IN VIVO ASSESSMENT OF SKIN CHEMICAL BURNS IN EXPOSURE TO VESICANTS AND THE EFFICACY OF AN ANTIDOTE FORMULA IN DIFFERENT PHARMACEUTICAL FORMS. AN EXPERIMENTAL APPROACH

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Abstract

The aim of this project was the histopathological and immunofluorescence evaluation of the cutaneous toxicity of the vesicant chemical compound 2-chloroethyl-ethyl sulphide (CEES), a yperite simulator, and the effectiveness of the applied antidote. A complex antidote formula was developed, the treatment offered 100% protection in case of exposure to 1DL50 chemical vesicant. The histopathological evaluation showed that the association of the newly developed antidote with antioxidant and anti-inflammatory actions with the antioxidant formulation, in the form of gel, with regenerating, moisturizing and epithelializing actions, is beneficial for 0.25 - 1 LD50 2-chloroethyl-ethyl sulphide concentrations. Immunofluorescence evaluation of the expression of anti-c-Ros-1 and anti-PARP-1 antibodies, respectively, highlighted the antidyplastic reepithelialising and nuclear stabilizing protective effect of the complex antidote on the skin lesions induced by the studied vesicant compound. The combination of the two complex curative antidotes (A1 formulated as a solution and A2 formulated as a hydrogel) developed within the project has superior therapeutic efficacy. It can guide the therapeutic conduct in case of exposure to vesicant chemicals.

Rezumat

Scopul acestui proiect a fost evaluarea histopatologică și prin imunofluorescență a toxicității cutanate a compusului chimic vezicant 2-cloroetil-etil sulfură (CEES), un analog de șturită și a eficacității antidotului aplicat. A fost dezvoltată o formulă complexă de antidot care a oferit protecție 100% în cazul expunerii la vezicant chimic 1DL50. Evaluarea histopatologică a arătat că asocierea antidotului nou dezvoltat cu acțiunile antioxidante și antiinflamatorii, sub formă de gel, cu acțiunile de regenerare, hidratare și epitelizare, este benefică pentru 0.25 - 1 DL50 2-cloroetil-ethyl sulfide concentrații. Evaluarea prin imunofluorescență a expresiei antiderapizante anti-c-Ros-1 și, respectiv, anti-PARP-1, a evidențiat efectul protector antidisplazic reepitelizant și stabilizator nuclear al antidotului complex asupra lezurilor cutanate induse de compusul vezicant studiat. Combinarea celor două antidoturi curative complexe (A1 formulat sub formă de soluție și A2 formulat sub formă de hidrogel) dezvoltată în cadrul proiectului are o eficacitate terapeutică superioară și poate ghida conduita terapeutică în cazul expunerii la substanțe chimice vezicante.

Keywords: vesicants, toxicity, 2-chloroethyl-ethyl sulphide, CEES, antidote

Introduction

Vesicants are composed of sulphur, nitrogen, arsenic and halogen-based structures that cause chemical burns to the skin, eyes, and lungs through direct contact or inhalation of vapours [18]. There are several types of vesicants: the most important are those containing nitrogen, known for their uses in chemotherapy, and those with sulphur, for military applications. [18, 21, 27]. The prototype of this category of compounds is mustard (sulphur mustard) with the chemical structure bis (2-chloroethyl) sulphide, which, when absorbed transcutaneous, binds to tissue proteins, and produces changes that are characteristic of chemical burns, highlighted by erythema, hyperpigmentation, oedema, vesicles (blisters) and phlyctenules [7, 8, 11]. Another vesicant with a similar chemical structure is 2-chloroethyl-ethyl...
sulphide (CEES). Due to its chemical and structural properties, similar to vesicant chemicals, and its lower toxicity, it is more suitable for laboratory studies [1, 12]. The molecular mechanisms involved in the appearance of oedema and vesicles induced by the sulphur-based vesicant chemicals listed above are not fully elucidated, which justifies the difficulty of identifying effective medical countermeasures for the treatment of skin lesions such as moderate/severe chemical burns [3, 20]. Combined therapy using topical drugs, oral anti-histamines, anti-inflammatory drugs and anti-TNF-alpha antibodies is an efficient option without remitting the lesions, which sometimes lead to neoplasms [4, 10]. To optimize effective therapeutic solutions, studies were performed using the chemical vesicant 2-chloroethyl-ethyl sulphide (CEES). It has a chemical structure similar to that of the class of sulphur-based vesicant chemicals and acts through similar pathogenic mechanisms of acute exposure (induction of oxidative stress and inflammatory response), but is less toxic, being a valid experimental alternative for detoxification and antidote studies [31]. Skin lesions caused by vesicant chemicals are characterized by oedema, inflammation, and cell death, mainly of the basal layer of keratinocytes, with varying immunological and pathological changes in the acute phase. Xerosis, hypo- or hyperpigmentation, scarring and, rarely, skin cancers are also long-term skin effects [20]. At the molecular level, the activation of anti-c-ROS-1 and anti-PARP-1 antibodies' expression was found. The expression of the c-ROS-1 antibody, a member of the tyrosine kinase receptor family, is correlated with cell growth and proliferation, including tumour proliferation, and is associated with in vitro sensitivity to tyrosine kinase inhibitors [5, 32]. It is a marker of various forms of cellular stress transmitted through the cytoplasm to the nucleus. It is mainly a marker of cellular aggression of various aetiologies, including tumoural. Secondary, it can also be interpreted as a marker of cell growth and proliferation. It is also a participant in the cell regeneration process, involved in many cellular activities, including transcription, signal transduction and immune response [6, 15, 16]. The presence of anti-c-ROS-1 has been correlated with a higher degree of nuclear atypia [28, 34]. The PARP-1 monoclonal antibody recognizes native and/or cleaved poly (ADP-ribose) polymerase. PARP cleavage is an early indicator of apoptosis (the cleaved form of caspase) as well as DNA repair [2, 29]. Intense DNA damage leads to overexpression of PARP-1, a key DNA repair enzyme. Moderate activation of PARP promotes cell survival through a repair mechanism [17, 19]. PARP is a factor with nuclear expression, and counter-marking with DAPI identifies the cells with viable nuclei [19, 35, 37]. In this context, the project aims to: histopathological and immunofluorescence assessment of skin toxicity of the 2-chloroethyl-ethyl sulphide (CEES) vesicant in order to develop a complex antidotic formulation that pharmacodynamically antagonizes the toxicity of this category of blistering agents.

Materials and Methods

Materials and reagents
The following materials and reagents were used for the experimental study: anti-ROS-1 antibody (Oncogene Tyrosine Kinase Receptor), Abexa, USA; anti PARP antibody. Invitrogen, USA; 2-chloroethyl-ethyl sulphur, Sigma Aldrich, Germany; dexamethasone, X’ian Mellon Chemical Technology, China; acetylcytistine, Merck, Germany; silver sulfadiazine, Sigma Aldrich, Germany; nicotinamide aminonucleotide, Sigma Aldrich, Germany; hyaluronic acid, Parchem, USA; doxycycline, Sigma Aldrich, Germany; collagen, Sigma Aldrich, Germany; anhydrous lanolin, Sigma Aldrich, Germany.

Laboratory animals
A number of 35 Wistar rats, male and female, with an average weight of 120 - 150 g kept in an appropriate microclimate were purchased from the accredited biobase of INCDMM “Cantacuzino”, Bucharest, Romania in compliance with the legislation in force.

Laboratory equipment
Confocal microscope, Zeiss 980 used to identify foci/sites of c-ROS-1 and PARP antibody expression on tissue using immunofluorescence techniques; Zeiss light microscope for histopathological examination of skin samples using haematoxilin-eosin staining; dedicated software for each piece of equipment. The experimental design went through the following stages.

The pharmaceutical formulation of the complex antidote
The antidotes were administered simultaneously, immediately after application of the vesicant compound, daily, for 7 days, having the following pharmaceutical formulations: curative antidote A1 formulated as solution, with percutaneous administration: acetylcytistine: 10% (antioxidant), dexamethasone: 4% (steroidal anti-inflammatory compound), nicotinamide nucleotide 10% (PARP inhibitor); excipients - PBS ad 100 mL buffer containing disodium phosphate, sodium chloride, potassium phosphate and potassium chloride achieving a pH of 7.8 required for faster transdermal absorption. Healing antidote A2 formulated as a hydrogel with epithelializing, regenerating, healing and antibiotic role: hyaluronic acid; silver sulfadiazine 1%; doxycycline 3%; collagen 1%; anhydrous lanolin 20%; sterile water.

In vivo assessment of toxicity of vesicant compound 2 chloroethyl-ethyl sulphide CEES and efficacy of antidotic treatment
Experimental studies on laboratory animals were carried out with the approval of the INCDMM “Cantacuzino” Ethics Committee. An experimental model of in vivo toxicity of CEES was performed using 7 groups of 5 rats each. According to the previous experiments and literature data that...
the range of average lethal doses for percutaneous application was 100 ± 7.5 µL. The different doses of CEES (0.5, 1 LD50) were administered percutaneously. The treatment consisting of the two antidotes was applied simultaneously on the same surface, immediately after applying the toxic compound, and daily for 7 days (Table I).

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>DL50</th>
<th>CEES Vesicant compound, 7.78 M concentration (µL/2cm²)</th>
<th>Treatment</th>
<th>A1 (200 µL/2cm²) + A2 (15 mg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group 1</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Group 2</td>
<td>0.5</td>
<td>50</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Group 3</td>
<td>0.5</td>
<td>50</td>
<td>A1</td>
<td>A1 + A2</td>
</tr>
<tr>
<td>4</td>
<td>Group 4</td>
<td>1</td>
<td>100</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Group 5</td>
<td>1</td>
<td>100</td>
<td>A1</td>
<td>A1 + A2</td>
</tr>
<tr>
<td>6</td>
<td>Group 6</td>
<td>0</td>
<td>0</td>
<td></td>
<td>A2</td>
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<tr>
<td>7</td>
<td>Group 7</td>
<td>0</td>
<td>0</td>
<td></td>
<td>A1</td>
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</table>

**Histopathological examination**
At the end of the treatment period, the animals were slaughtered, and the affected skin was harvested for histopathological and immunofluorescence studies to show the expression of ROS-1 and PARP-1 antibodies. The animals were euthanized with a ketamine overdose after 7 days of treatment, with previous anaesthesia. Skin fragments were collected for histopathological assessment of the severity of the lesions produced by CEES. The haematoxylin-eosin staining method was used for paraffin sections [13].

**Immunofluorescence detection of anti-ROS-1 and PARP antibody expression**
Immunofluorescence is one of the most widely used techniques in modern biology and medicine and was developed by Coons et al. and is a combination of immunofluorescence technique and morphological technology to develop immune fluorescent cells (or tissues). The protocol includes steps for the indirect immunofluorescence test according to the literature data [14].

**Results and Discussion**

*In vivo assessment of toxicity of vesicant compound 2 chloroethyl-ethyl sulphide CEES and efficacy of antidote treatment*

The complex treatment, applied daily for 7 days, offered 100% protection corresponding to an exposure of 1 LD50 2-chloroethyl-ethyl sulphide. **Histopathological evaluation of skin lesions caused by vesicant compound 2 chloroethyl-ethyl sulphide (CEES) and the efficacy of the combined treatment consisting of the two antidote formulations (solution A1 and hydrogel A2).**

They were evaluated by the classical histopathological method, under optical microscopy, using standard haematoxylin-eosin staining, tissue fragments representing skin samples from small rodent subjects (Wistar rats). The control, *study group no 1*, not exposed to the vesicant compound, presented classical histology without particularities. Reticulated and papillary dermis with vascular plexuses - present, intact, with rare cells and capillaries with free red blood cells, without stasis elements, rare fibrocytes.

**Figure 1.**
Haematoxylin staining, 4X

a - Group 1 non-intoxicated and untreated; b - Group 6 non-intoxicated and treated with A1 c - Group 7 non-intoxicated and treated with A2. - 10X - tegument that includes the three layers (epidermis, dermis and hypodermis), underlying muscles and fascia with vascular plexuses within normal limits.
Study groups 6 and 7, not exposed to 2 chloroethyl-ethyl sulphide and treated only with antidotes A1 (solution for percutaneous application) and A2 (hydrogel), respectively, showed aspects similar to those of the normal non-intoxicated and untreated group (Figures 1a, 1b and 1c). Study group 2, to which 0.5 DL50 and 50 µL of the technical solution of 2-chloroethyl-ethyl sulphides (CEES) of 7.78M concentration was applied percutaneously, the following were observed: continuity solution at epidermal level, extended to the hypodermis through a fibroblastic proliferation that replaces the damaged structures (reticulated and deep dermis). The reepithelialisation was scarred and incomplete, with the loss of the pilosebaceous units and the other adnexa. The partially detached crust can be seen at the junction of the lesion with the intact dermis. The sclerodermal reaction partly respects the hypodermis. No inflammatory elements adjacent to or underlying the area of liquefaction necrosis were identified, but slight vascular congestion in the capillary network of the underlying muscle tissue was noted (Figure 2).

Figure 2.
Group 2 exposed to 0.5 LD50 HE, 10 X – Continuity solution at the epidermis level that generates underlying scar fibroblasts, without neovases or cells participating in the granulation tissue, i.e. without repair elements. The upper part of the image shows the partially scaled fibrin-haemorrhagic crust, with a point of attachment in the continuity solution at the epidermal level.

Study Group 3, exposed to the same dose of CEES 2 and treated, shows a solution of continuity with active reepithelialisation area, acanthosis area in conjunction with the intact dermis and recruitment of neovases in the dermo-hypodermic junction (Figures 3a and 3b). Application of treatment to the lesions generated by exposure to CEES2 enriches the tissue with cellular and vascular elements, which shows the participation of the cellular and vascular immune system in tissue regeneration. Partial preservation of the adnexal structures is also noted.

Figure 3.
Group 3 exposed to 0.5 DL 50 and treated a) HE, 10X. Acanthosis in the dermis adjacent to the lesion and hyperkeratosis. The continuity solution, which is covered by hyperkeratosis, extends on an inframilimetric portion and presents underlying intact skin adnexa. b) HE, 10X. Detail of the area of reepithelialisation with acanthosis and overlying hyperkeratosis.
Figure 4.
Group 4 exposed to a 1 LD50 and untreated – HE, 10 X. Continuity solution is observed at the epidermal level, with parcellar detachment of the epidermis, with dermo-epidermal phlyctenule. Liquefaction necrosis is limited to the dermo-epidermal level, with loss/thinning of the adnexal structures. Inflammatory infiltrates in all layers of the dermis.

In the study group 4, exposed to CEES 3 (1 LD50) and 100 µL of the technical solution of 2-chloroethyl-ethyl sulphides (CEES) of 7.78 M concentration and untreated, we observed: solution of continuity at epidermal level, in-depth. The reticulated dermis contains inflammatory elements and signs of vascular congestion (Figures 4a and 4b).

In study group 5, exposed to CEES 3 (1 DL50) and treated, an initially reepithelialised lesion is observed, with disorganization and disappearance in some places of the pilosebaceous units and acanthosis. The lesion is noted by maintaining a superficial crust belonging to the epidermis through a minimal continuity solution (Figures 5a and 5b).

Figure 5.
Group 5 4X group exposed to 1 LD50 (CEES 3) and treated Fibrin-haematous crust that incorporates cellular detritus and covers an epithelium that presents continuity solutions with disorganization of skin anexa; no inflammatory elements are observed, but a complete reepithelialisation, which extends from the basal layer to the deeper ones, is evident. No blisters or continuity solution areas were identified on the examined segments.

Figure 6.
a - normal DAPI staining group; b - normal DAPI and c-ROS-1 group
Immunofluorescence evaluation of skin preparations taken from animals exposed to 2 chloroethyl-ethyl sulphide (CEES) and those exposed to CEES and treated aimed to study the expression of two markers: anti-c-ROS-1 and anti-PARP-1 antibodies.

Evaluation of the immunofluorescence reaction of anti-c1-ROS-1 For the control group unexposed to CEES, untreated, in all animals, the expression of the anti-c-ROS-1 antibody was positive in the stratum corneum, continuously, rare connective cells of the dermis (fibroblasts) and rare inflammatory cells in the keratinized or lipid structures of the pilosebaceous units. Counter-marking with DAPI discriminates the artifactual uptake of fluorochrome, and thus we can see, in distinct regions, the absence of expression of the anti-c-ROS-1 antibody (Figures 6a and 6b).

In group 4 exposed to CEES 1 DL50 and untreated, it can be observed that the discontinuity of the basal layer highlighted by DAPI staining is false positive for the anti-c1-ROS-1 antibody, which is also captured by metabolically inert structures (lacking a nucleus and implicitly necrotic). The positive immunofluorescence reaction for the anti-c antibody ROS-1, evidenced in cells co-positive for DAPI, is identified in the area of aggression at the level of the reactive elements both at the level of the skin anexa (in the areas that maintained pilosebaceous units) and in the underlying connective tissue. The underlying tissue, especially the hypodermic vascular plexus vessels, has a positive reaction for c-ROS-1, which demonstrates that deep vascular aggression is transmitted by indirect cellular signals, as there is no obvious continuity of ROS expression between the superficially attacked area and the deep answer. This aspect generates the hypothesis of specific ROS-1 intercellular signalling pathways. (Figures 7a and 7b).

In the case of the group exposed to the vesicant compound and treated, considering the participation of ROS in carcinogenesis, we can consider that, through a weaker expression of ROS, the potential anti-tumour properties of the therapeutic agent are demonstrated, and the cell contingent with dysplastic potential.

Figure 7.

a) Group 4 exposed to CEES and untreated (DAPI and ROS); b Group 5 exposed to CEES and treated (DAPI and ROS)

Figure 8.

a - 10X DAPI in the normal group; b - 10X DAPI and PARP-1 in the normal group
Evaluation of the expression of anti-PARP-1 antibody in the control group, not exposed to aggression or treatment, showed that PARP-1 is positive in rare cells of the basal layer - preapoptotic as well as in keratin of the stratum corneum, internal sheath of hair, rare dermal cells with a fusiform nucleus, most likely fibroblasts (Figures 8a and 8b).

In the case of group 4, exposed to CEES (1 LD50) and untreated, activation of the anti-PARP-1 antibody in the reepithelialisation area is observed as a response to DNA stabilization and repair. The administering of the optimized complex antidote treatment (group 5) correlates with the inhibition of PARP-1 expression. It can be explained by maintaining the integrity of the nuclear apparatus under the protection of the pharmacological agents (Figures 9a and 9b).

Figure 9.
a - 10X DAPI for the group exposed to CEES (1 DL50) and untreated; b - 10X DAPI and PARP-1 for the group exposed to CEES (1 DL50) and treated.

The expression of the anti-PARP-1 antibody in the treated group demonstrates the DNA regeneration promoting effects that the therapeutic substances can induce. Thus, the applied treatment also has a protective role against cellular events with dysplastic potential. In the case of the group exposed to CEES (1 LD50) and untreated, an activation of PARP-1 is observed in the reepithelialisation zone in response to DNA stabilization and repair.

Intoxication with blister agents encountered in war settings still represents a therapeutic challenge in clinical practice. The severity of lesions depends on the route of exposure, time and dose of the toxic. The damage is multi-systemic, involving the skin, eyes, respiratory and digestive systems. Moreover, large cutaneous exposure or multi-systemic involvement can lead to leukopenia and pancytopenia (Ghasemi). The cutaneous lesions in human subjects are typically represented by erythema, itching, vesicle, and bullae filled with a yellow fluid, which manipulation can cause harm and intoxication to healthcare workers if appropriate protective equipment is not worn [22]. The pathological mechanism involves the degeneration of the basal membrane of the epithelium, cellular apoptosis and inflammation.

The current therapeutical approach of skin lesions induced by blister agents is mainly symptomatic, and it is similar to that of thermic burns. After removing cloths and intensive decontamination using soap and water, the blisters are treated with debridement, antibiotic ointment, collagen-laminated nylon dressings, and fluid resuscitation, if needed. Initial decontamination of the exposed area with RSDL (Reactive Skin Decontamination Lotion) was reported to be effective in actively desorbing, retaining and sequestering the blister agent due to its active ingredient Dekon 139 [30]. Chloramine 3% solution and silver sulfadiazine were used to prevent secondary bacterial infection. The inflammation and itching may be diminished by calamine or local steroids [24, 25]. Alternatives to topical steroids with anti-inflammatory properties, such as curcumin, capsaicin, doxepin, aloe vera/olive oil combination, hydroxyzine, and phenol/menthol combination, were used by other authors, but with limited clinical evidence [24-26, 33]. General administration of antihistamines, and pain medication such as acetaminophen, morphine, and sedatives, are recommended as other treatment protocols [9, 36].

A complex antidote formula was developed by developing and testing a fixed combination of active substances formulated as a solution for dermal administration in combination with a hydrogel in order to antagonize the vesicants’ toxic effects pharmacodynamically. This treatment provided 100% protection in the event of exposure to 1 DL50 vesicant. Immunofluorescence evaluation of the expression of anti-c-ROS-1 and anti-PARP-1 antibodies, respectively, highlighted the antidysplastic, reepithelialising and nuclear stabilizing protective effect of the complex antidote on the skin lesions induced by the studied vesicant.
Conclusions

The hydrogel curative antidotes A2 combines efficiently the re-epithelizing and pro-cicatrizing properties of hyaluronic acid and collagen, with the antibacterial effects of doxycycline and silver sulfadiazine and the neutralizing and anti-inflammatory effects of N-acetylcysteine and dexamethasone. It proved to be safe and effective in the treatment of skin lesions induced by vesicants in the experimental setting. The results of the present study create favourable conditions for the use of the antidote in vesicants poisoning in human subjects and can guide the therapeutic course in case of exposure to vesicant chemicals.

Conflict of interest

The authors declare no conflict of interest.

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