine Bromide and Oxidative Stress

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## Abstract

In this chapter the following topics will be addressed: (1) actions of the cholinergic system in the nervous system, commenting on acetylcholine metabolism and acetylcholinesterase metabolism; (2) acetylcholinesterase inhibitors as subtitle in this topic: pharmacological characterization of pyridostigmine bromide, mechanism of action, and therapeutic effect of the drug; (3) use of pyridostigmine bromide in Persian Gulf War; and (4) potential effect of pyridostigmine bromide in oxidative stress, addressing as subtitle the influence of pyridostigmine bromide on the superoxide-hydrogen peroxide imbalance model. Studies indicate that the interaction between pyridostigmine bromide and stressors could trigger genotoxicity, the mechanism associated with the induction of oxidative stress that leads to this side effect of this drug; however, this discussion needs to be better elucidated and may be more discussed as there is interaction between the pyridostigmine bromide and an endogenous oxidative imbalance caused by it or even by the possible interaction of this with genetic variations present in the antioxidant metabolism.

**Keywords:** acetylcholinesterase inhibitor, oxidative stress, neurotoxicity, superoxide dismutase 2, neuromuscular junction

## 1. Introduction

Pyridostigmine bromide (PB) is a reversible acetylcholinesterase (AChE) inhibitor and the first line of choice for the treatment of symptoms associated with myasthenia gravis (MG) and other neuromuscular junction disorder prophylactic treatment in the Persian Gulf War, for prevention of post-traumatic stress and heat and pesticide exposure. However, evidence suggests that PB may be associated with Gulf War illness, characterized by the presence of fatigue, headaches, cognitive dysfunction, and respiratory, gastrointestinal, and musculoskeletal disorders [1–4]. However studies in animal models showed that if used without any association did not cause extensive cytotoxicity and genotoxicity to these animals. But the association of these drugs with other chemical or even physical agents caused cellular

apoptosis and genotoxicity in animals. These studies would suggest that this toxicity caused in association was due to oxidative stress [5, 6].

## 2. Actions of the cholinergic system in the nervous system

Within the neurotransmitters acting on the body's nervous system is the so-called cholinergic system associated with the release of the acetylcholine (ACh) molecule in the synaptic cleft [7–10]. ACh is considered to be one of the major chemical neurotransmitters of the peripheral nervous system being released by all preganglionic, parasympathetic, and some sympathetic postganglionic fibers, as well as by motor neurons that project to the skeletal muscles. It was the researcher Otto Loewi who discovered this molecule when he observed in his study the release of a biochemical substance by the parasympathetic nerve endings, which he called ACh [8, 11].

In cholinergic synapses, cholinesterases are present, which consist of a class of enzymes that catalyze the hydrolysis of ACh in acetic acid and choline in the synaptic cleft, and thus allow the cholinergic neuron to return to its resting state after activation. The most common cholinesterases present in the synaptic cleft are butyrylcholinesterase (BuChE) and AChE [12].

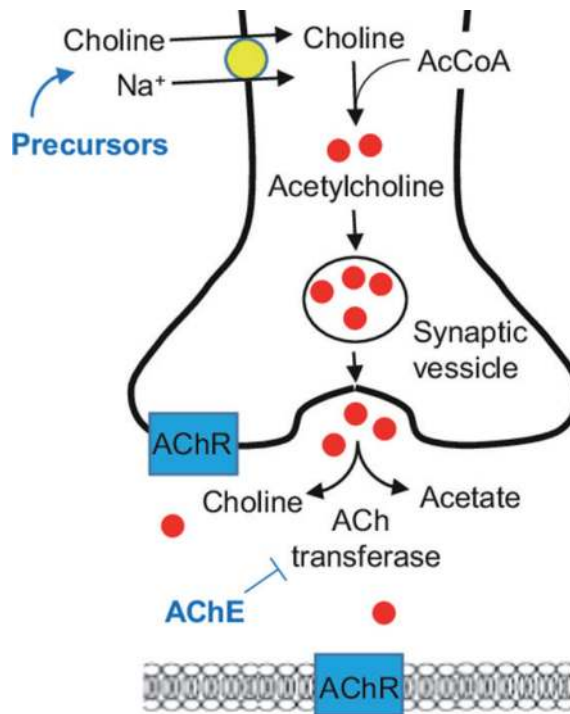
Although they are evolutionarily similar, these enzymes differ in their distribution in tissues, their kinetic properties, and the specificity of their substrates. AChE is found most abundantly in the central nervous system (CNS), in the skeletal muscles, and in the erythrocyte membrane, while BuChE is mostly found in blood plasma and is therefore also known as plasma cholinesterase [13].

Acetylcholinesterase and BuChE exhibit structural similarities, with their amino acids having approximately 50% homology. The other 50% heterogeneity among amino acids is responsible for the selectivity differences of both the substrates and the inhibitors of these enzymes. AChE preferentially hydrolyzes ACh, whereas BuChE is less selective and acts by hydrolyzing both ACh and butyrylcholine (BuCh) in comparable amounts [14].

In general, AChE is an enzyme that acts by hydrolyzing ACh in precursor molecules by rapidly closing the signaling of this molecule in the post-synaptic neuron or target tissue. Thus, AChE is a target enzyme in the treatment of various diseases, since anticholinesterase drugs act via their inhibition (**Figure 1**) [7–10].

Acetylcholine plays a crucial role in controlling numerous physiological processes in all divisions of the nervous system. However, it is also involved in various neurological and muscular dysfunctions. The apparently antagonistic action of ACh occurs due to the existence of different cholinergic receptors, which are present according to each type of target tissue. The knowledge of the various forms of ACh activity allowed the identification of causal mechanisms of several neuromorbidities associated with neuromotor plaque disorders, mainly related to changes in cholinergic receptors.

This knowledge, in turn, led to the development or understanding of the performance of drugs related to the control of symptoms of neurological diseases through differential modulation of the cholinergic system [7, 8, 10]. Among the morbidities with etiopathology associated with changes in cholinergic response, MG and other forms of myasthenic syndromes are prominent. In these diseases, AChE inhibitors are used to control clinical symptoms [16]. In addition to its role in MG, more recent studies indicate that ACh could be a key molecule in the progression and control of symptoms of other neurodegenerative diseases, such as Alzheimer's disease and other types of dementia [17, 18]. Because of its very specific physiological action, drugs associated with modulation of cholinergic neurons have also



**Figure 1.** Synthesis, storage, release, and degradation pathways of acetylcholine. Source: adapted Rang et al. [15]. AChR, acetylcholine receptor; ACh, acetylcholine; AChE, acetylcholinesterase.

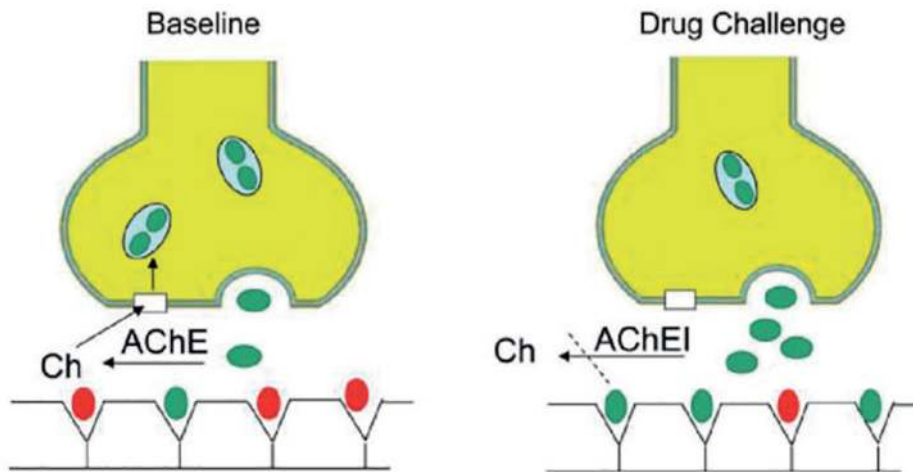
been prophylactically used to prevent populations subject to exposure to molecules potentially used in biological warfare, such as sarin gas [19]. For this reason, studies involving pharmacology related to the cholinergic system are considered clinical and epidemiologically relevant, in addition to their action in MG.

## 2.1 Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitory drugs are termed anticholinesterases, and these are therapeutically used to reverse the neuromuscular blockade promoted by depolarizing myorelaxants, in the treatment of neurological diseases such as MG and myasthenic syndrome, in smooth muscle atony, in strabismus, and in the treatment of symptoms of Alzheimer's disease, among others. An anticholinesterase drug delays the degradation of ACh, so the neurotransmitter spends more time in the synaptic cleft, thus intensifying cholinergic transmission, as can be observed in **Figure 2** [20–22].

Physostigmine, an alkaloid obtained from *Physostigma venenosum* L., was the first AChE inhibitor to be discovered. Thus, its cholinergic effects have been known for many years, and as early as 1923, the molecular structure of the active substance was elucidated. In 1929 Stedman demonstrated that the cholinomimetic effects of physostigmine were due to the reversible inhibition of AChE [24]. Although it is old, this drug is still in use and is currently used in the treatment of glaucoma and in cases of overdose by anticholinergic compounds, such as atropine, and tricyclic antidepressants such as amitriptyline [25]. Other drugs with inhibitory action of AChE have been developed, such as neostigmine and PB, which are simplified analog of physostigmine [20].

There are two classes of AChE inhibitors, based on their mechanism of action, and may be reversible or irreversible depending on the type of action with the active site of the drug. Reversible agents are still present in two groups: short-acting and intermediate-acting agents [26].



**Figure 2.** Cholinergic synapse in the absence and in the presence of an AChE inhibitor. Source: adapted Araújo et al. [23]. AChE, acetylcholinesterase; ch, choline.

Edrophonium, a quaternary ammonium compound that binds only to the anionic site of the enzyme, is one of the short-acting reversible anticholinesterases. The ionic bond formed is easily reversed, and the action of the drug is brief. It is mainly used as a diagnostic purpose, since the improvement of muscle strength observed with the use of an anticholinesterase is a characteristic of MG, but it does not occur when muscle weakness results from other causes [27]. In contrast, the anticholinesterases of intermediate duration include neostigmine, PB, and physostigmine that are composed of quaternary ammonium of clinical importance [26].

In chemical terms, all of these drugs are carbamoyl esters, once acetyl esters, and have basic groups that bind to the anionic site. The transfer of the carbamoyl group to the hydroxyl group of the serine from the esterase site occurs in the same way as with ACh, but the carbamylated enzyme undergoes hydrolysis much more slowly, taking minutes instead of microseconds. The anticholinesterase drugs are therefore hydrolyzed, but at an insignificant rate when compared to ACh, and the slow recovery of the carbamylated enzyme indicates that the action of these drugs is quite prolonged [12].

Irreversible anticholinesterases are compounds that have a pentavalent phosphorus containing a leaving group, such as fluoride, or an organic group. Upon binding the enzyme, this group is released, leaving the hydroxyl group of the serine of the enzyme phosphorylated. Most of these organophosphorus compounds have been developed to be used as a chemical weapon in the form of toxic gases, and as a pesticide, but also for clinical use. They interact only with the esterase site of the enzyme and do not have a cationic group. Ecotiopate is an exception, since it has a quaternary nitrogen group that also binds to the anionic site [12].

When AChE is in inactive phosphorylated form, this molecule is generally very stable. With drugs such as diflos, there is no appreciable hydrolysis, and the recovery of enzymatic activity depends on the synthesis of new molecules of the enzyme, a process that can take weeks. With other drugs like the ecotiopate, slow hydrolysis takes place in the course of a few days, so that its action is not strictly irreversible. The diflos and the parathion are apolar substances volatile with high lipid solubility, quickly absorbed through the mucous membranes and even through the integral skin and the cuticle of the insects. The use of these agents as a chemical weapon or as an insecticide is based on this property. The absence of a quaternary group that confirms specificity indicates that most of these drugs block other serine hydrolases, although their pharmacological effects stem mainly from inhibition of AChE [26].

Acetylcholinesterase inhibitors affect both peripheral, autonomic cholinergic synapses and CNS synapses. It is also important to note that some organophosphorus compounds are capable of producing a severe form of neurotoxicity leading to irreversible changes in the cholinergic system, especially triggering effects on autonomic cholinergic synapses. These implications mainly reflect increased ACh activity in parasympathetic postganglionic synapses (increased secretions of salivary, lacrimal, bronchial, and gastrointestinal glands, increased peristaltic activity, bronchodilation, bradycardia, hypotension, pupillary constriction, fixation of vision accommodation for near, drop in intraocular pressure). Larger doses are able to stimulate, and subsequently block, autonomic ganglia, producing complex autonomic effects. Blockade when it occurs consists of depolarization blockade and is associated with accumulation of ACh in plasma and in organic liquids. Neostigmine and PB tend to affect neuromuscular transmission more than the autonomic system, while physostigmine and organophosphates show the opposite pattern. The reason for this disparity is not clear, but therapeutic use takes advantage of this partial selectivity [26].

The main effect of these drugs is under neuromuscular junction; they increase the force of the contraction of a muscle stimulated by means of its motor nerve, thanks to the repetitive discharge in the muscular fiber associated with a prolongation of the action potential. Normally, ACh is hydrolyzed so quickly that each stimulus initiates only one action potential in the muscle fiber. However, when AChE is inhibited, there is a short series of action potential in the muscle fiber and, as a consequence, a greater tension. Much more important, however, is the effect produced when the transmission is blocked by a non-depolarizing blocking agent such as pancuronium. In this case, the addition of an anticholinesterase drug can dramatically restore transmission. When a large number of receptors are blocked, most ACh molecules will normally find AChE molecules and will be destroyed by them before reaching a vacant receptor. The inhibition of AChE gives ACh molecules a greater chance of finding a vacant receptor before being destroyed and as a consequence increases the action potential such that it reaches the threshold. Transmission does not occur in MG because there are very few ACh receptors, and in this case inhibition of AChE improves transmission [12].

Acetylcholinesterase inhibitors rarely fully induce symptom relief in myasthenic patients and do not affect disease progression; however, they may be sufficiently effective for proper management in certain patients with mild or purely ocular nonprogressive disease [28]. It is also important to note that people with MG are susceptible to presenting the so-called myasthenic crisis that involves weakness in respiratory muscles, upper airway muscles, or a combination of both muscle groups. Both inspiratory and expiratory respiratory muscles may be affected, manifesting as dyspnea. Respiratory dysfunction may also manifest as upper airway obstruction if bulbar or upper airway muscle weakness occurs. Signs of bulbar weakness include dysphagia, nasal regurgitation, nasal quality of speech, staccato speech, weakness of the mandible (closure of the mandible weaker than the opening of the mandible), bifacial paresis, and weakness of the tongue. Weakness of the upper airways can lead to failure by oropharyngeal collapse or tongue obstruction by increasing the work of already fatigued respiratory muscles. In epidemiological terms, it is estimated that 2/3 of myasthenic patients who present with myasthenic crisis need to be intubated and receive mechanical ventilation [29].

On the other hand, patients who ingest excess AChE inhibitors like PB may precipitate a cholinergic crisis characterized as muscarinic and nicotinic toxicity. Symptoms include increased sweating, lacrimation, salivation and pulmonary secretions, nausea, vomiting, diarrhea, bradycardia, and fasciculations. Although the cholinergic crisis is an important consideration in the evaluation of the patient

in a myasthenic crisis, it is quite uncommon in these patients. In the case of suspected cholinergic crisis, AChE inhibitors should be significantly reduced or discontinued [29].

In Ref. [30], the author described in his work that the natural course of MG, using only anticholinesterase drugs, with no other type of treatment, showed remission of symptoms in 20% of the patients and mortality in 25%. However, various therapies that involve thymectomy, immunosuppression, infection control, and others positively affect the natural history of the disease. Still in that decade, this author concluded that the mortality in patients with MG is practically zero and the great majority of the patients have normal life, thanks to the improvement in the assistive technology related to the management of myasthenic crisis.

## **2.2 Use of pyridostigmine bromide in Persian gulf war**

The use of chemical warfare agents is one of the greatest threats in the world today. Chemical warfare is based on the use of substances with toxic properties that are capable of killing, for mass destruction, and causing severe damage to the environment. The most prominent and dangerous chemical warfare agents are neurotoxic organophosphates which, due to their high toxicity, are sufficient in small amounts to cause seizures and death [31].

One of the biggest reasons for the use of chemical weapons in war and terrorist actions is that this war strategy ends up being cheaper than conventional weapons such as bombs, projectiles, and explosives. For example, to kill all people in an area of 1 km<sup>2</sup>, the use of chemical weapons can cost approximately 40% less than if traditional weapons were used. The other reason is that chemical weapons, in addition to causing death quickly and efficiently, also cause psychological problems to those who can survive intoxication, thus being more worrisome than other weapons of war [32].

The agents of chemical warfare were used several times in wars since antiquity, although being agents is not well defined nor very efficient. Already several more effective toxic agents received major importance in 1915, when the German army sent gases like chlorine and mustard against French troops during the First World War, causing countless losses in the enemy army. From that date the development of neurotoxic agents was more intense for several armies. Before World War II, the German army began the development of the first neurotoxic organophosphates as chemical warfare agents, especially tabun, sarin, and soman. Nevertheless, these agents, as well as mustard gas and other toxic substances, were not used during World War II. In the 1950s, the neurotoxic organophosphates of the V family were developed, which are more toxic and persistent in the environment, being that the first, called VX, was developed in England. Later similar compounds were created, especially in the former Soviet Union [32].

One of the first countries to use neurotoxic organophosphates was Iraq, under Saddam Hussein's command in the war against Iran between 1980 and 1988, leading to hundreds of deaths of Iranians [33]. In 1994, sarin was used in Japan against civilians in a terrorist attack that resulted in the death of 7 people and 200 intoxications [34, 35]. On the other hand, poisoning of American soldiers by sarin occurred during the Gulf War in 1991 [33]. Recently, chemical weapons were used in Syria, killing about 1300 people, especially civilians and children, making it one of the worst chemical weapon use events in the world.

Organophosphate, pesticides, carbamates, chemical agents such as sarin, and the drug PB all belong to a class of chemicals that inactivate the circulation of cholinesterase enzymes such as AChE, BuChE, paraoxonase, and neurotoxic esterase resulting in interference with the breakdown of ACh neurotransmitter among

other effects [36, 37]. Exposures lead to increased ACh in the brain and peripheral nerve endings, with overestimation resulting from cholinergic nerve receptors [38] and subsequent reduction of ACh available, as well as altered gene expression and late cognitive effects [39]. At high exposure doses, AChE inhibitors may be toxic or fatal and at lower doses may lead to long-term health effects [40], one of its mechanisms being oxidative stress [37]. The main symptoms secondary to AChE inhibition in people with deficiency in central and peripheral cholinergic function are similar to those reported by Gulf War illness soldiers, such as skeletal muscle fatigue, cognitive deficit, and gastrointestinal, sleep, and temperature regulation problems [1, 41].

Exposures to toxic agents in the Gulf War were considered contributors to numerous long-term health problems. Post-war effects include pesticide effects, uranium munitions, air contaminants from fires in Kuwait oil wells, and chemical nerve agents. PB was then used as a prophylactic measure against possible exposure to these nerve agents, and to other risks, such as psychologically stressful conditions and heat. A military who underwent several exposures in different combinations presented synergic effects that have not yet been determined in this population [4].

Gulf War illness is considered a chronic multi-symptom condition that affected 25–32% of soldiers who operated in the Gulf War. It is clinically characterized by the presence of fatigue; headaches; cognitive, respiratory, and musculoskeletal dysfunction; and gastrointestinal disorders [1–4]. Inflammation and increased oxidative stress associated with mitochondrial dysfunction may negatively affect cognitive function and mood, either directly or indirectly, through the reduction of hippocampal neurogenesis [42–44]. Therefore, chronic inflammation and oxidative stress are likely to be among the leading causes of Gulf War illness brain dysfunction.

Studies were conducted to identify possible causal factors, and evidence has suggested that PB may be associated with etiopathogenesis of Gulf War illness. One of the first studies carried out by [45] described the development of three syndromes associated with PB use: (1) impaired cognition, (2) confusion-ataxia, and (3) neuropathy. However, complementary investigations have also suggested that the use of PB without any other chemical or physical stressor in neuronal cells of animals does not cause great damages, such as decreased viability and increased cellular apoptosis [8, 46]. Thus, it appears that the interaction of PB with other endogenous or exogenous factors is what would trigger the Gulf War illness.

### **2.3 Potential effect of pyridostigmine bromide in oxidative stress**

Considering the results of epidemiological and in animal experiments, the data described so far reinforce the hypothesis that the interaction between PB and other drugs, such as organophosphates, or perhaps other stress factors, could contribute to the rupture of homeostasis neural, via amplification of oxidative stress and of chronic inflammatory conditions that would trigger systemic neural dysfunctions associated with Gulf War illness [42–44, 47–49].

In recent decades the role of reactive species in pathophysiological processes related to oxidative stress has been intensively investigated. The reactive species are molecules that contain one or more unpaired electrons in the last electron layer [50]. These reactive molecules are generally unstable and originate from oxygen, nitrogen, or sulfur [51]. When the generation of reactive species exceeds the anti-oxidant capacity of the organism, an imbalance occurs in the cellular redox state, promoting oxidative stress and subsequent oxidative damage [52].

Mitochondria are the main site of reactive oxygen species (ROS) production [53]. Much of the energy produced in the body is generated through oxidative

phosphorylation. Therefore, paradoxically, a fundamental process for the development of the life of eukaryotes (oxidative phosphorylation) is also one of the main responsible for the production of ROS. These species are also produced by other electron transfer reactions between different redox reactive agents, such as those involved in defense mechanisms against pathogens, for example, the case of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) [54].

The production of ROS in various metabolic processes plays an important role in the functioning of the organism. They are dose dependent, and some types of ROS when in low concentrations are considered important signaling molecules responsible for the transport of electrons in the respiratory chain [54]. ROS have a deleterious effect on the body when there is an excessive increase in its production or when there is a decrease in antioxidant agents. The three main types of ROS are superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $OH^{\bullet}$ ).

Among the ROS, the  $O_2^{\bullet-}$  radical is the most common, abundant, and quite diffusible both inside and between cells *in vivo*, the first ROS being formed by the reduction of oxygen by a single electron during oxidative phosphorylation that occurs in the mitochondria [52]. It is a poorly reactive ROS and has no ability to penetrate lipid membranes, thus acting only in the compartment where it is produced [55].  $H_2O_2$  is not a free radical, but an intermediate metabolite of oxygen, which in uncontrolled amounts becomes extremely deleterious, because it participates as an intermediate in the reaction that produces the  $OH^{\bullet}$  radical.  $H_2O_2$  has long life and is able to cross biological membranes. The  $OH^{\bullet}$  radical is considered the most reactive ROS in biological systems, being capable of causing more damage than any other ROS. It is formed from  $H_2O_2$ , in a reaction catalyzed by transition metal ions ( $Fe^{2+}$  or  $Cu^+$ ), called the Fenton reaction. This  $OH^{\bullet}$  radical can also initiate the oxidation of the polyunsaturated fatty acids of the cell membranes (lipoperoxidation) [55–57].

However, in order to maintain the balance in the ROS generation, there is the antioxidant system, but when the ROS are in excess and the antioxidant system cannot keep the balance, processes of damage to the organism can occur, and this situation of imbalance is denominated oxidative stress [58].

The antioxidant system has the function of inhibiting the oxidative damages caused by excess reactive species. These are divided into enzymatic or endogenous antioxidants and nonenzymatic or exogenous antioxidants, the latter being mainly acquired by the diet [59].

Nonenzymatic antioxidants include ascorbic acid (vitamin C), which inhibits the action of oxidized low-density lipoprotein (LDL) and protects against the action of ROS; phenolic acids; resveratrol; catechins;  $\beta$ -carotene (vitamin A), which protects against lipid peroxidation and damage to DNA;  $\alpha$ -tocopherol (vitamin E); copper (Cu); zinc (Zn); and others. As for enzymatic antioxidants, we have superoxide dismutase (SOD), which facilitates the conversion of the radical  $O_2^{\bullet-}$  into  $H_2O_2$ ; catalase (CAT), which converts  $H_2O_2$  to  $O_2$  and  $H_2O$ ; and glutathione peroxidase (GPx), which has the capacity to reduce  $H_2O_2$  to  $H_2O$  [60].

Oxidative stress is involved in several non-transmissible chronic diseases, such as atherosclerosis, hypertension, neurodegenerative diseases, cancer, and type II diabetes mellitus. In the latter, for example, excess reactive species have a detrimental influence on glucose uptake by muscle and adipose tissues, as well as decreasing insulin secretion, neuronal death, and apoptosis of various cells [61–64].

### 2.3.1 Influence of pyridostigmine bromide on the superoxide-hydrogen peroxide imbalance model

A large body of evidence suggests that oxidative stress is associated with cell aging, dysfunctions, and diseases [65]. However, it was long believed that ROS were



largely responsible for these deleterious processes. For this reason, about 20 years ago, studies were begun to investigate the beneficial health effects of supplementing large amounts of antioxidants. The results, surprisingly, were not good. In some studies, higher morbidity loads were reported in subjects supplemented with high-dose vitamin than in the placebo group. The explanation for this apparent paradox soon emerged: many ROS, in low concentrations, were actually signaling molecules of various cellular functions. Among these, nitric oxide (NO) and H<sub>2</sub>O<sub>2</sub> stand out, so the neutralization of these molecules by antioxidants influenced the cellular homeostasis processes [66].

It was hypothesized that maintenance of redox balance was a relevant aspect to avoid non-transmissible chronic morbidities or to decrease the side effects related to the ingestion of some drugs [67]. This hypothesis was tested and corroborated by genetic studies involving the imbalance of the endogenous antioxidant system. This is the case of the point polymorphism observed in the SOD2 enzyme gene called Val16Ala-SOD2 [68].

The enzyme SOD2 is synthesized from a nuclear gene located on chromosome 6, subregion 6q25, which codes for a homotetramer which binds to a manganese ion per subunit. This protein structure synthesized in the rough endoplasmic reticulum is still enzymatically inactive and has a peptide sequence known as the mitochondrial target sequence (MTS) that directs SOD2 into the mitochondria. As it passes through the pores of the inner mitochondrial membrane, the MTS peptide segment is cleaved by lysosomes, and the mature protein aggregates into an active form, making it a functional SOD2 enzyme [69, 70].

Previous studies have identified a single nucleotide polymorphism (SNP) in the MTS region of the SOD2 gene, in which a thiamine (T) is replaced by a cytosine (C) in exon 2, nucleotide 47. Substitution affects the codon 16, which encodes for amino acid 9, mutating a valine (GTT) in an alanine (GCT), and hence the polymorphism is called Val16Ala-SOD2 [69]. Therefore, this polymorphism is associated with the presence of two alleles alanine (A) and valine (V) and three possible genotypes: AA, AV, and VV. In phenotypic terms, the Ala-SOD2 variant generates a protein with  $\alpha$ -helix structure, thus being easily imported into the mitochondria. The Val-SOD2 variant, on the other hand, generates a protein with a partial  $\beta$ -lamina structure, which causes the inactive SOD2 protein to be partially retained in the pores of the mitochondrial inner membrane, as it is being imported into the mitochondria. In the presence of the two alleles that form the heterozygous genotype, the Ala-/Val-SOD2 protein presents helical structure [70, 71].

In vitro investigations have demonstrated that Ala-SOD2 is capable of generating SOD2 homotetramer with 30–40% greater enzymatic activity than the matrix processed with Val-SOD2 precursor [70]. Despite the increased efficiency of SOD2 produced from the A allele, many epidemiological studies have described association between this genetic variant and various types of cancer [72] including prostate [73], breast [74], and lung [75] cancer.

It is believed that this phenomenon occurs due to the higher efficiency of SOD2 that, if not accompanied by an increase in the levels of GPX and CAT, or of nonenzymatic antioxidant compounds stored in the cell, ends up generating excess H<sub>2</sub>O<sub>2</sub> that can react with transition metals via the Fenton reaction originating the strongly mutagenic OH<sup>•</sup> radical.

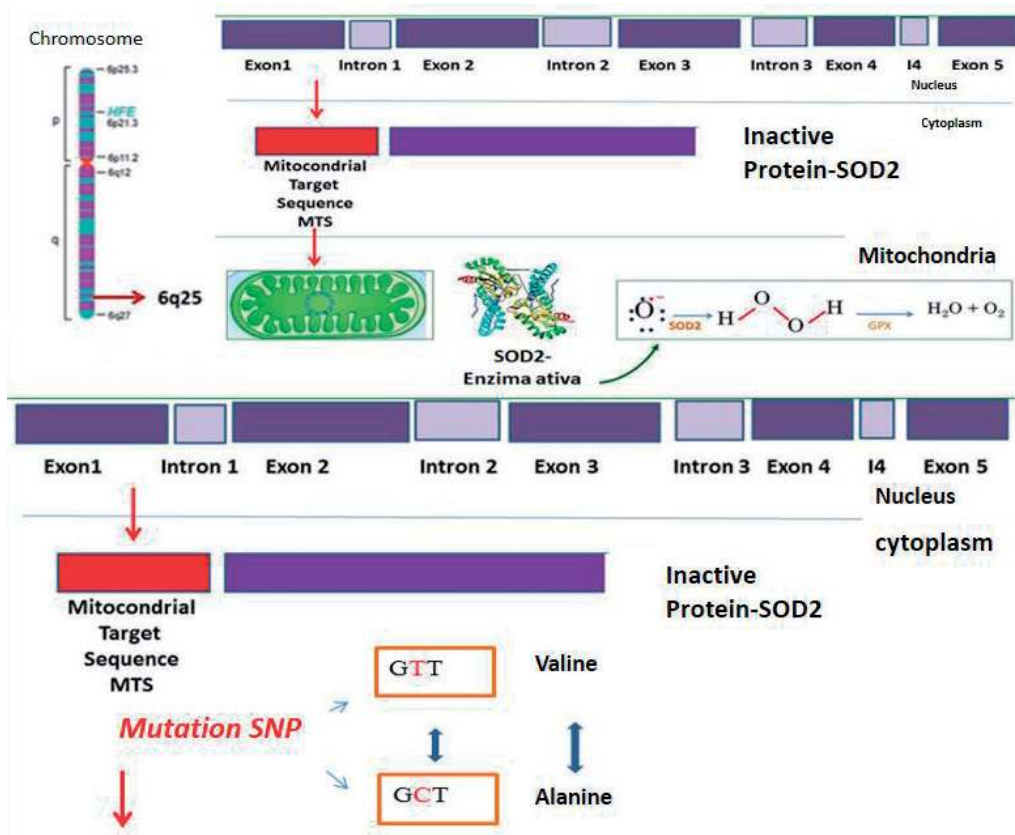
On the other hand, previous investigations related to the Val-SOD2 allele suggest that this allele and/or the VV genotype would increase the risk of some chronic non-transmissible diseases and also differential response to xenobiotic agents [68]. In fact, the VV genotype has a lower enzymatic efficiency of SOD2 and thus potentially leads to the basal accumulation of higher concentrations of the radical anion O<sub>2</sub><sup>•-</sup> within the mitochondria. This ROS is poorly permeable to membranes, and

highly reactive in the presence of NO, which leads to the production of peroxynitrite (ONOO<sup>-</sup>). In turn, this molecule has great affinity with lipids, thus causing an extensive oxidation of cell membranes, a phenomenon known as lipid peroxidation or lipid peroxidation. In addition, the excess of the radical anion O<sub>2</sub><sup>•-</sup> can lead to the production of other ROS that contribute to establish oxidative stress states [76].

Thus, the VV-SOD2 genotype has been associated with endothelial dysfunction, elevated oxidized LDL levels [77], the presence of microvascular complications associated with diabetes including retinopathies and nephropathies [78], elevated levels of inflammatory cytokines [79, 80], increased risk of developing obesity [81], hypercholesterolemia [82], and association between dyslipidemia and stroke [83]. Although AA genotype increases the risk of breast cancer, in certain populations, VV genotype appears to amplify tumor aggressiveness as it increases the potential for breast cancer metastasis [84, 85].

In addition, in vitro investigations have also shown that Val16Ala-SOD2 polymorphism differentially affects the toxicity of lymphocytes exposed to ultraviolet radiation [86], to the methylmercury heavy metal [87], and the pharmacological response of hypercholesterolemic patients to rosuvastatin [88]. This polymorphism also altered the antioxidant response of peripheral blood mononuclear cells (PBMCs) exposed to resveratrol [89] and to seleno-L-methionine [90].

Due to the importance of this genetic polymorphism for human health, an experimental pharmacological model was developed by [91], for prostate cancer,



**Figure 3.** This figure summarizes the Val16Ala-SOD2 polymorphism. This protein has a small peptide region known as the mitochondrial target sequence (MTS) that directs SOD2 protein into the mitochondria. Within this organelle, the enzyme SOD2 finally becomes active. This polymorphism causes a thymine to be exchanged for a cytosine at codon 16. This exchange leads to the substitution of the amino acid valine by the amino acid alanine. Thus, there are three possible genotypes related to this polymorphism: AA, VV, and AV. Source: adapted Barbisan et al. [93].

and by [92], for colorectal cancer, showing in tumor cells the difference in treatment by superoxide-hydrogen peroxide (S-HP) imbalance.

In this S-HP pharmacological imbalance model, two molecules, paraquat and porphyrin, were used. Paraquat is an  $O_2^{\bullet-}$  anion generator, whose higher levels of this molecule are observed in VV-like cells. On the other hand, porphyrin is a molecule that acts similar to SOD2 (SOD2-like), thus causing an increase in  $H_2O_2$  levels, as observed in cells with the genotype AA-like [91, 92], simulating in vitro the two genotypes of the polymorphism. A schematic summary of this genetic polymorphism is illustrated in **Figure 3**.

In summary, considering that PB seems to interact with other exogenous pro-oxidant agents, triggering symptoms recognized in Gulf War illness, the hypothesis of interaction between this drug and the Val16Ala-SOD2 polymorphism cannot be ruled out.

### 3. Discussion

Pyridostigmine bromide was used to treat changes in neuromuscular junction [21, 20] and was also used prophylactically in GWI for stress prevention and against chemical and physical agents, which soldiers were exposed to during the war [49]. However, this drug was associated with several adverse effects detected during and after the war in soldiers who prophylactically ingested 30 mg/3 times a day of this drug. Among these effects, the main ones were skeletal muscle fatigue, headache, attention deficit, cognition problems, gastrointestinal disorders, and sleep and temperature regulation problems, among other autonomic alterations [1, 2, 4].

These results suggested that BP could have a relevant cytotoxic effect on humans. However, previous animal cell studies have suggested that isolated exposure to CP would not cause extensive damage including decreased cell viability and cellular apoptosis [8, 46]. However, other studies have shown that when animals were treated with CP associated with other chemical molecules that were potentially used in biological warfare or in the prophylaxis of other diseases or even parasitic diseases, such as permethrin, used to prevent infestation by head lice, results were quite different. These results then indicated the interaction of BP with chemical agents or even physical factors such as intense physical activity and psychological stress such as oxidative stress and inflammation [6].

Considering that most of the agents in which BP interacts are factors that increase oxidative stress and body inflammation, an open question concerns the potential occurrence of interaction between BP and oxidative imbalances associated with individuals' genetic characteristics, as is the case of the Val16Ala-SOD2 polymorphism. This question is quite pertinent since, so far, doubts remain about the efficacy and safety of the use of BP as a drug and also about the fact that it was pointed as the cause of GWI disease.

So two studies were very important that showed that pyridostigmine bromide affected in vitro cytogenotoxicity and AChE enzyme activity of SHSY-5Y neural cells, in a concentration-dependent manner, showing decreased cell viability, increased oxidative stress, and apoptosis mainly when they were exposed to the highest concentration tested at 80 ng/mL. However, over a longer period of exposure, there was an increase in cell proliferation rate, suggesting that the oxidative effects triggered by CP exposure may be transient and reversible in these neural cells [94]. However, when exposed to different Val16Ala-SOD2 genotypes, the cytogenotoxicity and efficacy in inhibiting AChE induced by CP exposure were directly modulated by the Val16Ala-SOD2 polymorphism that alters the basal oxidative state of human peripheral blood polymorphonuclear cells. In this case, cells

with higher basal production of H<sub>2</sub>O<sub>2</sub> had higher cytotoxic sensitivity to CP, while cells with higher basal production of O<sup>2-</sup> anion showed higher resistance to inhibition of AChE enzyme. These results suggest a potential pharmacogenetic effect of S-HP imbalance on BP efficacy and safety [95].

These results found in the literature suggest that the efficacy and toxicity to CP are influenced by the interaction with oxidative imbalance by Val16Ala-SOD2 polymorphism, indicating potential toxicogenetic and pharmacogenetic effects of this drug. The data presented here may potentially contribute to elucidate the interaction between BP and oxidative stress-inducing agents and may also be relevant to the clinical and epidemiological field related to the use of AChE inhibitors as therapeutic agents [94, 95].

#### **4. Conclusion**

Studies indicate that the interaction between pyridostigmine bromide and stressors could trigger genotoxicity, the mechanism associated with the induction of oxidative stress that leads to this side effect of this drug; however, this discussion needs to be better elucidated and may be more discussed as there is interaction between the pyridostigmine bromide and an endogenous oxidative imbalance caused by it or even by the possible interaction of this with genetic variations present in the antioxidant metabolism.

This chapter was developed to show studies related to the toxicity of pyridostigmine bromide and its influence with oxidative stress, as we conclude that:

Results suggested that the exposure of neural cells to PB without other chemical and physical stressors does not cause extensive toxicity that could explain the clinical symptoms observed in GWI.

Study demonstrated that PB can transiently modulate redox metabolism in cells. However, factors that increase HP levels, such as the AA-SOD2 genotype, may affect PB efficiency and efficacy by inducing AChE inhibition and oxidative stress. Data from these in vitro studies may be useful for complementing population studies investigating PB or other AChE inhibitors.

Our results suggest that the efficacy and toxicity to CP are influenced by the toxicogenetic and pharmacogenetic interactions of this drug. The results presented here may potentially contribute to elucidate the interaction between CP and oxidative stress-inducing agents and may also be relevant to the clinical and epidemiological field related to the use of AChE inhibitors as therapeutic agents.

#### **Conflict of interest**

The authors declare no conflict of interest.

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