Current Topics in Toxicology
Vol. 15, 2019

Review

In the search for better radioprotective treatments: A risk-benefit equation between effectiveness and toxicity

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ABSTRACT

For decades, several compounds with radioprotective or mitigating capacity against the effects of ionizing radiation have been synthesized but their own toxicity has been the main limitation for their use. A program of synthesis and testing of radioprotectors was hence developed after the Second World War. A large number of preclinical and even clinical studies were carried out, including those on the use of some of these substances in cancer radiotherapy, intoxications, military emergencies and exposures during space flights. From those studies a radioprotector of acceptable efficacy, namely amifostine (WR-2721), arose. Despite its interesting qualities, it is far from being an ideal radioprotector due to its toxicity. The use of WR-2721 in humans has some important adverse effects, which prevent its repeated administration to achieve a sustained protective effect. An ideal radioprotector should sustain its effect for a reasonably long time and this implies that its toxicity should be low. Thus, the development of new radioprotectors, or their formulas, that act by preventing or mitigating the consequences of an exposure becomes very important. The initial idea behind our studies was to take advantage of the radioprotective capacity of amifostine when administered in a single low dose prior to radiation, but later on we continued with the administration of other substances of low toxicity that would reinforce the initial protective effect. This is how we successfully tested substances such as ethyl pyruvate and sodium butyrate, which proved to be efficient supplements for a low dose of amifostine. The results published recently by our laboratory generate a valuable antecedent about this hypothesis and open the panorama for therapeutic radiological and mitigating alternatives. The potential for clinical use of these treatments is high due to their low toxicity, which would facilitate approval for their use in humans.

KEYWORDS: ionizing radiation, X radiation, acute radiation syndrome, radioprotection, amifostine, WR-2721, sodium butyrate, ethyl pyruvate.

1. Introduction

1.1. Acute radiation syndrome: A complex multitarget toxicologic pathology

The International Agency for Research on Cancer (IARC), belonging to the World Health Organization, conducted a valuable evaluation in 2000 on the effects of ionizing radiation, which exhaustively detailed exposure events and their harmful effects with their degree of intensity [1]. Different feasible occasions in which exposures to ionizing radiation could be verified, causing harmful effects of varying intensity and consequences, have been evaluated in different institutions from many countries. Several examples have been derived from incidents in facilities of the nuclear industry

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(in power generation plants such as Chernobyl, Fukujima or Three Miles Island); the use of atomic energy for military purposes (Hiroshima and Nagasaki bombs, nuclear weapons tests and the resulting radioactive fallout, in the manufacture of the materials involved); from accidents during the use of nuclear medicine sources; and from occupational hazards (uranium mining, radon exposure, aircraft personnel and space flights, other industrial uses). In recent times there has been increasing concern about the use of radioactive sources for terrorist activities [1-3].

In 2001, a commission of experts in ionizing radiation belonging to different areas of the US government (for example, National Cancer Institute, Department of Energy, NASA, EPA, Uniformed Services University for the Health Sciences, Armed Forces Radiobiology Research Institute) met with the purpose of analyzing the problems derived from these accidental exposures and the alternatives of protection or treatment. In this meeting of specialists, the dose between 1 and 10 Gy was defined as a feasible one in treatments of patients with cancer, populations potentially exposed to accidental or intentional (terrorism) situations and for workers in the nuclear industry [3]. This range of exposure between 1 and 10 Gy involves health deleterious consequences, like the hematopoietic syndrome and some signs of the so-called gastrointestinal syndrome [3].

Acute Radiation Syndrome (ARS), also known as radiation toxicity or radiation sickness, is a name used to describe an acute illness caused by irradiation of the entire body (or significant partial-body) by a high dose of penetrating radiation delivered at high-dose rate (usually in a few minutes). The major cause of this syndrome is the depletion of immature parenchymal stem cells in specific tissues. ARS is subdivided into three subsyndromes: the hematopoietic, gastrointestinal and neurovascular syndrome but many other tissues can be damaged [4]. Both in humans and in experimental animals, time course and severity of clinical signs and symptoms are a function of the overall body volume irradiated, the inhomogeneity of dose exposure, the particle type, the absorbed dose and the dose rate.

Hematopoietic syndrome is caused by the destruction of the bone marrow, resulting in a severe leucocyte depletion, infection and hemorrhage. This is the earliest expression of the toxicity of ionizing radiation because bone marrow cells are extremely sensitive to this kind of oxidative damage.

Gastrointestinal syndrome occurs at doses of between 6 and 15 Gy. Clinical signs and symptoms are due to the lack of replacement of cells in the surface of the villi because the stem and proliferating cells located in the crypts are damaged by radiation and die in mitosis. Destruction of intestinal mucosa produces watery diarrhea, dehydratation and electrolyte loss, gastrointestinal bleeding and perforation. Breakdown of the mucosal barrier facilitates the entry of bacteria into the bloodstream. Immunosuppression associated with the hematopoietic syndrome favours opportunistic infections and thrombocytopenia favours hemorrhage. Death from the gastrointestinal syndrome is due to sepsis, bleeding, dehydratation and multisystem organ failure [4].

At higher doses the damage can also be expressed at the cardiovascular level and in the level of the central nervous system. It is important to note, however, that any of these subsyndromes can be the cause of mortality. The smaller the dose, the greater is the possibility of an effective treatment and, therefore, the targets involved are less.

Regardless of the source, exposure to ionizing radiation (X-rays or gamma rays) will have serious and generally irreversible consequences. These originate in the transfer of radiant energy to cellular molecules, which causes their ionization. Body water, because of its abundance, is the main target of radiation, but DNA, RNA, proteins and cellular lipids are also very susceptible. In the fraction of miliseconds all the free radical reactions generated from water are already complete. From that point and at different times, all the biochemical processes that lead to cell damage will have been completed. During these processes reactive species derived from oxygen (ROS) as well as nitrogen (post-induction of the enzyme nitric oxide synthetase) are generated [2]. These reactive molecules attack critical cellular components such as DNA, proteins, lipids, etc. In the course of these interactions, other free radicals are also produced in the cell macromolecules, which lead to the degradation of the critical cellular compartments. A clear example is the

formation of 4-hydroxynonenal from the lipids. This compound is considered to be mainly responsible for the damages caused by lipid peroxidation mediated by ROS [2]. The combined action of all these products derived from the action of radiation leads to cell death in different tissues. If the damage is not intense enough, in any case, many of these molecular alterations may lead, in longer times, to other pathologies (for example, mutations in DNA and cancer).

For decades, some compounds with radioprotective or mitigating capacity against the effects of ionizing radiation have been synthesized. Thus, a radioprotection synthesis and testing program was developed in the United States. A large number of preclinical and even clinical studies were carried out, including those on the use of some of these substances in cancer radiotherapy, intoxications, military emergencies and exposures during space flights [3]. From those studies, amifostine (WR-2721) emerged as a radioprotector of acceptable efficacy. This compound has also found applications in radiotherapy in cancer clinics, because of the selectivity in its effect on tumor tissue [5-8]. Despite its interesting qualities, it is far from being an ideal radioprotector due to its toxicity [9]. This led the United States Food and Drug Administration (FDA) to limit its use in patients against radiation damage after surgery for cancers of the head and neck and to prevent xerostomia [10-11]. Amifostine is only approved for intravenous administration, although some have used it subcutaneously [11]. This drug has very low liposolubility and its positive ionic charge at low pH makes oral absorption very difficult (for example, in the stomach) [12]. On the other hand, an ideal radioprotector should sustain its effect for a reasonably long time and this implies that its toxicity should be low. The use of WR-2721 in humans has some important adverse effects. Since the effective dose of amifostine differs little from its toxic dose, patients frequently present adverse effects that include hypocalcemia, diarrhea, nausea and vomiting. Some of those symptoms are also typical of exposure to ionizing radiation. In addition, other symptoms are emesis, hypotension and allergies [13].

The existence of these adverse effects prevents its repeated administration to achieve a sustained protective effect. What is desirable in a radioprotector is that it should be able to protect most of the exposed organs. Amifostine protects a wide variety of sites in the affected organism if it is administered for a short time before exposure to radiation but it has only a little effect when it is administered after exposure [14]. Only when this drug is present at the time of irradiation is it able to reduce mortality in a very significant way. It was even able to protect normal cells and not cancer cells after radiotherapy [10]. This virtue has enabled its extended application in the oncology clinic.

The mechanism of the radioprotective action of amifostine is basically related to its ability to trap free radicals, and to destroy some very reactive products that are formed from altered biological molecules, such as lipids and proteins, through the donation of hydrogen, or by reacting with them, as is the case with 4-hydroxynonenal [15]. This capacity depends on its previous conversion by enzymatic dephosphorylation to its active metabolite, WR-1065. This process is mediated by an alkaline phosphatase present in the cells of different organs and in the serum of different animal species and humans [10, 16]. This activation process is very fast, but it must still happen prior to the irradiation, in order to have the sulfhydryl compound in sufficient concentration when the free radicals of such a short life are generated.

Amifostine also has protective effects when it is administered after exposure to ionizing radiation. It has been less studied and its relevance has not been established [14]. The fact that the protective action of amifostine requires prior activation of its alkaline phosphatase-mediated sulfidrylic metabolite is critical and is supposed to be linked to its utility in clinical oncology. Amifostine has been shown to protect the normal tissue surrounding the cancerous one because the latter has a more acidic pH (Warburg effect).

The development of new radioprotectors, or their formulas, that act by preventing or mitigating the deleterious effects of an exposure becomes very important. This includes all clinical trials that are required for the approval of its use in humans. Clinical trials of a coadjuvant therapy with other non-toxic compounds have been reported extensively in the scientific literature [17]. The strategy of

some studies was to take advantage of the radioprotective capacity of amifostine administered in a single low dose prior to radiation and continuing with other substances of low toxicity that would reinforce the initial protective effect. It seems clear that hardly a single substance can block or mitigate the acute harmful effects of ionizing radiation and that it is more feasible to use formulations where each component contributes properties that are cooperative or complementary. This implies trials of new components with very different characteristics in their therapeutic potential and that, if effective, should be studied due to their compatibility with existing ones.

In our laboratory, different substances have been tested with the purpose of designing formulas in combination with amifostine, which preserve the radioprotective efficacy but without increasing toxicity. To achieve this objective, natural compounds or their derivatives that could be administered jointly with amifostine or subsequently to the exposure were selected. The latter is an aspect of particular relevance, since any therapy that can mitigate some of the deleterious effects caused by radiation will contribute to improve survival and because amifostine is no longer useful for this purpose.

2. Free radical scavengers

Free radical scavengers from the nitrone group has been proposed as drugs to treat pathologies where reactive oxidant species should be a therapeutic target in a biological system, either when oxidative stress accompanies the disease or is a causative factor [18]. Alpha phenyl-N-tert-butylnitrone (PBN) is a compound with good solubility either in water or lipid media, making it a suitable candidate for reaching all tissue compartments and trap oxigen radicals sparked by ionizing radiation. In our laboratory PBN was used to trap radicals generated by ethanol metabolism in several tissues [19].

The radioprotective effect of PBN at doses of 20 and 40 mg/kg (i.p. in saline, one hour before irradiation) has been tested in our laboratory [20, 21]. Non-irradiated group receiving the compound under test was also evaluated at the same time. Histology of irradiated animals showed inflammatory processes in the epithelia of the digestive tract and in the testis, with no changes detected in salivary glands. Leukocyte count was drastically reduced compared to the control values, presenting also an altered formula. The effect of PBN on the tested parameters was moderately protective when it was administered at the highest dose, highlighting the recovery of erythrocytes in males and the protection of the epithelium in the small intestine (both sexes) and in the testis. No statistically significant protection in the recovery of the level of leukocytes or leukocyte count was observed (both sexes). The genetic damage revealed in the irradiated animals by the Comet assay was not reversed by the treatment with PBN, neither a protective effect for survival was observed at any dose tested. In conclusion, this radical scavenger showed a moderate radioprotective action that can be improved by increasing doses or treatment times, as it is a substance with low toxicity.

3. Vitamin E

Vitamin E is the major lipid-soluble component in the cell antioxidant defence system and is exclusively obtained from the diet. It has numerous important roles within the body because of its antioxidant activity. It is an effective antioxidant, scavenging free radicals generated by ionizing radiation exposure. Vitamin E comprises of eight major analogs, collectively known as tocols (four tocopherols and four tocotrienols). They have been subject to active investigation for a long time as radioprotectors in patients undergoing radiotherapy and in the context of possible radiation accidents or terrorism scenarios [22].

Although the tocols are well recognized as potent antioxidants and are generally thought to mediate radioprotection through free radical quenching, recent studies have suggested several alternative mechanisms: most notably, an ‘indirect effect’ of tocols in eliciting specific species of radioprotective growth factors/cytokines such as granulocyte colony-stimulating factor (G-CSF). The radioprotective efficacy of at least two tocols has been abrogated using a neutralizing antibody of G-CSF [22].

Vegetable oils constitute one of the main sources of vitamin E dietary intake and an extensive bibliographic revision is available on the contents of vitamin analogs in each variety of vegetable oils [23]. On the other hand, there are precedents

in the scientific literature about the radioprotective effect of some vegetable oils rich in polyunsaturated fatty acids and vitamin E (tocopherols and tocotrienols) [24, 25].

In our laboratory we made studies comparing the radioprotective efficacy of various edible oils [26]. Oils (wheat germ, rice seed, palm, grape seed, chia, avocado and olive) were administered subcutaneously two hours before irradiation (X rays, 6 Gy) at a dose of 1 g/kg. For comparison, the effect of alpha-tocopherol was tested at a dose of 570 mg/kg (i.p.) (administered 4 hours prior to irradiation), and also for its acetate (given at a dose of 750 mg/kg (i.p.) 24 hours before irradiation). In irradiated animals the erythrocytes were significantly depleted (females, $p < 0.01$) and the white blood cell count was drastically reduced (both sexes, $p < 0.01$), also presenting an altered formula. In addition, Comet assay showed an important damage on DNA ($p < 0.01$ compared to control group). All of these treatments with the exception of avocado oil resulted in significant protection against DNA damage in both sexes (Comet assay, $p < 0.05$). Although the protective effect on the hematological parameters measured was complete in the survivors of both sexes (from day 30), no significant protective effect was observed with any of the treatments regarding survival. These oils showed ability to protect leukocyte DNA significantly but less than that of a radioprotectant such as the toxic amifostine. They could be useful as coajuvants of amifostine for preventing or mitigating the acute harmful effects of ionizing radiation [26].

4. Lipoic acid

Alpha-lipoic acid is a compound found naturally in human body with a primary role as a cofactor, converting glucose into energy through a process referred to as aerobic metabolism. It is also considered an antioxidant, having the ability to neutralize harmful compounds like free radicals. It is a compound that is soluble in both water and fat, reaching any compartments in the tissues and cells easily. It can also recycle other antioxidants, including vitamin C, vitamin E, and glutathione. In this way, alpha-lipoic acid helps in restoring the antioxidant capacity of the cell by absorbing excess electrons and converting them back to their active forms. In addition, it has anti-inflammatory action, independently of its antioxidant activity [27].

In our laboratory, radioprotective action of lipoic acid was tested by the administration as a single dose of 550 mg/kg (p.o.) suspended in 0.5% xanthan gum, three hours before irradiation. Amifostine was tested simultaneously for comparison at a dose of 100 mg/kg (i.p. in saline 30 minutes before exposure to X rays). In the irradiated animals erythrocytes were depleted (females, $p < 0.01$), and white blood cell count was drastically reduced with respect to the control (both sexes, $p < 0.01$), also presenting an altered formula. The effect of lipoic acid on the parameters tested was protective, with a complete recovery of erythrocytes in females. However, no statistically significant protection was observed in the recovery of the leukocyte level or the leukocyte formula, either with lipoic acid or amifostine (both sexes). Genetic damage revealed in leukocytes from irradiated animals was significantly reduced by treatment with lipoic acid (both sexes, $p < 0.01$). However, no significant protective effect was observed for survival [28].

5. Calcium chelators

Increased intracellular calcium is a factor known to be involved in the process leading to cell death. Changes in calcium homeostasis are relevant to the late stages of cell injury. Sodium alizarinsulfonate (ASR) has a potent action as calcium chelator, thus interfering with the entrance and action of this element in the cell. It is our interest to develop less toxic radioprotectors, either by themselves or as adjuvants to drugs approved for their use in humans. Using an experimental model of Sprague-Dawley rats (both sexes) exposed to X radiation (6 Gy, whole body), we studied the radioprotective effect of sodium alizarinsulfonate (3,4-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt) [29]. Groups of 8 rats were exposed at a dose of 6 Gy. At 48 hours post exposure blood samples were obtained by tail puncturing, followed by sampling at 7, 15, 21, 30 and 60 days. The haematological parameters (erythrocyte, leukocyte and leukocyte formula) were measured. In these animals, survival curves up to 60 days were also generated. Genotoxic

effects in leukocytes were assessed by the Comet assay (one hour post irradiation). The effects of sodium alizarinsulfonate were tested following its administration as a single dose of 100 mg/kg (i.p. in saline), one hour before irradiation. In the irradiated animals erythrocytes were depleted (females, $p < 0.01$), and the white blood cell count was drastically reduced with respect to the control (both sexes, $p < 0.01$), also presenting an altered formula. Genetic damage revealed by the Comet assay was significantly reduced by treatment with sodium alizarinsulfonate ($p < 0.01$). The effect of sodium alizarinsulfonate on blood parameters tested was protective in the recovery of erythrocytes in females ($p < 0.01$). No statistically significant protection was observed in the recovery of the leukocyte level or the leukocyte formula (both sexes). However, a significant protective effect was observed for survival ($p < 0.05$).

In previous studies ASR was shown to significantly protect against hepatic necrosis caused by carbon tetrachloride, a compound with radiomimetic action [30]. These studies were significant because they were the first in the literature to show that it was feasible to prevent irreversible cell damage *in vivo* by using specific calcium chelators, such as ASR, calcion or arsenazo III [30-32]. Currently, the critical role of calcium in the mechanisms of cell death is an established fact [33, 34]. An increase in calcium content was observed in cell cultures and in irradiated lymphocytes [35, 36].

Since the effective dose of amifostine differs little from its toxic dose, patients frequently present adverse effects that include hypocalcemia, diarrhea, nausea and vomiting [37]. A probable reason for hypocalcemia could be derived from the phosphatase activity present in different organs and its relationship with the metabolism of amifostine towards its active radioprotective form [16]. Phosphate produced in high concentrations could precipitate calcium, generating hypocalcemia and its consequences. Our experiments showed that this calcium chelator, which has the ability to act within the cell and decrease calcium levels [30], is also capable of causing severe damage in the small intestine [29]. It is of interest to mention that the damage by ASR observed in the duodenum is comparable in intensity to that caused by ionizing radiation, both in females and in males. Moreover, ASR significantly increased the damage caused by X radiation (6 Gy) during the whole observation period of toxic effects. The damage in the intestine was observed at 48 hours and seven days later in both sexes, although with greater intensity in the males. Crypt number in the irradiated animals of both sexes that survived 60 days had improved but not totally. However, the effect of ASR alone on the intestinal crypts always remained significantly altered. This deleterious effect was of the order of magnitude that the ionizing radiation has at 6 Gy. This damaging effect of the chelator could be explained in molecular terms, as it is able to interfere in the regulation of calcemia [38].

Previous studies had provided evidence that it was possible to prevent hepatic damage caused by carbon tetrachloride using different calcium chelators and that this supported the concept that preventing the intracellular accumulation of this element could prevent damage [30-32]. These results seem to agree with the hypothesis on the relevance of calcium homeostasis in relation to the cellular damage caused by different toxicants that altered it [33, 34]. Although carbon tetrachloride is considered a radiomimetic compound with respect to its toxicity, since it generates free radicals and oxidative stress, its action mainly occurs in the liver. On the other hand, exposure of the entire body to ionizing radiation generates deleterious effects throughout the body. For this reason, it is difficult to analyze the fact that ASR has apparently contrary actions; severely damages intestinal crypts but also tends to restore survival at sixty days in irradiated animals. This positive action on survival could be related to protective effects on the initial stage after irradiation, which is however critical for the development of toxicity. This effect is notorious in both sexes but more important in males. Several factors may be involved in this initial stage, modulated by the ASR. One of them could surely be damage to DNA, as shown by the Comet assay in white blood cells. Indeed, this test allows to establish the occurrence of a process of oxidation in DNA bases, triggered by the free radicals of water during irradiation [39]. The radioprotective effect of substances such as amifostine is largely due to

its ability to trap free radicals, particularly hydroxyl. The chemical structure of ASR, containing quinone and phenolic groups, should make it very reactive towards hydroxyl radical. On the other hand, these functional groups are related to toxicity, as is known for other aromatic compounds that generate quinones in their biotransformation [40]. In the case of ionizing radiation for a whole body exposure, the protective capacity of ASR can be explained by its ubiquitous ability to trap the hydroxyl radical. While, the chelator toxicity can be expressed only in certain organs, for example, related to antioxidant defense capacity of each tissue. The efficacy of compounds such as glutathione to neutralize quinone metabolites is known. Such could be the case of ASR, which in previous studies showed no toxicity to the liver [30].

In fact, in these studies, it was observed that exposure to the chelator caused a significant decrease in the glutathione levels of the duodenum. This favors the generation of oxidative stress with the consequent tissue damage. These findings are consistent with those noted in [38] such as intestinal damage by other compounds, poor calcium absorption etc. In those cases, the damage produced by substances that generate oxidative stress in the intestine and decrease glutathione could be reversed with antioxidants [41]. Another factor that could have a role in improving the survival of animals irradiated and treated with ASR is the moderate hypothermia produced by the chelant, as observed by others [42].

6. Ethyl pyruvate

Ethyl pyruvate is a scavenger of free radicals and reactive oxygen species, which has some properties that can compensate for the problems and limitations of the use of amifostine as a radioprotector. A previous study in the literature mentions that ethyl pyruvate protects against the damaging effect of ionizing radiation, behaving as a mitigating agent of the lethal effect of it when it was administered after irradiation [43]. These studies were limited by their short duration. Ethyl pyruvate can be administered by different routes, has a very low toxicity, allowing its repeated administration for longer periods of time in relation to the survival that is sought to be determined after exposure to important doses of ionizing radiation.

On the other hand, ethyl pyruvate was shown to be an effective protector for a wide variety of pathologies and particularly beneficial in the management of animal models of sepsis and inflammatory processes [44-49]. Precisely, severe inflammatory processes and damage to the cells of the intestinal crypts are observed after exposure to ionizing radiation and their lethal effects are very relevant [50].

Due to its liposolubility and rapid absorption, ethyl pyruvate can reach all tissues easily and thus exert its protective action. For example, in murine models of Parkinson's disease, it acted as a neuroprotector [44-46]. In summary, it was considered relevant to perform studies related to the joint use of amifostine and ethyl pyruvate in situations of acute exposure to ionizing radiation [51]. The combination of both substances was considered helpful even if the ethyl pyruvate also protects the cancer cells while amifostine does not.

To study the potential protective effects of both compounds on the early damage of ionizing radiation, alterations in leukocyte DNA were made one hour after irradiation of the animals. The Comet test allows to establish if a hydroxylation process has occurred in the DNA bases. Both substances, amifostine and ethyl pyruvate, showed a significant protective effect on this type of damage. However, the action of amifostine was more important than that of pyruvate although it did not fully reverse the effect of radiation [51, 52]. These would suggest feasible alternatives to improve the protective effect, by increasing the dose. In the case of amifostine, however, it would not be desirable to do so since it could cause an increase in its adverse effects. Ethyl pyruvate, due to its low toxicity, would not present this risk at higher doses [45, 46, 49]. Even its rapid absorption would allow oral administration, which was not tested in these experiments since the objective was to test both compounds under equal conditions in terms of their administration, against a damage as fast as that caused by ionizing radiation on the DNA of the leukocytes [51, 53].

Whole body exposure to ionizing radiation has detrimental effects on the central components of blood, erythrocytes and leukocytes. Not only were the total values of both significantly altered, but alterations were also observed in the relative leukocyte formula. Drastic falls were observed in the proportion of lymphocytes accompanied by a significant increase in neutrophils, eosinophils, basophils and monocytes. These results are expected for a human exposure at doses between 1 and 8 Gy, and linked to the hematopoietic syndrome [54]. The recovery of the number of erythrocytes by the use of amifostine combined with ethyl pyruvate was particularly adequate and comparable with the values of the controls. This joint treatment suggests that pyruvate protects against the adverse effects of amifostine on erythrocytes [51]. It is interesting to mention that previous studies by Giannopoulou and Papadimitriou using WR-2721 suggested that this compound increased the proliferation of human endothelial cells and that this effect was reversed by sodium pyruvate [55]. Moreover, they suggested that this could be due to the fact that WR-2721 acted on these cells producing hydrogen peroxide or NO, since sodium pyruvate is able to react with both [55, 56]. Our results would indicate that this hypothesis can also explain the beneficial effect of the combined treatment of amifostine with ethyl pyruvate, compared with the treatment with amifostine alone. The production of H_2O_2 and NO would damage the erythrocytes by hemolysis, and ethyl pyruvate would protect them by efficiently trapping these reactive molecules [51].

Treatments with amifostine, with ethyl pyruvate or with the mixture of both were not able to prevent the decrease that the radiations produced on the total number of leukocytes. On the other hand, they did produce important differences in the relative leukocyte formula. The biological significance of these effects is not clear from the experiments carried out.

A central aspect of our experiments with ethyl pyruvate was to study the impact of treatments with radioprotective potential on animal health, particularly survival and the percentage change in weight versus irradiation. Since the objective of these studies was aimed at reducing the risks derived from the adverse effects of amifostine, it was considered relevant to test the preventive effect of an initial dose of this substance and continue the treatment with another low toxic compound such as ethyl pyruvate, with a potential mitigating action and that could be administered orally continuously for a considerable time. Thus, during a month after the irradiation and application of the amifostine dose, ethyl pyruvate was administered in the drinking water as a 0.3% solution. Three different doses of amifostine were tested to assess the radioprotective efficiency as a function of the dose administered and then a dose that could be combined with ethyl pyruvate was chosen to evaluate its efficacy. Radioprotective action of amifostine depended markedly on the dose used. Thus, with the highest dose (200 mg/kg) 90% of the animals of both sexes irradiated survived until the 60th day of the trial and the deaths happened within the first month. With the other two doses tested (100 and 50 mg/kg) mortality increased substantially, with only 25% of the animals surviving at 60 days post irradiation. The beginning of the deaths was anticipated, which occurred from day 3. The remaining animals survived the two months of the trial. With the lowest dose of amifostine (50 mg/kg) mortality in treated males increased drastically and occurred at earlier times than those observed for the highest dose. Only 35% of the males survived at 10 days [51]. By the end of the study, 25% had survived. In the case of females, the results were qualitatively and quantitatively different with regard to the onset and magnitude of mortality. In contrast to what was observed for males, the radioprotective effect of amifostine was greater in the females, although without statistical significance with respect to the irradiated group without treatment [51].

As a consequence of this evaluation, it was decided to use the intermediate dose of amifostine of 100 mg/kg to continue studies on the adjuvant and mitigating effect of ethyl pyruvate orally in both sexes. Although treatments with ethyl pyruvate or with amifostine (100 mg/kg) failed to significantly improve survival in either sex, in the case of females, the combined treatment of ethyl pyruvate with amifostine produced a statistically significant recovery.

It was also considered important to study the behavior of treatments on the weight variation of

animals of both sexes by possible adverse or favorable interactions, taking into account that the same ionizing radiation severely affects growth. In the females none of the treatments tested produced a significant change in the evolution of the weight in the period of two months. The administration of these same treatments in the non-irradiated males did not alter the weight variation curve either.

Irradiation severely affected the growth of both females and males although the effect was more significant in the latter [51]. In the 20 days after irradiation, a weight loss was observed in the survivors of both sexes, which is consistent with the mortality that occurred in those days.

The treatments in the irradiated animals produced a different behavior between the sexes. In the females, the treatments did not restore the growth values from the decrease caused by the X radiation, although they suggest a tendency to improve after approximately 40 days. In males, treatment with WR-2721 or with ethyl pyruvate did not significantly improve the fall in the growth curve due to the action of ionizing radiation. However, combined treatment of both drugs increased the relative weight variation almost up to control levels. Joint analysis of the results of the effects of the treatments on survival with respect to the effects on relative weight variation, suggests that both are due to unrelated or even inversely related factors.

7. Sodium butyrate

Butyrate anion is the main energy source for intestinal microbiota and intestinal epithelial cells, and plays an important role in maintaining the stability and integrity of both. It also has antitumor properties, since it can inhibit cell proliferation, induce cell differentiation, promote apoptosis and reduce the invasiveness of tumor cells, thus playing an important role in colon health [57-62]. In the colon, short chain fatty acids are products of the bacterial degradation of the unabsorbed starch and the polysaccharide (fiber) that does not contain starch. They are important molecules in colonic light, which affect both morphology and the function of colonocytes. The three main acids (acetate, propionate and butyrate) stimulate the absorption of fluids and sodium in the colon and exert proliferative effects on the colonocyte [60, 62].

Experimental studies in animals have shown that they promote adaptive responses to small bowel resection and colonic anastomosis. In particular, butyrate has been shown to be the preferred energy substrate for the colonocyte and is a potent differentiating agent in cell culture. The butyrate anion may also have a role in the prevention of certain types of colitis. A diet low in starch and resistant fiber will result in a low production of these short chain acids in the colon, which may explain the high incidence of colonic disorders in Western countries [60].

As mentioned above, acute radiation syndrome involves, among other targets, the bone marrow and gastrointestinal epithelia. The combination of a failure in the immune system with an alteration of the absorption of nutrients and permeability to the bacteria leads to an acute condition that is a cause of high mortality. In this regard, it was considered that the butyrate anion could be an effective mitigating agent to reduce the toxic impact. This substance has been tested as a mitigant of the severe effects of radiotherapy for prostate cancer on the colon, with ambiguous results [63, 64].

In our laboratory, the potential of sodium butyrate as an adjunct to amifostine and as a mitigant of the acute adverse effects of ionizing radiation for a whole-body irradiation in rats was studied [65]. It is important to note that the butyrate anion does not have an important reactivity towards free radicals, and hence it was not considered as a radioprotective agent but as a mitigating agent.

Results obtained showed a significant protective effect of amifostine in the dose used on DNA damage in both sexes. However, its action was less important than that shown in previous studies, for radiation doses and shorter exposure times [51]. These differences can be interpreted as a function of the irradiation time, related to the rapid pharmacokinetics of this substance, which in the present condition should be at lower effective concentrations against damage as fast as that caused by ionizing radiation on the leukocyte DNA [53]. Our data showed the damaging effects of ionizing radiation on erythrocytes and leukocytes

during whole-body exposure. Not only were the total values of both significantly altered, but significant alterations in the relative leukocyte formula were also observed. There could be drastic decreases in the proportion of lymphocytes accompanied by a significant increase in neutrophils, eosinophils, basophils and monocytes. As mentioned above, these results are comparable with those reported for a human exposure at doses between 1 and 8 Gy, and linked to the hematopoietic syndrome [54]. The treatment with amifostine combined with sodium butyrate was effective for the recovery of the number of erythrocytes, reaching values comparable to those of the controls (in the case of females the recovery was after a month, and for males it took a little more time) [65].

The treatment with amifostine and sodium butyrate was not able to prevent the severe decrease that the radiation produced on the total number of leukocytes in the first few days. However, it allowed the survival and slow recovery of the leukocyte levels until reaching the control values at 21 days in males and 28 days in females. Regarding the relative leukocyte formula, in both sexes a complete recovery was observed in the survivors at day sixty [65].

The impact of X-radiation and subsequent treatment with a radioprotective potential agent on survival and percentage variation of weight in both sexes was also studied. As expected, the radioprotective action of amifostine depends markedly on the dose used [51]. Thus, with the dose of 100 mg/kg mortality was very high, with only 10% of the animals of both sexes surviving 60 days after irradiation, similar to the effect caused by radiation alone. However, the combination treatment of amifostine at that dose with sodium butyrate significantly improved survival in both sexes. Both in the case of females and males deaths occurred before eleven days, a time similar to that of radiation alone.

It was also considered important to study the effect of the treatment on the weight variation of the animals of both sexes due to possible adverse or favorable interactions, taking into account that the same ionizing radiation severely affects body weight. Irradiation with 6 Gy drastically affected the weight of both females and males and caused high mortality in a few days [65]. This same situation was observed for the group irradiated and treated with amifostine only. In the females, the treatment with amifostine and sodium butyrate managed to compensate the weight loss in 20 days after the irradiation while the males could recover before (on day 9). It is likely that these variations in weight are due to several factors: the malabsorption caused by the damage to the intestinal epithelium, the general decline in the health of the animals, the loss of fluids due to diarrhea and the protective effect of the treatment. In particular, the absence of diarrhea in the treated animals was notorious, which may be related to the protective effect of butyrate, added to the fact that this substance contributes useful calories in a rapidly absorbable form. In any case, the combined treatment of both drugs never caused the weight to reach up to the control levels. Finally, it should be noted that in both females and males none of the treatments tested *per se* produced a significant change in the evolution of the weight in the two-month period.

Ionizing radiation produces very severe alterations in the epithelium of the small intestine and this effect could be verified in the experimental model studied. A quantitative way to evaluate it is through the number of crypts per intestinal circumference [65]. Results showed that combined treatment of amifostine with sodium butyrate managed to recover the number of crypts significantly in both sexes, although without reaching the control values. On the other hand, the histological alterations observed and also described by other authors, such as lower villous height, edema or vascular damage, were also reduced by the treatment, as could be observed in the specimens taken from the surviving animals at sixty days [65].

8. Conclusions

The goal of an ideal radioprotective treatment is still far from being achieved. Several causes can be identified to explain the difficulty in achieving a successful outcome. In the first place, the nature of the toxic process of the syndrome, very intense and with several critical target organs affected, makes difficult the choice of radioprotective substances that can achieve effective concentrations in short times. For example, many compounds

known for their antioxidant capacity and efficiency for reacting with free radicals were not effective when tested *in vivo* [17]. Behind this several reasons can be found, such as poor bioavailability, an inadequate route of administration, instability in biological media and, of course, the self toxicity. Second, the ideal radioprotective effect should be sustained for a time after exposure. This in turn is linked to two aspects: the containment of cellular damage processes following the action of free radicals and the repair of affected cell structures and functions. This stage, which in the radiation protection therapeutics is called mitigation of the damage, is not easy to accomplish either because there are several critical deleterious processes that condition survival.

In our laboratory, many substances and natural products were tested for their radioprotective potential, separately or in combination with amifostine. Several of those trials failed, illustrating what was mentioned above. Substances that are efficient scavengers of free radicals *in vitro* could not improve survival, even though administered prior to irradiation. Others, with known mitigating capacity against harmful effects that ionizing radiation shares with many toxic substances, revealed toxicity *per se*, which is undesirable particularly in those organs most affected by radiation (eg, calcium chelating agents and intestinal epithelium). Two of the treatments tested were successful and promising, and share characteristics that are obviously critical to the therapeutic efficacy, i.e. a very low toxicity and good bioavailability and ability to mitigate the gastrointestinal syndrome due to their antibiotic properties. This seems to be a critical step in the development of ARS, by giving time for the recovery of the immunogenic capacity of the bone marrow. Thus, both ethyl pyruvate and sodium butyrate could cooperate with the radioprotective action of a low dose of amifostine to improve the survival significantly, against important doses of radiation.

Beyond the effectiveness of each of these compounds to prevent or mitigate the acute harmful effects of ionizing radiation it seems clear that a single substance can hardly achieve it and it is more convenient to use formulas whose components provide properties that are cooperative or complementary [17, 41, 50]. This not only allows us to approach the problem from different aspects of the pathology, but in practice it can mean a decrease in the dose of each compound, if they have any toxicity. These initial radiation protection studies attempted to develop treatments that reduce or prevent the observed damages, despite the fact that in the case of humans, for doses between 1 and 6 Gy the recovery prognosis was good [54].

All personnel involved in the containment of a radiological incident should have an early therapeutic alternative for eventual exposure to relevant doses of ionizing radiation. In this sense, it is obvious that the low toxicity of the treatment is crucial in order not to cause an additional problem to be solved [17].

In summary, in our working hypothesis we considered relevant to develop and deepen studies related to the joint use of amifostine with adjuvants in situations of acute exposure to ionizing radiation. The goal is to identify and characterize natural molecules (or derivatives thereof) with very low toxicity, which allow to substantially reduce the dose of amifostine as a radioprotector (or replace it completely) and continue with a mitigating therapy. These treatments have a high potential for transfer to clinics due to their low toxicity, which would facilitate their approval for use in humans [7].

ACKNOWLEDGEMENTS

We wish to thank CITEDEF, MINDEF (PIDDEF 11/12 and 03/16), CONICET and UNSAM for their financial support to do the research.

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflicts of interest regarding the research mentioned in this article.

REFERENCES

1. IARC-WHO. 2000, IARC Monographs on the evaluation of carcinogenic risk to humans. Volume 75. Ionizing radiation. Part 1: X- and gamma-radiation and neutrons, IARC Press, Lyon, p. 59-101.
2. Riley, P. 1994, Int. J. Rad. Biol., 65, 27.

3. Coleman, C., Blakely, W., Fike, J., MacVittie, T., Metting, N., Mitchell, J., Moulder, J., Preston, R., Seed, T., Stone, H., Tofilon, P. and Wong, R. 2003, Radiat. Res., 159, 812.
4. Macia i Garau, M., Calduch, A. and Casanovas López, E. 2011, Rep. Pract. Oncol. Radiother., 16, 123.
5. Rose, P. 1996, Semin. Oncol., 23, 83.
6. Kligerman, M., Turrisi, A. 3rd., Urtasun, R., Norfleet, A., Phillips, T., Barkley, T. and Rubin, P. 1988, Int. J. Radiat. Oncol. Biol. Phys., 14, 1119.
7. Winczura, P. and Jassem, J. 2010, Cancer Treat. Rev., 36, 268.
8. van der Vijgh, W. and Peters, G. 1994, Semin. Oncol., 21(5 Suppl. 11), 2.
9. Cairnie, A. 1983, Radiat. Res., 94, 221.
10. Maier, P., Wenz, F. and Herskind, C. 2014, Strahlenther Onkol., 190, 745.
11. Jensen, S., Pedersen, A., Vissink, A., Andersen, E., Brown, C., Davies, A., Dutilh, J., Fulton, J., Jankovic, L., Lopes, N., Mello, A., Muniz, L., Murdoch-Kinch, C., Nair, R., Napeñas, J., Nogueira-Rodrigues, A., Saunders, D., Stirling, B., von Bültzingslöwen, I., Weikel, D., Elting, L., Spijkervet, F. and Brennan, M. 2010, Support Care Cancer, 18, 1039.
12. Bardet, E., Martin, L., Calais, G., Alfonsi, M., Feham, N., Tuchais, C., Boisselier, P., Dessard-Diana, B., Seng, S., Garaud, P., Aupérin, A. and Bourhis, J. 2011, J. Clin. Oncol., 29, 127.
13. Gu, J., Zhu, S., Li, X., Wu, H., Li, Y. and Hua, F. 2014, PLoS One, 9, e95968.
14. Ormsby, R., Lawrence, M., Blyth, B., Bexis, K., Bezak, E., Murley, J., Grdina, D. and Sykes, P. 2014, Cell Biol. Toxicol., 30, 55.
15. de Toranzo, E. and Castro, J. 1994, Free Rad. Biol. Med., 17, 605.
16. Valles, E., de Castro, C. and Castro, J. 1995, Exp. Mol. Pathol., 63, 101.
17. Weiss, J. and Landauer, M. 2009, Int. J. Radiat. Biol., 85, 539.
18. Floyd, R., Towner, R., He, T., Hensley, K. and Maples, K. 2011, Free Radic. Biol. Med., 51, 931.
19. Castro, J. and Castro, G. 2002, Methods in Molecular Biology, "Oxidative stress biomarkers and antioxidant protocols", D. Armstrong (Ed.), Humana Press Inc., Totowa, 88.
20. Maciel, M., Quintans, L., Costantini, M., Formosa Lemoine, F., López, G., Montalto de Mecca, M., Díaz Gómez, M., Castro, J. and Castro, G. 2015, Appl. Res. Toxicol., 1(Suppl.), 75.
21. Maciel, M., Quintans, L., Costantini, M., Formosa Lemoine, F., López, G., Montalto de Mecca, M., Díaz Gómez, M., Castro, J. and Castro, G. 2015, Bioquímica y Patología Clínica (Suppl. Agosto 2015), 114.
22. Singh, V., Beattie, L. and Seed, T. 2013, J. Radiat. Res., 54, 973.
23. Câmara Grilo, E., Nunes Costa, P., Santos Sânzio Gurgel, C., de Lima Beserra, A., de Souza Almeida, F. and Dimenstein, R. 2014, Food Sci. Technol., 34, 379.
24. Barakat, I., Abbas, O., Ayad, S. and Hassan, A. 2011, J. Am. Sci., 7, 664.
25. Said, U., Saada, H., Shedid, Sh., Mahdy, E. and Shousha, W. 2008, Egypt J. Biochem. Mol. Biol., 26, 101.
26. Castro, G., Maciel, M., Díaz Gómez, M., Costantini, M., Formosa Lemoine, F., Montalto de Mecca, M., López, D. and Castro, J. 2017, Medicina, 77(Suppl. 1), 456.
27. Moura, F., de Andrade, K., dos Santos, J. and Goulart, M. 2015, Curr. Top. Med. Chem., 15, 458.
28. Castro, G., Montalto de Mecca, M., Maciel, M., Díaz Gómez, M., Costantini, M., Formosa Lemoine, F., López, D. and Castro, J. 2017, Medicina, 77(Suppl. 1), 296.
29. Castro, G., Maciel, M., Díaz Gómez, M., Costantini, M., Montalto de Mecca, M., Formosa Lemoine, F., López, D. and Castro, J. 2017, Medicina, 77(Suppl. 1), 349.
30. de Ferreyra, E., Villarruel, M., Bernacchi, A., de Fenos, O. and Castro, J. 1992, Exp. Mol. Pathol., 56, 197.
31. de Ferreyra, E., Villarruel, M., Bernacchi, A., Fernández, G., de Fenos, O. and Castro, J. 1989, Arch. Toxicol., 63, 450.
32. de Ferreyra, E., Bernacchi, A., Villarruel, M., de Fenos, O. and Castro, J. 1993, Exp. Mol. Pathol., 58, 194.
33. Orrenius, S., Nicotera, P. and Zhivotovsky, B. 2011, Toxicol. Sci., 119, 3.
34. Zhivotovsky, B. and Orrenius, S. 2011, Cell Calcium, 50, 211.

35. Szumiel, I., Sochanowicz, B. and Buraczewska, I. 1990, Int. J. Radiat. Biol., 58, 125.
36. Hallahan, D., Bleakman, D., Virudachalam, S., Lee, D., Grdina, D., Kufe, D. and Weichselbaum, R. 1994, Radiat. Res., 138, 392.
37. Zhang, H., Hao Yan, H., Xiaoliang Zhou, X., Wang, H., Yang, Y., Zhang, J. and Wang, H. 2017, BMC Complement. Altern. Med., 17, 410.
38. Díaz de Barboza, G., Guizzardi, S. and Tolosa de Talamoni, N. 2015, World J. Gastroenterol., 21, 7142.
39. Tice, R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. and Sasaki, Y. 2000, Environ. Mol. Mutagen., 35, 206.
40. Cavalieri, E., Rogan, E. and Zahid, M. 2017, Int. J. Cancer, 141, 1078.
41. Shadad, A., Sullivan, F., Martin, J. and Egan, L. 2013, World J. Gastroenterol., 19, 199.
42. Alva, N., Palomeque, J. and Carbonell, T. 2013, Oxid. Med. Cell Longev., 957054.
43. Epperly, M., Jin, S., Nie, S., Cao, S., Zhang, X., Franicola, D., Wang, H., Fink, M. and Greenberger, J. 2007, Radiat. Res., 168, 552.
44. Fink, M. 2003, Crit. Care Med., 31(1 Suppl.), S51.
45. Fink, M. 2007, Curr. Drug Targets, 8, 515.
46. Fink, M. 2008, Curr. Opin. Anaesthesiol., 21, 160.
47. Kao, K. and Fink, M. 2010, Biochem. Pharmacol., 80, 151.
48. Olek, R., Ziolkowski, W., Flis, D., Fedeli, D., Fiorini, D., Wierzba, T. and Gabbianelli, R. 2013, J. Nutr. Sci. Vitaminol. (Tokyo), 59, 232.
49. Fedeli, D., Falcioni, G., Olek, R., Massi, M., Cifani, C., Polidori, C. and Gabbianelli, R. 2007, J. Appl. Toxicol., 27, 561.
50. Berger, M., Christensen, D., Lowry, P., Jones, O. and Wiley, A. 2006, Occup. Med. (Lond.), 56, 162.
51. Maciel, M., Quintans, L., Díaz Gómez, M., Costantini, M., Formosa Lemoine, F., Montalto de Mecca, M., López, G., Castro, J. and Castro, G. 2016, Acta Bioquím. Clín. Latinoam., 50, 733.
52. Müller, A., Pigorsch, S., Beyer, C., Lautenschläger, C. and Dunst, J. 2004, Strahlenther Onkol., 180, 517.
53. Wang, Y., Xu, C., Du, L., Cao, J., Liu, J., Su, X., Zhao, H., Fan, F., Wang, B., Katsube, T., Fan, S. and Liu, Q. 2013, Int. J. Mol. Sci., 14, 22449.
54. International Atomic Energy Agency. 1997, Effects of ionizing radiation on blood and blood components: A survey, IAEA, Vienna, IAEA-TECDOC-934, 7.
55. Giannopoulou, E. and Papadimitriou, E. 2003, Free Radic. Res., 37, 1191.
56. Liu, J., Litt, L., Segal, M., Kelly, M., Pelton, J. and Kim, M. 2011, Int. J. Mol. Sci., 12, 6469.
57. Wang, Y., Hu, P., Ma, Y., Fan, R., Gao, F., Zhang, J. and Wei, L. 2016, Ultrastruct. Pathol., 40, 200.
58. Böcker, U., Nebe, T., Herweck, F., Holt, L., Panja, A., Jobin, C., Rossol, S., B Sartor, R. and Singer, M. 2003, Clin. Exp. Immunol., 131, 53.
59. Wu, X., Wu, Y., He, L., Wu, L., Wang, X. and Liu, Z. 2018, J. Cancer, 9, 2510.
60. Scheppach, W. 1994, Gut, 35(1 Suppl.), S35.
61. Peng, L., Li, Z-R., Green, R., Holzman, I. and Lin, J. 2009, J. Nutr., 139, 1619.
62. Bach Knudsen, K., Lærke, H., Hedemann, M., Nielsen, T., Ingerslev, A., Gundelund Nielsen, D., Theil, P., Purup, S., Hald, S., Schioldan, A., Marco, M., Gregersen, S. and Hermansen, K. 2018, Nutrients, 10, 1499.
63. Mete, L., Assisi, D. and Casale, V. 2007, Dig. Liver Dis. Supp., 1, 23.
64. Maggio, A., Magli, A., Rancati, T., Fiorino, C., Valvo, F., Fellin, G., Ricardi, U., Munoz, F., Cosentino, D., Cazzaniga, L., Valdagni, R. and Vavassori, V. 2014, Int. J. Radiat. Oncol. Biol. Phys., 89, 518.
65. Costantini, M., Díaz Gómez, M., Formosa Lemoine, F., Montalto de Mecca, M., López, G., Castro, J. and Castro, G. 2019, Acta Bioquím. Clín. Latinoam., in press.