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REDOX STATUS ASSESSMENT IN THE BLOOD TISSUE IN A CONTRAST MEDIA INDUCED NEPHROPATHY ON A RABBIT MODEL

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"Alle Ding sind Gift und nichts ohn' Gift,
allein die Dosis macht, das ein Ding kein Gift ist."
All things are poison and nothing (is) without poison,
only the dose makes that a thing is no poison

Paracelsus, 1493-1541

Contents

Abstract

Introduction: Contrast Media (CM) are widely used substances in every day hospital clinical practice either in diagnostic or in interventional procedures, with a few adverse effects. Iopromide, the substance of choice, is a low osmolar, non-ionic X-ray contrast agent for intravascular administration. Contrast-induced nephropathy (CIN) is thought to be a hospital acquired nephropathy, in cases which no other underlying cause is observed. The pathophysiology of CM is not yet completely known. Three main mechanisms have described to be involved in that: a) hemodynamic effects, b) increase in oxygen free radicals and reactive oxygen species (ROS), and c) direct CM molecule tubular cell toxicity. The aim in the present study is to assessment the reduction-oxidation status in blood tissue in a rabbit model after contrast media administration and the possible correlation with a potential renal injury. The majority of the studies conducted in rodent models. Rabbit is an easy to handle animal and susceptible to nephropathy.

Methods: Ten New Zealand White healthy rabbits were used in this study, divided in three groups. In Control group n=3, was administrated only NaCl 0,9% iv. appr. 2 ml/minute in a 30 minute time period and the animals were euthanized 48 hours after the end of the administration. In group N1Dx, n=4, was administrated 8 gr/kg iv iopromide, in a 30 minute period and the animals were euthanized in 24 hours after the end. In group N2Dx, n=3, was administrated 8 gr/kg iv iopromide, in a 30 minute period and the animals were euthanized in 48 hours after the end. Blood sampling was conducted at 0 hours (before administration), 2, 10, 24 and 48 hours (only in Control and N2Dx) after the end of the administration. Urea and Creatinine were analyzed in blood serum by a standard absorbance photometry. For the histopathological examination of the kidneys, hematoxylin and eosin stain was used and evaluated under light microscopy. Total Activity Capacity (TAC) was measured by the method of Janaszewska and Bartosz, Catalase by the method of Aebi et al., TBARS by a slightly modified assay of Keles et al. and Protein Carbonyls by the method of Patsoukis et al. Also, hemoglobin concentration determined by hemiglobincyanide (HiCN) method.

Results: a) Blood serum Creatinine and Urea started to elevate at two hours after administration in both groups, over 25%, and return to baseline in 48 hours, b) histopathological evaluation and scoring presents a similar type of necrosis in the tubular cells in both groups where the alterations reached a certain plateau, c) bivariate Pearson correlation indicates that there are significant correlations between 24 hours TAC and CREA and UREA (r=0,912, p=0,088 and r=0,916, p=0,084 resp.), 48 hours TBARS with CREA and UREA (r= -0,981, p=0,003 and r= -0,985, p=0,002 resp.), also in control TAC with 24 hours TAC (r=0,955, p=0,045) and control CAT with 24 hours CAT (r=0,987, p=0,013), d) in an ANOVA statistical analysis, TBARS decreased in both groups at 10 hours after administration 37% (p=0,050) in N1Dx group and 55% (p=0,042) in N2Dx group respectively, and remain at the same levels in 24 and 48 hours. Plasma's protein carbonyls in N2Dx group increased

significantly in 2 hours after administration, 67% (p=0,000), and in 24 and 48 hours dropped significantly in a percentage 27% and 29% respectively (p=0,003 and p=0,002) in comparison with the 2 hours.

Conclusion: High doses of iopromide seem to compromise the natural renal function in a healthy population, in which endogenous mechanisms overcome the impairment. Reduction-oxidation disturbance plays an important role in the pathophysiology of Contrast Induced Nephropathy. Even though, enzymatic and nonenzymatic systems are present in every living organism to regulate the injury, an assistant is needed. Systemic alterations stimulate further investigation.

Key words: Contrast media, blood, redox status, rabbit, iopromide, kidney, oxidative stress

Abbreviations: im, intramuscular; iv, intravenous; CM, Contrast Media; NO, nitric oxide, HOCM, high osmolar contrast media; IOCM, iso-osmolar contrast media; LOCM, low osmolar contrast media; CIN, contrast induced nephropathy; CI-AKI, Contrast-Induced Acute Kidney Injury; LD50, lethal dose; ROS, reactive oxygen species; TAC, Total Antioxidant Capacity; CAT, catalase; PCT, proximal convoluted tubule; TBARS, thiobarbituric acid–reactive substances;Crea,creatinine

Introduction

Since the time of Hippocrates, physicians have sought better tools to diagnose disease. Röntgen's discovery of x-rays enabled physicians to use this modality in improving diagnosis of disease in patients. Radiology has rapidly evolved, and the improvement has partly been successful thanks to different types of contrast media that have been invented to better visualize tissues[1].

Contrast medium, *plural contrast media*, is a substance, such as barium or air, used in radiologic studies to increase the contrast of an image. In x-ray imaging, a positive contrast medium absorbs x-rays more strongly than the tissue or structure being examined, a negative contrast medium, less strongly. (*By The American Heritage® Science Dictionary Copyright © 2002. Published by Houghton Mifflin*).

The first radiographic contrast medium to be used in clinical practice was sodium iodide, which was introduced in the 1920s. However, high toxicity and poor radiographic contrast limited the clinical use of this preparation.[2] In the 1950s ionic monomers were developed, in which for every three atoms of iodine, two particles in solution exist (ratio between atoms of iodine and particles in solution, 3:2=1.5). The osmolality of such agents is five to eight[2,3] times the osmolality of the plasma, and they are usually called "high-osmolality" contrast media.[4] Torsten Almén a Swedish radiologist, noted that the pain with angiography was associated with the osmolality[5]. Research aimed at the reduction of osmolality led to the synthesis of dimeric ionic contrast media (molecules containing six atoms of iodine but actually dissociating in solution into an anion and a cation, ratio 6:2=3). Both monomeric non-ionic compounds and dimeric ionic agents are named "low-osmolality" contrast media, however, their osmolality it is approximately twice as that of plasma[4]. In the 1980s non-ionic dimeric contrast media were introduced. Two non-ionic tri-iodinated benzoic rings were attached, giving an iodine: particle ratio of 6:1 since there are six iodine atoms and only one active particle in each molecule. The osmolality of this class of contrast media is similar to that of the blood.[2] The iodinated agents are positive contrast agents because iodine atoms, which are incorporated into their chemical structures, are efficient absorbers of x-rays in the diagnostic energy range[6].

Physicochemical Properties of Contrast Media

Contrast media have traditionally been classified by their physical and biochemical properties[7,8]:

S t r u c t u r e is related to the number of benzene rings per molecule (degree of polymerization). The basic structure of all currently used CM consists of a 2, 4, 6 tri-iodinated benzene ring. The structural composition of iodinated CM is either a single tri-iodinated

benzene ring (monomer) or two bound benzene rings (dimer). Monomers and dimers can be either ionic or nonionic depending on their side chain constituents.

I o n i c i t y refers to the conjugation of the benzene ring structure (anion) with a non-radio-opaque cation resulting in a water-soluble compound. Ionic monomeric CM dissociate (ionize) in solution (i. e., in the bloodstream) into 1 anion and 1 cation, resulting in an iodine-to-particle ratio of 3:2 (3 iodine atoms for 2 ions). Nonionic monomeric CM consist of tri-iodinated benzene rings with hydrophilic hydroxyl groups and organic side chains placed at the 1, 3, 5 positions, which do not ionize in solution, resulting in an iodine to particle ratio of 3:1. Dimeric CM can be composed of either 2 bound nonionic monomers or a bound nonionic and ionic monomer, resulting in iodine-to-particle ratios of 6:1 and 6:2, respectively. The iodine-to-particle ratio and the concentration of iodine-bearing molecules in solution affect the osmolality and amount of radio-opacity of a given CM, respectively.[9]

O s m o l a l i t y refers to the concentration of osmotically active particles in a solution. The normal osmolality of blood is 280–295 mOsm/kg H_2O.[10] Contrast media used in procedures are often referred to as high osmolar (HOCM, typical osmolality 1400–2016 mOsm/kg H_2O), low osmolar (LOCM, typical osmolality 600–844 mOsm/kg H_2O) or iso-osmolar (290 mOsm/kg H_2O)

V i s c o s i t y refers to the intrinsic resistance of a material to changing form and is determined primarily by the chemical structure of CM, differences in organic side chain composition, iodine concentration and temperature[8]. Factors, such as molecular size and complexity of side chains, may lead to steric hindrance of bond torsion angles, restricting rotation and resulting in a more rigid molecule with higher viscosity. In general, viscosity is directly related to particle size and inversely related to osmolality[11]. As with osmolality, CM may be categorized as high-viscosity CM (HVCM) or low-viscosity CM (LVCM)

Iodinated water-soluble contrast agents are of four types[12]:

- High-osmolar ionic monomers (for example amidotrizoate (diatrizoate) iodamide, iotalamate, ioxitalamate, metrizoate)
- Low-osmolar ionic dimers (for example ioxaglate)
- Low-osmolar non-ionic monomers (for example iobitridol, iohexol, iomeprol, iopamidol, iopromide, ioversol)
- Iso-osmolar non-ionic dimer (for example iodixonal, iotrolan).

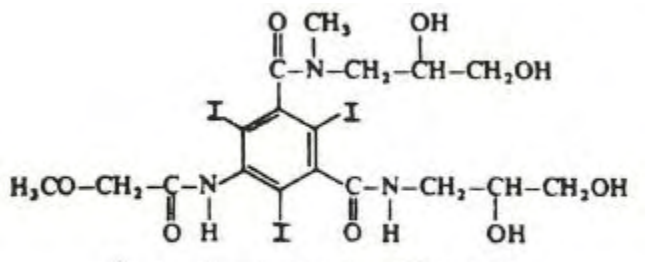
In this study the contrast media of choice was iopromide.

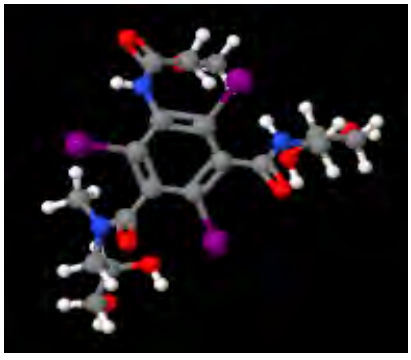
Iopromide

Iopromide is a low osmolar, non-ionic X-ray contrast agent for intravascular administration. It functions as a contrast agent by opacifying blood vessels in the path of flow of the contrast agent, permitting radiographic visualization of the internal structures until significant hemodilution occurs. Iopromide is used in radiographic studies such as intra-arterial digital subtraction angiography (IA-DSA), cerebral and peripheral arteriography, peripheral venography, excretory urography, brain computer tomography (CT), coronary arteriography, left ventriculography, visceral angiography, and aortography.

Technical Information[13]

Formal Name	N^1,N^3-*bis*(2,3-dihydroxypropyl)-2,4,6-triiodo-5-[(2-methoxyacetyl)amino]- -N^1-methyl-1,3-benzenedicarboxamide
CAS Number	73334-07-3
Synonyms	Ultravist
Molecular Formula	$C_{18}H_{24}I_3N_3O_8$
Formula Weight	791.1
Purity	≥98%
Formulation	A crystalline solid
λ_{max}	241 nm





Tolerance[14]:

LD50 mouse, iv (g l/kg) = 16.5

LD50 rat iv, (g l/kg) = 11.4

Pharmacokinetics in rat after intravenous injection[14]**:**

Excretion via the urine (% of the dose in 3 hr)…..82

Excretion via the bile (%of the dose in 3 hr)……..6

Renal elimination half-life (min)……..16

In principle, contrast media should be injected and leave the body immediately after use in the same condition or undergo natural metabolism without making any harm to the patient. The ideal contrast agent should be totally inert causing no interactions with the organism at any level. Furthermore, the excretion should be rapid and complete[2]. Like all other pharmaceuticals, however, these agents are not completely devoid of risk.[15]

Side effects of Contrast Media

Side effects of radiographic contrast media range from a mild inconvenience to a life-threatening emergency[16]. Main side effects are[17]:

- a) Mild hypersensitivity reactions (<3%), moderate to severe reactions (<0,04%) including life-threatening cardiac arrhythmias, pulmonary edema, seizures, syncope etc. Mortality[18] due to CM reactions is less than one death per 100000 patients. Hypersensitivity reactions to contrast media include both Ig E and non-Ig E-mediated anaphylaxis, with activation of mast cells, coagulation, kinin and complement mechanisms, inhibition of enzymes, and platelet aggregation[19]. Delayed adverse reactions might occur[20], usually mild[21].
  b) Infusion of contrast material in the rabbit leads to histamine release, a decrease in complement, and a fall in microarteriolar pressure, mainly in HOCM[22],[23].
- CM exposure may be associated with development of either hyperthyroidism or hypothyroidism, presumably due to the effect of free, biologically active iodide ions present in the CM preparation.
- Chemotoxic reactions[21]: a) minor: nausea, flushing, warmth, pain at the injection side, b) major: hypotension, dysrhythmias, depressed myocardial contractility, myocardial ischemia (mainly due to HOCM).
- Thrombosis, hemodynamic effects, CM-induced pulmonary edema all might be induced due to the endothelial injury that cause the CM, especially the HOCM.[24]
- Contrast-induced nephropathy (CIN) is a well known adverse reaction associated with the use of intravenous or intra-arterial contrast material[17].

Contrast-induced nephropathy (CIN)

Contrast-induced nephropathy (CIN) is a common hospital-acquired acute kidney injury. Published studies on this condition have dramatically increased in recent years[25].

The **term** contrast medium induced nephropathy refers to the reduction in renal function induced by contrast media. It implies impairment in renal function, an increase in serum creatinine by more than 25% (relative increase) or 44 μmol/L (or more than 0,5 mg/dL, absolute increase) from baseline, occurred within 3 days following the intravascular administration of contrast media and the absence of alternative etiology. Most authors use

that definition which was endorsed by the committee of European Society of Urogenital Radiology (ESUR) in 1999[2,26,27].

There has been no definitive answer to the pathogenesis of CIN in the literature. Human and animal studies have demonstrated some pathophysiological changes in the renal parenchyma after the administration of the contrast media. However, the definite answer to its development is yet to come[28]. Also, is referred as Contrast-Induced Acute Kidney Injury (CI-AKI) which is defined as a sudden deterioration in renal function associated with the administration of iodinated contrast media, in the absence of another etiology[29,30].

When radiographic contrast media are injected intravenously or intra-arterially, they pass from the vascular compartment through capillaries into the extracellular space. They are eliminated almost entirely by glomerular filtration, concentrated in the tubular lumen by water tubular reabsorption, thereby visualizing the urinary tract[17,31]. Thus, the pharmacology of iodinated CM is simple. CM distribute quick within the extravascular space, there is no metabolization and excrete solely by glomerular filtration[32].

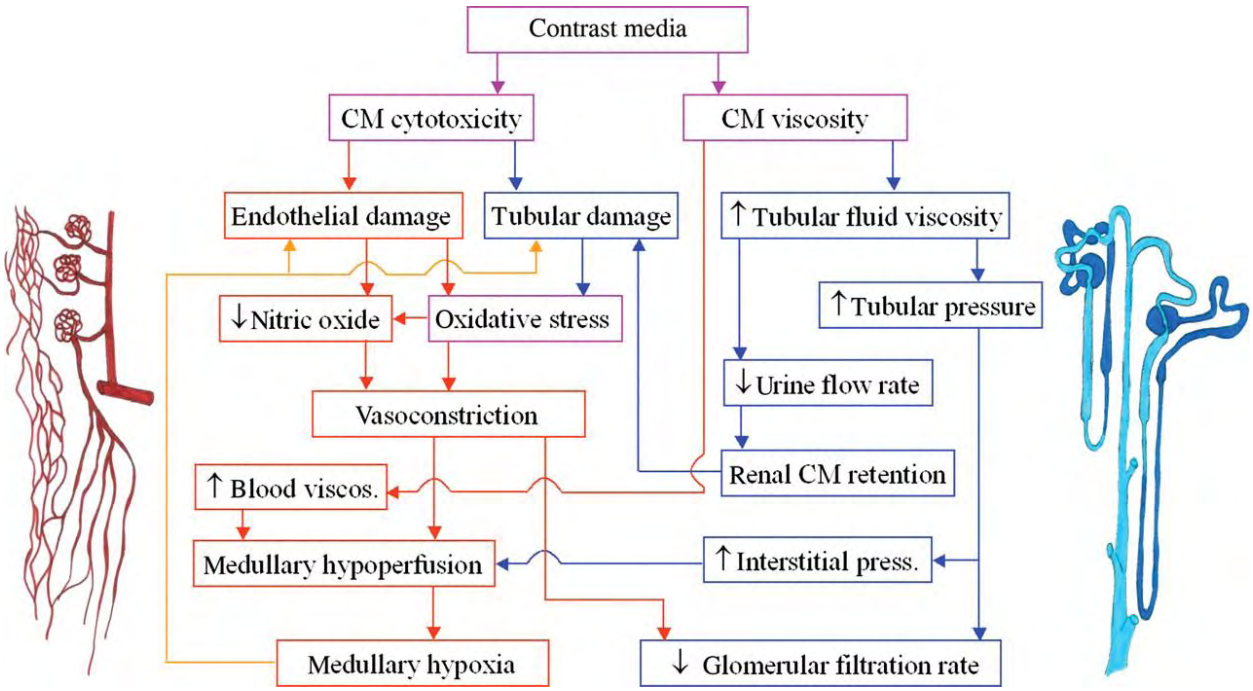
On the contrary, in the pathophysiology of CIN, three major distinct, but potentially interacting pathways are recognized: a) hemodynamic effects, b) increase in oxygen free radicals and reactive oxygen species (ROS), and c) direct CM molecule tubular cell toxicity[33,34].

A) Hemodynamic effects: a biphasic hemodynamic response to contrast injection has been observed in animals[35]. Responses in rabbit and dog renal arteries were similar to those in human renal arteries and could serve as models for investigating CM-induced renal vasoconstriction[36]. Under physiologic resting conditions, a percentage of the cardiac output, via the renal artery, is directed toward the kidneys, and the greater part of it toward the cortex, to optimize glomerular filtration and reabsorption of water and salts. The blood flow toward the medullary region is low, in which its function is to preserve osmotic gradients and enhance urinary concentration. Under physiologic circumstances, oxygen partial pressure (PO_2) levels of the renal cortex are approximately 50 mm Hg, whereas PO_2 levels of the renal medulla can be as low as 20 mm Hg[33]. The injection of contrast induces an initial, transient increase and then a more prolonged decrease in renal blood flow. Alterations in the metabolism of prostaglandin, nitric oxide, endothelin, or adenosine may play a role.[37] It was reported by various studies that adenosine is an important factor involved in the hemodynamic response of the kidney to CM administration and is connected to the generation of vasoconstriction[29,35,38]. Endothelin and adenosine are strong vasoconstrictors in the kidney whereas nitric oxide (NO) and prostaglandin PGE_2 are potent vasodilators[39]. With the decreased renal blood flow coexists a reduction in oxygen consumption which induce hypoxia, more importantly in the outer medulla at the region of the thick ascending limbs of the loop of Henle, the most vulnerable part[33]. The outer medulla is particularly susceptible to ischemic injury because of its high metabolic activity and low prevailing oxygen tension[35]. Low

oxygen supply to the outer medulla is due to the great distance between descending vasa recta (DVR) that supply the medulla with blood[40].

B) Free radicals are atoms or molecules that contain one or more unpaired electrons[34]. The most common oxygen radicals are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). $O_2^-$and OH^- are more reactive than H_2O_2, which is not a radical, but exhibits a greater membrane permeability[41]. Under physiological conditions, medullary tubular ROS formation plays an important role in cellular signaling processes, regulation of regional microcirculation and tubular transport by its effects on nitric oxide (NO) concentration and tubular transport activity. The intense vasoconstriction and loss of autoregulatory capacity can contribute to additional renal injury through the release of ROS[35]. In CM administration, ROS, in combination with medullary hypoxia, leads to cellular ischemia – reperfusion injury. Also, ROS affects mitochondrial and nuclear DNA, membrane lipids and cellular proteins, additionally alters renal microcirculation due to increased in vasoconstriction induced by angiotensin-II and endothelin-I. Furthermore, bioavailability of the vasodilatative NO is reduced by ROS[32]. Nitric oxide (NO) produced by the endothelium (from L-arginine by the enzyme NO synthase[35]) and so, damage in the membrane of the endothelial cells by increased superoxide levels leads to a NO deficiency[42]. Damage to endothelial cells by CM has been reported after injection of CM into rabbits and rats[43]. The result is increased cell injury leading to increased formation of oxygen free radicals and ROS, creating a vicious cycle.

C) The contribution of direct tubular cell toxicity caused by CM is the least understood. This direct cytotoxicity is best studied in vitro, because interactions with hypoxia and other systemic mechanisms cannot be excluded in vivo[44]. All types of CM are concentrated inside the tubules. The extent of direct cytotoxic damage is related to the duration of the exposure to the CM[29]. The osmolality and viscosity of CM probably play a more important role in the functional disturbances of tubules *in vivo* than *in vitro*. In general, the toxic effects of HOCM are more pronounced than the effects of LOCM and IOCM, but all types of CM have negative effects on cell cultures[42]. Prolongation of retention is in part related to the induction of vacuoles in proximal tubular cells[45]. Vacuole formation is a rather non-specific reaction to several drugs and substances and is also described in osmotic nephrosis. Nevertheless, osmolarity does not seem to play a role in the appearance of vacuoles associated with CM. It has also been speculated that vacuolization could represent pinocytosis of CM by tubular cells. Vacuolization can also be a sign of cell membrane damage, indicating an overload of the membrane repair system[42]. More effects of CM on tubular cells have been described, including redistribution of membrane proteins, reduction of extracellular Ca^{2+}, DNA fragmentation, disruption of intercellular junctions, reduced cell proliferation, apoptosis and altered mitochondrial function[44]. Likewise, in direct tubular cell toxicity, the medullary hypoxia in combination with the renal blood are factors that have important role in the mechanism of induction CIN[44].



Simplified scheme depicting major mechanisms of CM-AKI pathophysiology. Contrast media effects that primarily affect the nephron are depicted in blue (see stylized nephrons with glomeruli, tubules and collecting duct at the far left), effects that primarily affect blood perfusion and tissue oxygenation are depicted in red (see stylized vasculature including afferent and efferent arterioles, tufts of glomerular capillaries, peritubular capillaries and descending vasa recta at the far right), and contrast media properties and effects that affect both in pink. The orange arrows indicate a feedback that may result in a vicious cycle: medullary hypoxia aggravates cellular damage that, by several factors, increases vasoconstriction.

Seeliger, Erdmann, Sendeski, Mauricio, Rihal, Charanjit S., Persson, Pontus B., : Contrast-induced kidney injury: Mechanisms, risk factors, and prevention, European Heart Journal,2012,

Oxidative Stress

The concept of oxidative stress has been introduced for research in oxidation-reduction (redox) biology and medicine in 1985[46]. Oxidative damage inflicted by reactive oxygen species has been called “oxidative stress”. Biological systems contain powerful enzymatic and nonenzymatic antioxidant systems, and oxidative stress is an imbalance between pro-oxidants and anti-oxidants in favor of the former[47],[48]. Oxidative stress is the result of the disruption of redox signaling and control in the organism and can cause damage to biomolecules, such as DNA, lipids and/or proteins[48],[49]. Therefore, increased oxidative damage can result not only from excess in oxidative stress, but also from failure of repair or replacement systems[50]. Oxidative stress may be involved in processes such as mutagenesis, carcinogenesis, membrane damage, lipid peroxidation, protein oxidation and fragmentation, as well as carbohydrate damage[51].

Cells, tissues, and body fluids possess powerful defense systems that help counteract oxidative imbalance[52]. Oxidative stress exceeding the antioxidant capacity level may induce oxidative damage, but low-level stress may enhance the defense capacity[53].

The three major antioxidant enzymes are the superoxide dismutases, catalase and glutathione (GSH) peroxidases[51].

At the cellular level, the cells react to oxidative stress in different ways, depending of the type of the cell and the severity of the oxidative stress. Consequences may be any or a combination of any of the following: a) increased proliferation, b) adaptation to the damage completely, partially or overcorrected, c) cell injury, mainly, involve damage to some or all biomolecules, e.g. lipids, proteins, DNA d) senescence of the cells, e) cell death by apoptosis, necrosis, or other type of death[50].

Reduction – Oxidation, **Redox**, reactions are thought to play key role in intracellular signaling, intercellular signaling (e.g. NO) and in regulation of the organ and whole body responses to stress[50].

Free radicals were first described by Moses Gomberg more than a century ago[54,55].

In general, free radicals[56] can be defined as any chemical species that contains unpaired electrons. Unpaired electrons increase the chemical reactivity of an atom or molecule, because are highly unstable. The chemical reactions involved in free radical damage occur almost instantaneously so that direct measurement it is not possible, only the consequences of those actions. Free radicals are formed in large amounts as an unavoidable by-product of many biochemical processes and in some instances, deliberately, such as in activated neutrophils. Furthermore, many different biochemical processes within the body occur that produce free radicals, such as reduction of molecular oxygen during aerobic respiration, production of superoxide and hypochlorous acid (HOCl), which is a powerful

oxidant by activated phagocytes, nitric oxide (NO) production by vascular endothelium and other cells, e.g. in CIN. The simplest free radical is atomic hydrogen, $H^{\bullet}$, since a hydrogen atom has only one electron, which must therefore be unpaired[50].

Reactive Oxygen Species (**ROS**) is a collective term that includes not only the oxygen radicals but also some non-radical derivatives of O_2. The term reactive species has been expanded to include reactive nitrogen, chlorine, bromine, and sulphur species[50].

ROS are either radicals, e.g. hydroxyl radical $OH^{\bullet}$, peroxyl radical $RO_2^{\bullet}$, superoxide radical anion, alkoxyl radical $RO^{\bullet}$, carbonate radical, carbon dioxide radical or reactive non-radical compounds such as singlet oxygen, peroxynitrite $ONOO^{-a}$, hydrogen peroxide H_2O_2, hypochlorous acid HOCl, ozone O_3. Their half-lives vary from a few nanoseconds for the most reactive compounds to seconds and hours for rather stable radicals. ROS react with biologically relevant molecules and modification or destruction of DNA, lipids, carbohydrates and proteins has been related to the pathogenesis of several degenerative diseases[57]. Under normal conditions, living organisms maintain a basal steady-state (stationary) ROS level within a certain range, due to highly efficient ROS-neutralizing intrinsic systems[58], based on the principles of prevention, interception, and repair[52].

On the contrary, ROS seem to be vital to the functions of a living organism, since their total elimination prove to be fatal. Golstein, N. had showed that mice and rats kept in a chamber with $O_2^{\bullet -}$ - free air die within three weeks[59,60]. Also, ROS are important in the innate immune system for the activation of the phagocytic cells to promote the response in a microbial invasion[61,62].

Total antioxidant does not mean all the antioxidants in the defense system in vivo, but **TAC** means the capacity of free radical scavenging by the radical scavenging antioxidants contained in the test samples. The TAC values have been measured also for plasma to assess the in vivo antioxidant and nutritional status[63].

Animal **catalase** contain four subunits, each of which has Fe(III)-haem at its active site. The heme-containing enzyme catalase transforms H_2O_2 into water and oxygen:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

It is widely accepted that the use of only one type of modification to assess oxidative damage during oxidative stress is not sufficient. That is due to the different sensitivity, dynamics, and nature of ROS-promoted modifications.

Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with ROS in the microenvironment and change in response to increased redox

stress. DNA, lipids, including phospholipids, proteins and carbohydrates are examples of molecules that can be modified by excessive ROS in vivo[64].

In determining whether to use lipids, DNA, or proteins as a marker of oxidative stress, the nature of the ROS will play a significant role[65].

ROS react with multiple, long-chain, unsaturated fatty acids in cell membranes to generate lipid peroxidation products, particularly malondialdehyde (MDA)[66]. Lipids are susceptible targets of oxidation because of their molecular structure abundant with reactive double bonds. Two of the most well studied markers of lipid peroxidation are isoprostanes (IsoPs) and malondialdehyde (MDA)[64]. MDA is typically quantified from plasma samples with the most popular method being a colorimetric assay based on the reaction between MDA and thiobarbituric acid (TBA). The impact of **lipid peroxidation** in membranes is that compromises their function, decreases fluidity, inactivates the membrane-bound receptors and enzymes and increases the permeability to certain ions[67].

Oxidative Stress-induced Protein Oxidation. Elevated levels of oxidized protein are present in animals and cell cultures following their exposure to various conditions of oxidative stress. Thus, exposure of animals or cell cultures to either hyperoxia, forced exercise, ischemia-reperfusion, rapid correction of hyponatremia, paraquat toxicity, magnesium deficiency, ozone, neutrophil activation, cigarette smoking, x-radiation, chronic alcohol treatment, or mixed function oxidation systems leads to an increase in the level of oxidized protein[68].

Carbonyl (CO) groups, aldehydes and ketones, are produced on protein side chains when they are oxidized, especially of Pro, Arg, Lys, and Thr. These moieties are chemically stable, which is useful for both their detection and storage. Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation. Accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease, diabetes, inflammatory bowel disease, and arthritis[65].

The membrane of erythrocytes consists of about 44% lipids, 49% protein and the rest of carbohydrate, and the cytoplasm is rich in protein, where the 33% of it is hemoglobin. Erythrocytes must preserve their membrane intact for a long period of time. Their lack of mitochondria and the ability of synthesized new proteins and lipids make them unable to replace their membrane in case of oxidation[50].

Hemoglobin is normally an intracellular protein in red blood cells (see Blood paragraph). When erythrocyte passes through the lung capillaries heme binds O_2. Heme iron in deoxyhemoglobin is in the Fe^{+2} state, so sometimes a molecule of oxyhemoglobin releases $O_2^{\bullet-}$, an H_2O or an OH^- replacing the O_2 [heme-Fe^{+2} – O_2 → $O_2^{\bullet-}$ + heme-Fe(III)]. Fe(III) product, methemoglobin, cannot bind O_2 [50]. When hemoglobin is exposed to excess H_2O_2 or

lipid peroxides is degraded, releasing heme and iron ions, from heme ring breakdown. Heme and iron can stimulate lipid peroxidation, and iron ions can form $OH^{\bullet}$ from H_2O_2. At lower H_2O_2 **:** protein ratios, hemoglobin is converted to heme ferryl species. Heme and hemoglobin outside their normal location are potentially damaging molecules. Thus, erythrocytes carry inside a potential pro-oxidant.

Kidney tissue

The kidney consists of three distinctive regions: renal cortex, renal medulla and the renal pelvis. The renal medulla, furthermore, is divided in to outer medulla and inner medulla, and the outer medulla in to the outer stripe and the inner stripe.

The nephron[69] is the basic functional unit of the kidney. It is the smallest part of the kidney that can carry out its basic functions. Each nephron is composed of a renal corpuscle, a proximal convoluted tubule, a loop of Henle, a distal convoluted tubule and collecting duct.

The cortex contains the cortical labyrinth, which includes the renal corpuscles, distal and proximal convoluted tubules, and the medullary rays, which are comprised of the straight tubules and collecting ducts. The outer medulla of the kidney contains in the outer stripe straight tubules and collecting ducts and in the inner stripe thin tubules. The inner medulla contains thin tubules and collecting ducts[70].

The renal corpuscle is located in the cortex of the kidney, and it consists of the glomerulus (tuft of glomerular capillaries) and Bowman's capsule. Bowman's capsule is a double-walled capsule, with a parietal (outer) layer and a visceral (inner) layer, where the visceral layer is made up of podocytes. The podocytes covering the capillaries have spaces between them, creating a permeable layer through which fluid and dissolved substances can pass during filtration. The space between the visceral and parietal layers is the capsular space. The function of the renal corpuscle is to filter blood in the first stage of urine production. The fluid that is filtered out of blood is called the glomerular filtrate.

The proximal convoluted tubule (PCT) is a continuation of the capsular space of Bowman's capsule. It is the longest part of the tubular system of the nephron. The epithelial cells that line the PCT are cuboidal and have a brush border, which help in the production of the tubular filtrate or primitive urine.

The loop of Henle continues from the PCT, descends into the medulla of the kidney, makes a U-turn, and heads back up into the cortex. The distal convoluted tubule (DCT) is a continuation of the ascending part of the loop of Henle. Collecting ducts are a series of tubules that carry tubular filtrate through the medulla into the calyces, which lead to the renal pelvis. Also, they regulate the acid-base balance control, potassium levels, urine volume through the action of antidiuretic hormone (ADH).

The renal pelvis is a urine collection chamber that forms the beginning of the ureter.

Also, the macula densa, mesangial cells and granular cells construct the juxtaglomerular apparatus, which is located at the vascular pole of the renal corpuscle. The

macula densa controls the composition of the fluid and provides feedback to cells to control the blood flow through the glomerulus. The mesangial cells believed to be phagocytic and the granular cells produce renin.

Each kidney has a very large blood supply. Up to 25% of the blood pumped by the heart goes to the kidneys. Blood enters the kidney through the renal artery which branches as the interlobar artery, which continues as the arcuate artery and then branches between lobules as the interlobular artery. The interlobular artery continues as an afferent glomerular arteriole which forms the glomerular capillary bed and then exits as the efferent glomerular arteriole, a second capillary bed. These capillaries are known as the peritubular capillaries that surround the nephron and converge to form venules that in turn converge to form larger veins that eventually become the renal vein. Superficial and midcortical glomerular blood vessels provide a capillary network surrounding the tubules of the cortex, while juxtamedullary glomeruli form the vasa recta capillary bed surrounding the straight and thin tubules of the medulla, the loop of Henle[69,70].

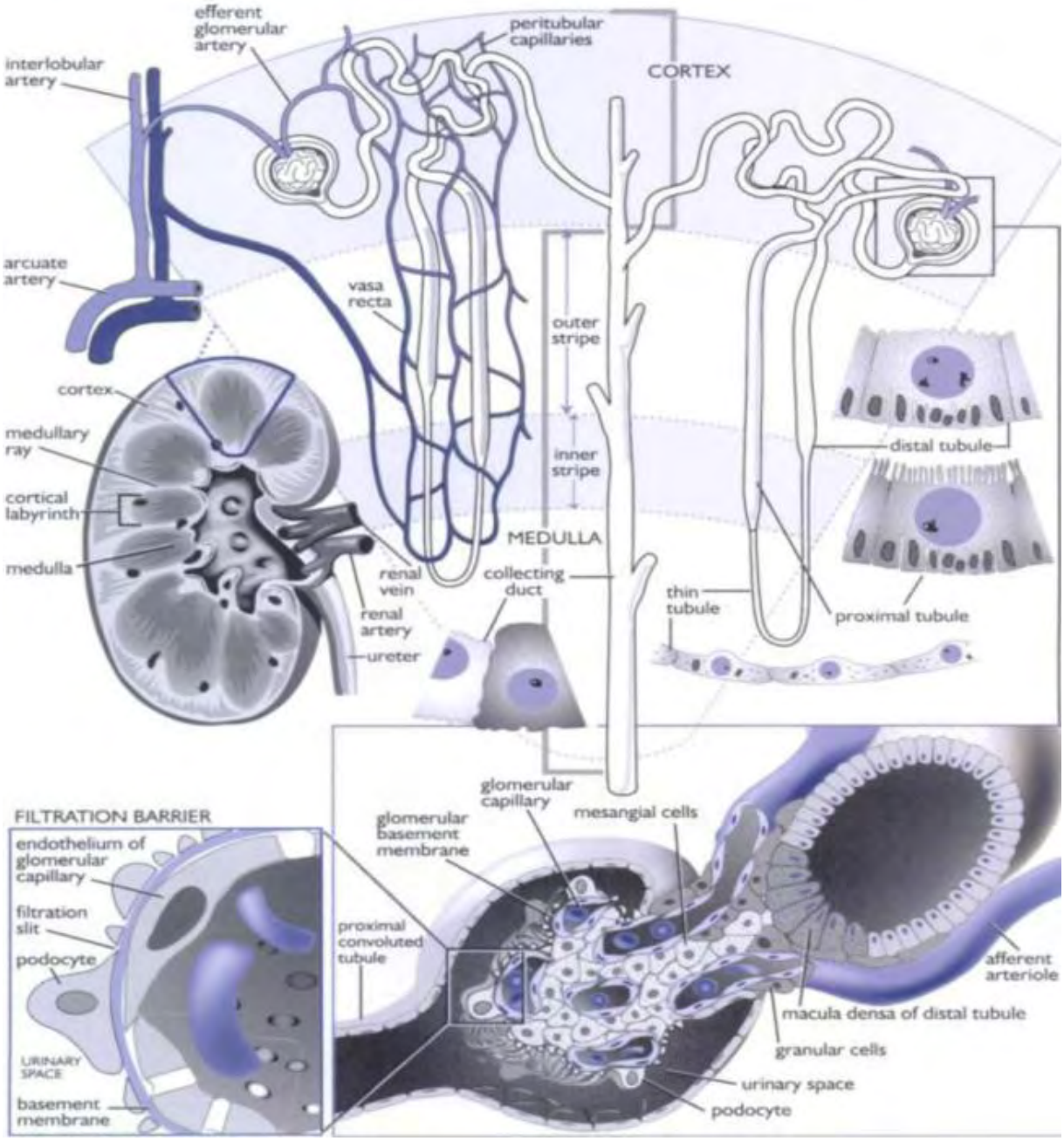
The rabbit has been used as a test animal for evaluating nephrotoxicity, particularly with cephalosporin antiinfectives and cyclosporine. Morphological changes and mechanistic studies have also been conducted in the rabbit[71]. Rabbits are very susceptible to the effects of acid–base disturbances, pain, stress, anorexia and dehydration because of their renal responses. The role of the rabbit kidney in calcium homeostasis results in the excretion of large amounts of calcium that form a calcium carbonate precipitate in the alkaline urine, giving it a turbid appearance in healthy animals[72].

The **rabbit's kidney** is unipapillate with extensive evaginations of the pelvis into the medullary tissue. The rabbit is the only known mammal in which the tubules can be separated from kidney slices with the tubular epithelium intact. Rabbit kidneys are therefore used for many in vitro studies of renal function[72]. Each kidney has approximately 30000 nephrons. The number of glomeruli increases after birth. In the kidney there is a short neck region of distinct morphology that connects the glomerulus to the proximal convoluted tubule (PCT), not noted in humans or rats. The PCT can be divided in three segments. The PCT-S1 segment includes the first two-thirds of the PCT, the PCT-S2 includes the last third of the PCT and the first part of the straight tubule (pars recta) and the PCT-S3 the terminal part of the pars recta. In rabbit, the PCT-S2 segment covers greater region of the pars recta. In the loop of Henle, 60% of the loops are long and 40% are sort[71].

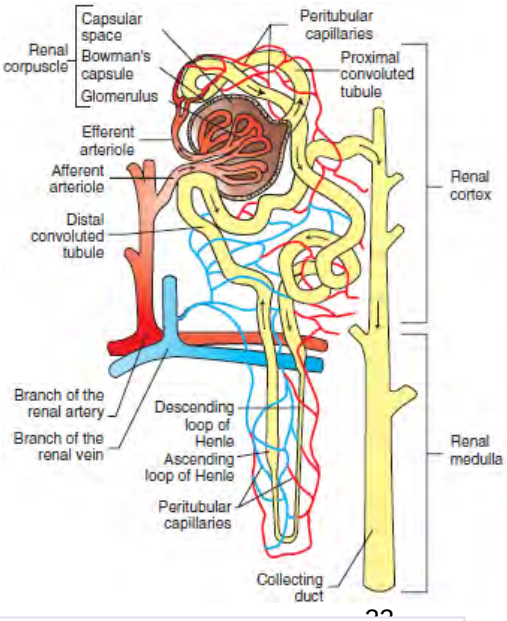
In toxicology pathology are important the histologic and ultrastractural cytologic characteristics of the : glomerulus, proximal convoluted tubule, pars recta, loop of Henle, thick ascending tubule, juxtaglomerular apparatus, and distal convoluted tubule , collecting ducts and interstitium[73].

The three main mechanisms by which the kidneys carry out their waste elimination role are filtration of the blood, reabsorption of useful substances back into the bloodstream, and secretion of waste products from the blood into the tubules of the nephron.

The kidneys play a major role on maintaining homeostasis in animals: a) in times of negative balance preserve water and electrolytes, and increase water and electrolyte elimination in times of positive balance, b) excrete or preserve hydrogen ions to maintain normal limits of blood pH, c) conserve nutrients, d) remove waste products of nitrogen metabolism, such as urea, creatinine and allantoin, e) produce renin, erythropoietin and prostaglandins and aid in vitamin D activation[74].



Eurell JAC. *Veterinary Histology*. Teton NewMedia; 2004.

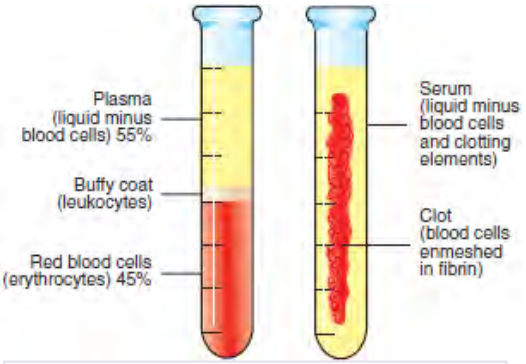


Colville TP, Bassert JM. *Clinical Anatomy and Physiology for Veterinary Technicians*. 3rd ed. 2015.

Blood tissue

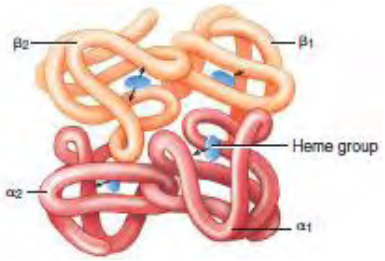
Blood connects the body systems together bringing the needed oxygen, nutrients, hormones and other signaling molecules and removing the wastes.

As rabbits are used extensively for toxicological and physiological studies, there is a lot of information about the effects of experimental infections, drugs and toxic substances on hematological and biochemical parameters. Rabbit's blood hemolyses easily and clots quickly. Blood tissue consists of the fluid portion called plasma and the cellular portion composed of red blood cells (erythrocytes), white blood cells (leukocytes), and platelets. Blood can be separated into its major components by centrifugation. The heavier portion (red blood cells) goes to the bottom, and the lighter portion (plasma) rises to the top of the hematocrit tube. A very thin middle layer, the buffy coat zone, is composed of leukocytes and platelets. The plasma consists of 90% water and the rest 10% includes large protein molecules, electrolytes, antibodies, nutrients, waste products and clotting factors. Serum is the fluid that remains if the blood left clot in a tube without any anticoagulant. When blood clots, one of the dissolved plasma proteins, fibrinogen, is converted to insoluble fibrin, which is entwined in the blood cells, therefore converts plasma to serum. In a word, plasma is whole blood minus the cells and serum is whole blood minus the cells and clotting factors[69].



From Palton KT, Thibodeau GA: Anatomy& physiology ed 8, St Louis, 2013, Mosby

Erythrocytes are the numerous cellular components of the blood. They carry a molecular of hemoglobin, which is the mediator of the oxygen transport. Hemoglobin[75] (Hb) is transported in erythrocytes whose membrane, shape, cytoskeleton, and metabolic processes ensure survival of the cell against the stresses of circulation and various injurious substances. It consists of heme and globin. The heme unit is the pigmented portion of hemoglobin. Each heme moiety contains iron in the Fe^{2+} state, which is the only form[50] that can bind O_2 .Therefore, one molecule of hemoglobin can carry four molecules of oxygen. Hemoglobin that has oxygen bound to it is referred to as oxyhemoglobin. When hemoglobin gives up its oxygen to tissues in the body, it is becomes deoxyhemoglobin[69]. A globin chain of specific amino acid sequence is attached to each heme group. The complete hemoglobin molecule is a tetramer, containing four heme units and four globin chains. The globin chains are identical pairs (dimers), designated as α-chains or ß-chains. Hemoglobin also helps to control pH in body fluids.



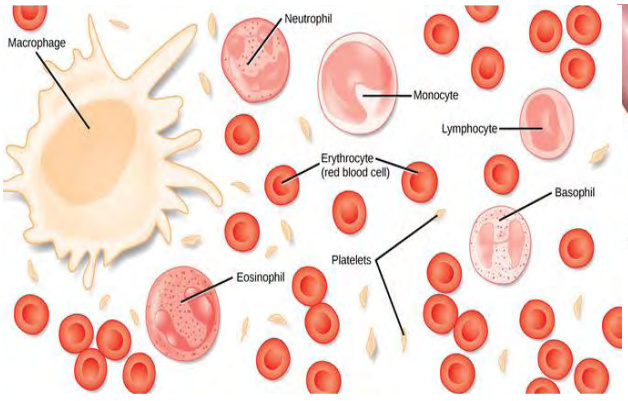
Colville TP, Bassert JM. *Clinical Anatomy and Physiology for Veterinary Technicians*. 3rd ed. 2015.

Rabbit's erythrocytes appear as a biconcave disk and vary in diameter within a range of 5.0-7.8 μm. Normally, 1-4 % of circulating erythrocytes are reticulocytes. Also, polychromasia and reticulocytes in rabbit blood smears have been attributed to the short life span, 57-67 days, and high random destruction rate of erythrocytes of 0,5 % per day[71]. Nucleated red cells and Howell–Jolly bodies can also be found occasionally[72]. Erythrocytes, also, participate in the transportation of carbon dioxide to the lungs and provide more membrane surface area for the diffusion of oxygen and carbon dioxide.

All leukocytes participate in body defense, but each is functionally independent. Three of the leukocytes types have granules visible within their cytoplasm, thus, they are called granulocytes. Rabbit neutrophils[72] have an almost colorless cytoplasm and contain two types of granules, which has led to a different nomenclature. They are called heterophils, pseudoeosinophils, acidophils or amphophils, depending on the text. They function, primarily, as phagocytes and are important in infectious conditions and inflammation. Eosinophils[72] have greater size than neutrophils and large acidophilic granules. The main function of eosinophils is detoxification by either inactivation of histamine or histamine-like toxic materials. Eosinophils are important in the allergic response and are capable of phagocytosis. In contrast to other laboratory species, basophils are frequently found in the circulation of rabbits in small to modest numbers, even as high as 30% with no apparent abnormalities[76].

The agranulocytes are lymphocytes and monocytes. Small lymphocytes[72] are seen more commonly than large lymphocytes and are mainly involved in immunological responses. The lymphocytes are round cells with round nuclei and small cytoplasm. Activated lymphocytes (immunocytes) typically have a more intensely stained cytoplasm. Monocytes[77] are large nucleated cells. Once they leave the blood and enter the tissues, they turn into macrophages. The monocyte - macrophage system plays a major role in the body's immune response. In rabbits, increased monocyte counts can be associated with chronic bacterial infection.

Platelets[77] or thrombocytes are fragments of megakaryocytes. They are an essential component of the blood's ability to clot in mammals.



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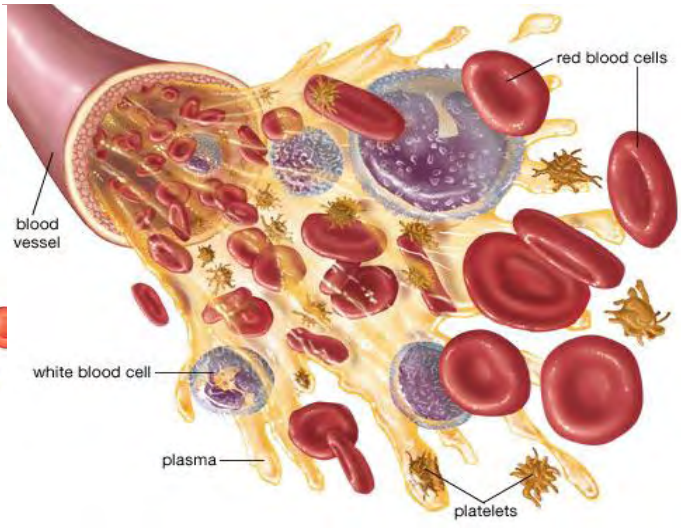


https://fatimachs.files.wordpress.com/2013/11/blood-diagram.gif

Biochemical Parameters

Creatinine

Creatinine is a nitrogenous waste product that is transported in the blood to the kidney, through the renal glomeruli, where it is excreted in the urine. Creatinine is the endogenous product of creatine breakdown, which is a substance in the form of phosphocreatine[74], present in the muscle and is involved in high energy metabolism. Any changes in blood creatinine concentrations are due to changes in excretion and are a reflection of renal function. Concentrations rise quickly at the outset of renal disease and decrease when an improvement of renal function takes place[72]. Additionally, creatinine levels may be increased in severe muscle injury[71] and are not affected by high protein diet[74].

Urea

Urea is a nitrogenous waste product formed in the liver as the end product of deamination of amino acids. It is transported in the blood to the kidney, through the renal glomeruli, where it is excreted in the urine. In rabbits, many physiological factors influence the concentration of urea in the blood, including circadian rhythm, amount and type of protein in the diet, nutritional status, liver function, intestinal absorption and hydration status[78]. The rabbit's urea metabolism is further complicated by urea utilization (urease activity) by caecal microflora during catabolism or during periods of dietary excess. As in other species, elevated blood urea values in rabbits are associated with renal insufficiency[72] and below reference range indicate hepatic insufficiency or muscle mass loss[78]. Blood urea values are 2.14 times greater than Blood Urea Nitrogen (BUN), urea/2.14=urea nitrogen. BUN is commonly used in domestic animals to assess renal function[71].

Urea vs Creatinine

Rabbits have a limited ability to concentrate urine, so few hours without drinking or diarrhea could elevate urea and creatinine levels, suggesting renal failure. Levels rapidly return to normal once dehydration is corrected. Blood urea and creatinine values can be high in cases of prerenal azotemia in rabbits and can occur with stress, fright, water deprivation, dehydration, heat stroke, toxic insults and gastrointestinal hemorrhage due to increased protein digestion[72],[78]. Renal azotemia is associated with acute or chronic renal failure, clinical Encephalitozoon cuniculi infection, chronic interstitial nephritis, glomerulonephritis, pyelonephritis, renal lithiasis, renal cysts and lymphosarcoma. Postrenal azotemia occurs in obstruction of urine flow due to hypercalcinuria or other causes, in urolithiasis and in rupture of the urinary tract. Variable increases are noticed in isosthenuric urine[78]. The accumulation of urea in the blood is called uremia, whereas the buildup of creatinine is called azotemia. Also,

both are insensitive at detecting modest renal damage, as values remain within normal reference ranges until 50-75% of renal function is lost, making early diagnosis of renal disease difficult. Creatinine may be more reliable indication of renal function because is somewhat less influenced by external factors than urea[71].

Aspartate aminotransferase (AST)

In rabbits, AST is found in liver, heart, skeletal muscle, kidney and pancreas, with the highest activity in the liver and skeletal muscle. AST is an indicator of tissue damage. Physical exertion, stress or tissue damage during blood collection can elevate results. The most common causes of increased blood levels of AST are hepatic disease, muscle inflammation or necrosis and spontaneous or artifactual hemolysis[74]. Raised AST in combination with normal Creatine Kinase levels is good evidence to suggest the AST is not of muscular origin[72].

Aim

The aim of this study is to investigate the response in a contrast media - administration in a rabbit model and a possible underlying mechanism, especially the role of oxidative stress.

It is known that a CM - administration induce nephropathy in humans and animal models, such as rats and mice, mainly in individuals with a primary disease. The exact pathophysiology of CIN is not completely determined. The substance of choice, iopromide, is a widely used CM in every day clinical practice, in both diagnostic and interventional procedures. The goal is to investigate in a healthy population, in vivo, the linkage between CIN and oxidative stress, in a tissue much accessible, the blood tissue.

Rabbits are animals easy to handle and can be easily manage and maintain under environmental controlled conditions. Also, it is a model that is well established that is susceptible in CIN.

Materials and Methods

Animals and Administration Protocol

Ten healthy New Zealand White male rabbits three months old, weighing 3,2 - 3,8 kg, drug-free at least a month before experimentation, were used in this study. The animals were housed under controlled environmental conditions (12-hour light/dark cycle, temperature 20–23 °C) in standard single cages. They were fed with commercial rabbit pellets ad libitum and provided with drinking (tap) water. As confirmed by daily inspection, animals were consuming all water made available to them (250 ml approx. of tap water per day).

The animals were divided into three groups, two groups consisting of three animals and one of four animals.

- The first group was the control group (control, C) in which ≈2 mL/min sterile saline solution NaCl 0,9% was administered, once, intravenously through catheterization in marginal ear vein, over a period of 30 minutes and was euthanized 48 hours after the end of the administration.
- The second group was the N1Dx group (N1Dx, x=1,2,3,4) in which iopromide 8 gr I/kg body weight (Ultravist® , 370 mg/ml, 37% iodine, Bayer) was administered, once, intravenously through catheterization in marginal ear vein, over a period of 30 minutes and was euthanized 24 hours after the end of the administration.
- The third group was the N2Dx group (N2Dx, x=1,2,3) in which iopromide 8 gr I/kg body weight was administered, once, intravenously through catheterization in marginal ear vein, over a period of 30 minutes and was euthanized 48 hours after the end of the administration. For catheterization were used 24-gauge intravenous catheters.

All the animals were weighed and during the time of administration were anesthetized with a combination of xylazine 4 mg/kg im (Sedaxylan, 20 mg/ml, Dechra) and ketamine hydrochloride 40 mg/kg im (Imalgene 1000,100 mg/ml, Merial).

Blood Collection

Blood samples were drawn at 0 time point (time before administration), and 2 hours, 10 hours, 24 hours and 48 hours after the end of the administration. The 48-hour blood samplings were taken only from the control and N2Dx group. The blood was collected mainly from the external jugular vein by 24-gauge, 1 inch, needle puncture.

The total amount of blood of each collection was 4 ml, in which 3 ml placed in ethylenediaminetetraacetic acid (EDTA) tubes and 1 ml in Serum-separating tubes. Blood samples in EDTA were centrifuged immediately at 3000 g for 10 minutes and the plasma was

collected and used for measuring TAC, TBARS and protein carbonyls. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020 *g* for 15 minutes at 4 °C and the erythrocyte lysate was collected for the measurement of hemoglobin and catalase activity.

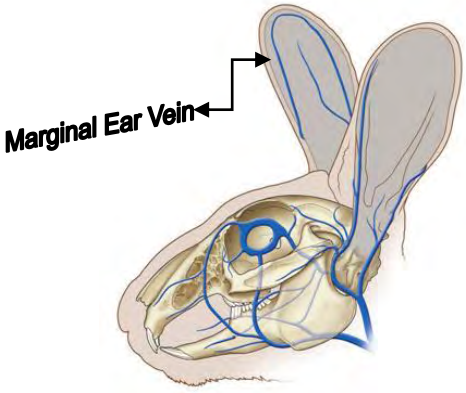
The animals were euthanized, after the last blood sampling, according with the Guidance of Animal Welfare. They were anesthetized as above and euthanized with 5 ml pentobarbital sodium 20 % (Dolethal, 200 mg/ml solution for injection, Vetoquinol) iv through catheterization in marginal ear vein. Necropsy procedure [79] was conducted and all the organs were removed and stored in 10% buffered formalin solution. The kidneys were prepared for histopathological evaluation. Left kidneys were longitudinally sectioned and right kidneys were transversely sectioned.

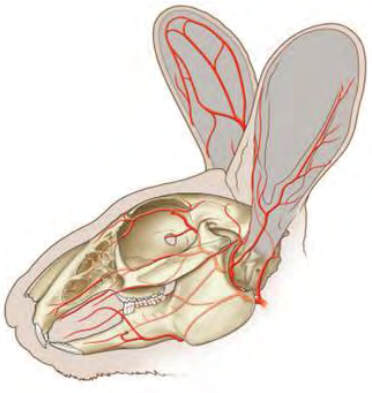
All the procedures took place under the ethics of good Veterinary Clinical Practice and according the Laws and Guidance of Animal Welfare of Greece and European Union. Furthermore, all procedures were conducted to minimize the exogenous and excessive stress in the animals.

Serum-separating tube

Blood sample in EDTA

iv. Catheter





Protocols of Oxidative Stress Biomarkers

1. Total Antioxidant Capacity (TAC)

There are two different ways of approaching the quantification of antioxidant activity. The first is the sum of the antioxidant capacity of the individual antioxidant of plasma. This is a very difficult effort and the results will not be accurate as there are a lot of molecules that contribute to the antioxidant capability of plasma. The second one is the measurement of the total capacity as a whole that provide an overview of the biological interactions between antioxidant species.

The determination of TAC was based on the method of Janaszewska and Bartosz [80]. Total Antioxidant Capacity (TAC) of blood serum is evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH). In the presence of a hydrogen donor existing in the serum, the free radical DPPH• is reduced to the corresponding hydrazine. The depletion of the radical is photometrically evaluated by the decrease in absorbance at 520 nm.

Phosphate buffer 10 mM (pH 7.4)

MW (KH_2PO_4): 136 and MW (Na_2HPO_4): 178

To make 500 mL of phosphate buffer, make 100 mL KH_2PO_4 (10 mM) and 400 mL Na_2HPO_4 (10 mM). For KH_2PO_4 weight 0.136 g and dissolve it in 100 mL water (this solution has initial pH ~4.57). For Na_2HPO_4 weight 0.712 g and dissolve it in 400 mL water (this solution has initial pH ~9.11). In a heating glass mix together the 400 Na_2HPO_4 and the KH_2PO_4 solutions. If required, correct it with NaOH or HCl, 1 N, until pH 7.4.

DPPH (2,2-diphenyl-1 picryl hydrazyl) 0.1 mM. MW (DPPH): 394.32 (stored at -20 C)

Dispense 0.02 g DPPH in 5 mL methanol and mix them in the stirrer (10 mM solution). Then dilute 100 times with methanol and mix in the stirrer, for example, dilute 200 μL of the 10 mM DPPH solution into 19.8 mL methanol (it is enough for 10 samples, plus the Blank and the Positive Control). Because of the dilution, the initial volume of 5 mL is always enough for the determinations. Cover the vessel with aluminum foil to avoid photolysis. DPPH must be made fresh on the day of the experiment.

Ascorbic acid 10 mM.

The solution in the freezer is ready to use.

To obtain comparable sets of data, scavenging activities can be correlated in a dose-response curve, with standard antioxidants such as ascorbic acid, allowing the calculation of AEAC (ascorbic acid equivalent antioxidant capacity). Normally, the value of TAC of the positive control, the sample containing ascorbic acid, should be lower than the value of sample and of course the blank. The reason is the presence of ascorbic acid, which is a potential antioxidant molecule. So, sample absorbance values should fall between positive control (smallest value) and blank (greatest value)

Add the following volumes in eppendorf tubes

	Blank	Positive control	Sample
Phosphate buffer 10 mM, pH 7.4	500 μL	495 μL	480 μL
DPPH 0.1 mM	500 μL	500 μL	500 μL
Ascorbic acid 10 mM	—	5 μL	—
Plasma	—	—	20 μL

Invert the eppendorfs several times and incubate them in the dark for 60 minutes. Centrifuge for 3 min at 20000 g at 25 ^{o}C in the eppendorf centrifuge. Transfer 900 mL of the supernatant using pipette from each eppendorf into a clean plastic cuvette and measure the absorbance at 520 nm. TAC is a sample's scavenging ability toward 2,2-diphenyl-1picryhydrazyl (DPPH) radical. The reaction occurs during the 60 minutes of the incubation in the dark. The centrifugation offers a better colour for the sample. In that way, the spectrophotometric results are better and more reliable.

Calculations

Results can be expressed as:

i) % absorbance reduction in reference to blank, i.e.

% Abs reduction = (Abs blank – Abs sample) / Abs blank $\times$ 100

ii) μmol DPPH scavenged / mL serum = [(% Abs reduction / 100) $\times$ 50 $\times$ 50] **/** 1000

a) We divide by 100 in order to convert percentage absorbance decrease into absorbance decrease.

b) We multiply by 50 because the concentration of DPPH into cuvette is 50 μmol/L of cuvette.

c) We multiply by 50 because dilution of the serum in cuvette is 50-fold (1000 μL in the cuvette / 20 μL of serum of the sample in the cuvette = 50).

d) We divide by 1000 in order to convert L of serum into mL of serum.

2. Hemoglobin determination

Due to the fact that the results of markers in erythrocyte lysate were given as per mg Hb, it was appropriate to determine the Hemoglobin value of samples. Hemoglobin concentrations were determined by the hemiglobincyanide (HiCN) method[81]. Briefly, 5μL of the red blood cell lysate (RBCL) was added in 1 ml of working hemoglobin reagent R1. Then, samples were vortexed and incubated into dark for 10 minutes, because R1 is light-sensitive, and was measured at 540 nm. The 10 x concentrated hemoglobin reagent has to be diluted 10 - fold to give the working reagent R1. In each experiment, the sample alone in R1 was used as a blank. For the calculation, absorbance of each sample was multiplied by 29.4 and the final hemoglobin concentration expressed by g/dL.

3. Catalase (CAT)

Catalase activity was determined by using the method of Aebi et al. [82]

Reagents

Phosphate buffer 67 mM (pH 7.4)

MW (KH_2PO_4): 136 and MW (Na_2HPO_4): 178

To make 500 mL of phosphate buffer make 100 mL KH_2PO_4 (67 mM) and 400 mL Na_2HPO_4 (67 mM). For KH_2PO_4 weight 0.91 g and dissolve it in 100 mL water (this solution has initial pH ~4.57). For Na_2HPO_4 weight 4.77 g and dissolve it in 400 mL water (this solution has initial pH ~9.11). In a heating glass mix the 400 Na_2HPO_4 and the KH_2PO_4 solutions. If required, correct it with NaOH or HCl, 1 N, until reach pH 7.4.

30% hydrogen peroxide (H_2O_2)

Hydrogen peroxide is ready to use.

Assay

Add the following volumes in the plastic tubes:

	Sample
Phosphate buffer 67 mM, pH 7.4	2991 μL
RBCL diluted 1/10 in water	4 μL

1. Vortex the tubes at a moderate speed and incubate them in the oven at 37 ^{o}C for 10 min. It is more practical to work with (incubate) 2 samples at a time to make sure that the samples are measured shortly after the incubation.

2. Transfer the content of one plastic tube into the UV glass cuvette.

3. Add 5 μL 30% hydrogen peroxide into the UV glass cuvette, invert the cuvette three times (using a parafilm plastic piece on the top of cuvette), and measure the absorbance immediately at 240 nm for 130 sec. Proceed each sample separately.

Calculations

Catalase activity (U/mg Hb) = (Δ Abs_{sample} per min / 40) $\times$ (750 $\times$ 1000 $\times$ 10 $\times$ 2) / Conc. Hb (mg/mL)

Where the 40 (mol/L) is the molar extinction coefficient of H_2O_2 which is being multiplied with 1000 to convert mol/L into μmol/mL, 750 is the dilution factor which results by dividing the final volume (3000 μL) to RBCL volume (4 μL) (3000 / 4 = 750), 10 is the dilution of the sample (1/10) and 2 is the dilution of red blood cells with H_2O when they are lysed.

Concentration of hemoglobin is converted into mg/mL multiplying the value determined spectrophotometrically by 10×2. Hemoglobin concentration is determined spectrophotometrically in g/dL. Multiplying by 10 it is converted into g/L (mg/mL) and by 2 the 1/1 dilution during erythrocyte lysis is taken into account.

U = μmol/min

Δ Abs (min) = the change in absorbance in a min.

Δ Abs_{blank} is always zero so there is no need for the blank to be measured.

The concentration of H_2O_2 in cuvette is about 16 mM.

4. Thiobarbituric - Acid Reactive Substances (TBARS)

For thiobarbituric acid–reactive substances (TBARS) determination, a slightly modified assay of Keles et al.[83] was used.

TBARS are expressed in terms of malondialdehyde (MDA) equivalents. Malondialdehyde (MDA) forms a 1:2 adduct with Thiobarbituric acid (TBA), which can be measured by spectrophotometry. TBARS return to normal levels over time, depending upon the presence of antioxidants.

Before starting the experiment switch on the water bath and adjust the temperature to 95 ^{o}C.

Reagents

Tris-HCl 200 mM (pH 7.4)

MW (Tris): 121.14

MW (HCl): 36.46 (stock 37%) [equal to 10.1 N]

To make ≈100 mL of Tris-HCl buffer make 25 mL Tris (200 mM) and 42 mL HCl (0.1 N). For Tris weight 0.61 g and dissolve it in 25 mL water. For HCl dilute 0.42 mL of the stock 37% HCl (equal to 10.1 N) up to 42 mL water. In a heating glass pour the 25 mL of Tris solution and add slowly the 42 mL HCl, and then add water up to 100 mL. Check pH to be 7.4.*

Tris is an abbreviation of the trivial name, trishydroxymethylaminomethane, for 2-amino-2-hydroxymethyl-1,3-propanediol. It is widely used as a component of buffer solutions, such as in TAE and TBE buffers, with an effective pH range between 6.5 and 9.7.

Solution Na_2SO_4 (2 M) – TBA (55 mM)

MW (TBA): 144.1

MW (Na_2SO_4): 142.04

Instructions for 10 mL solution: This solution should be made fresh in the day of the experiment. Weight 2.84 g Na_2SO_4 and 0.08 g TBA. Transfer them in a heating glass and add 10 mL water. Heat and stir them until fully dissolve.

TCA 35%: Weight 35 g of TCA and dissolve it in distilled water up to a final volume of 100 mL of water (stable at room temperature).

TCA 70%: Weight 70 g of TCA and dissolve it in distilled water up to a final volume of 100 mL of water (stable at room temperature).

Assay

1. In Falcon (15 ml) tubes add 100μL plasma (for the samples) or distilled water (for the blank).

2. Add 500 μL TCA 35% and 500 μL Tris-HCl and vortex.

3. Incubate for 10 min at room temperature.

4. Add 1 mL Na_2SO_4 – TBA and incubate at 95 ^oC for 45 min in the waterbath.

5. Transfer the tubes into the ice and let them cool for 5 min.

6. Add 1 mL TCA 70% and vortex.

7. Transfer 1 mL into eppendorf tubes and centrifuge at 11200 g (10000 rpm) at 25 ^oC for 3 min at batches of 12 eppendorfs.

8. Transfer by pipette 900 μL into a clean cuvette and measure the absorbance at 530 nm.

TCA is being added to the serum in order the serum proteins to be precipitated.

Tris-HCl is a buffering agent, helping for enzymatic reactions to be made.

Na_2SO_4 is used as a drying agent for organic solutions.

The thiobarbituric acid reactive substances (TBARS) test is a quick and easy way to assess lipid peroxidation as the levels of derivatized Malondialdehyde (MDA). The product is the MDA $(TBA)_2$ adduct, whose absorbance is measured at 530 nm.

Calculations

Concentration of TBARS (μmol/L) = (Abs sample – Abs blank) / 0.156 $\times$ 31, where 31 is the dilution factor, which results from the division of the final volume (3100 μL) to the serum volume (100 μL) (3100 / 100 = 31). The 0.156 results from the molar extinction coefficient* of MDA which is 156000 (mol/L) divided by 10^{-6} in order to convert mol/L to μmol/L.

*The molar extinction coefficient of a substance is the absorbance of this substance at the concentration of 1 mol/L.

5. Protein Carbonyls

Protein carbonyls were determined based on the method of Patsoukis et al. [84]

The measurement of protein carbonyls following their covalent reaction with DNPH was pioneered by Levine et al. and has become the most widely utilized measure of protein oxidation in several human diseases.

Carbonyl formation is usually detected by the reaction with 2,4-dinitrophenylhydrazine (DNPH) and the conversion into 2,4-dinitrophenylhydrazone (DNP - hydrazone).

Reagents

HCl solution 2.5 N

HCl: MW 36.46; stock 37% (equal to 10.1 N)

To make 100 mL 2.5 N HCl solution, add slowly 24.6 mL of the 37% HCl (equal to 10.1 N HCl) stock to ≈70 mL distilled water and brought it up to 100 mL with distilled water.

A great deal of attention should be paid during the preparation of 2.5 HCl solution because the stock 37% (10.1 N) HCl is very erodent. Work always under the hood and wear gloves.

DNPH 14 mM

DNPH: 2,4-dinitrophenylhydrazine; MW: 198.1

To make 100 mL 14 mM DNPH dissolve 0.2833 g DNPH into 100 mL 2.5 N HCl.

It is made fresh in the day of measurement. Cover with aluminum foil, light sensitive, 0.5 mL per sample is needed. Prepare a blank for every sample.

Urea 5 M (pH 2.3), (MW: 60.06)

To make 100 mL 5 M urea (pH 2.3, adjusted with 2N HCl), dissolve 30 g urea in ≈70 mL distilled water and brought it to 100 mL with distilled water.

Assay

1. In 50 μL of plasma add 50 μL 20% TCA in eppendorf tubes and vortex (every sample has a positive blank)* (manually suspend pellet).

20% TCA is being added to the plasma in order the plasma proteins to be precipitated. TCA (Trichloroacetic acid also known as trichloroethanoic acid) is widely used in biochemistry for the precipitation of macromolecules such as proteins, DNA and RNA.

2. Incubate in ice bath for 15 min and centrifuge at 15,000 × g for 5 min at 4 °C.
3. Discard the supernatant.
4. Add in the pellet 0.5 mL of 14 mM DNPH (in 2.5 N HCL) for the sample or 0.5 mL 2.5 N HCL for the blank (every sample has a positive blank), manually suspend the pellet using pipette tip, vortex and incubate in the dark at room temperature for 1 hour with intermittent vortexing every 15 minutes. Centrifuge at 15,000 × g for 5 min at 4 °C.
5. Discard the supernatant.
6. Add 1 mL of 10% TCA, vortex (manually suspend pellet with a pipette tip, if necessary) and centrifuge at 15,000 × g for 5 min at 4 °C.
7. Discard the supernatant.
8. Add 0.5 mL ethanol and 0.5 mL ethyl acetate (ethanol/ethyl acetate, 1:1 v/v), vortex and centrifuge at 15,000 × g for 5 min at 4 °C.

The pellets are washed with 10% TCA followed by three ethanol–ethylacetate (1:1) (v/v) washes to remove any unreacted DNPH.

9. Repeat steps 7 and 8 two more times.
10. Discard the supernatant.
11. Add 1 mL 5 M urea (pH 2.3), vortex and incubate at 37 °C for 20 min.

Urea, which is used at the final step of the method, is a powerful protein denaturant. This property can be exploited to increase the solubility of proteins. Urea effectively disrupts the noncovalent bonds in proteins.

12. Centrifuge at 15,000 × g for 3 min at 4 °C.
13. Transfer by pipette 900 mL into a clean cuvette and measure the absorbance at 375 nm.

*(Every sample has a positive blank. The blank should contain everything except for the 0.5 mL DNPH, which is replaced by 0.5 mL plain HCL 2.5 N. The blank should be treated as a sample).

Calculations

The formula for protein carbonyl calculation is:

Protein carbonyls (nmol/mL) = A_{sample} - A_{blank} / 0.022 × 1000/50.

The molar extinction coefficient of DNPH is 22 mM · cm^{-1}.

0.022 is calculated as follows: 22 mmol/L equal to 22 μmol/ml, equal 0.022 nmol/ml. Where 1000 / 50 is the dilution factor (1000 μl in the cuvette / 50 μl of the sample),

Normalization for protein concentration can be done by the following formula:

Protein carbonyls (nmol/mg) = carbonyl conc. nmol/mL **/** protein conc. mg/mL

Protein concentration = 70 mg/mL.

Hematoxylin and Eosin (H&E) Staining Protocol

Principle

The natural dye, hematoxylin obtained from the logwood, Hematoxylon campechianum, that originated in the Mexican State of Campeche,[85] is the most important and most used dye in histology, histochemistry, histopathology and in cytology[86]. Hematoxylin itself is not a stain. The major oxidization product is hematein, a natural dye that is responsible for the color properties[85] . Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction.

Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining[87] . Eosin is a fluorescent red dye resulting from the action of bromine on fluorescein. It can be used to stain cytoplasm, collagen and muscle fibers for examination under the microscope. Structures that stain readily with eosin are termed eosinophilic.[88]

The renal tissue harvested from animal was washed with 0.9% saline, fixed in 10% buffered formalin solution and then embedded in 10% paraffin. Replicate sections were cut at 5 μm thickness on a rotary microtome and stained with hematoxylin and eosin.

Staining Procedure [89],[90],[88]

1. Deparaffinize sections, 2 changes of xylene, 7 minutes each.
2. 96% alcohol for 5 minutes
3. 80% alcohol for 1 minutes
4. 70% alcohol for 1 minutes
5. Wash briefly in distilled water for 2 minutes
6. Stain in hematoxylin solution for 6 minutes.
7. Wash in running tap water for 2 minutes.
8. Differentiate in 1% acid alcohol for few seconds. (one dip)
9. Wash in running tap water for 2 minutes.
10. Counterstain in eosin solution for 4 minutes.

11. Wash in running tap water for 2 minutes.
12. Dehydrate through 70% alcohol for 2 dips.
13. 80% alcohol for 2 dips.
14. 96% alcohol for 2 dips
15. 100% alcohol for 2 dips
16. Clear in xylene, 5 minutes.
17. Mount with xylene.

Results

Nuclei: blue. Cytoplasm and matrix: varying shades of red and pink. Erythrocytes: pink-red

Biochemical Parameters

Creatinine (Crea), Urea and Aspartate Aminotransferase (AST) were analyzed by a standard absorbance photometry in the blood serum by the COBAS INTEGRA® 400 plus analyzer, Roche.

Statistics

The statistical analysis of the results was conducted by the statistical program SPSS 20.0 (Statistical Package for Social Sciences Inc., Chicago, IL, USA, SPSS). Initially, for the statistical evaluation, in every sample were calculated the mean, the standard deviation and the standard error. The results were evaluated by one-way ANOVA, followed by Tukey's and Dunnett's correction tests for multiple comparisons per pairs. The level of statistical significance was set at $p<0,05$. The data were expressed as mean ± SEM. These two tests are used because it is non-parametric tests, as we do not know if we have a normal distribution, due to the small number of samples.

In the end, comparisons were conducted between biochemical parameters and redox biomarkers using Pearson bivariate correlation in SPSS, measured the association (strength) of the relationship between two variables.

ANOVA test tells you whether you have an overall difference between your groups, but it does not tell you which specific groups differed – post hoc tests do. Because post hoc tests are run to confirm where the differences occurred between groups, they should only be run when you have a shown an overall statistically significant difference in group means (i.e., a statistically significant one-way ANOVA result). Post hoc tests attempt to control the experiment wise error rate (usually alpha = 0.05) in the same manner that the one-way ANOVA is used instead of multiple t-tests. Post hoc tests are termed *a posteriori* tests, that is, performed after the event, the event in this case being a study.

Results

Animals

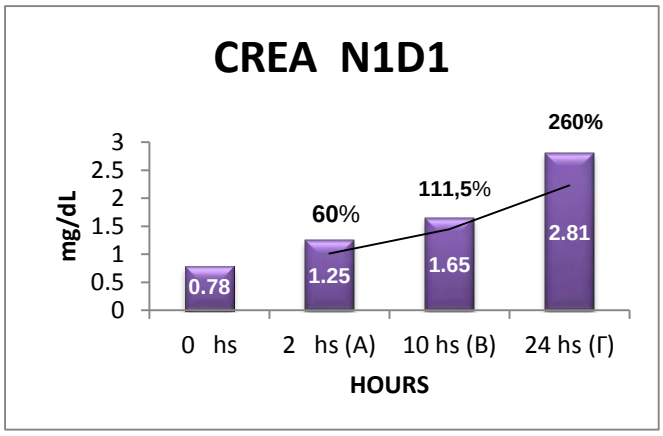
In general, animals didn't show any sign of discomfort after the CM administration and remain healthy until the last day. Daily inspection, at least twice a day, shows no anorexia or dehydration. No weight changes were observed.

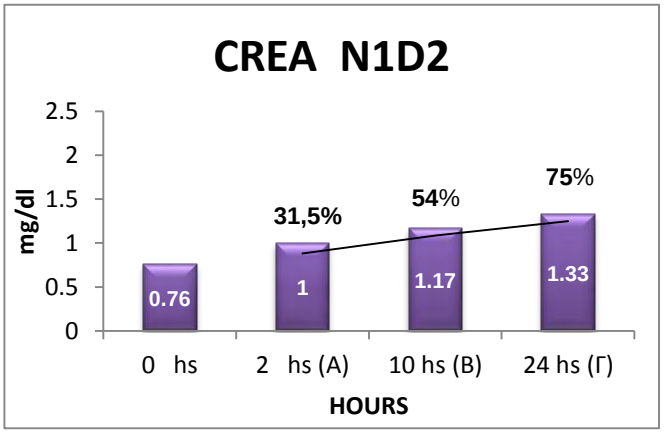
Biochemical Parameters

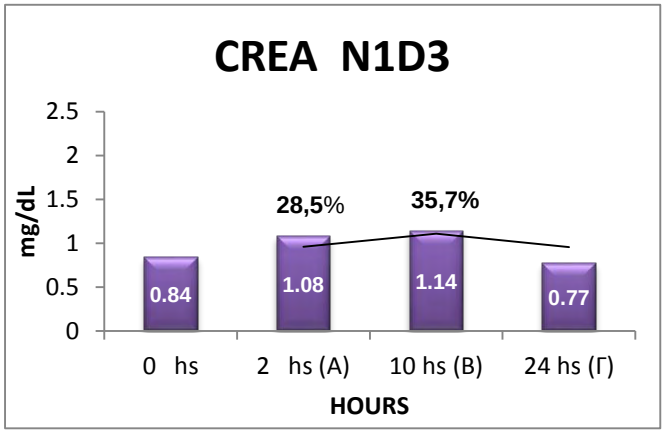
GROUP N1Dx (CM24)

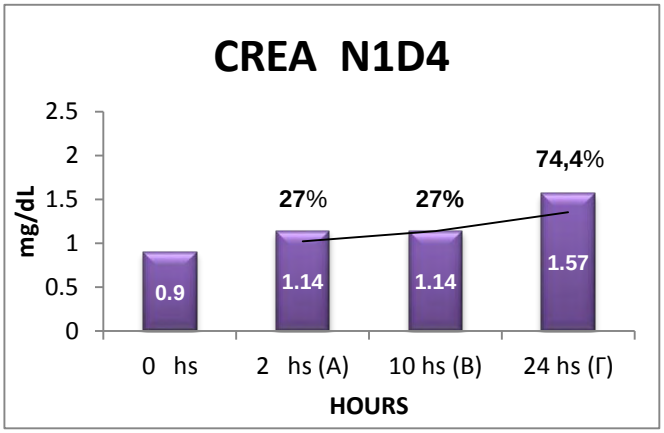
Creatinine

The diagrams of Creatinine (CREA) levels in each animal of the group N1Dx shows that the administration of Contrast Media raise the level of CREA in a percentage >25%, which indicates impairment in renal function, according ESUR[26].



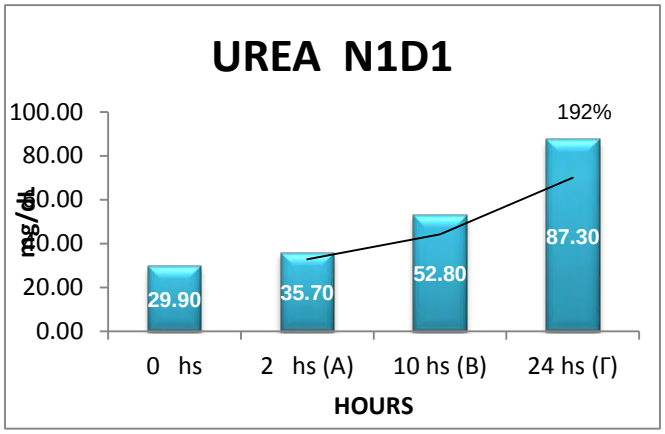


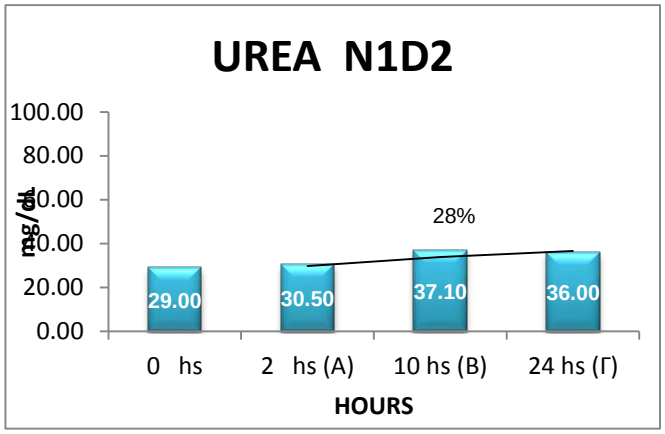


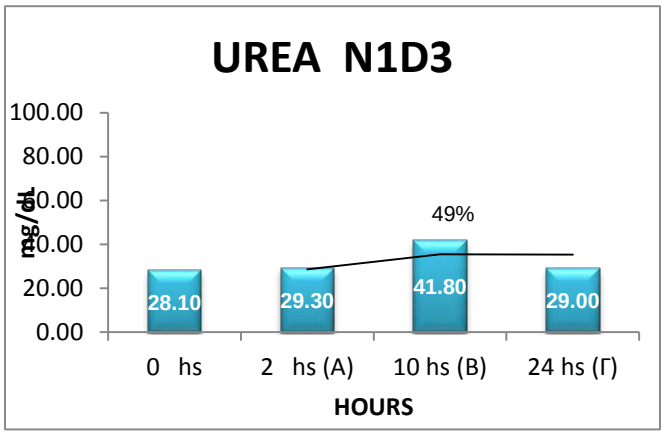


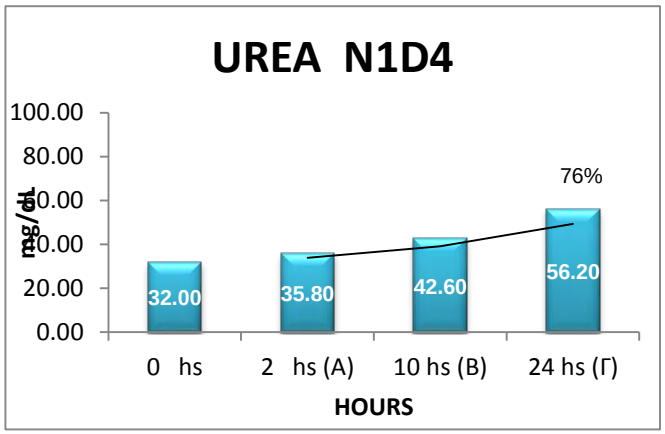
Urea

UREA levels seem to follow the same chances pattern as CREA, which is natural, since UREA is another parameter that characterizes renal activity.





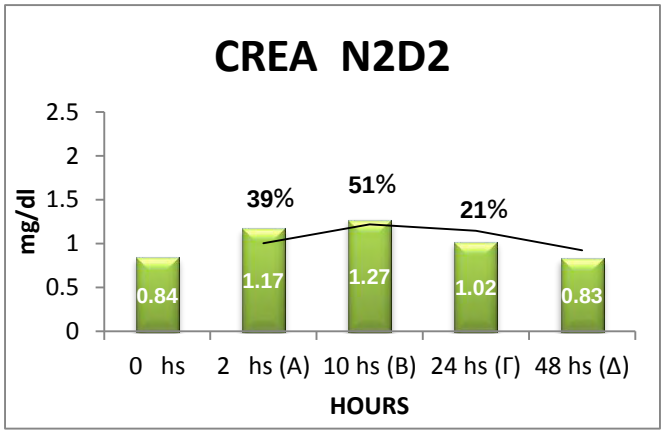


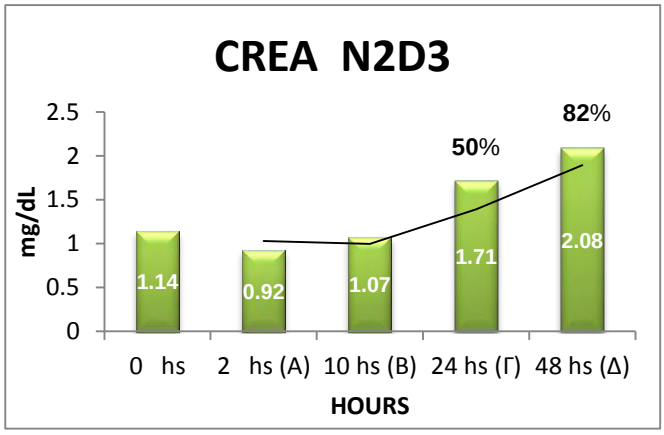


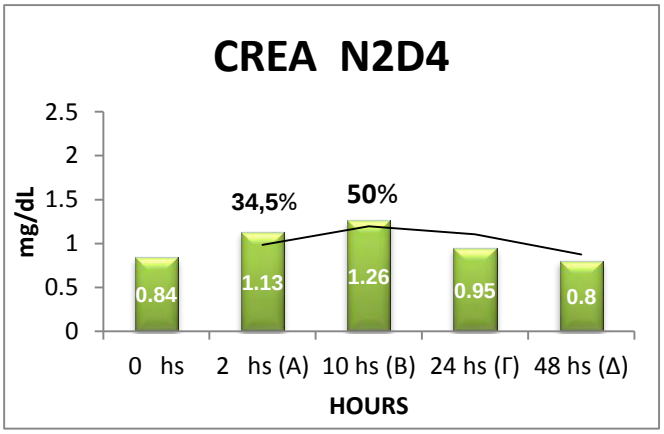
GROUP N2Dx (CM48)

Creatinine

As well, the diagrams of Creatinine (CREA) levels in each animal of the group N2Dx shows that the administration of Contrast Media raise the level of CREA in a percentage >25%, which indicates impairment in renal function, according ESUR[26], and at the time point of 48 hours the levels of CREA seems to return at the baseline of 0 hours, time reference before the administration.

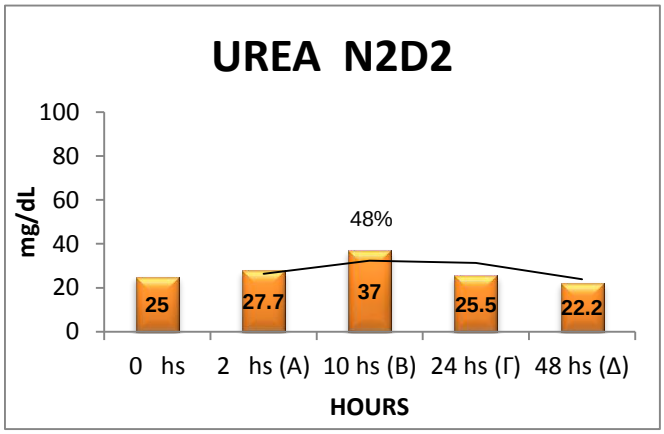


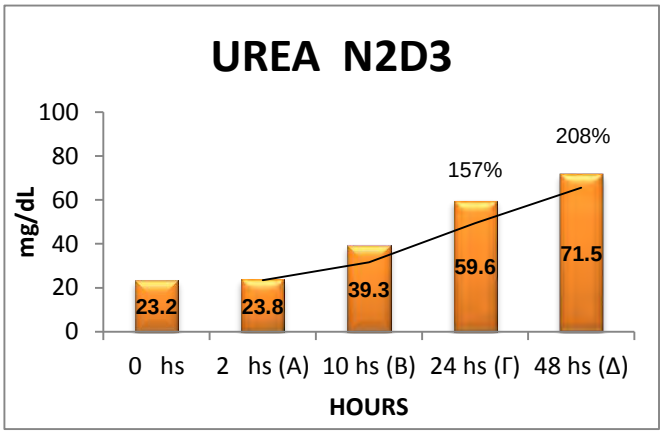


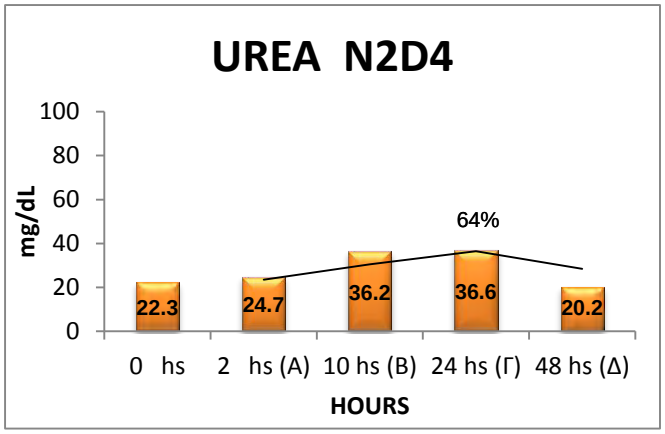


Urea

As well, UREA levels seem to follow the same chances pattern as CREA, which is natural, since UREA is another parameter that characterizes renal activity.







Oxidative Stress Biomarkers VS Biochemical Parameters

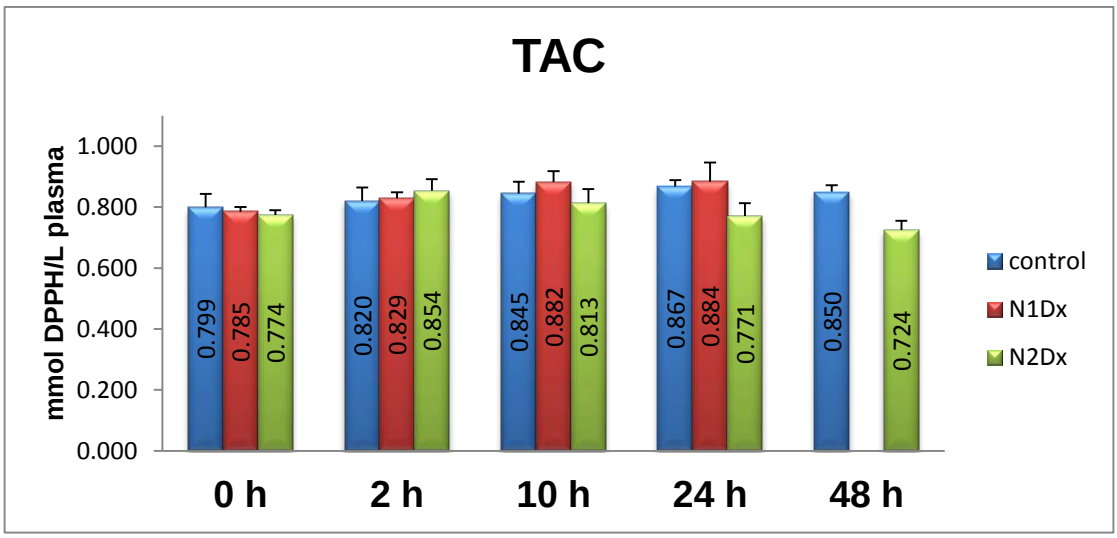
Correlation between Redox biomarkers and Biochemical parameters

Significant and nearly significant only (Pearson bivariate Correlation)

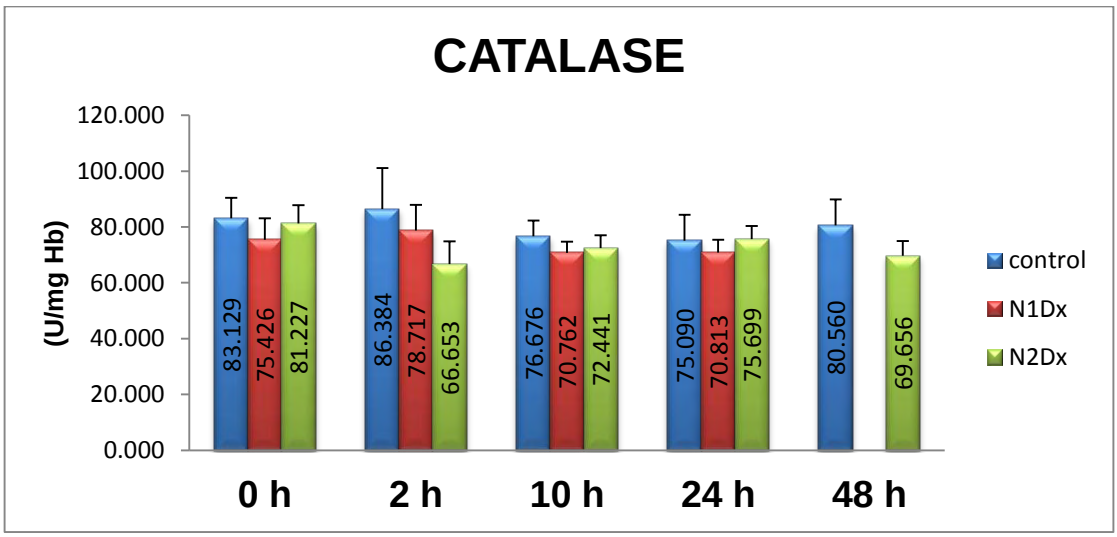
Correlated parameters	Correlation coefficient, r	*P* value
Control TAC – CM24 TAC	0,955	0,045
CM24 TAC – CM24 CREA	0,912	0,088
CM24 TAC – CM24 UREA	0,916	0,084
CM48 TBARS – CM48 CREA	- 0,981	0,003
CM48 TBARS – CM48 UREA	- 0,985	0,002
Control CATALASE – CM24 CATALASE	0,987	0,013
Control CARBONYLS – Control CREA	0,918	0,028
Control CREA – Control UREA	0,865	0,058
CM24 CREA – CM24 UREA	0,962	0,038
CM48 CREA – CM48 UREA	0,947	0,014
CM=Contrast Media , CREA=Creatinine		**p<0,05**

Redox Status Biomarkers

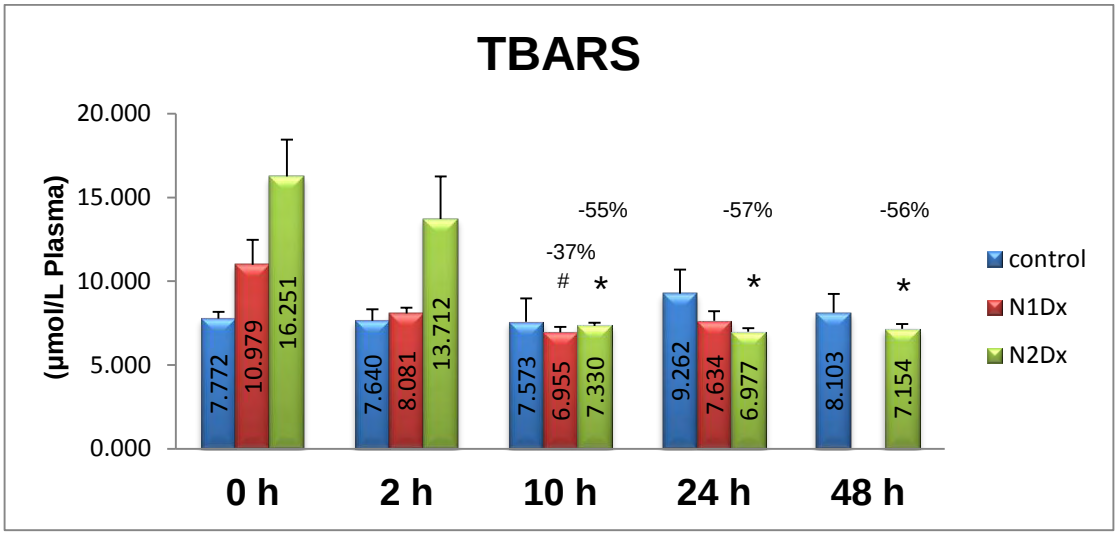
Total Antioxidant Capacity (TAC)



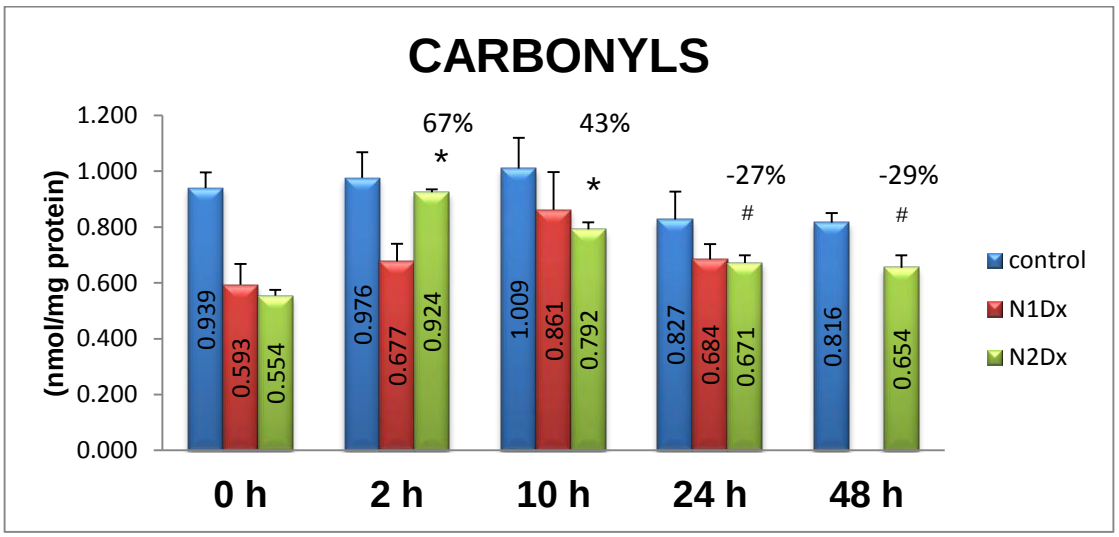
Catalase (CAT)



Thiobarbituric - Acid Reactive Substances (TBARS)



Protein Carbonyls (CARB)



Histopathological Examination

The histopathological evaluation was conducted under light microscopy and lesions were graded by a scoring system. Multiple sections in each field were evaluated at 20x and 40x magnification. Representative sample photomicrographs (40x) were taken from all groups from cortical and medullary regions of the kidney.

Histological evaluation:

Cortex Area:

Overall, tubular epithelial cells in N1Dx and N2Dx groups have maintained their position in the tubules, but many have lost their integrity. Cytoplasmic membrane in many cells appears disrupted. Cytoplasmic vacuolation is diffuse. Abnormalities in the nucleus it's been observed in some cells, mostly pyknotic nucleus or displacement due to vacuolation. A very few nephrons seem to be compromised, especially in N2Dx group animals. Glomerular changes did not observe.

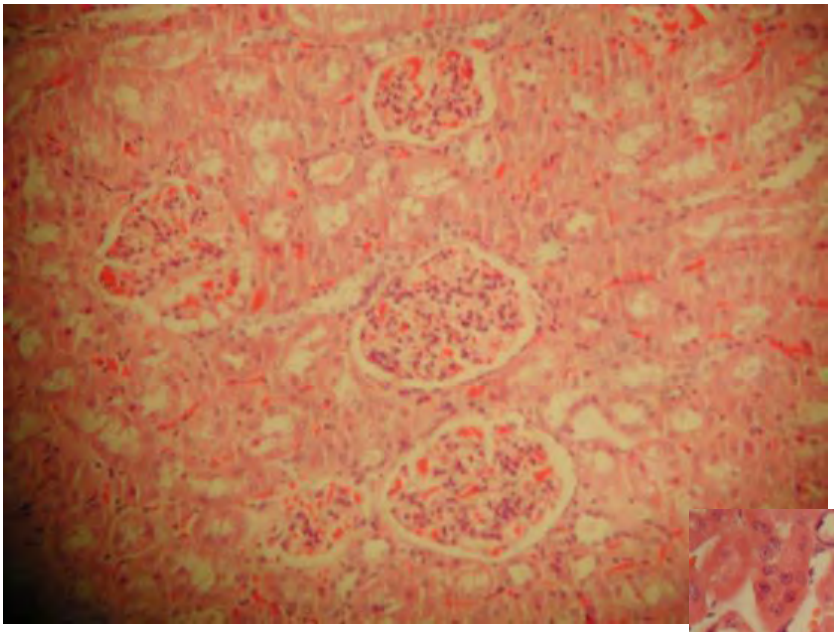
Medulla Area:

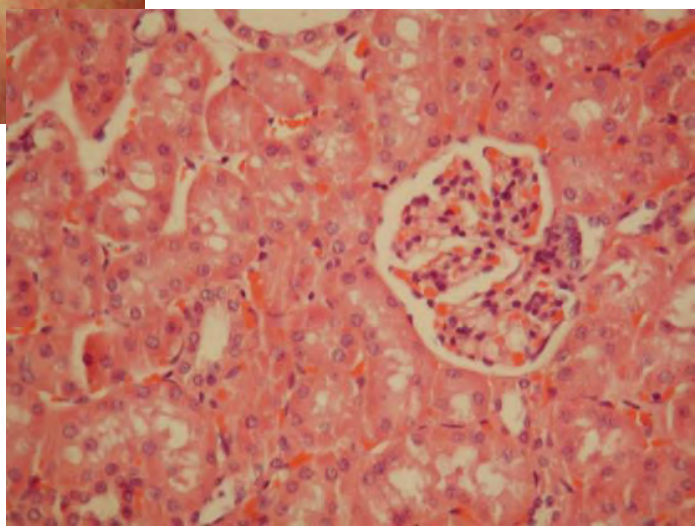
Outer part (outer – inner stripe): Have the same alterations as cortex with more intense vacuolation and hyaline casts present in the intratubular space. Hyaline casts contain proteinaceous material derived from the increase permeability of the glomeruli. Hyaline casts dye pink in the H&E stain[91]. In general, tubular cells appear to have a degree of dilation.

Inner part: Tubules and tubular cells appear normal.

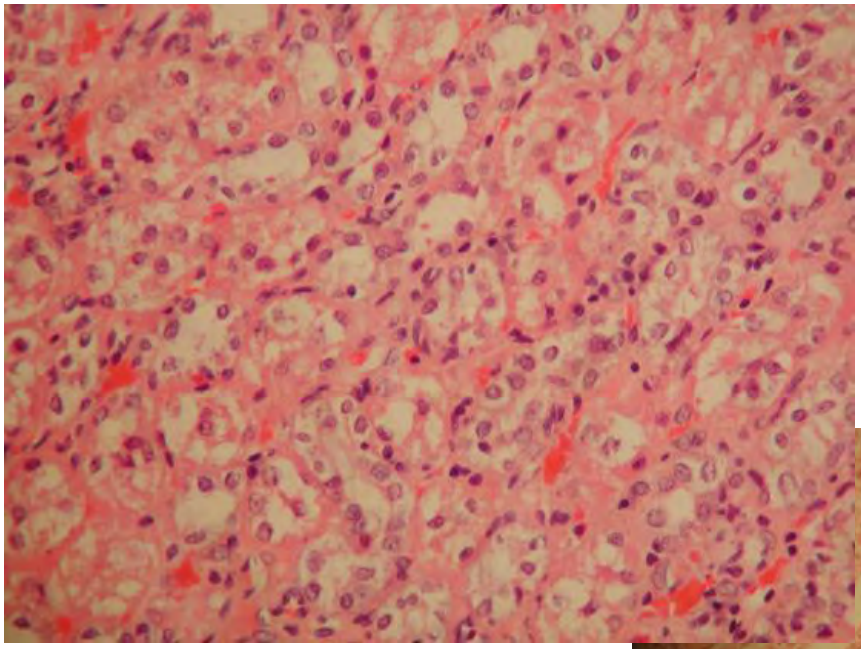
	Necrosis Cortex	Necrosis Outer Medulla	Necrosis Inner Medulla	Vacuol. Cortex	Vacuol. Outer Medulla	Vacuol. Inner Medulla	Proteinc. Casts Medulla
C2	0	0	0	0	0	0	0
C3	0	0	0	0	0	0	0
C4	0	0	0	0	0	0	0
N1D1	3	3	1	3	3	2	2
N1D2	4	4	1	3	3	2	3
N1D3	3	3	1	3	3	2	2
N1D4	3	3	1	4	4	3	2
N2D2	4	4	1	3	3	2	2
N2D3	4	4	1	3	3	2	3
N2D4	3	3	1	3	3	3	3

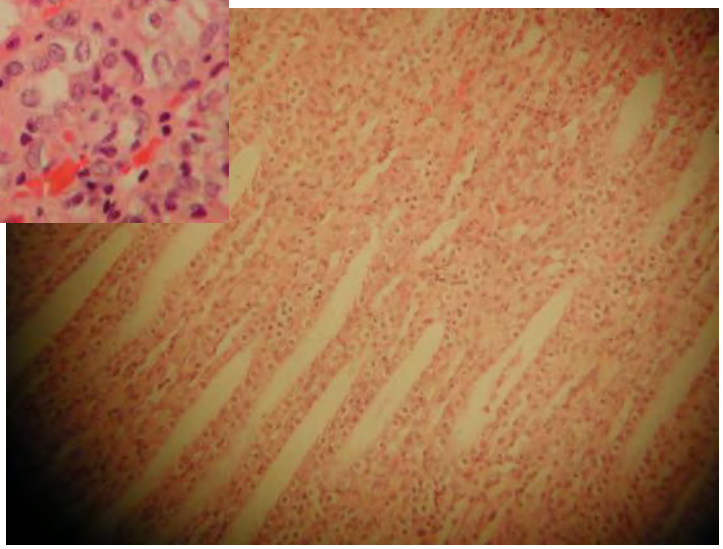
0: no damage, **1:** mild (damage less than 25%), **2:** moderate (damage between 25 and 50%), **3:** severe (damage between 50 and 75%), **4:** very severe (more than 75% damage)

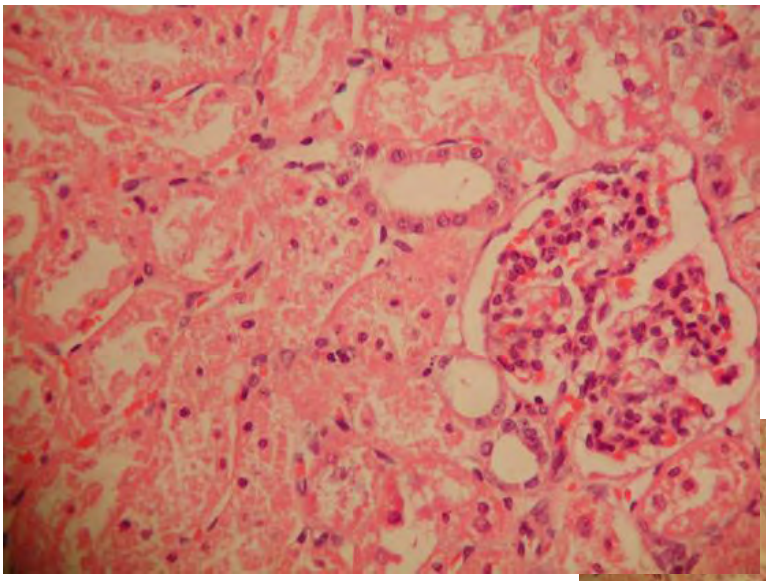


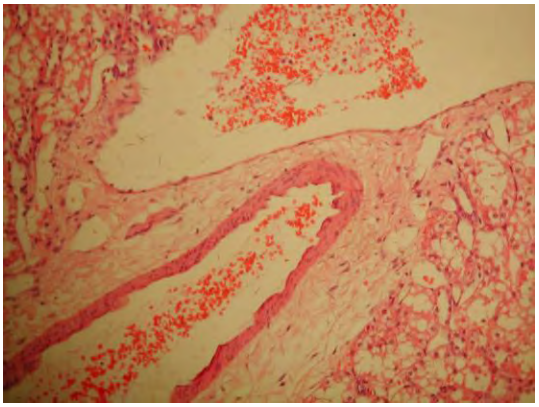


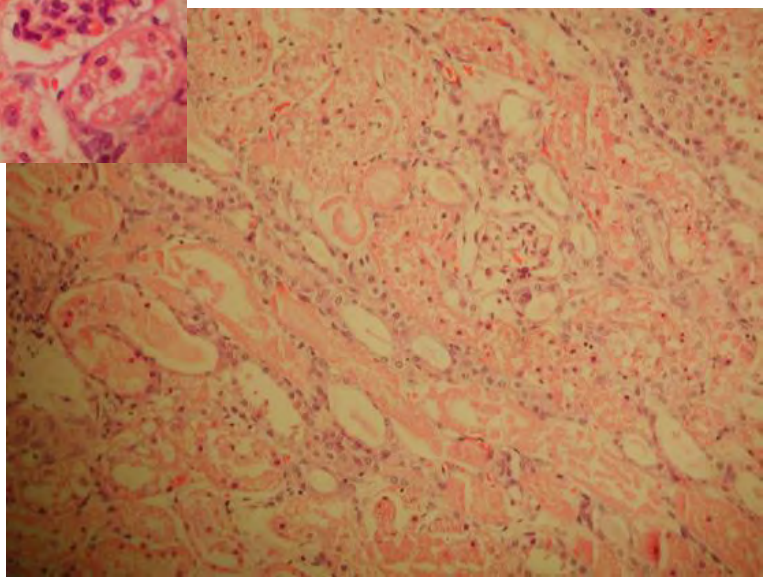
control group



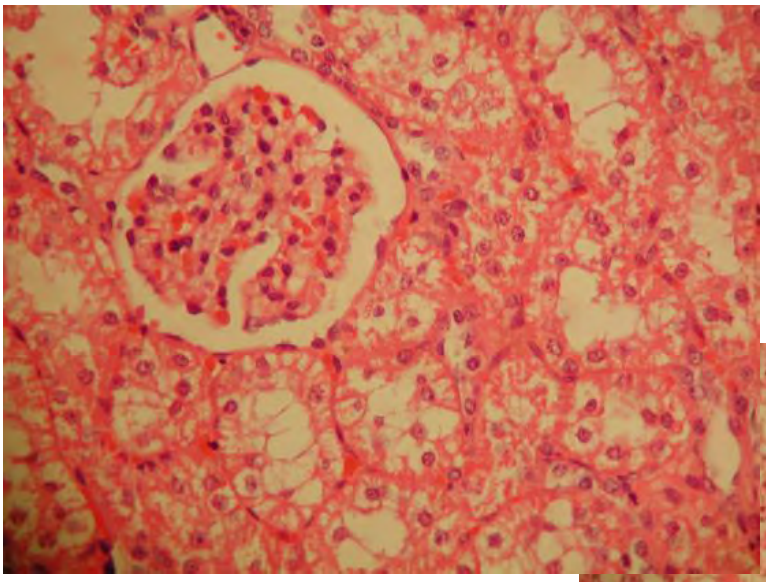




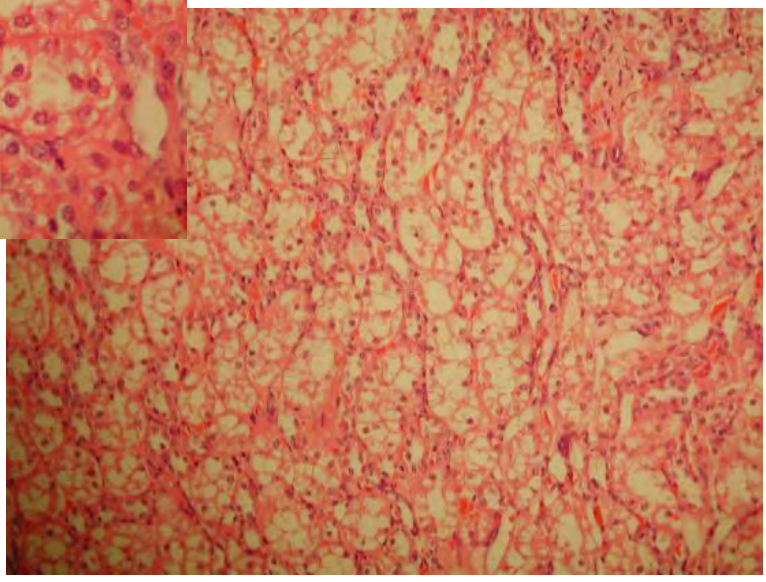




N1Dx Group



N2Dx Group



Discussion

The results in this experimental study could be summarized in four main points: a) changes in the blood's biochemical parameters, b) changes in the redox status in the blood tissue, c) changes in the histological appearance of the kidney, and d) the correlation among them.

Contrary to other studies, no water deprivation, dehydration, or any other procedure was conducted to induce nephropathy in the animals. Studies, as Bhargava et al[92].1990 have shown that rabbits are sensitive to certain CM and more than rats. Unlike Tumlin's et al[35], 2006 opinion that it is difficult to induce CIN with contrast medium alone in animals with normal renal function, present's study results indicates that is easy to induce renal injury alone with a high dose CM administration.

Iopromide is a common substance in every day clinical practice and is preferred as non ionic Low Osmolality Contrast Medium with minimum effects in renal function, especially with patients with compromised urinary track system or diabetes. Although, in high doses, as current study indicates, seems to have negative effects in renal function. Pettersson et al[93] 2002, Davidson et al[8]. 2006 and Baumgart et al[94]. 1997, they refer that in clinical practice, in certain procedures in humans, it's been used a high dose of CM that induce a slight impairment in renal function, even in patients with normal initial renal function.

Despite the limitation of the small number of treated animals in each group, evidences of alterations in renal structure and functions were observed by CM administration.

As any other study, the creatinine levels raised after the administration of CM. In the N1Dx group, CREA started to increase over 25% at 2 hours after administration and remained elevated. This indicates renal impairment. In the N2Dx group, respectively, CREA started to increase at 2 hours after administration, but at 48 hours after administration the levels of CREA seem to return at the baseline of 0 hours time point of the first blood sampling. This is an indication that the kidney posses innate mechanisms to counterbalance the action of the substance. The same pattern of changes, in both groups, follows the levels of urea. As mentioned above, CREA and UREA are indicators of renal function, thus, it is obvious the similar changes in the levels of urea.

In the correlation between biochemical parameters and redox status biomarkers, there are significant variations in bivariate Pearson correlation.

The blood's Total Antioxidant Capacity (TAC) in the 24 hours seems to be affected from the CM administration in a relation with the increase of CREA ($r=0,912$, $p=0,088$) and UREA ($r=0,916$, $p=0,084$). This is an indication that the renal damage compromises the systematic activity of TAC, as the organism try to counterbalance the injury. Also, TAC increases significantly with the administration of CM in relation with the Control ($r=0,955$, $p=0,045$) at 24 hours as an endogenous response to the xenobiotic.

The blood's TBARS in the 48 hours correlate significantly with the CREA (r= -0,981, p=0,003) and UREA (r= -0,985, p=0,002).

Additionally, CREA and UREA correlate significantly at 24 hours time point (r=0,962, p=0,038) and at 48 hours time point (r=0,947, p=0,014), as at previous results has mentioned.

Respectively with TAC, seems to correspond Catalase (CAT) in 24 hours in relation with the Control (r=0,987, p=0,013). Catalase as a very important antioxidant enzyme contributes to the CM administration repair system.

In redox status biomarkers in blood tissue there are not any changes with statistical significance in TAC and Catalase, using ANOVA statistical test.

On the contrary, TBARS in blood tissue (plasma) seems to be affected in a CM administration. In 10 hours after the administration, TBARS decrease in both groups in a percentage 37% (p=0,050) in N1Dx group and 55% (p=0,042) in N2Dx group respectively, and remain at the same levels in 24 and 48 hours.

Plasma's protein carbonyls in N2Dx group increased significantly in 2 hours after administration in a percentage 67% (p=0,000), and in 24 and 48 hours dropped significantly in a percentage 27% and 29% respectively (p=0,003 and p=0,002) in comparison to the 2 hours. These alterations are an indication that in CM administration there is an elevation at protein oxidation systematically. Since the organism possesses endogenous mechanisms in ROS invasion, the decrease is a sign that is trying to compensate the injury in proteins.

According with the histopathological evaluation, the kidneys in both CM-groups appear to have obtained a type of necrosis in the tubules, as have been evaluated in H&E stain. These type of lesions are consistent with the alterations presented in the study of Lauver et al[95]. 2014. No glomerular changes observed are consistent with a previous study of Zhao et al[96]. 2011. The scoring results signify that both groups have reached a certain plateau of morphologically alterations. This corresponds to the CREA and UREA levels, in which at 24 hours have reached the highest point and in 48 hours the kidneys seems to try to counterbalance their damage. Thus, it is possible that the lesions had appeared in an earlier stage, since the biochemical changes had occurred earlier. As in Zhao et al[96]. 2011 study presents in rats, the histopathological lesions were observed in 12 hours after administration.

Conclusion

Contrast Media, as xenobiotic substances, induce free radical release provoking a disturbance in the reduction-oxidation balance status, systematically. Even in a healthy population of rabbits, it is possible to compromise the natural renal function, hence much need to be considered in a use in a delicate population. The alterations in a healthy population seem to be short term and reversible, since the organism possesses innate counterbalance mechanisms to overcome the invasion of a xenobiotic.

Even though iopromide designed to be a much safer contrast medium, it is not free of adverse effects. The exact mechanisms of Contrast – Induced Nephropathy need to be investigated on a greater scale, in order to prevent the adverse effects with a safer contrast medium or with a substance similar to an antioxidant.

It is obvious that oxidative stress plays an important role in the pathophysiology of CIN. Changes in redox status in the blood tissue indicate systemic injury and the alterations in the kidney, local injury. Thus, further investigation in that direction is essential, both in humans and animals.

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