Review

Impact of melatonin effects on toxicology of vesicant chemical warfare agents: When science meets reality

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Running title: Melatonin treatment for vesicants-induced damage

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ABSTRACT

In this review, we focused our attention on sulfur mustard [bis(2-chloroethyl) sulphide], the principal vesicant chemical warfare agent (CWA). It has been widely used in different military conflicts, including World War I and the Iran-Iraq war. Indeed, the evolution of the recent Iraq and Syria conflicts suggests that terrorist groups are aware of the notable psychological and media effects that the mere attempt to use CWAs would be generated. Sulfur mustard can alkylate macromolecules bearing sulfhydryl groups, such as DNA or proteins. These vesicants can also produce free radicals able to develop toxicity in the exposed areas, such as the eyes, skin, respiratory tract (inhalation) and gastrointestinal tract (ingestion). Melatonin is a broad-spectrum multipotent molecule with a high free radical scavenging potential. In this respect, we propose three salvage mechanisms for melatonin, that may facilitate the neutralization of induced toxicity by sulfur mustard. We also speculate that the long-term effects of varying severity can appear after acute poisoning. Besides, melatonin-based therapy strategies can modulate epigenetic mechanisms and become highly suitable to victimized patients from a clinical point of view. However, the utilization of melatonin as a "therapeutic bullet" addressed to counteract the vesicant CWAs needs much more additional *in vitro* and systematic animal studies as well as controlled translational clinical trials.

Key words: melatonin, vesicants, oxidative stress, inflammation, epigenetics, DNA damage.

1. INTRODUCTION

The North Atlantic Treaty Organization (NATO) defines chemical agents, commonly known as chemical warfare agents (CWAs), like chemical substances intended for military operations to kill, seriously injure, or incapacitate personnel through their physiological effects (1). The first CWAs were chemicals widely used in the chemical industry, such as chlorine and phosgene, two lung-damaging agents. Afterward, other CWAs with no industrial use were developed. This is the case of sulfur mustard, a blister agent used in World War I, or nerve agents, discovered just before the start and developed during World War II (2).

Vesicant CWAs include sulfur mustards (H, HS or HD), nitrogen mustards (HN), lewisites (L), and phosgene oxime (CX) (Figure 1). The most relevant blister agent is sulfur mustard, commonly known as "mustard gas". Although it is referred to as a "gas," sulfur and nitrogen mustards and lewisites are liquids at room temperature. CX, by contrast, is a highly volatile crystalline solid. The main problem of lewisites as weapons is that they quickly hydrolyze and hence would be deactivated at high humidity conditions (3). Sulfur mustard is a persistent agent that remains in the attacked area and affects not only the respiratory tract but the whole-body surface. The blisters that appeared in affected troops in World War I were the reason why sulfur mustard and similar agents are called blister or vesicant agents. On the other hand, lewisites and nitrogen mustards (Figure. 1) did not provide any particular advantages to sulfur mustard (4). Thus, the available information regarding the clinical effects of vesicants pertains mostly to sulfur mustard and is mainly derived from World War I and the Iran-Iraq war experience (5, 6).

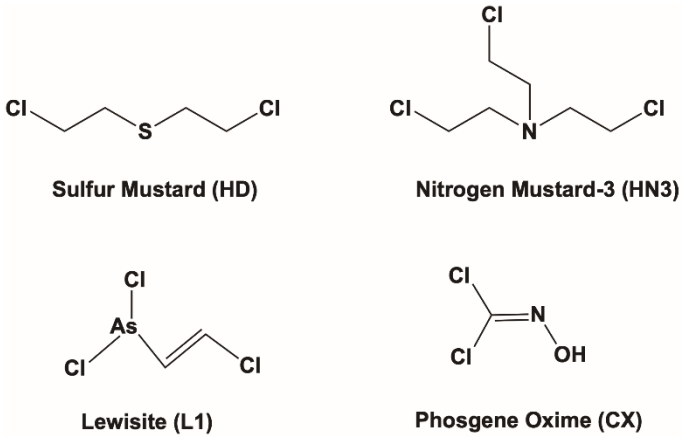


Fig. 1. Chemical structure of vesicant agents.

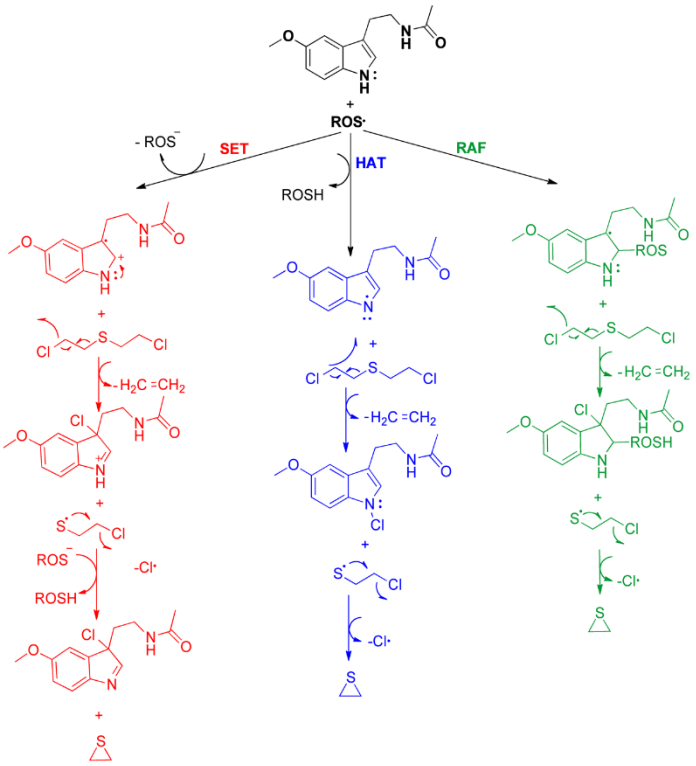
CWA´s lipophilic character allows that more than 80% quickly access to the organism through the skin surface and exposed moist mucosa (ocular and respiratory), reaching the blood and opening the door to the involvement of any other tissue. CWAs are extremely reactive vesicants, exposure to these agents causes acute (time/dose-dependent) macro- and micro-blistering in skin and eyes (7). Besides, they also involve burning lesions of the tracheobronchial tract (8, 9), which are the principal pathological burden post-exposure, usually leading to a fatal outcome in the short term (10). Sulfur mustard is absorbed through the skin and eyes, reaches the lungs by inhalation and the gastrointestinal tract after ingestion (11, 12), at high temperatures, this process is enhanced. The systemic effects occur mainly in bone marrow, gastrointestinal tract and central nervous system (CNS). Notwithstanding, long-term symptomatology of varying severity may appear after acute poisoning and persist decades after (13). When death occurs in the first 24 h is usually due to an acute respiratory failure resulting from obstruction of the bronchial tree by pseudomembranes and laryngospasm (14).

Melatonin is an excellent and broad-spectrum antioxidant agent, widely distributed in the body at appropriate concentrations (15), easily transported across morphophysiological barriers (16) and endowed with very low toxicity (17). The many biochemical pathways in which the molecule acts as a regulator brings the indolamine to the position of a pleiotropic modulator involved in cell protection and survival (18) and endowed of capacities against the damage caused by blister agents (19).

Unfortunately, total immediate decontamination after vesicant CWAs exposure is difficult to achieve nowadays, and there are no completely effective antidotes nor effective treatments. In this review, we try to highlight that the physiological importance of melatonin exceeds the circadian control traditionally attributed to this pineal hormone. Its biological potential may include interesting medical countermeasures against CWAs-induced injury, providing promising therapeutic strategies.

2. MECHANISMS OF THE REACTION OF MELATONIN WITH SULFUR MUSTARD AND SIMILAR CHEMICAL WARFARE AGENTS

The recognized free radical scavenging capacity of melatonin facilitates the neutralization of sulfur mustard induced toxic damage. Three mechanisms could be proposed to achieve this action (Scheme 1).



Scheme 1. Three alternative free radical-mediated mechanisms for quenching sulfur mustard toxic agent exerted by melatonin.

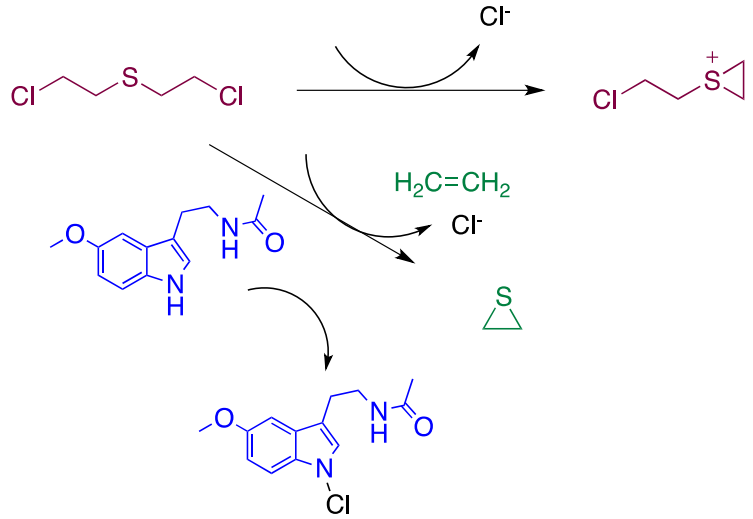
Previous computational calculations point out hydrogen-atom transfer (HAT) as the suitable mechanism to quench sulfur mustard, furnishing the much less toxic thiirane (19). In this regard, melatonin would reduce reactive oxygen species (ROS) mostly by transferring the hydrogen at N1. Afterwards, radicalic melatonin attacks sulfur mustard by radical nucleophilic substitution, and the reactive intermediate collapses by releasing ethylene and subsequently a chlorine atom thus forming thiirane (Scheme 1). Otherwise, single electron transfer (SET) and radical adduct

formation (RAF) (20) are alternative mechanisms that produce the same by-product when neutralizing sulfur mustard. In these last mechanisms, pyrrole double bond quenches ROS either forming an adduct (RAF mechanism) or transferring it an electron (SET mechanism). In both cases, the reactive melatonin derivative intermediates neutralize sulfur, similarly to HAT. Indeed, the analysis of the final melatonin derivatives formed could clarify which of these mechanisms is more plausible.

3. MELATONIN HYPOTHESIS TO COUNTERACT DNA DAMAGE INDUCED BY VESICANTS

Regarding sulfur and nitrogen mustard-induced injury, several toxicological mechanisms of action have been proposed (21-23), still, alkylation of macromolecules seems to be the main one. Sulfur and nitrogen mustards undergo intramolecular cyclization reactions giving rise to sulfonium or immonium ions, respectively (24). These intramolecular reactions are favored by the presence of water and high temperatures (25). Thus, moistly body areas are the most susceptible. Sulfonium and immonium cations are highly electrophilic and able to alkylate nucleophilic molecules such as the enzymes that contain sulfhydryl groups, which are responsible for regulating Ca^{+2} homeostasis in the cell (26). Such processes would increase the intracellular Ca^{+2} concentration, disrupting the microfilaments responsible for cell integrity, with the subsequent activation of endonucleases, proteases, and phospholipases, that finally induce apoptosis (25). Moreover, mustards interact with glutathione (GSH) and increase the concentration of free radicals which, through peroxidation of membrane lipids, affects the integrity and function of the membrane (25, 27).

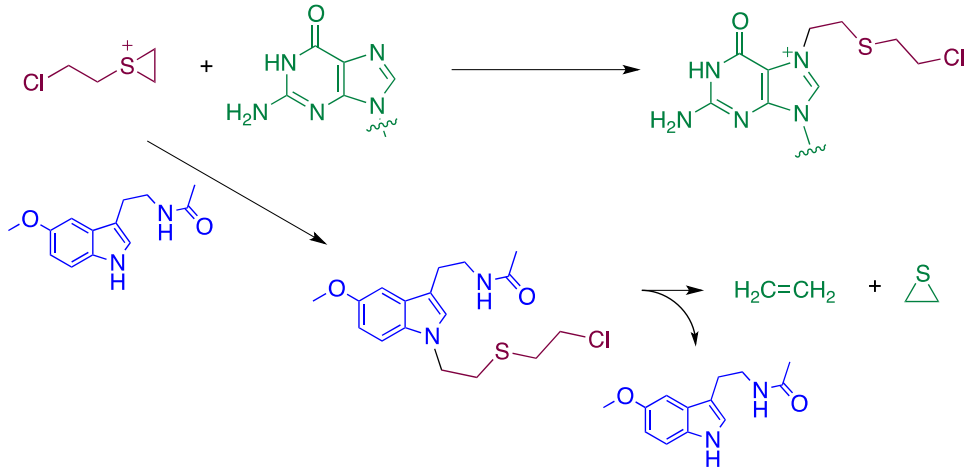
DNA alkylation produces cross-linking and breakage of strands, and polymerases such as poly (adenosine-ribose diphosphate) polymerase (PARP) are activated leading to depletion of the substrate nicotinamide adenine dinucleotide (NAD+), inhibition of adenosine triphosphate (ATP) synthesis and finally apoptosis (28-30). Therefore, fastest-dividing cells are the most affected targets of mustards (25). On the other hand, sulfur mustard appears to have a specific affinity for nitrogen at position seven of guanine (30, 31). As anticipated above, melatonin or its reactive species transform sulfur mustard in the much less toxic thiirane (Scheme 2).



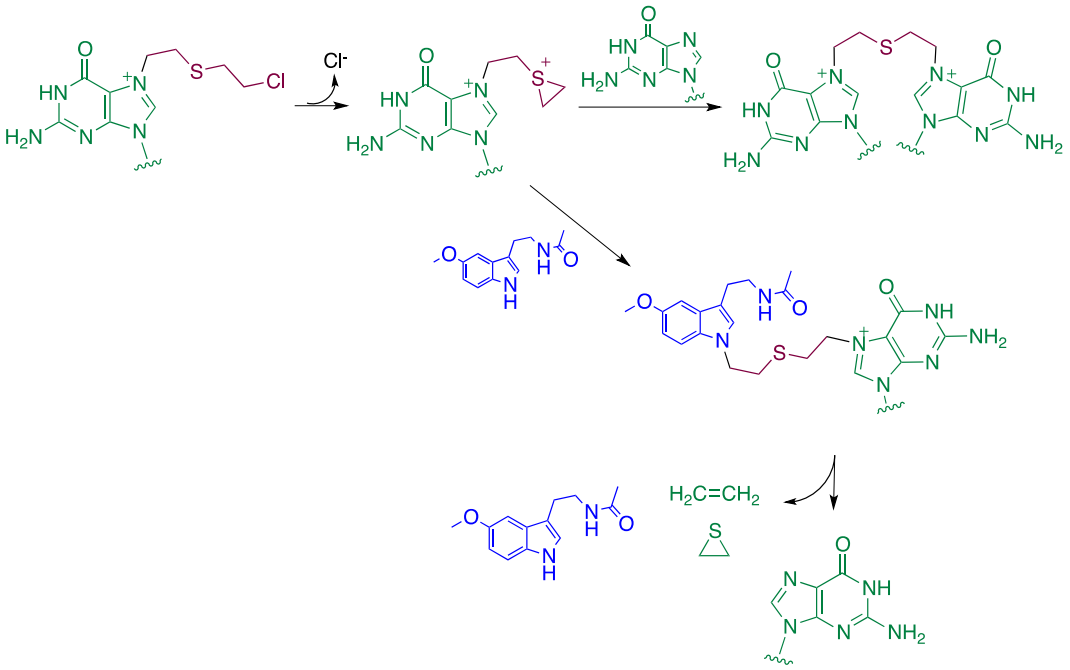
Scheme 2. First cyclization reaction that forms the highly electrophilic sulfonium cation.

Melatonin prevents this generation by alternatively forming the less toxic thiirane (a HAT-type mechanism is represented as an example).

Scheme 2 shows how melatonin could dissipate the generation of the highly electrophilic sulfonium cation. Otherwise, this reactive species is thought to form an adduct with guanidine at N3 (Scheme 3). In this scenario, melatonin could sequester the sulfonium cation, turning it in thiirane gas, thus preventing the formation of the guanidine-sulfur mustard adduct (Scheme 4).



Scheme 3. Formation of reactive sulfur mustard and interaction with guanine in DNA.
Melatonin would be able to trap the sulfonium cation, avoiding such adduct formation.



Scheme 4. Second cyclization and alkylation of complementary DNA base resulting in a DNA crosslink.

It is worthwhile mentioning that the toxicity mechanism of the sulfur mustard-guanidine adduct comprises DNA-cross-linking with another guanidine base (Scheme 3), through the formation of an intermediate adduct that presents the corresponding cyclic sulfonium cation structure. In this situation, melatonin likely counteracts cross-linking by reacting again with the sulfonium cation, herein bond to a guanidine. If that happens, melatonin comprises the potential to catch the sulfur mustard bridge, thus removing it from the guanidine.

4. IMMUNE-INFLAMMATION AND OXIDATIVE STRESS INDUCED BY VESICANT CHEMICAL WARFARE AGENTS: ANTAGONIST MECHANISMS REGULATED BY MELATONIN

The world has witnessed the indiscriminate use of chemical weapons for more than a century, from the First World War to the recent Syrian Civil War (32). Unfortunately, despite an intensive quest for strategies against their acute toxicity, the pharmacopeia has no effective choices for preventing the appearance of chronic disabilities. The fact that the Chemical Weapons Convention has not yet achieved universality and the suspicion that some nations have CWA capabilities (33), urges to find novel treatments for post-exposure disorders. In response to this challenge, ROS scavenging, oxidative/nitrosative stress mitigation, anti-inflammatory capability, preservation of membrane and intracellular macromolecules, and abrogation of mitochondrial anoikis equip melatonin for counteracting the cytotoxicity of alkylating agents leading to a promising therapy option (34-38). Accordingly, melatonin has demonstrated biochemical and morphological protection against the respiratory tract, the bladder and the kidney damage induced by mustard vesicant agents (36, 39-43). Likewise, studies in rodent models have reported that melatonin ameliorates several detrimental actions of lewisites on account of its metal-chelating capacity (44) and perhaps based on its well-known antioxidant and anti-inflammatory properties (45, 46).

The balance between ROS and antioxidant defense mechanisms is disturbed after sulfur mustard exposure leading to oxidative stress. Redox imbalance and severe immune reactions are significant events involved in the pathogenesis of sulfur mustard toxicity. Although sulfur mustard does not generate a chemotaxis-inducer effect, during the initial unspecific immune mast cell post-exposure response, a specific secretion of metalloproteinase 9 (MMP-9), prostaglandin, cyclooxygenase (COX) and subsequent leukocyte infiltration occur, which contributes to vesication (47) (Figure 2). In this context, the capacity of melatonin to modulate the inflammation-immune axis (48, 49) should mitigate the pathological features induced by sulfur mustard through the modulation of the inflammation-immune axis. Some studies have suggested that the enhancement of oxidative species production or weakening of antioxidant mechanisms are crucial in the acute toxicity of sulfur mustard and long-term effects following acute poisoning (22, 38, 50-53). The powerful nitrosating agent peroxynitrite ($ONOO^{\bullet}$) is involved in detrimental effects of all mustards as it covalently modifies all the principal types of biomolecules including membrane lipids, thiols, proteins and purine and pyrimidine bases (54-56). $ONOO^{\bullet}$ also activates metalloproteinases (MMPs) promoting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and proinflammatory responses (57). Hence, higher $ONOO^{\bullet}$ concentrations induce necrosis rather than apoptosis through the activation of the DNA repair enzyme poly (ADP ribose) polymerase-1 (PARP-1) (57). In addition to DNA repair, the activities of the enzymes are involved in the maintenance of genomic stability, the regulation of gene expression and DNA replication. Experimental evidence has shown that cell death induced through the PARP-1 pathway plays a pivotal role in tissue damage and organ dysfunction during acute mustard-induced toxicity (58, 59). The increase in $ONOO^{\bullet}$ will induce acid sphingomyelinase and therefore ceramide (60). Increased ceramide is relevant to the apoptosis associated with sulfur mustard (61). Prior to apoptosis, ceramide induces mitochondrial dysfunction, including in immune cells, with immune dysfunction a significant contributor to the pathophysiological changes driven by sulfur mustard. It is also of note that ceramide suppresses the 14-3-3 protein levels, and therefore may act to decrease the levels of local melatonin production, which is dependent upon 14-3-3 stabilization of arylalkylamine *N*-acetyltransferase. As such, the effects of sulfur mustard may be intimately linked to a decrease in local melatonin production, with consequences for mitochondrial function across a wide range of cell types (62).

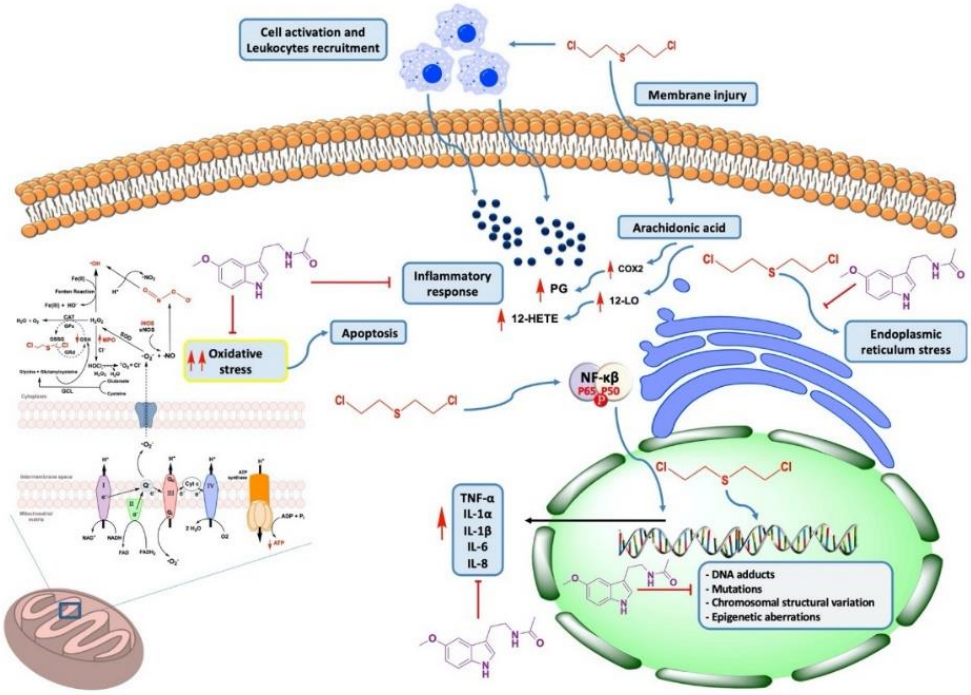


Fig. 2. Cellular and molecular mechanisms of sulfur mustard in the presence of melatonin.

Sulfur mustard triggers mast cell degranulation, which contributes to inflammatory response and edema formation. This generates the up-regulation of proinflammatory transcription factor NF-κB and further its eventual translocation to the nucleus and binding to DNA, thereby increasing the expression and secretion of different proinflammatory cytokines. Sulfur mustard increases arachidonic acid secretion from cell membrane which induces overexpression of COX-2 and 12-LO enzymes to produce bioactive lipid mediators with inflammatory properties (PG, 12-HETE). Melatonin exerts effective protection modulating multiple points in the complex inflammatory cascade, furthermore it scavenges ROS and upregulates the expression of antioxidant enzymes. After sulfur mustard exposure, mitochondrial $O_2^{\bullet-}$ levels can reach the cytosol from the intermembrane space through voltage-dependent mitochondrial anion channels. The resulting high levels of $O_2^{\bullet-}$ coupled with the activation of NO-producing iNOS and increase the likelihood of $ONOO^{\bullet}$ formation. Consequently, melatonin inhibits mitochondrial dysfunction preventing the activation of apoptosis. Sulfur mustard as a nucleophilic and alkylating agent causes a wide variety of adverse effects on DNA. At this point, melatonin may revert these effects acting as an epigenetic modulator. Due to sulfur mustard-dependent protein alkylation, an accumulation of improperly folded proteins leads to endoplasmic reticulum stress. Melatonin restores the endoplasmic reticulum homeostasis regulating the linking between endoplasmic reticulum stress and inflammatory response. Stimulation (blue colored) or inhibition (red colored) by melatonin and sulfur mustards are also shown. CAT = catalase; COX-2 = cyclooxygenase-2; GCL = glutamylcysteine ligase; GPx = glutathione peroxidase; GRd = glutathione reductase; 12-HETE = 12-hydroxyeicosatetra-enoic acid; H_2O_2 = hydrogen peroxide; $HO^{\bullet}$ = hydroxyl radical; iNOS = inducible nitric oxide synthase; 12-LO = 12-lipoxygenase; MPO = myeloperoxidase; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; $O_2^{\bullet-}$= superoxide anion; $ONOO^{\bullet}$ = peroxynitrite; PG = prostaglandin; SOD = superoxide dismutase.

The efficacy of histone deacetylase (HDAC) inhibitors in attenuating sulfur mustard-induced symptoms, would indicate a role for a decrease in gut microbiome-derived butyrate in

the emerging symptomatology of sulfur mustard victims. Butyrate is a HDAC inhibitor that can induce an increase in the activation of the melatonergic pathway (63). This provides another route for a decrease in the positive regulation of melatonin in the pathophysiology of sulfur mustard.

Antioxidant therapy has been postulated as a promising approach to efficiently control mustard-induced acute toxicity (64). GSH is involved in the defense against metal cations, oxyradicals, and xenobiotics, among others. Thus, the depletion of GSH has been defined as a principal mechanism of sulfur mustard pathology (65) (Fig. 2). In this regard, GSH depletion and markers of pulmonary inflammation have been found in Iran-Iraq War victims after sulfur mustard exposure in the 1980s (66, 67). Moreover, experiments in animal models had shown that several antioxidants protect the liver and lung from oxidative mustard-induced damage (68). Consequently, the excellent capacity of melatonin as the main scavenger of both oxygen and nitrogen-based radicals, including $ONOO^{\bullet}$ (69-71) has been extensively proven. Indeed, both melatonin and its metabolites have relevant advantages over "classical antioxidants", as the iNOS inhibition and $ONOO^{\bullet}$ scavenging exhibited against mustard induced acute toxicity (39) (Fig. 2). $ONOO^{\bullet}$ is thought to be a primary event triggering the inflammatory cascade and tissue injury (54). Since chemical poisoning inflammation has been related to many different diseases, it is necessary to identify new pharmacological approaches to reduce the inflammatory response after nitrogen and sulfur mustard intoxication. Although inflammatory cells contribute to the protection and repair processes, the proinflammatory mediators released may exert harmful effects. In this regard, anti-inflammatory melatonin regulates different molecular pathways (72).

Altogether, it has been reported that melatonin reduces oxidative stress, inflammation and kidney toxicity induced by the bifunctional alkylating agent mechlorethamine [bis(2-chloroethyl)methylamine] (42). Likewise, sulfur mustard leads to the production of different cytokines such as IL-1α, IL-1β, IL-6, IL-8, and TNFα, among others, and as a consequence enhances intracellular oxidative stress, antioxidant defense mechanisms (73) (Fig. 2) and markers of oxidative stress, including malondialdehyde, 8-hydroxydeoxyguanosine, 4-hydroxynonenal and heme oxygenase-1 (74). In this respect, melatonin administration *in vivo* counteracted oxidative stress and oxidative stress markers after sulfur mustard exposure (36) and attenuated lung injury and oxidative stress induced by nitrogen mustard in rodents (39). The accumulation of misfolded and aggregated proteins results in cellular stress. Sulfur mustard generates the accumulation of improperly folded proteins that trigger the activation of compensatory mechanisms focused on the restoration of endoplasmic reticulum (ER) homeostasis (75). Regarding this observation, melatonin significantly reduces aberrant accumulation and aggregation of disease-specific proteins (76) (Fig. 2). Thereby, it would be necessary to explore the capacity of melatonin to regulate the linking between ER stress pathways and sulfur mustard exposure. In an interesting review article, Korkmaz et al. (77) proposed that delayed sulfur mustard toxicity may be due to epigenetic disruption. It can be hypothesized then that melatonin counteracts delayed sulfur mustard toxicity by epigenetically modulating the mRNA expression of the antioxidant enzymes, both in physiological conditions and in increased oxidative stress conditions (78).

5. EPIGENETICS IN THE MANAGEMENT OF LONG-TERM TOXICITY OF VESICANTS: A GREAT NEW DEAL FOR MELATONIN THAT AWAITS FURTHER RESEARCH

The precise molecular details of the cellular pathophysiology triggered by acute sulfur mustard intoxication or by repeated low-dose exposure still wait to be examined (79). Nevertheless, as for other toxics chemical modification of DNA and other macromolecules,

inflammation and apoptosis induction, ROS imbalance, depletion of antioxidant defenses and the subsequent emergence of oxidative/nitrosative stress are the most plausible cytotoxic mechanisms postulated (38, 80). Conversely, the incapacitating complications and progressive health deterioration after sulfur mustard/nitrogen mustard injury currently lack delineated mechanistic support (81, 82). However, inflammatory response and characteristic late-onset disablements include dermal and ocular lesions and a severe lung disease denominated "mustard lung" (10, 83, 84) debut accompanied by an increased incidence of a plethora of other detrimental features (85). At this point, the possibility that part of this time-delayed morbimortality comes from changes in the cellular epigenome is attracting growing attention over the past years (35, 77, 86). The epigenotoxicity might affect germline cells and thus could lead to the transmissibility of physical anomalies and reproductive pathology (82, 87, 88). There are still few objective examples of mustard vesicant agents impacting the epigenetic profile, although alterations from standard healthy epigenetic configuration is an established etiopathogenic framework for a broad collection of disorders (89-92). However, some observational clues are making the panorama evolve towards the incrimination of epigenetics in the origin of systemic disabilities after the initial challenge by vesicant agents. Pathologies from the sulfur mustard spectrum such as the inflammatory response and the cardiovascular, respiratory and neoplastic disease have been associated with epigenetics (35, 93-95). Thus, the inflammatory cascade gathered in normal human epidermal keratinocytes (NHEK) by sulfur mustard at 200 μM induced the expression of p38 mitogen-activated protein kinase (MAPK) and soon after the p38-regulated MAPK activated kinase 2 (MK2) phosphorylation previous to release the inflammatory modulators TNFα or IL-6 and IL-8 (96). Additionally, the suppression of antioxidant and anti-inflammatory genes that characterizes the "mustard lung" has been assigned to the upregulation of histone deacetylases (86). In this context, the proof-of-concept in lung tissue from sulfur mustard-victimized individuals of more than 50 oxidative stress and antioxidant-related genes with their expression altered (97) has reinforced the assumption of epigenetic involvement in the respiratory pathogenicity and shown the oxidative origin of traditional pulmonary sequelae afflicting those patients. For this reason, the search for an effective intervention strategy against sulfur mustard/nitrogen mustard side-effects has focused on several known antioxidants (41, 64, 80), with melatonin in a prominent place as discussed in previous Sections.

The best characterized epigenetic mechanism is the methylation of cytosines into the ~30,000 CpG dinucleotide rich sequences (CpG islands) present in the human genome at gene promoters (98). Usually, these sequential motifs remain unmethylated in the transcriptionally active genes while their extensive methylation results in the transcriptional silencing of the corresponding coding genes. Particularly sulfur mustard-sensitive murine early endothelial cells (EEC) elicited global dose-dependent DNA hypermethylation on a 78-gene panel of epigenetic modulators by acute micromolar sulfur mustard, thus leading to the downregulation of most of them (99). Noteworthy, one of the few up-regulated genes was the DNA-methyltransferase 1 gene, which has been related to *de novo* DNA methylation and therefore reinforced the functional upregulated methylotype caused by sulfur mustard. Interestingly, a human patient that exposed himself unintentionally to "a few drops" of pure sulfur mustard was included in the study. He presented significant cytosine hypermethylation on skin affected areas compared to non-exposed areas like the observed in EEC model (99), showing the biological relevance of *in vitro* findings and the relative stability over time of environmental epigenetic impacts. Further research on this same experimental system showed minor impacts on histone acetylation (H3-K9, H3-K27, H4-K8) and di-methylation (H3-K9, H3-K27, H3-K36) (100), even though the transcriptional output of histone modifications is currently hard to predict and remains a matter of study (100-102). Regarding histone modification, this second epigenetic mechanism comprises a series of post-translational changes including acetylation

and methylation, but also phosphorylation and other enzyme-dependent chemical derivatives, which result critical for nucleosome stability and the structuration of the open (active) and closed (inactive) transcriptional configurations of chromatin (103). There is evidence that gene silencing by DNA methylation is relatively stable over time while histone modifications are more dynamically reactive in response to inputs from nutritional or environmental status (104). DNA methylation and histone modification, both pivotal mechanisms in the control of genome functionality, are actively involved in mustard-delayed lung pathology, as the above-mentioned *in vitro* studies have reported and preliminary *in vivo* evidence begin to highlight. Indeed, the severe pulmonary disease induced in rats by acute injection with nitrogen mustard-prototype mechlorethamine (MEC) was significantly avoided by coadministration of HDAC inhibitor Trichostatin A and contrarily exacerbated by MEC combined with decitabine, a DNA methyltransferase inhibitor (86).

The third epigenetic mechanism called to play a significant role in the etiopathogenesis of sulfur mustard is the ~2300 human (105) ~23-nucleotide non-coding RNAs, the so-called miRNAs. Binding target mRNAs, these short antisense miRNAs regulate post-transcriptional gene expression, in general by translational repression through the degradation of miRNA-mRNA duplexes (106). The up-regulation of miRNA-203 and miRNA-210 has been implicated in functional impairments of NHEK cells exposed to sulfur mustard (107), revealing two potential targets of future-directed countermeasures against the epithelialization defects caused by blistering agents. Similarly, sub-lethal micromolar sulfur mustard doses perturbed the expression of 10% of more than 600 miRNAs assayed in EEC and showed that some of them (e.g. miRNA-92a) could mediate the chronic failure of wound healing and endothelial regeneration in sulfur mustard-injured victims (108). Analogously, it has been reported in the sulfur mustard-resistant HaCaT human keratinocyte line (109) that changes of miRNA profile are pivotal for sulfur mustard-resistance (110) and that some of them are also critical for resistance to alkylating agent cisplatin (111). Another study addressing miRNA-9 and miRNA-143 in the urine of 32 sulfur mustard-injured patients and 32 healthy individuals reported their significant reduced excretion correlated with the ongoing post-exposure pathogenicity (112). These two miRNAs have been assigned to the regulation of central signalling pathways (MAPK, TGF-β, Wnt, etc) and their down-regulation specifically associated with the reduction of the inflammation suppressing factor TGF-β, thus conferring mechanistic support to the inflammatory response of mustard-induced pathology.

The multifunctionality of melatonin exceeds the capacities above mentioned and such as initially hypothesized Korkmaz and Reiter (113) and further evidenced the up-regulation of histone H3 acetylation and histone deacetylase isoforms HDAC3, HDAC5 and HDAC7 (114), the molecule performs gene control through epigenetic on/off mechanisms (115-118). What's more, the melatonergic system has a biunivocal relationship with the epigenetic control of biological processes. On the one hand, expression of the high-affinity melatonin receptors can be induced by epigenetic reprogramming, as found in rat C6 glioma cells in which the epigenetic modulator valproate induced MT1 expression through the histone H3 hyperacetylation along with the MT1 gene promoter (119). On the other hand, melatonin signaling participates in multiple pathways under epigenetic control, such as revealed that administration to pregnant rats elicited an epigenetic program in the kidney of litters that activated more than 400 genes to successfully prevent neonatal and protect from adult-programmed hypertension (120). Moreover, melatonin seems to produce epigenetic modifications in oocytes through its nuclear receptors and thereby carry out adaptive changes in offspring by epigenetic inheritance (121). Thus, DNA methylation, histone modifications, and subsequent chromatin remodeling, as well as the expression of regulatory non-coding RNAs take part in the effective downstream mechanisms of melatonin (117). Indeed, this indoleamine has access to the epigenetic edition and gene expression reprogramming and

thereby to the achievement of highly dynamic adaptations that avoid the emergence of long-term disease. In this regard, the epigenetic involvement increasingly endorsed in the cell, animal and human melatonin studies confer absolute plausibility that adequate administration regimens may prevent the insidious pathology that vesicants establish throughout the years and which is partially suspected of epigenetic origin. Thus, patients with a documented encounter with sulfur mustard manifest chronic respiratory disabilities that determine the common sleep disorders afflicting to mustard-injured victims. Some recent evidence highlighted the benefits of melatonin for these patients; in a cohort of 30 sulfur mustard-exposed males from the Iran-Iraq War displaying sleepless along with mild to moderate respiratory difficulties and nocturnal melatonin declines in serum (122), melatonin supplementation was effective improving sleep quality, particularly sleep latency and duration (123). This clinical benefit of melatonin may come from changes in the epigenome as recently reported for sleep disturbances in job exhaustion, which have been found related to a variant of melatonin receptor MT1 and loss of DNA methylation at promoter sites (124, 125).

Epigenetic modulators such as melatonin allow dynamic optimization of cell homeostasis and fitness. In this regard, melatonin involvement in the remediation of epigenetic disturbances produced by blistering agents has been barely investigated and therefore its therapeutic applicability is currently hypothetical (94, 126). In agreement with this scarce research, literature raising the suitability of melatonin is partially based on indirect evidence, as occurs with protection of DNA and macromolecules, prevention of oxidative stress or cell and tissue homeostasis restoration that are core capacities of the indoleamine and simultaneously have leading roles in the acute mustard pathology (35, 77). Given that some clinical phenotypes classically ameliorated with melatonin, such as chronic obstructive pulmonary disease (COPD), share pathogenic mechanisms with mustard lung (84), some authors have inferred the potential benefits of melatonin administration for respiratory and other late-onset toxicity of vesicant agents (77). A similar theoretical inference has led researchers to propose the epimutations involved in COPD pathogenesis with those allegedly operating in the mustard lung (93). Besides, a recent study discussed the possibilities of next-generation sequencing to fulfill the catalog of chromosomal, genetic and epigenetic aberrations induced by sulfur mustard and screening biomarkers and therapeutic targets to address acute and chronic mustard disease (95).

Certain epigenetic modifications related to sulfur mustard toxicity deserved special consideration (92, 94, 127-129), it would be interesting to investigate the impact of vesicants in the epigenome to search of preventive and/or palliative measures for the exposed population. The goal is not a chimera since several patented epigenetic remedies are currently in use or clinical trials for the treatment of certain tumors (94, 130-132). In this context, melatonin displays relevant epigenetic actions against the emergence, progression and metastatic spreading of tumors (133). Indeed, studies with tumor lines and BALB/c female mice have isolated genes regulated epigenetically by melatonin (134) and profiled some of its oncoprotective effects (135). Thus, it urges continuing investigation with melatonin in the hope of finding a new therapeutic alternative for the management of long-term pathogenicity of vesicant agents.

The current understanding of complex pathogenesis gathered by vesicant agents is insufficient to explain in mechanistic terms their long-term derived pathology (136) and shed light on the presumed driving role that the epigenotype plays. Moreover, epigenetic and biochemical markers for the monitoring of affected patients and the selection of therapeutic targets are lacking despite rapid progress being made (137). Therefore, the potential of epigenetic melatonin to shield against blistering agents needs additional *in vitro* research as well as systematic animal studies and controlled clinical trials to fulfill the translational gap between animal models and clinical trials (118) and design new therapeutic strategies. In the case of the much less studied organ-arsenic lewisite compounds the therapeutic importance of

epigenetic melatonin has not even been postulated, although we trust they be included in future studies on this issue. Taken as a whole, once profiled epigenetic aberrations in vesicant-injured individuals and elucidated the map of epigenetic actions deployed by melatonin, new translational opportunities dealing with acute and long-term pathology and based on melatonin administration or adjuvant combination will be probably in our hands.

6. CONCLUSION AND PERSPECTIVE

In this review, we have tried to provide an overview of the complexity of the molecular and cellular mechanisms involved in mustard toxicity, which however are still not well understood. In this scenario, we propose melatonin as a therapeutic option, since this molecule is able of modulating, through a plethora of biochemical cascades, multiple pathways involved in physiopathological mechanisms activated after exposure to mustards. The high capacity to reduce of oxidative damage, the role as an immunomodulatory agent in response to inflammation or the prominent function regulating epigenetic mechanisms make melatonin a suitable molecule to be part of the arsenal of medical countermeasures for the prophylaxis against vesicant CWAs poisoning and the development of post-exposure treatments in the near future.

It is also important to highlight that epigenetic aberration promoted by vesicant CWAs have not been addressed systematically. Pleiotropic melatonin is active in the control of the genome through the modulation of epigenetic mechanisms and everything seems to indicate that this capacity may represent an extraordinary opportunity for the clinical treatment of victimized patients. Unfortunately, *ad hoc* studies in this field and a research program in depth are lacking.

To advance in the knowledge of the therapeutic value of melatonin against vesicant CWAs, two concerns should be addressed: first, an effective dosage of melatonin supplementation should be established. Second, new galenic formulations or prolonged-release preparations should be investigated given the absorption variability, short biological half-life, and extensive first-pass metabolism of melatonin when orally administered. This would allow modifying melatonin`s pharmacokinetic characteristics.

Altogether, accumulated experimental evidence points to melatonin as a real protective shield against the acute distress caused by acute exposure to vesicants. Moreover, melatonin influences on epigenetic marks and epigenetic inheritance system and some *in vitro*, animal and clinical reports suggest that melatonergic therapy could also have beneficial effects on long-term pathology, which is suspected epigenetic in origin. However, despite its biochemical and epigenetic potentiality, melatonin seems capable of counteracting blistering-related toxicity, thus, much more research is needed before the translational applicability in the form of new melatonin treatments becomes a reality.

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AUTHORSHIP

AR and RP conceived the idea and they have made substantial contributions to conception, design, supervision and writing-original draft preparation and acquisition of funding. AJ, JE, FLM and ER critically revised the manuscript. EGM contributed to conception and design, drafted and critically revised the manuscript. CDLR collaborated on the conception and design of the chemical reactions, and writing-original draft preparation. All co-authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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