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Ischemia–reperfusion injury: assessment and treatment, part II

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Abstract

Objective: To review the current scientific literature on ischemia–reperfusion (IR) injury in both human and veterinary medicine and to describe the assessment of IR injury, the available testing methods, and the options available for treatment.
Data sources: Data sources include scientific reviews and original research publications in both human and veterinary medicine.
Summary: The assessment of IR injury includes measuring products formed by the reaction of reactive oxygen species (ROS) with biological membranes, measuring levels of endogenous antioxidants, and measuring ROS themselves. Testing depends on the laboratory used, the test method chosen, the sample submitted (i.e., plasma, urine, tissue, etc.), and the timing of the test in relation to sample collection. For this reason, testing is not standardized and pharmacological data on antioxidant effectiveness are not available. Antioxidants and drugs tested have included single agents as well as 'cocktails' consisting of several agents working at different key points in the injury cascade.
Conclusions: There are several new testing methods as well as new strategies for attempting to ameliorate the damage inflicted upon reperfusion and this article is intended as a review of the assessment and treatment of IR injury.

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Keywords: glutathione, hydroxyl radical, isoprostanes, lipid peroxidation, malondialdehyde, reactive oxygen species, superoxide

Introduction

The assessment of ischemia–reperfusion (IR) injury includes measuring products formed by the reaction of reactive oxygen species (ROS) with biological membranes (i.e., markers of lipid peroxidation), levels of endogenous antioxidants, and ROS.[1] Testing for markers of IR injury is not standardized and is fraught with inaccuracies depending upon the test used, the laboratory method, the parts sampled (i.e., tissue, serum, urine, cerebrospinal fluid, etc.), and the timing of the test in relation to sample collection. This last point is crucial since auto-oxidation *ex vivo* occurs in many of the testing methods and samples used.[2,3] Newer, more accurate testing methods appear promising and may be significantly more sensitive and specific than many of the methods currently in use.[4,5]

Numerous treatment strategies have been tried in an attempt to alleviate some of the pathologic manifestations of IR injury. Again, because testing is not standardized, the results of treatments vary dramatically and it is difficult to discern what role, if any, the treatment played. The timing of treatment has ranged from treating before the onset of ischemia, treating after ischemia but before reperfusion, and treating after reperfusion. Single agents as well as combinations consisting of several agents working at different key points in the injury cascade have been used. Much of the research in this area has focused on laboratory animals treated pre-ischemia and the success seen in the laboratory is often not replicated in a clinical scenario. Ischemic preconditioning involves exposing the animal to brief periods of ischemia before the

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'ischemic event'. Although this has been shown to offer some benefits in laboratory animals, the clinical relevance in veterinary medicine is limited.[6]

There are several new testing methods as well as new strategies for attempting to ameliorate the damage inflicted upon reperfusion and this article is intended as a review of those as well as a review of the pathophysiology of IR injury.

Assessment of oxidative stress

Elucidating the specific damage caused by ROS in various diseases and measuring the effect of treatment with exogenous substances is challenging. Over the last 20 years, one of the greatest needs regarding measuring ROS injury has been the development of sensitive and specific, non-invasive, standardized tests. Because of this lack of standardization, the clinical pharmacology of antioxidants cannot be effectively studied. In addition, much of the research in IR injury involves methodologies that are either not directly applicable or are not practical in clinical situations.

Histological samples can be assessed for changes that reflect oxidative damage (i.e., determination of XO and XD activities) before and after treatment via tissue biopsy. This method is rarely practical in clinical situations.

More clinically applicable measures of oxidative injury include quantification of products formed by reaction of ROS with biological membranes, concentrations of ROS, and amounts of endogenous antioxidants.

Measurement of reaction products

Lipids are a common substrate for attack by ROS and are the most extensively studied for markers of oxidation.[7] Breakdown products of lipid peroxidation include malondialdehyde (MDA), conjugated dienes, short-chain alkanes, and lipid hydroperoxides (Figure 1). When endoperoxides combine with unsaturated fatty acids and free iron, MDA can be formed.[8] The most commonly used test to assess MDA concentrations is the thiobarbituric acid reactive substances (TBARS) test. This test was first used by chemists to measure the rancidity of fats.[9] There are numerous problems with this test. Up to 98% of MDA that reacts with TBARS is formed after collection, during the incubation period of the assay.[2] There is an inconsistent level of sensitivity and specificity when these tests are applied *in vivo*. In addition, there are artifactual increases of lipid peroxidation products outside of the body occurring in many testing methods.[10] Tests must be conducted expediently to minimize spontaneous oxidation *ex vivo*, falsely increasing levels of oxidation by-products.

MDA is not a specific product of lipid peroxidation. It can also be formed as a result of thromboxane synthesis, which becomes significant when serum or plasma are used as samples, as there is likely some

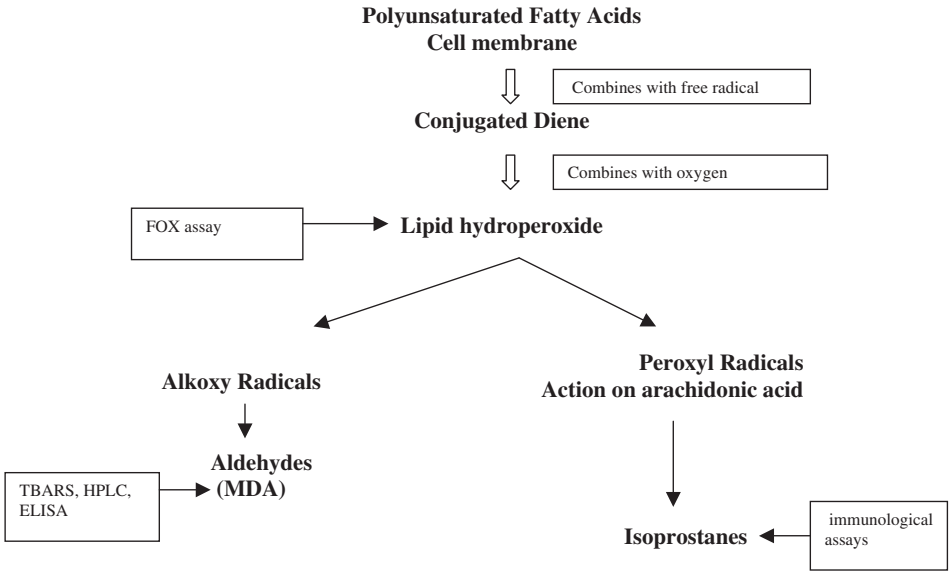

Figure 1: Methodologies for testing in reperfusion injury. Schematic representing the breakdown products of ROS damage on biological membranes. Polyunsaturated fatty acids in the cell membrane are damaged by free radicals. Conjugated dienes are formed that combine with oxygen to form lipid hydroperoxide, which can be analyzed using the FOX assay. The aldehydes formed can be measured using TBARS, HPLC, and ELISA methodologies. Isoprostanes can be measured using immunological assays. MDA, malondialdehyde; FOX, ferrous oxidation of xylenol; TBARS, thiobarbituric acid reactive substances; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay.

degree of platelet activation.[11] The results of tests for lipid peroxidation are highly dependent on the specific details of the testing procedure. Although TBARS is applicable *in vitro*, the specificity is improved *in vivo* if the TBARS-MDA adducts are measured after separation by high-performance liquid chromatography (HPLC) or gas chromatography and then identified by mass spectrometry (MS).[12] Most important discoveries on the pathological consequences of increased lipid peroxidation were made using the TBARS test,[9] and it is still the most frequently used test to measure lipid peroxidation.[13] Recently, several laboratories have developed immunological assays, such as the enzyme-linked immunosorbent assay (ELISA), for determination of proteins modified by lipid peroxidation products (i.e., MDA levels).[9] This is an improvement over the previously cumbersome method, using the TBARS assay.[7]

When ROS attack arachidonic acids on cell membranes, isoprostanes are formed.[14] These prostaglandin-like compounds are created via 3 precursors into 4 classes. They are produced *in vivo* independent of the cyclo-oxygenase enzyme by free radical-catalyzed peroxidation of arachidonic acid.[15] These are chemically stable compounds that reportedly have high sensitivity and specificity regarding measurement in several diseases including IR injury.[4,16,17]

The isoprostane most commonly studied is the αF_2-isoprostane. Much evidence now exists indicating that the F_2-isoprostanes are a reliable, non-invasive way to measure lipid peroxidation *in vivo* compared with other methods.[18] In one study, F_2-isoprostanes increased dramatically in an animal model of oxidant stress and correlated with the degree of tissue damage.[19] The administration of antioxidants has been shown to inhibit the formation of F_2-isoprostanes in both animal models of oxidant injury and in humans.[20,21] Isoprostanes can be detected in all types of biological fluids and tissues; the free form can be measured in urine and plasma, esterified complexes can be measured in tissue, or metabolites can be measured in urine.[2,15] In addition, the liver, testes, heart, brain, skeletal muscle, aorta, kidney, lung, eye, bile, cerebrospinal fluid, and gastric fluid have all been shown to contain detectable levels of F_2-isoprostanes.[15]

Currently, up to 64 different isoprostanes arising from the oxidation of arachidonic acid have been discovered. As mentioned, the isoprostane studied most extensively is αF_2-isoprostane or 8-iso$PGF_2\alpha$. There is disagreement as to the correct nomenclature of the isoprostanes. Several research groups refer to them as iso-prostaglandins, while others recommend referring to them as isoprostanes. The reference to isoprostanes is to distinguish them from the cyclo-oxygenase-derived prostaglandins.[15] One potential problem with detection is auto-oxidation occurring in lipid-containing samples during processing and storage. Because blood contains appreciable quantities of arachidonic acid, it may not be the best medium for testing oxidation. This is not a problem with urine from humans and dogs due to the low levels of lipid. When human urine was incubated at 37 °C for 1 week, there was no increase in F_2-isoprostanes.[22] In several studies, urinary excretion of F_2-isoprostanes correlated with oxidative stress *in vivo*.[23]

Much recent research suggests that the measurement of urinary F_2-isoprostanes as a non-invasive marker of lipid peroxidation may be one of the best markers of oxidative damage *in vivo*.[7] A study evaluating oxidative stress in sled dogs during endurance trials showed significantly increased levels of F_2-isoprostanes in plasma compared with controls.[24] Interestingly, there appears to be a trend for F_2-isoprostanes to increase with age in humans, adding credence to the hypothesis that aging is due to increased oxidation of biological membranes with age.[25]

At least 2 isoprostanes may actually cause oxidative injury, in addition to their function as markers.[26] While highly accurate, the current methodology to assess F_2-isoprostanes, the mass spectrometric method, is labor intensive and not readily available.[4] Testing is expected to gain wider acceptance as immunoassays for specific isoprostanes become more available.[27] The author has recently reported elevated F_2-isoprostanes in the urine of dogs with intervertebral disc disease compared with healthy control dogs.[28]

The ferrous oxidation of xylenol, or FOX, assay is based on the oxidation of ferrous iron to ferric iron. The ferric ions can be detected with xylenol, a ferric-sensitive dye. This assay is believed to give highly reproducible signals in biological fluids, but as mentioned in part I, there are numerous pathways to produce the ferrous ion.[2]

Oxidation of ω-3 polyunsaturated fatty acids (PUFA) results in increased exhalation of ethane gas, and oxidation of ω-6 PUFA results in increased exhalation of pentane gas. The measurement exhaled of these gases has been studied as a marker of oxidation in several diseases including rheumatoid arthritis,[29] myocardial infarction,[30] and ARDS.[31] Several limitations exist with this methodology, including contamination by air pollution,[32] contamination by isoprene gas, which is present in human breath and interferes with testing,[33,34] and production of the gases by bacteria.[35]

Measurement of ROS

Owing to their brief half-life, ROS are very difficult to measure in biological systems. Electron paramagnetic

resonance (EPR) spectroscopy appears to be a direct method of detecting free radicals. The unpaired electron present in free radicals gives rise to a typical absorbance spectrum when placed in a magnetic field. A special technique, called spin trapping, forces free radicals to react with a scavenger or spin trap to produce a more stable free radical that has a longer half-life and a spectrum that can then be identified using EPR spectroscopy.[36]

In a study on ischemic muscle flaps of dogs exposed to 4 hours of ischemia, EPR signals characteristic of free radical adducts were only detected in 5 of 9 dogs.[37]

Measurement of endogenous antioxidants

Measurement of specific antioxidants in blood or tissues can be used as an indicator of oxidative stress if the levels of antioxidants are low.

Glutathione peroxidase is a tripeptide molecule, comprised of glycine, glutamine, and cysteine, which is an essential part of the endogenous antioxidant defense system. Glutathione, the substrate used by the enzyme glutathione peroxidase, exists in 2 forms, reduced glutathione (GSH) and the oxidized form, glutathione disulfide (GSSG). During oxidative damage, GSH is oxidized to GSSG. Measurement of the ratio of GSH to GSSG can be used to assess oxidative damage via depletion of GSH and has been reported in dogs and cats.[38] This method is also susceptible to spontaneous oxidation *ex vivo* and artificially elevated GSSG levels. In one study, the plasma concentration of GSSG increased by 500–1000% after an ischemic challenge, and this was correlated with increased lipid peroxidation.[39] Depletion of GSH can cause irreversible cell damage and death.[40] Center et al.[38] reported decreased levels of GSH from liver biopsies of dogs and cats with necroinflammatory disorders, extrahepatic bile duct occlusion, and feline hepatic lipidosis compared with healthy dogs and cats.

In sled dogs, α-tocopherol was shown to decrease significantly after an exercise run, suggesting that the endogenous antioxidant capacity may not be adequate for the challenges of vigorous racing.[24] In another study aimed at decreasing oxidative damage in racing sled dogs, Baskin et al.[41] reported that supplementation with α-tocopherol, β-carotene, and lutein increased plasma concentrations of these antioxidants significantly in a population of Alaskan sled dogs.

Treatment of IR injury

The treatment of IR injury involves blocking the formation of ROS, scavenging ROS after they are formed, blocking neutrophils, and preventing platelet activation. Some other treatment modalities include Na^+/H^+ exchange inhibitors, nitric oxide (NO) donors or NOS antagonists, treatment with adenosine, endothelin receptor antagonists,[42] angiotensin receptor antagonists,[43] propofol,[44] grape extract,[45] and hyperbaric oxygen.[46]

The treatment designed to block neutrophils results in decreased lipid peroxidation, suggesting that the neutrophil-derived ROS are responsible for a large portion of the damage in humans, rats, and dogs.[47–52] However, the administration of inhibitors of XO-derived ROS also appears to attenuate the damage inflicted upon reperfusion, suggesting that the XO-derived ROS are responsible for a portion of the damage.[52] Xanthine oxidase-derived ROS do not appear to be a major factor in rabbit intestinal injury or horse large intestinal IR injury.[53,54]

The major derivation of ROS (i.e., XO or neutrophil derived) most likely differs between species and also between organs. In addition, the major endogenous antioxidants may differ between organs and species. Glutathione peroxidase has been shown to be the major cardiomyocyte antioxidant.[55,56]

It is likely that the best treatments will encompass a combination of drugs that target several steps in the IR injury cascade. In the past 20 years, approximately 1000 types of interventions have attempted to ameliorate IR injury.[56] Some of the current treatment strategies that appear promising are highlighted.

Blocking neutrophils

As stated previously, neutrophils appear to play a significant role in IR injury. Since resident or invading neutrophils appear to be responsible for much of the damage, which occurs upon reperfusion, blocking them should be beneficial. Blockade of leukocyte and endothelial adhesion molecules has been shown to decrease tissue injury in several animal models of reperfusion. Pretreatment with monoclonal antibodies against ICAM-1 prevents hepatic IR injury in rats.[57] Prevention of neutrophil infiltration reduces IR injury in skeletal muscle, heart, lungs, liver, and kidney.[47–52] Pretreatment of cats with a monoclonal antibody against β_2 integrins decreases neutrophil influx, as assessed by myeloperoxidase (MPO) activity, after IR injury.[58] However, clinical trials of anti-adhesion molecules that block neutrophil adhesion have been disappointing.[59–62] In a clinical trial of CD-18 blockade in patients with myocardial infarct, stroke, and traumatic shock, there were no significant differences in the endpoints between placebo and controls.[63] There were 2 prospective, double-blind, placebo-controlled trials in human traumatic shock victims assessing the effective-

ness of the CD-18 monoclonal antibody and there were no differences in the primary endpoints including mortality between the 2 groups.[64,65] There are several possible explanations for the disappointing results. There may be a pathway of neutrophil adhesion to the endothelium that does not involve the β_2 integrins. It is also possible that neutrophils are not a significant component of IR injury in a clinical setting. It has been suggested that these laboratory animal models may not be adequate models for human clinical disease.[63]

Blocking formation of ROS

Glutathione can act both as a chain-breaking antioxidant, inhibiting lipid peroxidation, and as a metal chelator, preventing the formation of the hydroxyl radical.[66] GSH is synthesized in all mammalian cells with the rate of synthesis dependent upon cysteine stores in most organs except the liver. In the liver, GSH can be synthesized from either cysteine or methionine and the liver is the primary site for GSH synthesis, supplying up to 90% of circulating GSH.[67,68] When GSH is given exogenously it cannot penetrate cell membranes.[69] Cysteine is the rate-limiting amino acid in the formation of GSH and treatment with *N*-acetylcysteine (NAC) enables continued production of GSH. NAC is also a powerful scavenger of both the hydroxyl radical and hypochlorous acid.[70] The protective effects of NAC are believed to be associated with the sulfhydryl groups trapping electrophilic intermediates by acting as a nucleophile.[71] Treatment with NAC is protective against endotoxin challenge, radiation-induced injury, and lung injury from toxic gas.[72–74] In a rat model of IR injury, NAC blocked NFκB activity in addition to scavenging ROS.[75] It has attenuated IR injury during cardiac catheterization and has shown cardioprotective effects during ischemia.[76] NAC has also shown some benefit in both sepsis and ARDS patients.[77,78]

Vitamin E, composed of tocopherols and tocotrienols, is a lipid-soluble vitamin that antagonizes the peroxidative injury of membrane lipids and inhibits the propagation of cell membrane destruction. It converts the alkylperoxyl radicals to hydroperoxides and then to tocopheroxyl radicals. The tocopheroxyl radicals are then reduced by vitamin C. Vitamin E, C, and ubiquinol destroy ROS involved in the 'chain reaction' of lipid peroxidation.

Vitamin C (ascorbic acid) is a water-soluble vitamin that allows the regeneration of vitamin E for continued antioxidant effects. Ascorbic acid reduces the tocopheroxyl radical back to the antioxidant tocopherol. It also has pro-oxidant properties and can act as an oxidant during times of increased free iron such as blood transfusions or inflammation. Vitamin C reduces ferric iron to ferrous iron, which under normal conditions improves the absorption of iron from the GI tract. Under conditions of ischemia or increased availability of free iron, vitamin C can function as a pro-oxidant by providing more ferrous iron for the generation of a hydroxyl radical (via the Haber–Weiss reaction).[79] In patients with coronary artery disease, endothelial dysfunction was attenuated by the administration of vitamin C and this appears to be due to superoxide scavenging by vitamin C.[80] However, it appears that vitamin C must be given in very high concentrations to compete effectively with NO for superoxide.[81]

Ubiquinol appears to act as an antioxidant but the exact mechanisms are not clear. It appears to prevent both the initiation and propagation of lipid peroxidation.[82]

Calcium channel blockers have several theoretical benefits. Blocking calcium influx may prevent the conversion of XD to XO, decreasing XO-derived ROS. In addition, since intracellular calcium can be cytotoxic, blocking its influx may prevent cell death. Several studies have shown beneficial results in experimental models. In adult rabbits, improved blood flow and reduced infarct size were seen in focal ischemia when the calcium channel blocker, nimodipine, was used.[83] However, most animal studies have only shown a benefit when the drug is given before the onset of ischemia.[83,84] Severe systemic hypotension was seen in a study on human neonates given the calcium channel blocker nicardipine.[85] In several studies, calcium channel blockers were beneficial in treating the 'no flow' phenomenon, and were able to increase flow in those areas previously blocked.[86–88]

Allopurinol is a structural analog of hypoxanthine that competitively inhibits XO, preventing the formation of superoxide. Cats pretreated with allopurinol had attenuation of microvascular permeability and decreased neutrophil infiltration in several models of IR injury.[89–91] Improved kidney function after transplantation was shown with allopurinol and it is now a component of the preservative solution in most transplant centers.[92,93] Since allopurinol appears to work best with pretreatment, its utility in veterinary medicine is limited.

Scavenging ROS

Superoxide dismutase exists on the extracellular surface, in the cytosol, and in the mitochondria. It scavenges superoxide anion and converts it to hydrogen peroxide. If there is not sufficient catalase available

to convert the hydrogen peroxide to water, then hydrogen peroxide will accumulate and contribute to the formation of the hydroxyl radical. In this case, SOD can be considered to be a pro-oxidant. A defect in the gene for SOD exists in the familial form of amyotrophic lateral sclerosis (i.e., Lou Gehrig's disease), a degeneration of motor neurons in the spinal cord, brain stem, and motor cortex of humans. People with this disease have a buildup of superoxide due to lack of SOD.[94]

Exogenous SOD has been shown to be protective in many models of IR injury. Its short half-life may be a factor in the studies that showed no improvement.[95,96] In renal transplants, it has been shown to decrease acute rejection and improve 4-year graft survival.[97,98]

Catalase converts hydrogen peroxide to water and oxygen. Pretreatment in cats with IR injury of the small intestine decreased neutrophil infiltration.[99] It is essential that catalase be present along with SOD to convert the hydrogen peroxide produced by SOD to water and oxygen. The paired administration of SOD and catalase conjugate has been shown to be effective in attenuating IR injury in several models.[100,101]

Since free iron is central to the formation of the hydroxyl radical, many treatment strategies attempt to block iron. However, iron is essential to many biological processes and iron chelation therapy can have potentially toxic side effects when they interfere with normal iron metabolism. Most strong chelating agents remove ferric iron from proteins (i.e., transferrin) and can interfere with iron incorporation into hemoglobin.[102] Deferoxamine chelates ferrous iron and has been shown to reduce IR injury in several models.[103–105]

One study in 24 dogs, with experimentally created GDV, used deferoxamine (an iron chelator) and dimethylsulfoxide (DMSO) as potential treatments for IR injury. Although there was improved survival in the deferoxamine group, there were no direct measures of oxidative damage.[106]

Several studies evaluating deferoxamine have been unrewarding most likely due to the toxic side effects and its short half-life in circulation in humans ($\sim$5 minutes).[107] Trials using deferoxamine complexed to high-molecular-weight species (e.g., dextrans) to prolong the half-life are underway.[108] Galey [102] is working on iron chelators that circulate in an inactive form and then are activated by ROS, making them active at the site where they are needed most.

DMSO scavenges the hydroxyl radical and the metabolite that is formed traps other ROS. It permeates cell membranes to get to intracellular sites of ROS formation, and is also thought to inhibit platelet aggregation and increase vasodilation. It can lead to the formation of the methyl radical, which can then react with PUFAs to form methane gas or can react with oxygen to form methyl peroxyradicals. Pretreatment with DMSO decreased microvascular permeability and neutrophil infiltration in both cat and rat models of IR injury.[99,109,110] However, no improvement was seen when DMSO was given before ischemia or after reperfusion in horses with IR injury.[111,112] It is believed that the levels of DMSO needed to scavenge the hydroxyl radical may be high enough to cause damage to healthy cells.[113] There are no studies documenting beneficial effects with DMSO in IR injury in horses.

The 21-aminosteroids (lazaroids) are a modification of glucocorticoids. They were created to enhance the antioxidant effects (increase ROS scavenging), while minimizing the glucocorticoid and mineralocorticoid activity. Some also bind and inactivate iron. A 21-aminosteroid, U74006F, given before reperfusion decreased tissue MDA concentrations but not plasma MDA concentrations in a GDV model in dogs, after reperfusion.[114] Although they have great promise, there has been a long delay in getting these drugs onto the market and the eagerly awaited anticipation in the medical community seems to have waned.

NO is a vasodilator that inhibits platelet aggregation, downregulates cellular adhesion molecules, and blocks monocyte migration. Molsidomine, a NO donor, significantly increased survival in a rodent model of IR injury when given 30 minutes prior to reperfusion.[115]

NO scavengers also play a role in IR injury. When NOX-100, a NO binder, was given to a rat cardiac transplant model in combination with cyclosporine A, there was increased graft survival and decreased NFκB activity.[116]

During the breakdown of ATP during ischemia, there is a buildup of adenosine. Adenosine is also released by neutrophils, endothelial cells, and myocytes. Interestingly, adenosine, in high concentrations, is believed to be responsible for the benefit seen with ischemic preconditioning.[117] In addition to stimulating A1, A3, and potassium ATP channels, adenosine may inhibit the conversion of XD to XO during ischemic periods. If this is true, then the accumulation of adenosine during ischemia would result in decreased ROS formation.[117] Adenosine is also believed to decrease the release of superoxide radical by neutrophils[118] and to decrease leukocyte adhesion.[119] Adenosine increases the synthesis of NO via A2 receptor binding. In a canine hepatic IR injury model, adenosine attenuated oxidative damage to proteins. There was also inhibition of neutrophil accumulation, superoxide production, and a decrease in the rise of aspartate aminotransferase and alanine transaminase.[120] Adenosine, however, can cause hypotension and atrioventricular block when administered intravenously.[6,121]

Na^+/H^+ exchange inhibitors

Intracellular pH drops during the ischemic phase, leading to the activation of the Na^+/H^+ pump.[6] Hydrogen ion leaves the cell in exchange for sodium. The intracellular sodium continues to increase during ischemia. The Na^+/K^+ ATPase is inhibited under acidotic conditions and cannot extrude sodium. The Na^+/Ca^{2+} exchanger is then activated in order to eliminate sodium and intracellular calcium increases.[6] This is the rationale for the use of Na^+/H^+ exchange inhibitors for IR injury. In a randomized, multi-center, placebo-controlled trial in humans with myocardial infarction, a Na^+/H^+ exchange inhibitor attenuated left ventricular dysfunction postreperfusion.[122]

Conclusion

The pathology of IR injury is widespread and diverse and is seen in a number of diseases affecting companion animals. A high priority has been the development of sensitive and specific, non-invasive tests that can be standardized and used to assess the damage that occurs during IR injury. This would allow assessment of the effectiveness of antioxidants clinically. Many of the methodologies used to assess IR injury in the past are not practical in clinical cases. The most extensively studied breakdown product of lipid peroxidation is MDA. There are many pitfalls with assessing MDA concentrations including spontaneous oxidation *ex vivo* in the samples. Isoprostanes, a product of ROS action on arachidonic acids, have been shown to be good indicators of oxidative stress in several experimental and clinical trials. Sled dogs had higher concentrations of plasma isoprostanes during endurance trials[24] and dogs with intervertebral disc disease had a higher concentration of isoprostanes than normal dogs.[28] Other methods of evaluating oxidative stress include the FOX assay and the exhalation of pentane gas. Both of these methods have serious limitations. Depletion of GSH is another method used to assess oxidative damage and decreased concentrations of GSH have been reported from liver biopsies of dogs and cats with hepatic disease.[38] Measurements of antioxidants can be used to assess the endogenous store, and supplementation with α-tocopherol, β-carotene, and lutein was shown to increase plasma concentrations of these antioxidants in a population of Alaskan sled dogs.[41]

Owing to the enormous complexity of the IR injury disease process, a treatment that encompasses just one target is likely to show conflicting results. At this time, no single agent has been shown to ameliorate IR injury completely. The best treatments will, most likely, include a combination of therapies that target several steps in the IR injury cascade. Treatments that have been attempted include supplementation with antioxidants, blocking neutrophils, calcium channel blockers, ROS scavengers, NO scavengers, adenosine, and Na^+/H^+ exchange inhibitors.

Treatments that have shown some promise in small animals include neutrophil blockers in cats treated before IR injury,[58] allopurinol administered to cats before IR injury, and deferoxamine and DMSO given to dogs with experimentally created GDV.[106]

In the future, testing may become more standardized as immunological assays become more widely available. Ideally, once testing is standardized, clinical trials can begin to assess the efficacy of a number of antioxidant compounds.

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