The Role of Nitric Oxide in the Modulation of the Leukocyte-Endothelial Cell Interactions during Ischemia and Reperfusion

Fernando López-Neblina

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THE ROLE OF NITRIC OXIDE IN THE MODULATION OF THE LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS DURING ISCHEMIA AND REPERFUSION

by

Fernando López-Neblina

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Faculty of The Graduate College
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Department of Biological Sciences

Western Michigan University
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Fernando López-Neblina
Nitric oxide (NO) is involved in several metabolic pathways and physiological phenomena. The role of NO in inflammation after ischemia and reperfusion (I/R) is controversial. The early phases of inflammation include the leukocyte-endothelial (L/EC) cell interactions which have been divided in rolling, activation, adhesion and migration.

The first experiment was to demonstrate the role of NO in a model of renal I/R. We demonstrated that exogenous NO improves survival and renal function tests after renal I/R, while endogenous NO does not appear to be an important contributor to renal I/R. We also demonstrated that NO modulate the infiltration of neutrophils (migration) in postischemic damaged kidneys, by counting the neutrophils and by measuring the activity of myeloperoxidase in renal tissue. Finally, using the model of intravital microscopy, we demonstrated in a model of mesenteric I/R in the rat, that exogenous NO down-regulated the L/EC interactions (rolling and adhesion).

These results suggest that NO exerts a beneficial effect in I/R through its ability to down-regulate the rolling, adhesion and further migration of neutrophils into the ischemically damage tissue.
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CHAPTER I

INTRODUCTION

Statement of Problem

The growing interest regarding nitric oxide (NO) and NO synthases (NOS) can be easily observed. First, NO received the distinction as "The Molecule of the Year 1992" by the journal Science. Second, if we look at the literature addressing both molecules, it can be seen that the interest in these molecules has increased in the last years, particularly to NOS which was isolated just 5 years ago (Table 1).

Table 1

<table>
<thead>
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<th>Year of Publication</th>
<th>Papers Related to NO</th>
<th>Papers Related to NOS</th>
</tr>
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<tbody>
<tr>
<td>1987</td>
<td>110</td>
<td>0</td>
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<tr>
<td>1988</td>
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<td>1992</td>
<td>1229</td>
<td>330</td>
</tr>
<tr>
<td>1993</td>
<td>1595</td>
<td>600</td>
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As a transplant surgeon I am interested in the role of NO in I/R because it is present in harvesting, preservation and transplantation. The role of NO in I/R is however controversial. Some data suggest that NO can be deleterious because of its reactivity with oxygen free radicals (Stamler, 1992). These free radicals, and NO itself, are very reactive, and they may produce the damage seen at reperfusion (Padmaja, 1993).
Other studies suggest that NO could give protection of I/R injury, however there is a lack of information regarding the mechanism of protection. Some studies attribute the protective effect of NO to its potential free radical scavenging activity (Gaobury, 1993). Other reports have found that NO decreases the adhesion of platelets to endothelium and these anti-adhesion properties are related to its ability to inactivate the superoxide anion (Kubes, 1993).

Therefore the first few questions are: What is the role of NO in I/R?, Does it play any role?, Is it beneficial?, Is it detrimental?. The next question that arises in any case is: How does NO work?

After ischemia, at the time of reperfusion, the ischemic endothelium and the leukocytes are the first cells to come in contact. They develop several interactions during inflammation. The interaction between leukocytes and Endothelial Cells (EC) has been divided in four steps: (1) Rolling, (2) Activation, (3) Adhesion, and (4) Migration. In every step, different molecules are involved, some on the surfaces of the leukocytes and some on the EC.

Given the above information, and that NO could modify the inflammatory response, another question to be addressed is: Does the mechanism of NO work through the modulation of the L/EC interactions?

Purpose of the Study

The aim of this study was to answer the questions mentioned previously. We divided this study in several steps:

1. Find out if NO is important in I/R.
2. Is the role of NO beneficial or detrimental?
3. Is a mechanism of action through L/EC interactions?
4. Utilize the information to develop a potential clinical application.
Organization of the Study

In order to achieve these aims, we designed a series of experiments, based on models that had already been tested to induce I/R (López-Neblina, 1994). This model was only useful to partially answer our questions, therefore we developed another model that allowed us the measurement of L/EC interactions. We organized this study in a defined way to orderly answer the questions of the above stated problems. First, using the model of renal I/R, we studied the role of NO. Then in the same model we studied part of the mechanism of action. To study the rest of the leukocyte-EC interactions we used the model of intravital microscopy in mesenteric I/R. Finally to study a potential clinical application we returned back to the renal model and used different times of exposure to study a potential clinical scheme of dose administration.
CHAPTER II

REVIEW OF RELATED LITERATURE

Ischemia/Reperfusion Basics

The compromise of blood flow and oxygen delivery to tissues is one of the major causes of human disease. An example in the USA, is the primary cause of, coronary heart disease, which as a part of its physiopathology involves I/R damage. Another important source of I/R is the loosely called “vascular collapse” or shock which involves several types (cardiogenic, hypovolemic, septic, neurogenic) of I/R injury. Another field of interest is the transplantation. In order to perform a transplant an organ suffers a necessary time of warm and cold ischemia, then when it is anastomosed to the recipient, it suffers the revascularization (or reperfusion) damage.

The phenomena involved in the ischemic damage and the reperfusion injury has been studied extensively. Every study has its own approach, therefore, multiple physiopathological and pharmacological manipulations have been utilized. In order to understand the differences, I will first review pathophysiology components of the I/R injury.

Pathophysiology: Ischemia

As mentioned above, the first component of this injury is an interruption of blood supply that prevents the delivery of oxygen and nutrients to the ischemic tissue. This lack of oxygen or anoxia is characterized by the conversion of cellular metabolism to anaerobic pathways in other words, the cell switches its metabolism to further fermentation excluding the Krebs cycle. In the normal well oxygenated tissue, the
metabolism in the cytoplasm starts with the fermentation of substrate to form pyruvate, at this step, the oxygen is needed to convert pyruvate into acetyl-CoA which via the Krebs cycle goes to complete oxidation in the form of carbon dioxide and water. In the case of anoxia (anaerobiosis) the pyruvate forms lactic acid as a terminal product. Herein where the problem starts. First the production of ATP via the aerobic pathways is around 18-fold higher (36 mol ATP/mol of substrate) than in anaerobic conditions (2 mol ATP/mol of substrate) (Mathews & van Holde, 1990). Then the oxygen debt and the cellular energy depletion leads to several biochemical alterations including dysfunction of the membrane transport system, and atypical activation of cytoplasmic systems. One important example of these changes is the well known increase in intracellular $\text{Ca}^{++}$ concentration $[\text{Ca}^{++}]_i$. This increase is augmented during reperfusion (see below). $[\text{Ca}^{++}]_i$, acts as a “second messenger”, and triggers activation of several enzymatic systems important to the production of the mediators of inflammation. As a result many researchers have studied the effect of calcium ($\text{Ca}^{++}$) channel blockers on I/R (López-Neblina, 1994).

Not just the $[\text{Ca}^{++}]_i$ is altered in ischemia, the lack of ATP also decreases the function of the ATPases involved in maintaining the other ion gradients ($\text{Na}$, $\text{K}$, $\text{H}$) across cell membranes. This fact results in a depolarization of the cell membrane and a larger accumulation of $\text{Ca}^{++}$ occurs within the cell. This dysfunctional membrane, leads to accumulation of fluid within the cell and results in cell swelling.

Among the $\text{Ca}^{++}$-sensitive enzymes are the cell-associated phospholipases (i.e. phospholipase A2) which stimulate the subsequent degradation of membrane phospholipids and release of free fatty acids. Rupture of the damaged membrane is an important factor in the pathogenesis of irreversible ischemic injury (Figure 1).

The effect of the ischemia is different in different organs. Every organ and every ischemic condition displays different degree of tolerance of an ischemic period.
Note: Although reduce oxidative phosphorylation and ATP levels have a central role, ischemia causes direct membrane damage (*). Source, Kumar (1991).

Figure 1. Sequence of Events in Ischemic Injury.

For example, the more active a cell is metabolically, the faster it will expend its energy reserves. It is well known that hypothermia retards the cellular metabolism and thus serves to increase tissue tolerance to ischemia. Cardiac muscle for example, due to its more active metabolism tolerates a shorter period of ischemia than does the kidney. In addition to the distinctive physiologic properties of parenchyma cells in an organ, the EC lining the local capillary and postcapillary vascular network also plays an important role in the pathophysiology (Figure 2) .
Pathophysiology: Reperfusion

Although the previously mentioned cellular events occurring during ischemia have long been recognized as contributing to the pathogenesis of organ injury, the contribution of the reperfusion to the pathogenesis of injury has only recently been recognized. The paradox of reperfusion injury is that the re-establishment of the normal vascular supply can intensify the tissue injury.

Experimental studies suggest that alterations in selected enzyme systems in ischemic cells and tissues, predispose the formation of reactive oxygen intermediates. Included among these toxic substances is the superoxide anion (O\textsuperscript{2-}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), the hydroxyl radical (OH\textsuperscript{-}), and other free radical species and oxidants (Figure 3).

It is well known that stimulated phagocytic cells can produce O\textsuperscript{2-}, and H\textsubscript{2}O\textsubscript{2} secondary to the activation of an NADPH oxidase present in cell membranes. These agents as well as, endogenous enzymes in ischemic tissues may also be other sources of these reactive compounds during reperfusion. Reactive oxygen intermediates formed within reperfusion postischemic tissue lead to a variety of microvascular alterations including EC swelling and increased capillary permeability.
Reactive Oxygen Metabolites

\[
\text{O}_2 + e^- \rightarrow \text{O}_2^- \\
\text{O}_2^- + \text{O}_2^- + 2\text{H}^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH} \\
\text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \xrightarrow{\text{Myeloperoxidase}} \text{H}_2\text{O} + \text{OHCl} \\
\text{H}_2\text{O}_2 + \text{Fe}^{3+} - \text{Heme} \rightarrow \text{H}_2\text{O} + \cdot\text{Fe}^{4+} - \text{Heme} = 0 \\
\text{Hydrogen peroxide} \\
\text{Hydroxyl radical} \\
\text{Hypochlorous acid} \\
\text{Ferryl-heme radical}
\]

Antioxidant Enzymes

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{Superoxide dismutase}} \text{O}_2 + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2 \\
\text{H}_2\text{O}_2 + 2\text{GSH} \xrightarrow{\text{Glutathione peroxidase}} 2\text{H}_2\text{O} + \text{GSSG}
\]

Figure 3. Reactive Metabolites of Oxygen and Antioxidant Enzymes.

As we mentioned in the previous section, in addition to the direct effects of the oxygen metabolites in the membrane of the EC, an important reperfusion phase is the increase in [Ca\(^{++}\)] that results in nonspecific protease and phospholipase activation. These enzymes trigger several cellular processes, many of which lead to the production of mediators of the inflammatory process. The factors activated include Platelet-Activating Factor (PAF), and the metabolites of the arachidonic acid cascade (eicosanoids) such as leukotriene, thromboxane and prostaglandin species (Peplow, 1990). These substances are lipids, which, unlike the transcribed peptides, can be generated rapidly via enzymatic transformation of cell membrane phospholipids or cytoplasmic free fatty acids. Of these molecules the most important ones in reperfusion injury are PAF, Leukotriene B4 (LTB4) and thromboxane A2 (TXA2). In addition to their primary functions, PAF and LTB4 can also amplify the inflammatory response by
increasing the more delayed production of peptide or protein mediators (vr. gr.: Cytokines such as tumor necrosis factor (TNF) and interleukine-1 (IL-1).

**Eicosanoids**

After ischemia, as an inflammatory response, EC (ECs), tissue mast cells, monocytes, platelets and circulating neutrophils can produce eicosanoids. TXA2 and LTB4 are produced by two different enzymes from arachidonic acid: cyclooxygenase and lipooxygenase, respectively (Figure 4).

![Cell membrane phospholipids](image)

**Figure 4. Arachidonic Acid Metabolites Involved in Inflammation.**

These compounds have properties that may contribute to I/R injury. LTB4 is a potent leukocyte chemoattractant that enhances neutrophil adhesion to the endothelium, emigration from the bloodstream, and release of both free radicals and proteolytic enzymes (Dahlen, 1981 & Lehr, 1991) (Figure 5). The effect of LTB4 on neutrophil-EC interaction is mediated by the CD11/CD18 receptor (Lindstrom, 1990) (a member of the integrins, one of the three families of intercellular adhesion molecules that will be discussed later (Albelda, 1990).
Figure 5. Ischemic Damage Due to Neutrophil Infiltration.

The rest of the metabolites from the arachidonic acid cascade may also participate in the inflammatory response secondary to I/R. LTD4 and LTE4 cause leakage of large molecules in reperfusion postcapillary venules (Lehr, 1991). TXA2 induces neutrophils to release oxygen reactive species (Paterson, 1989) and mediates diapedesis through regulation of the CD11/CD18 receptor activity (Goldman, 1991). TXA is also a potent vasoconstrictor and platelet aggregator and thus increase impairment of capillary flow during reperfusion (Kadowits, 1980).

Platelet-Activating Factor (PAF)

PAF a lipid, and a potent mediator of inflammation at concentrations as low as 10-12 µM (Prescott, 1990). In fact, its ability to increase vascular permeability is 1000-fold more potent than histamine. It is produced by many cells involved in inflammatory and allergic responses such as neutrophils, monocytes, macrophages, eosinophils, basophils, mast cells and EC (Sturk, 1989). PAF synthesis is induced by a variety of stimuli such as H₂O₂, thrombin, LTC₄, LTD₄, IL-1, histamine, bradykinin, ATP, and elevated [Ca++]i (Prescott, 1984; McIntire, 1985; Bussolilno, 1986; Lewis, 1986 &
Peptide Mediators of Reperfusion Injury

More than 20 years ago, the first system implicated as a mediator of ischemia injury was complement activation (Hill, 1971). Since then, many studies have demonstrated that complement activation is involved in I/R (Rubin, 1990). The results of those studies show the complement as an activator of leukocyte adhesion (Zimmerman, 1985; Marks, 1989; Carden, 1991).

Researchers have now turned their attention to the activity and function of the cytokines during reperfusion. Tumor Necrosis Factor (TNF) and Interleukine-1 (IL-1) release may occur during post-ischemic reperfusion in various organs including skeletal muscle, intestine and liver (Caty, 1990; Welbourn, 1991 and Suzuki, 1993&1994). These cytokine molecules may influence reperfusion injury through their adhesive activity (Nathan, 1989; Pober, 1986 & 1988; Suzuki, 1994).

Leukocytes vs. Endothelium: Targets of I/R Injury

The above mentioned production of mediators of inflammation secondary to ischemia influence the activity of regionally circulating leukocytes. Of the leukocytes, neutrophils are the first activated after I/R. Neutrophils through adhesion, emigration and proteolytic enzyme degranulation, contribute a great deal to the acute inflammatory injury of reperfusion. Earlier work focused on verifying the role of neutrophil infiltration in postischemic tissue (Romson, 1982; Rubin, 1989; Lee, 1992) and established this cell type as a primary generator of oxygen free radical species during
the reperfusion phase (Romson, 1983; Hernandez, 1987; Litt, 1989; Lee, 1990 &1992). The interactions between leukocyte and endothelium are crucial to the I/R injury. In general there are two mechanisms that have been described to explain the inflammatory leukocyte-EC interactions based upon either the neutrophil or the EC as the primary activated target.

**Neutrophil-dependent Adhesion**

As was mentioned before, the first cell activated is the neutrophil. In the serum after I/R, all the mediators, such as LTB4, TXA2, or components of the complement cascade, influence the leukocyte adhesion to the endothelium through modulation of the CD18 receptor activity. This process is rapid with peak of adhesiveness for neutrophils around 10 minutes and decaying over a 45-minute period (Lindstrom, 1990).

**Endothelium-dependent Adhesion**

The second general mechanism is primarily that of EC (EC) activation, this, initiates the inflammatory response after I/R. In this model, the circulating neutrophil will be attracted, activated and become adherent in response to signals derived from activated ECs. As discussed above, at reperfusion, the reactive oxygen species cause both direct vascular injury as well as production and release of lipidic and peptide inflammatory mediators. This endothelium-dependent process involves two steps in the inflammatory response: the early and the late EC-dependent adhesion.

**The Early Endothelial Cell-dependent Adhesion**

The substances involved here are reactive oxygen species (particularly H2O2), thrombin, LTB4, PAF, and components of the complement cascade. These products can initiate the early response mechanism that induce the well known rapid leukocyte-
EC adhesion (Gimbrone, 1984; Marks, 1989; Zimmerman, 1990; Lorant, 1991). This is associated not only with the previously mentioned complementary activation of neutrophil CD11/CD18 receptors, but also with the expression of the EC of the granule membrane protein 140 (GMP140), in the endothelial membrane. GMP140 is a member of the selectin family of adhesion molecules that will be described later. PAF, and neutrophil receptors (Zimmerman, 1990; Lorant, 1991) are also increased this time. This rapid response of endothelial-dependent adhesion, peaks in activity at 5-10 minutes and decays over a 30-45 minute period.

**The Late Endothelial Cell-dependent Adhesion**

In contrast, the effects of the late-response period, peaks at 4-6 hours following the stimulus, and persists for up to 24 hr. It includes the stimulation of the endothelium by the newly transcribed cytokines (such as TNF and IL-1) and the synthesis and expression of membrane endothelial leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1). ELAM-1 is another member of the selectin family of adhesion molecules, and ICAM-1 is a member of the immunoglobulin superfamily of adhesion molecules. These molecules are involved in a variety of leukocyte-EC interactions that play an important role in reperfusion, this is described in detail in the next section.

**The Leukocyte-Endothelial Cell Interactions**

The knowledge of the inflammatory interactions between leukocytes and EC are not new. Specifically, Cornelius Conheim (1839-1884) like many others in its time studied thin membranes such as the frog’s mesentery and tongue. In those preparations, Conheim observed a series of changes that occur in venules secondary to
the irritation caused by its exposure under the microscope. The description by Conheim was as follows:

This stage having been reached, the vessels are seen to be all of them very wide; a multitude of capillary which were formally hardly perceptible can now be clearly distinguished... But it is the veins rather than the capillaries that attract the notice of the observer, for slowly and gradually there is developed in them an extremely characteristic condition; the originally plasmatic zone becomes filled with innumerable colorless corpuscles. The plasmatic zone of the veins, you will remember, is always occupied by scattered colorless blood-corpuscles, which owing to their globular form and low specific gravity are driven into the periphery of the stream, and whose adhesiveness makes it difficult for them to escape from the wall once they have come into contact with it. It is obvious that this difficulty will be enhanced in proportion to the slowness of the blood stream; and thus it is not surprising that a gradual accumulation of large numbers of colorless corpuscles should take place in the peripheral zone, and here come to be comparatively motionless... Yet this does not lessen the striking contrast presented by the central column of red blood corpuscle, flowing on in an uninterrupted stream of uniform velocity... the internal surface of the vein appears paved with a single but unbroken layer of colorless corpuscles without the interposition at any time of a single red one. It is the separation of the white from the red corpuscles that gives the venous stream in these cases that characteristic appearance, anything analogous to which you will look for in vain in the other vessels... [From Harlan, 1992. pp 118-119].

Conheim also emphasized the molecular changes in the endothelium which promote the emigration of leukocytes. In contrast, Metchnikoff (1893) believed “...that the migration is effected by the amoeboid power of the leukocytes..” citing the studies of Leber (1888) who was the first to use the term chemotaxis for the directed movement of the leukocytes (Harlan, 1992).

Today all these inflammatory interactions between leukocyte and EC are well described and they have been classified into four phases: rolling, adhesion activation and migration (Harlan, 1985). See Figure 6.

Only in the past decade has the molecular bases of these adhesive interactions been established. In every step, different molecules, some on the surfaces of the leukocytes on one side and some on the EC on the other side, are involved. The cellular adhesion molecules or CAMs associated with inflammation have been classified into three groups: The immunoglobulin super family (ICAM-1, ICAM-2, ICAM-3, VCAM-
1) (Osborn, 1990), the integrins (LFA-1, Mac-1) (Hynes, 1987) and the selectins (E-Selectin, P-Selectin and L-Selectin) (Springer, 1991) (see Figure 7 and Appendix I).

Figure 6. Leukocyte-EC Interactions.

Rolling

This is the first interaction between leukocytes and EC. It is rapidly induced upon surgical preparation and tissue handling. Intravital microscopy (the technique that exposes tissues in vivo under the microscope), has demonstrated that leukocyte rolling precedes adherence at sites of inflammation. Several studies have suggested the role for selectin receptors in leukocyte rolling (Ley, 1991).

Activation

In this phase which can not be seen in intravital microscopy, the leukocytes decrease their velocity of rolling on the endothelium. Thus they are exposed to the mediators of the reperfusion injury. And as mentioned before, cellular adhesion molecules (CAMs) are expressed on the surface of the leukocytes and EC.
Adhesion

The important CAMs in this step are the CD11/CD18 molecules (Mac-1 and LFA-1) in the leukocytes and ICAM-1 in the endothelium which is up-regulated in
response to inflammatory stimuli. ICAM-1 recognizes Mac-1 and LFA-1. These CAMs are responsible for the next step of the leukocyte-EC interactions: Adhesion. Use of monoclonal antibodies against either CD11a (anti-LFA-1-la) or CD54 (Anti-ICAM-1) have demonstrated inhibition of stimulated adherence. Also CD11b (anti-Mac-1a) monoclonal antibodies also produce significant but weaker inhibition. (Argenbright, 1991).

**Migration**

Finally, after transient adhesion (rolling) and later, firm adhesion (sticking), adherent leukocytes then move across the EC surface, diapedese between EC, and migrate through the subendothelial matrix to the extravascular site of inflammation. This phenomenon is dependent on CD11/ CD18 CAMs. Monoclonal antibodies to CD11a, CD11b and ICAM-1 have been used to inhibit neutrophil emigration to tissues in several models of inflammation including I/R (Hernandez, 1987; Vedder, 1990; Ma, 1990; Welbourn, 1991; Horgan, 1990; Granger, 1991).

**Nitric Oxide**

As early as 1916, there was evidence to suggest a NO pathway in mammals including the rat, pig and human. This finding was that urine contained more nitrate than was present in their diets (Mitchel, 1916). Before 1981 NO biosynthesis was established only in microorganisms. In 1981, it was reported that the studies with $^{15}$NO$_3^{-}$ showed as the source of the excess nitrate in urine, the endogenous biosynthesis (Green, 1981). The same group demonstrated that injection of endotoxin enhanced the biosynthesis of mammalian nitrate (Wagner, 1983). Other important studies were done in 1985 by Stuehr and Marletta in which they demonstrated that activated macrophages, stimulated in vitro with lipopolissacharides (LPS) express a
nitrogen oxide synthase activity producing nitrite and nitrate. (Stuehr & Marletta, 1985).

Further studies have shown that macrophages form nitrites and nitrates are produced by enzymatic oxidation of the guanidino nitrogen of the amino acid L-arginine and that citrulline is a coproduct (Figure 8).

Also in 1987 it was known that macrophage-mediated tumor cell cytostasis was L-arginine dependent and that \( \text{N}^\text{G} \)-methyl-L-arginine was a potent, reversible and stereoselective inhibitor of this tumor cytostatic activity. It also was found to be an inhibitor of nitrite and nitrate synthesis by macrophages (Hibbs, 1987).

\[
\begin{align*}
\text{L-Arginine} & \rightarrow \text{NOS} \rightarrow \text{Citrulline} \\
& \quad \text{O}_2 \quad \& \text{NO}^\cdot 
\end{align*}
\]

Figure 8. Production of Nitric Oxide by Nitric Oxide Synthase.

Crucial evidence given by Palmer and Moncada showed that EDRF which had been studied by others (Furchgot and Zawadzki, 1980) was actually NO.

All of those studies yielded an impressive amount of information that we can just mention in this review briefly concerning the production of NO from L-arginine. These studies were carried out in neutrophils (McCall, 1989), platelets (Radomski, 1990), megacariocytes (Lelchuk, 1991), endocardium (Schulz, 1991), vascular smooth muscle (Perrella, 1993), adrenal gland (Palacios, 1989), retina (Montes, 1993), myocardium (Balingand, 1993), Kupffer cells and hepatocytes (Geller, 1993 and Billiar, 1989), non-adrenergic/non-cholinergic nerves (Guillespie, 1989), mesangial cells (Nicholson, 1993), fibroblasts (Pein, 1993), mast cells (Salvemini, 1990),

The list is still growing and probably by the completion of this thesis new findings will be discovered showing other organs, cells and systems in which the L-arginine pathway (or NO production) plays a specific role. One new finding is that lately is involved in apoptosis of macrophages (Sarih, 1993).

Nitric Oxide Synthase Isoforms

It has become apparent that the release of NO for physiological transduction purposes (basal levels) is due to the activity of one NOS while the immunostimulated NO formation is due to a different NOS (Moncada, 1991 and Stuehr, 1992). The first has been called constitutive NOS and the second one the inducible NOS. All the mammalian species studied up to now exhibit constitutive activity and the more analyzed, the more the inducible NOS has been also demonstrated. Cells that express NO activity are listed in Table 2. The general features of the constitutive and inducible isoforms of NOS are summarized in Table 3.

Physiology of Nitric Oxide

All the previous studies have established a common metabolic pathway, the synthesis of NO from L-arginine. The biological effects of endogenous NO depends upon the amount of NO that is produced and the amount of NO that reaches the target cells. When NO is produced in small amounts, it acts through the activation of soluble guanylyl cycles (Waldman, 1987 and Ignarro, 1990). This activation by NO is the one involved in vascular tone (Gold, 1990), platelet function (Radomski, 1987), renal function (Marsden, 1990) and neural signaling (Knowles, 1989).
Table 2
Tissues and Cells That Express Nitric Oxide Synthase (Several Sources)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Source</th>
<th>Isoform</th>
<th>Species</th>
<th>Cells</th>
<th>Source</th>
<th>Isoform</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery and Vein</td>
<td>Constitutive</td>
<td>Cow, Rat, Pig,</td>
<td>Macrophages</td>
<td>Both</td>
<td>Human</td>
<td></td>
<td>Mouse, Cow, Pig</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human, Dog</td>
<td></td>
<td></td>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea pig</td>
<td></td>
<td></td>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric vessels</td>
<td>Constitutive</td>
<td>Rat</td>
<td>Endothelial cells</td>
<td>Both</td>
<td>Mouse, Pig, Cow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpus cavernosum</td>
<td>Constitutive</td>
<td>Rabbit, Rat</td>
<td>Hepatocytes</td>
<td>Both</td>
<td>Rat, Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>Constitutive</td>
<td>Rabbit, Guinea pig</td>
<td>Neutrophils</td>
<td>Both</td>
<td>Rat, Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Constitutive</td>
<td>Pig, Rat</td>
<td>Platelets</td>
<td>Constitutive</td>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Both</td>
<td>Rat, Human</td>
<td>Astrocytes</td>
<td>Constitutive</td>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenals</td>
<td>Constitutive</td>
<td>Rat</td>
<td>Mesangial Cells</td>
<td>Inducible</td>
<td>Cow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Inducible</td>
<td>Rat</td>
<td>Endocardial Cells</td>
<td>Inducible</td>
<td>Cow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>Inducible</td>
<td>Rat</td>
<td>Smooth Muscle Cells</td>
<td>Inducible</td>
<td>Cat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Inducible</td>
<td>Rat</td>
<td>Microglial Cells</td>
<td>Constitutive</td>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Constitutive</td>
<td>Human</td>
<td>Renal Tubule Cells</td>
<td>Inducible</td>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye</td>
<td></td>
<td>Rat, rabbit</td>
<td>Chondrocytes</td>
<td>Inducible</td>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>Constitutive</td>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td></td>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When NO is produced in larger amounts, it has different actions: it inhibits the ribonucleotide reductase (Stuher, 1989), and cellular DNA synthesis (Busconi & Michel, 1993); blocks oxidative phosphorylation by inactivating iron-sulfur centers in aconitase and complex I and II of the mitochondrial electron transport chain (Boutelle, 1993 and Nakane, 1993), and decreases the synthesis of proteins by an unknown mechanism (Masaki, 1993).
Table 3
General Features of the Nitric Oxide Synthase Isoforms

<table>
<thead>
<tr>
<th>Features</th>
<th>Inducible</th>
<th>Constitutive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototypic Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td>EC</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td></td>
<td>Cerebellar cells</td>
</tr>
<tr>
<td>Tumor cells</td>
<td></td>
<td>Platelets</td>
</tr>
<tr>
<td>Dependent on Calcium/Calmodulin</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Tetrahydrobiopterin</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FAD</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FMN</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>Inhibited by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>Partially</td>
<td>Completely</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>N^w-substituted arginines</td>
<td>NAA=NMA&gt;NNA</td>
<td>NAA=NNA&gt;NMA</td>
</tr>
<tr>
<td>Diphenyleneiodonium</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>NAA= N^w-amino-L-arginine; NMA=N^w-Methyl-L-arginine; NNA=N^w-nitro-L-arginine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Role of Nitric Oxide in I/R

The role of NO in I/R remains controversial. There are papers that suggest that NO can be deleterious because of its reactivity with oxygen free radicals (Stamler et al 1992). These free radicals and NO which itself is considered a free radical, are very reactive, and they may produce the damage seen at reperfusion (Padmaja, 1993).

On the other hand, some authors suggest that NO could give protection from ischemia/reperfusion injury. There is a lack of information regarding the mechanism of protection. Some attribute the protective effect of NO to its potential free radical scavenging activity (Gaobury, 1993). Others have found that NO decreases the adhesion of platelets to endothelium and these anti-adhesion properties are related to its ability to inactivate the superoxide anion (Kubes, 1993).
I/R is a very complex phenomenon in which several systems are involved and work in a delicate, organized manner. Basically, at the time of ischemia the energy falls down and the systems that maintain the homeostasis of the cell suffer. At the time of reperfusion, there is a production of a number of inflammatory that induce leukocyte and EC interactions. These interactions have been divided in four steps: (1) Rolling, (2) Activation, (3) Adhesion, and (4) Migration. In every step, different molecules, some on the surfaces of the leukocytes and others on the EC, are expressed. They are the ones responsible for these interactions.

Nitric Oxide (NO) is known to occur in a variety of mammalian cells. It has been shown that two enzyme systems are necessary for NO biosynthesis.

The constitutive enzyme that generates NO in small amounts appears to function primarily as a short-range messenger molecule. The inducible enzyme produces large quantities of NO. The role of NO in I/R (I/R) is controversial. After (I/R), NO can be deleterious because of its reactivity with oxygen free radicals. These radicals, along NO itself are very reactive, and they may produce the damage seen at reperfusion. However, recent studies have shown a potential beneficial effect of NO in I/R.
CHAPTER III
DESIGN, MATERIAL AND METHODS

Experiment # 1: Role of Nitric Oxide in Ischemia and Reperfusion

Hypothesis

In order to answer the questions discussed in the introduction, we developed the next hypothesis:

$H_1$: Nitric oxide plays an important role in I/R

$H_{1.1}$: NO is beneficial

$H_{1.2}$: NO is detrimental

$H_0$: Nitric oxide does not play any role in I/R.

In this case we approached our first experiment as a two tailed study (NO could or could not play an important role and this role could be beneficial or detrimental). Basically this study attempted to define the role NO played in renal I/R.

Model of Rat Renal I/R

We have chosen this model because we had experience in handling the rat and based on previous experiments, we reached a “dose” of renal I/R which caused death to 50% of the animals submitted to that injury. Our ischemic time (IT) 50 or IT$_{50}$ was 75 minutes of renal pedicle ligation (including artery and vein) (López-Neblina, 1994).

In more detail, Sprague-Dawley rats (Charles River Laboratories, Portage MI), weighing 250-300 g were subjected to 75 minutes of warm ischemia as follows: under intraperitoneal sodium pentobarbital injection (Butler Co., Columbus, OH) (60 mg/kg
body weight) and through a midline abdominal incision, the intestines were displaced to the right side. The left renal vascular pedicle including artery and vein were dissected out, and the perirenal fat was preserved. The renal vascular pedicle was temporarily ligated with 2-0 silk, the intestines were repositioned in the abdominal cavity, and the incision was covered with a moist polyethylene sheet (Appendix C).

**Experimental Design**

This model with a $IT_{50}$ allowed us to test if certain medications or treatments were beneficial (increment in survival) or detrimental (decrease in survival). The animals were divided into six groups. Twelve animals per group were used for survival and laboratory tests: Group I, ischemic control (IC), received normal saline; group II, L-$N^G$-monomethyl-arginine (MeArg) 50 mg/kg; group III, L-Arginine (300 mg/kg); group IV, Na-Nitroprusside (Na-NP)(50 mg/kg) group V, the combination of MeArg + Na-NP at the same dosages; and group VI, the sham group. This last group was exposed to the same conditions as the other groups such as laparotomy, nephrectomy, dissection of the renal pedicle, intravenous infusion of saline solution 0.9% but with no ligation of the renal vessels. A rationale for this design is presented in Table 4.

All animals received the drug through the penile vein 60 minutes prior to the ischemic event. MeArg and L-Arg were obtained from Sigma Lab (St. Louis, MO) and Na-NP from Abbott Labs. (North Chicago, IL). At the end of the ischemic period, the abdominal cavity was re-entered, the ligature was removed, and a right contralateral nephrectomy was performed. The abdominal cavity was closed with 4-0 silk. The animals received standard pellet diet (Harlan Sprague-Dawley, Indianapolis, IN), water ad libitum, and were observed for survival for seven days (Appendix D). A schematic of the design is shown in Figure 9.
Table 4
Design and Rationale of the Experiment #1

<table>
<thead>
<tr>
<th>Group</th>
<th>Substance and Dose</th>
<th>Function of Substance</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I Ischemic Control</td>
<td>0.9 % Saline</td>
<td>Just as a control group</td>
<td>To observe the changes induced by I/R</td>
</tr>
<tr>
<td>Group II Antagonist</td>
<td>L-N\textsubscript{G}G monomethyl-arginine (MeArg) 50 mg/kg</td>
<td>Competitive antagonist of the arginine that blocks the NOS</td>
<td>Study the changes observed by the blocking of NOS and NO after I/R</td>
</tr>
<tr>
<td>Group III Substrate</td>
<td>L-Arginine 300 mg/kg</td>
<td>Substrate for the NOS to produce NO</td>
<td>To assure enough substrate for the enzyme</td>
</tr>
<tr>
<td>Group IV NO-donor</td>
<td>Na- Nitroprusside 50 mg/kg</td>
<td>Exogenous NO-donor</td>
<td>In case the endogenous NO was not enough</td>
</tr>
<tr>
<td>Group V Combination of II and IV</td>
<td>Same substances and doses as in groups II and IV</td>
<td>Intentionally block endogenous NO by giving a exogenous NO donor</td>
<td>To observe the function of the exogenous NO alone</td>
</tr>
<tr>
<td>Group VI Sham</td>
<td>0.9% NaCl No ischemia</td>
<td>Normal changes due to the surgery without I/R</td>
<td>To observe the effect of the surgical technique without I/R</td>
</tr>
</tbody>
</table>

Variables of the Study, Samples and Procedures

The variables are: Renal function, lipid peroxidation, tissue I/R damage. The renal function was assessed with laboratory studies including blood urea nitrogen (BUN) and serum creatinine (SCr), obtained at 24, 48 and 72 h. These samples were obtained by drawing 1 ml of central venous blood from a catheter placed from the jugular vein through the right atrium in the heart and then replacing it with the same amount of lactated Ringer’s solution. The BUN and SCr samples were processed in a Hitachi 717 automatic analyzer (Boehringer Mannheim, Indianapolis, IN).
Lipid peroxidation was assessed at 10 minutes of reperfusion in five more animals per group. The amount of malondialdehyde (MDA) formed in the reaction with thiobarbituric acid was measured. After 10 minutes of reperfusion, the kidney was flushed out through a tube placed in the abdominal aorta with 0.9% NaCl. Once washed out, the kidney was homogenized 4°C 0.9% NaCl and stored at -20°C until assay.

The assay was performed according to Tappel & Zalin (1959), 0.5 ml of the whole homogenized sample was mixed with 10% Trichloroacetic Acid. It was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was mixed with 0.67% of TBA and the mixture was boiled for 10 minutes and cooled to room temperature in ice. Samples were spectrophotometrically read at 532 nm and nanomoles of malondialdehyde calculate from a standard curve using malondialdehyde (F.W. 164.2).
The protein content was detected with the Lowrey method, using bovine serum albumin (BSA) as standard. The MDA level was expressed as nmol/mg protein. Absorbance was obtained using a Beckman DU 650 UV/VIS programmable scanning spectrophotometer (Beckman Instruments, Fullerton, CA).

Five more animals per group were used for histological studies. Kidney samples obtained 24 hr after I/R. 5-mm sections were stained with hematoxylin and eosin for histological analysis. They were evaluated using a prior described scale (Jablonski 1983) to assessed the severity of the lesion. The lesion was graded from 1 to 4, according to the following criteria: Grade 1, mitoses and necrosis of individual cells; grade 2, necrosis of all cells in adjacent proximal convoluted tubules with survival of surrounding tubules; grade 3, necrosis confined to the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner cortex; and grade 4, necrosis affecting all three segments of the proximal convoluted tubule.

Statistical Analysis

The statistical analysis included the use of the Student-Newman-Keuls analysis of variance (ANOVA) test at critical values for the variance ratio F at p=0.05. The survival at each point (every day) was analyzed using the Fisher’s exact test, and the histological samples were analyzed with the Kruskal-Wallis test comparing with critical values at 0.05.

Results

Survival

There were significant differences between the Na-NP group and all other groups (p<0.05) at 72 and 96 h. and at 7 days. There were no significant differences
between the sham group and the Na-NP group. No differences were noted among the rest of the other groups (Figure 10).

![Graph showing survival rates of different groups](image)

**Legend:**
- IC = Ischemic Control
- MeArg = L-NG-monomethyl-arginine
- L-Arg = L-Arginine
- Na-NP = Na-Nitroprusside
- MeArg+Na-NP
- Sham

Figure 10. Survival of All Experimental Groups.

**Kidney Function Tests**

Figure 11 shows the SCr levels at 24, 48 and 72 hrs. The lowest levels were obtained in the Na-NP group at all points on the curve. There were no significant differences among the other groups. The BUN results were similar to those observed with the SCr, with the best finding noted in the Na-NP group.

**Lipid Peroxidation**

The MDA levels (Figure 12) were high in the Na-NP group compared to the other groups. The MeArg group had a significant decrease in the MDA levels when
compared to the other groups. There were no significant differences between the IC and the L-Arg groups.

![Graph showing serum creatinine and blood urea nitrogen levels across different groups.]

Figure 11. Serum Creatinine and Blood Urea Nitrogen of Experiment 1.

**Histology**

In the IC (median = 4) and the MeArg (median = 4) groups, there was necrosis affecting all three segments of the proximal convoluted tubules (Figure 13-A and 13-B). In contrast, the ischemic damage was significantly (P<0.05) diminished in the Na-NP group (median = 2), showing necrosis of individual cells, with survival of
surrounding tubules. The necrosis was limited to some areas of the inner medulla and outer medulla (Figure 13-D).

![Figure 12. Malondealdehyde Activity in Experiment 1.](image)

**Discussion**

This study demonstrated that exogenous NO excerts an important beneficial role in renal I/R injury. Other investigators have suggested that NO could have a deleterious effect in I/R, supposedly through the interaction between NO and superoxide free radicals (Saran, 1990; Ischiropoulos, 1990; Hogg, 1992). There is evidence that the production of nitric oxide from L-arginine contributes to the cytotoxicity of macrophages (Hibbs, 1987 & 1988; Becherman, 1993; Granger, 1988; Styer 1989). NO has been studied mainly in the septic model (Minnard, 1994). NO has been evaluated in ischemia/reperfusion *in vitro*, in macrophages and EC (Hibbs, 1988 & Schoror, 1991). Most of the *in vivo* ischemia studies are in cat and dog hearts (Weyrich, 1992), and in cat intestines (Carey, 1992). In those studies, it was found that L-Arg relieves the damage seen after I/R injury. In the same model, NO donors shown antishock and endothelial protective actions (Weyrich, 1992). There is only one
Figure 13. Histological Samples of the Experimental Groups.
study *in vivo* using MeArg and L-Arg in the rat kidney (Cristol, 1993). When the endogenous production of nitric oxide was blocked, the recovery from I/R injury was prevented. In another study, using the isolated perfused kidney, after 25 min. of ischemia, it was concluded that the endothelial derived relaxing factor activity was impaired following I/R (Cristol, 1993). It was suggested that this phenomenon may be an important mechanism contributing to postischemic vasoconstriction in the renal vasculature and associated damage (Cristol, 1993).

This is the first study in the literature so far using Na-NP in ischemic kidneys *in vivo*. In this study, after severe ischemia, we found that endogenous NO does not play an important role in renal I/R, but exogenous NO does have a significant beneficial effect. These results agree with recent findings of inhibition of nitric oxide synthesis during endotoxemia (Minnard, 1994). In the septic model, it was found that the inhibition of NO is detrimental during endotoxemia.

The inhibition of NO diminished its protective effect (Cristol, 1993 & Lieberthal, 1989). Others (Wright, 1992) have found in the same septic model, however, that the use of low doses of non-isofom selective inhibitors or of higher doses of relatively selective inhibitors of the inducible isoform of NOS have beneficial effects. In our experiment, we found a significant protective and beneficial effect from exogenous NO, in the renal I/R model.

We did not know the mechanism of action of exogenous NO, but interestingly, it appears to be independent of lipid peroxidation. This was probably due to a very high lipid metabolism and recovery rate of the kidney compared to other organs. On the other hand, we thought that the beneficial effect of NO was more than a vasodilating effect, since in other experiments, allopurinol or a Ca²⁺ channel blocker, Verapamil (López-Neblina, 1995), demonstrated some beneficial effect, but not of the same magnitude encountered with the Na-NP group.
Others suggest the possible modulating mechanism of NO in the adhesion of platelets and neutrophils (Johnson, 1991). Thus, the beneficial effect observed in our study may be due to the interaction between NO and EC and/or leukocytes and not only due to a vasodilating effect.

Experiment #2: Modulation of Neutrophil Infiltration by Exogenous NO

Hypothesis

Exogenous nitric oxide (NO) exerts an important protection in renal I/R. It was not clear what is the mechanism of this protective effect. The purpose of this experiment is to determine whether exogenous NO has any effect in neutrophil infiltration of severely ischemic damaged kidneys.

H₁: Exogenous NO decreases neutrophil infiltration after renal I/R
H₀: Exogenous NO does not affect neutrophil infiltration after renal I/R

In this case, we choose a single tailed study (specifically looking for a decrease in neutrophil infiltration). We used the same model as in the previous experiment: rat renal I/R.

Experimental Design

Forty-eight male Sprague-Dawley rats weighing 250-300 g, were subjected to 75 minutes of total warm ischemia (detailed technique in the previous experiment). The animals were divided into two groups. Twelve animals per group were used for survival and kidney function tests. Group I, the ischemic control (IC), received saline solution in the same amount as the treated one, and Group II, the experimental group, which received Na-nitroprusside (NP) 50 mg/kg. The drug was administered
immediately after the ligation of the renal pedicle, through the penile vein, and before reperfusion (Figure 14).

![Diagram](image_url)

**Ischemia**  
**75 minutes**  
**Reperfusion**

Figure 14. Design of Experiment #2.

**Variables of the Study, Samples and Procedures**

Laboratory studies included Blood Urea Nitrogen (BUN) and Serum Creatinine (SCr), assessed at 24, 48 and 72 hr. by obtaining 1 ml of central venous blood and replacing it with same amount of normal saline. The BUN and SCr levels were analyzed using the Hitachi 717 automatic analyzer (Boehringer Mannheim, Indianapolis, IN). The amount of malondialdehyde (MDA) produced was measured in the same way as in the previous experiment. Neutrophil infiltration was measured by
myeloperoxidase activity in tissue, which is an enzyme specific for neutrophils. The method used was that of Bradley (1982) as modified by Mullane et al (1985). MPO tissue-levels were used as an index of neutrophil accumulation in the kidney. After the period of I/R, the aorta was cannulated and the kidney was flushed out in situ with cold saline solution, immediately removed and frozen in liquid nitrogen and stored at -70 °C until assay was performed. Kidney samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical Co., St. Louis MO), dissolved in 50mM potassium phosphate buffer with 0.146% of EDTA (Sigma Chemical Co., St. Louis MO) at pH 6.0. Homogenates were centrifuged for 30 minutes at 12,500 g. The supernatants were decanted and incubated at 60°C for two hours, 0.167 mg/ml of O-dianisodine dihydrochloride (Sigma Chemical Co., St. Louis MO), and 0.005% hydrogen peroxide in 50mM phosphate buffer at pH 6.0 were then added. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1mmol peroxide/min. at 25°C.

Five animals per group were used for histological studies. Kidney histology samples were obtained 24 hours after ischemia/ reperfusion. These were embedded in paraffin, cut, stained with hematoxilin and eosin and evaluated using the previously described scale (Jablonsky, 1983) that assesses the severity of the rat renal injury after I/R. The evaluation of the histological damage was done blindly.

Statistical Analysis

To test our hypothesis we used the non paired Student's t test rejecting the null hypothesis at α=0.05. The survival at each point (every day) was analyzed using the Fisher exact test and the histological samples were analyzed with the Mann-Whitney U
test comparing with critical values at 0.05, and significance was established at this level.

Results

Figure 15 demonstrates statistically significant differences in survival (p<0.05) between the two groups at all points. The survival of the NP group was better than the IC group.

There were statistically significant differences (p<0.05) between the BUN and SCr levels of the two groups at 24, 48 and 72 hr. The BUN and SCr levels of NP group were lower than the IC group (Figures 16 and 17). The MDA levels were significantly higher (p<0.05) in the NP group compared to IC. In contrast, the values for MPO activity were significantly lower (p<0.05) in the NP group compared to those of the IC group (Table 5).

In the IC, there was necrosis affecting all three segments of the proximal convoluted tubules (Figure 18-A, median value = 4). In contrast, the ischemic damage was significantly reduced in the NP group (p<0.05), showing necrosis of individual
cells, and survival of surrounding tubules, with the necrosis limited to some areas at the inner medulla and outer medulla (Figure 18-B, median value=2).

![Image of SCr Levels of Experiment #2.](image)

**Figure 16.** SCr Levels of Experiment #2.

![Image of BUN Levels of Experiment #2.](image)

**Figure 17.** BUN Levels of Experiment #2.

**Discussion**

In this experiment, we demonstrated that NP significantly modulates the infiltration of neutrophils following renal I/R injury. This was the first study to identify such a mechanism of action for NP. It appears that NP works through a mechanism independent of lipid peroxidation. The protective effect observed in our studies may be
### Table 5

MDA Values and MPO Activity in Kidney Tissue Samples, Experiment #2
(Values Expressed as Mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>MPO (Abs/g)</th>
<th>MDA (nM/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic Control</td>
<td>0.196 (± 0.017)</td>
<td>4.4 (± 0.3)</td>
</tr>
<tr>
<td>Na-Nitroprusside</td>
<td>0.113 (± 0.015)*</td>
<td>8.4 (± 0.3)*</td>
</tr>
</tbody>
</table>

p<0.05 compared to control group

---

Figure 18. Histological Samples, Experiment #2.
due to the interaction between NO and leukocytes, because the NP was given after the ligation of the renal pedicle. In this way, the treatment is selectively affecting the leukocytes, and the contact between exogenous NO and endothelium is prevented. At the time of reperfusion, even though the effect of exogenous NO (which lasts 2-3 minutes) was not present, we found diminished neutrophil infiltration and MPO activity in the tissue, which correlated well with animal survival. Thus, NO plays a modulating role in the migration of neutrophils, probably through downregulation of adhesion molecules, such as β2 integrins and/or selectins, and its added membrane expression.

Experiment #3: Exogenous Nitric Oxide and Leukocyte-Endothelial Cell Interactions

We had already demonstrated that NO modulate the infiltration of neutrophils (migration) in postischemically damaged kidneys. But with our model of renal I/R it was not possible to measure the other leukocyte-EC interactions. The early phases of inflammation can be easily studied using the model of intravital microscopy. In this model we can actually measure the leukocyte-EC interactions such as rolling and adhesion to endothelium.

In this experiment, we studied the early phases of the inflammation cascade rolling and adhesion when animals received the nitric oxide releaser Na-nitroprusside as a pretreatment. We tested the hypothesis that exogenous nitric oxide prevents early phases of inflammation secondary to mesenteric ischemia.

\[ H_1: \text{Exogenous NO decreases Rolling and/or Adhesion After Mesenteric I/R} \]

\[ H_0: \text{Exogenous NO does not affect Rolling and/or adhesion} \]

Model of Intravital Microscopy and Mesenteric Ischemia and Reperfusion

Sprague-Dawley rats, weighing 250-300 g, were subjected to 45 minutes of mesenteric ischemia. Under intraperitoneal sodium pentobarbital anesthesia (Butler
Co., Columbus OH) and through a midline incision, the abdominal contents were very gently displaced to the right side. The superior mesenteric artery were dissected out, and a 2-0 silk was placed around the artery. The silk was exteriorized through the posterior abdominal wall of the rat using an endocath as a tunnel. The animal was then heparinized with 50 Units of Na-Heparin and the last 8-10 cm of the ileum where exteriorized and placed in a special board that can be placed under the microscope (Figure 19). The rest of the abdominal content was replaced into the abdominal cavity and the incision closed with 4-0 silk. The preparation was always superfused with 37°C ringer lactated and the preparation was allowed to stabilize for 5 minutes. A suitable (35-40 microns of diameter) venule was localized and then, the mesenteric artery was occluded for 45 minutes pulling the 2-0 silk. At the end of the ischemic period, the ligature was removed and the measurements were recorded using a color videocamera (Sony CCD-IRIS, DX-107A, Japan) and saved in a VCR (Mitsubishi HS-V58) to be analyzed later. Some pictures were taken with the Full Color Printer System (Panasonic AG-EP70 Video-Printer, Japan).

Experimental Design

The animals were divided into 2 groups. Group I, the ischemic control (IC, n=5), received one ml of 0.9% saline solution and Group II, the experimental group, received Na-nitroprusside (NP) 50 mg/kg in one ml of 0.9% saline solution (Abbott Labs. North Chicago IL) through the penile vein, 60 minutes before ischemia.
Variables of the Study, Samples and Procedures

Rolling

The rolling was measured using a 10 micron window. The number of rolling cells in 60 seconds was counted. The time of rolling was measured and defined as the time that takes a leukocyte to roll 100 microns. Twenty leukocytes were measured per animal in a random fashion.
Adhesion

Adhesion was defined as the number of cells that remain motionless (adhered) for more than 30 seconds in a 100 microns length on the venule. Measurements were performed 20 times in each animal in different areas of the venule. After measurement, the animals were sacrificed.

Statistical Analysis

The statistical analysis included unpaired Student’s t test to compare the control group and the experimental group. The comparisons were done at alpha critical values of 0.05, and significance was established at this level.

Results

Figure 20 shows time of rolling: The maximum expression of rolling was at 3 minutes of reperfusion and it was maintained until 30 minutes postreperfusion. In contrast, in the NP group there was a peak at 3 minutes, but it decreased to a normal values after 5 minutes. The increments were much more lower than those observed in the ischemic control (IC) group. There were statistically significant differences (p<0.05) the two groups at all points in time.

A picture taken at 5 minutes of reperfusion (Figure 21) shows the comparison of adhesion observed between the ischemic control (A) and the NP group (B). It shows an important attachment of leukocytes in the IC group (arrows) compared to almost no attachment of neutrophils in the NP group.

Figure 22 shows the graph of adhesion obtained. In the IC group, the peak of adhesion was at 5 minutes, and decreases as the time increases never returning to normal values. In contrast, in the NP group a significant difference can be seen.
Adhesion (p<0.05) also peaks at 3-5 minutes, but it returned to basal levels at 30 minutes postreperfusion.

Figure 20. Rolling in Mesenteric Ischemia Experiment.

Figure 21. Adhesion at 5 Minutes of Mesenteric Ischemia.
Figure 22. Graph of Adhesion After Mesenteric Ischemia.

Discussion

This study demonstrated that NP had a modulating effect in the prevention of leukocyte rolling and adhesion. In our previous studies, we had demonstrated that the beneficial effect of NP was through the inhibition of neutrophil infiltration (migration) into the ischemically damaged kidney. The beneficial affect of NO appears to go beyond its early vasodilating effect, since the administration of NP was 60 minutes prior to the ischemic event. It is well known that the early phases of interaction between leukocyte and adhesion molecules is due to the expression of adhesion molecules (Harlan, 1992). Exogenous nitric oxide may be acting through modulation of adhesion molecules, such as β2 integrins and/or selectins. Our work as well as the work of others (Gauthier, 1994) has supported the theory of nitric oxide as a modulator of the interaction of leukocytes and EC. Early phases of inflammation have been related to expression of selectins. P-selectin expressed on the surface of activated EC and platelets, is an adhesion receptor for leukocytes. It appears to play an important role in
early phases of leukocyte interactions with the vessel wall. It is also important in the early steps of leukocyte recruitment, such as rolling and early adhesion, at sites of inflammation (Mayadas, 1993). Other cellular adhesion molecules such as ICAM-1 and ICAM-2 on the endothelial surface and 2-integrins in the leukocytes surface interact to continue the next step in inflammation, leukocyte migration. Migration has been related to another set of specific adhesion molecules (Harlan, 1992).

**Experiment #4: A Potential Clinical Approach**

No one knows at what moment an ischemic event will occur. Thus, it is difficult to give drugs as a “pretreatment”. Ideally a drug that limits ischemic damage administered as close as possible to the reperfusion or even after reperfusion.

In order to find out if this NO donor could be given in a more realistic approach we performed the following experiment. We administered the drug after ischemia and several times before reperfusion. We tested the hypothesis that exogenous nitric oxide, even when administered after the ischemic event and after reperfusion, has beneficial effects.

$H_1$: Exogenous NO can be administered near reperfusion and still be beneficial.

$H_0$: Exogenous NO is not beneficial when administered near reperfusion.

In this experiment, we studied the ideal time of administration of exogenous NO, through Na-Nitroprusside, in the ischemically damaged rat kidney.

**Model of Renal Ischemia Reperfusion**

We studied the same model of renal I/R described at the beginning of this paper.
Design and Variables and Samples

One hundred and sixty-two male Sprague-Dawley rats, weighing 250-300 g, were subjected to 75 minutes of left renal warm ischemia. The animals were divided into 5 groups. Group I, the ischemic control (IC, n=44), received saline solution in the same amount as the treated ones. The experimental groups received Na-nitroprusside (NP), 5 mg/kg (Abbott Labs. North Chicago IL) through the penile vein, after the ligation of the renal pedicle, at 75 (NP75, n=12), 30 (NP30, n=12), 15 (NP15, n=12) and 5 (NP5, n=12) minutes, before reperfusion. At the end of the ischemic period, the animals were observed for survival for seven days. Laboratory studies included blood urea nitrogen (BUN) and serum creatinine (SCr), assessed at 24, 48 and 72 hr. by obtaining 1 ml of central venous blood and replacing it with same amount of normal saline. The BUN and SCr samples were processed in the same manner as previously discussed. Neutrophil infiltration was measured in 6 animals per group (n=30) by the MPO activity in tissue in the same way as previous experiments. Four animals per group were used for histological studies (N=20). Kidney histology samples were obtained 24 hours after ischemia/reperfusion and were evaluated using the previously described scale. The evaluation of the histological damage was done blindly. A scheme of the experimental design is presented in Figure 23.

Statistical Analysis

The statistical analysis included ANOVA with F values of significance at $\alpha=0.05$. The survival at each point (every day) was analyzed using the Fisher’s exact test. The histological samples were analyzed with the Kruskall-Wallis test comparing with critical values at 0.05, and significance was established at this level.
Results

Figure 24 demonstrates statistically significant differences in survival (p<0.05) between the ischemic control group and the groups of NP75, NP30 and NP15 at all points of the graph. There were no significant differences between the IC and NP5 groups.

Figure 25 shows the statistically significant differences in serum creatinine (p<0.05) levels between the control group and the treatment groups of NP75, NP30 and NP15 at 24, 48 and 72 hr. Again, there were no significant differences between IC and the NP5 groups. The NP75 group had the lowest BUN levels when compared to
the NP30, NP15, NP5 or IC groups at all points studied. No significant differences between the IC and NP5 groups were observed at any time.

Figure 24. Survival in Experiment #4.

Figure 25. SCr in the Experiment #4.
Figure 26 demonstrates the values for MPO activity in renal tissue as a marker of neutrophil infiltration, which correlates well with survival and kidney function tests. The MPO activities were significantly lower (p<0.05) in the NP75, NP30 and NP15 groups compared to those of the IC or NP5 groups.

Figure 26. MPO Activity in Renal Tissue, Experiment #4.

Figure 27 shows the histological findings in which the IC group had necrosis affecting all three segments of the proximal convoluted tubules (Figure 5-A and 5-C, median value = 4). In contrast, the ischemic damage was significantly reduced in the NP75 group (p<0.05), as well in the NP30, and NP15 groups (Fig 5-B and 5-D, median value=2), showing necrosis of individual cells, and survival of surrounding tubules, with the necrosis limited to some areas of the inner medulla and outer medulla. There were no differences in the NP5 group compared to the IC group.
Figure 27. Histological Findings, Experiment #4.
Discussion

This study demonstrated that NP had a time-of-administration dependence in the prevention of neutrophil infiltration and secondary protection of ischemic kidneys. The protective effect observed in our studies may be due to the interaction between nitric oxide and leukocytes, since the NP was given after the ligation of the renal pedicle. In this way, the treatment is selectively affecting the leukocytes, and the contact between exogenous nitric oxide and endothelium is prevented. Nitroprusside, through NO, exerted a modulating effect in the migration of neutrophils.

The time-of-administration is very important. NO, released through NP, when administered at 75, 30 and 15 minutes after reperfusion, exerted a beneficial effect related to diminished neutrophil infiltration or migration. Migration is the last step in the inflammatory interaction between leukocytes and EC, and this interaction is secondary to expression/activation of cellular adhesion molecules. Thus, exogenous nitric oxide may be acting through modulation of adhesion molecules, such as β2 integrins and/or selectins, because of its time-of-administration dependence. This study, as well as that of others (Carey, 1992; Gauthier, 1994), supports the theory of nitric oxide as a modulator of the interaction of leukocytes and EC.
CHAPTER IV

CONCLUSIONS

Based on the results that we have obtained we can answer the questions raised at the beginning of this thesis. We have found that

1. Although endogenous NO does not appear to play an important role after ischemia reperfusion in vivo, exogenous NO excerts a beneficial effect in survival, and kidney function tests.

2. The best results appear to be in relation to the modulation of the infiltration of neutrophils by exogenous NO. This is a plausible mechanism of action that has been demonstrated in these studies.

3. Not only migration (infiltration), but the other leukocyte-EC interactions, including rolling and adhesion, are lowered by exogenous NO releasers in I/R.

4. Exogenous NO exerts its beneficial effect even when it administered after ischemia and as close as 15 minutes before reperfusion. Closer administration (e.g. 5 minutes before or at reperfusion) times had no significant effect. This demonstrates the potential clinical application of this compounds after ischemic events and as close as 15 minutes before reperfusion.

Future Lines of Research

Although Gauthier (1994) found that by blocking NOS it is possible to augment the expression of ICAM-1, more work is necessary regarding other CAMs and the role of exogenous-NO in their expression.
It will be of great interest to know the potential role of nitric oxide donors in controlling CAMs. Is this control is at the transcriptional level or just is at the activation level on the membrane?

Another interesting field of potential research is the use of other NO donor with less potential toxicity than Na-Nitroprusside such as molsidomine, nitroglycerin, etc.

Knowing how to control intercellular interactions, allow the improved survival of organs and individuals who have suffered an ischemic event. The application of this knowledge in the field of transplantation has the potential of avoiding the ischemic damage always present to some degree with this kind of surgery. This knowledge can be expanded, not only to inflammation secondary to I/R, but also to inflammation secondary to other causes such as sepsis, trauma and infectious diseases.
Appendix A

Adhesion Molecules
### Table 1 Immunoglobulin Superfamily

<table>
<thead>
<tr>
<th>CAM</th>
<th>Cell Expression</th>
<th>Ligand Molecule</th>
<th>Cell of Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1*</td>
<td>Endothelial/Monocyte/Linocyte T&amp;B/Dendritic</td>
<td>Mac-1</td>
<td>Leukocyte/Monocyte</td>
</tr>
<tr>
<td>ICAM-2**</td>
<td>Endothelial/Monocyte/Dendritic</td>
<td>LFA-1</td>
<td>Leukocyte</td>
</tr>
<tr>
<td>VCAM-1*</td>
<td>Endothelial</td>
<td>VLA-4</td>
<td>Lymphocyte/Monocyte</td>
</tr>
</tbody>
</table>

* Inducible by Cytokines  
** Constitutive

### Table 2 Integrin Family

<table>
<thead>
<tr>
<th>CAM</th>
<th>Cell of Expression</th>
<th>Ligand Molecule</th>
<th>Cell of Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1*</td>
<td>Leukocyte</td>
<td>ICAM-1</td>
<td>Endothelial</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICAM-2</td>
<td></td>
</tr>
<tr>
<td>Mac-1*</td>
<td>Monocyte/Neutrophil</td>
<td>ICAM-1</td>
<td>Endothelial</td>
</tr>
</tbody>
</table>

* Inducible by Cytokines  
** Constitutive

### Table 3 Selectin Family

<table>
<thead>
<tr>
<th>CAM</th>
<th>Cell of Expression</th>
<th>Ligand</th>
<th>Cell of Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Selectin*</td>
<td>Lymphocyte</td>
<td>Mucine</td>
<td>Endothelial</td>
</tr>
<tr>
<td>E-Selectin**</td>
<td>Endothelial</td>
<td>Fucosilated or Sialylated Molecules</td>
<td>Neutrophil/Monocyte</td>
</tr>
<tr>
<td>P-Selectin***</td>
<td>Platelets/Endothelial</td>
<td>Fucosylated or Sialylated Molecules</td>
<td>Platelet/Neutrophil/Monocyte</td>
</tr>
</tbody>
</table>

* Decreased by Cytokines  
** Inducible by Cytokines  
*** Released by Cytokines
Appendix B

Federal Animal Welfare Act
FEDERAL ANIMAL WELFARE ACT

Passed in 1966 and amended in 1970, 1976, 1989, and 1991. The standards were written and inspections are carried out by the U.S. Department of Agriculture (USDA). This law applies to warm blooded animals except birds, rats, mice, and farm animals.

REGULATIONS

Licensing and Registration

A. Animal dealers, exhibitors and operators of auction sales must be licensed.

B. Research facilities, carriers and intermediate handlers must be registered.

Research Institutions

A. Must be registered with USDA.

B. Must appoint an Institutional Animal Care and Use Committee (IACUC).

1. Membership:
   Minimum of three people — Chairman, veterinarian and non-affiliated person.

2. Functions:
   a) Review animal program, inspect facilities and make recommendations twice each year; provide written report to Vice President of Research.
   b) Review and investigate complaints of noncompliance.
   c) Review and approve research projects using animals to ensure:

      1) Care and use is in accordance with AWA standards.
      2) Procedures minimize pain and discomfort.
      3) No unnecessary duplication of protocols.
      4) Appropriate sedatives, anesthetics, and analgesics will be used, veterinarian is involved in the planning, and no paralytic will be used.
      5) Animals in chronic pain or distress will be euthanized at end of procedure.
      6) Medical care is provided by qualified veterinarian.
      7) Personnel on projects are trained and qualified.
      8) Appropriate pre- and post-operative care is planned.
9) No animal is used in more than one major surgical procedure unless justified in writing.

10) Methods of euthanasia will produce rapid unconsciousness and death without evidence of pain or distress.

d) Notify Principal Investigator, Vice President of Research, USDA, and federal funding agency if a project is disapproved or suspended.

C. Personnel Qualifications

1. Personnel involved in animal care and use must be qualified.

2. Training and instruction in animal care and use must be available.

3. Training must be provided in:

   a) Humane methods of care and use pertaining to the basic needs, proper handling, pre- and post-procedure care, and aseptic surgery.

   b) Use of alternate methods.

   c) Proper use of anesthetics, analgesics and tranquilizers.


   e) Utilization of literature search services.

D. Veterinary Care

1. Must have attending veterinarian.

2. If veterinarian is part time, he/she must have regular visits and written program of veterinarian care.

3. Attending veterinarian must be a member of IACUC.

4. Program of veterinary care must include:

   a) Appropriate facilities, personnel, equipment, and services to meet AWA standards.

   b) Daily observation of animals to assess health.

   c) Appropriate methods to prevent, control, diagnose, and treat diseases and injuries.

   d) Available emergency weekend and holiday care.
e) Guidance to personnel on handling, immobilizing, anesthesia, analgesia, tranquilization, and euthanasia.

f) Adequate pre- and post-procedural care.

E. Recordkeeping Requirements (Retain for 3 Years)

1. Minutes of IACUC meetings and activities.

2. Records on each dog and cat:
   a) Name and address of person from whom obtained.
   b) Date of acquisition.
   c) Description and official tag number.
   d) Name and address of recipient.
   e) Method of transportation and person responsible.

F. Annual Report

Director of Research ensures that:

1. Professionally acceptable standards are followed.

2. Alternatives have been considered.

3. AWA standards are being met.

4. Location of all animals is known.

5. Common name and number of animals used in the following categories are recorded:
   a) Held but not yet used.
   b) Used involving no pain or distress.
   c) Used involving pain or distress; given appropriate drugs.
   d) Used involving pain or distress; drugs withheld for scientific reasons approved by the IACUC.

G. Police may inspect for missing animals.

H. Veterinary inspectors may treat or destroy mistreated animals.

I. Must comply with the facilities and operating standards described below.
1. **Facility and Operating Standards** (these vary for each species):

   a) Facilities — General
      
      1) Structurally sound and maintained in good repair.
      
      2) Water and electrical power must be available.
      
      3) Storage for food and bedding must protect supplies; opened packages of food and bedding must be stored in sealed containers and containers must be properly identified as to the contents.
      
      4) Waste disposal must minimize vermin infestation, odors and disease hazards.
      
      5) Washing facilities for caretakers must be provided.

   b) Facilities — Indoor
      
      1) Heat must be provided.
      
      2) Ventilation is necessary.
      
      3) Illumination must be adequate for inspection and cleaning.
      
      4) Building surfaces must be impervious to moisture.
      
      5) Drains must be functional.

   c) Facilities — Outdoor
      
      1) Shade must be provided.
      
      2) Shelter from rain, snow, and cold weather must be provided.
      
      3) Drainage must rapidly eliminate excess water.

   d) Primary Enclosures (cages or pens)
      
      1) Maintained in good repair to protect animals and keep predators out.
      
      2) Constructed and maintained to keep animals dry and clean.
      
      3) Litter box and resting surface must be provided for cats.
      
      4) Specific space requirements for each animal species are maintained.
5) An exercise program must be implemented for dogs.

2. **Animal Health and Husbandry Standards**

   a) Feeding
      
      1) Clean food must be available daily.
      2) Food containers must be clean.
   
   b) Watering — Clean water must be available each day.
   
   c) Sanitation
      
      1) Excreta removed on a schedule to keep the animals clean.
      2) Cages and pens must be sanitized at least every two weeks and between animal groups.
      3) Premises must be kept clean.
      4) There must be an effective pest control program.
   
   d) Employees
      
      There must be enough employees to maintain the prescribed husbandry program.
   
   e) Classification and separation
      
      Animals must be maintained in compatible groups.

3. **Transportation Standards**

   a) Animals can be accepted for shipment not more than 4-6 hours prior to shipment.
   
   b) The carrier must attempt to notify the consignee of arrival at least once every six hours after arrival.
   
   c) Animals must be shipped in containers that meet many specific requirements.
   
   d) The health, safety and comfort of the animal(s) must be ensured during transportation.
   
   e) Food and water must be provided at specified intervals. Instructions for feeding and watering must be posted on the cage.
   
   f) Animals must be observed at specified intervals during
transportation.

g)  Terminal facilities must be temperature controlled and clean; animals must be protected from rain, snow and excessive sunlight.

h)  Primary enclosures must be handled carefully.

i)  The temperature within the primary enclosure must not exceed 95°F or be less than 45°F for more than 45 minutes (varies by species).
A. Research facilities that use animals (living vertebrates) must register with the Michigan Department of Public Health.

B. Research and animal care must be supervised by a qualified person.

C. Care and use of animals must conform with the standards in the “Guide for the Care and Use of Laboratory Animals.”

D. Careful consideration must be given to the physical comfort and welfare of the animals and the safety of the animal care staff.

E. Anesthesia, analgesia and tranquilization must be in accord with the “Guide.”

F. Post-experimental care shall minimize pain and discomfort.

G. Animals that cannot live without long-term pain or discomfort must be humanely killed at the conclusion of the experiment.

H. Animals must be humanely treated while being transported.

I. Each facility shall be inspected at least annually.
BURGESS MEDICAL CENTER
PRINCIPLES FOR THE CARE AND USE OF LABORATORY ANIMALS
(Adapted from U.S. Government Principles for the Utilization and Care of Vertebrate
Animals Used in Testing, Research, and Training)

A. The transportation, care, and use of animals should be in accordance with the
ANIMAL WELFARE ACT and other applicable federal and state laws,
guidelines and policies.

B. Procedures involving animals should be designed and performed with due
consideration of their relevance to human or animal health, the advancement of
knowledge, or the good of society.

C. The animals selected for a procedure should be of an appropriate species and
quality and the minimum number required to obtain valid results. Methods such
as mathematical models, computer simulation, and in vitro biological systems
should be considered.

D. Proper use of animals, including the avoidance or minimization of discomfort,
distress, and pain when consistent with sound scientific practices, is imperative.
Unless the contrary is established, investigators should consider that procedures
that cause pain or distress in human beings may cause pain and distress in
animals.

E. Procedures with animals that may cause more than momentary or slight pain or
distress should be performed with appropriate sedation, analgesia, or
anesthesia. Surgical or other painful procedures should not be performed on
unanesthetized animals paralyzed by chemical agents.

F. Animals which would otherwise suffer severe or chronic pain or distress that
cannot be relieved should be painlessly killed at the end of the procedure or, if
appropriate, during the procedure.

G. The living conditions of animals should be appropriate for their species and
contribute to their health and comfort. Normally the housing, feeding and care
of all animals used for biomedical purposes must be directed by a veterinarian
or other scientist trained and experienced in the proper care, handling, and use
of the species being maintained or studied. In any case, veterinary care should
be provided.

H. Investigators and other personnel shall be appropriately qualified and
experienced for conducting procedures on living animals. Adequate
arrangements shall be made for their in-service training, including the proper
and humane care and use of laboratory animals.

I. Where exceptions are required in relation to the provisions of these Principles,
the decisions should not rest with the investigators directly concerned but
should be made by an appropriate review group such as an institutional animal
research committee. Such exception should not be made solely for the purpose
of teaching or demonstration.
Appendix C

Summary of Policies Regarding the Use of Animals in Research and Teaching
PROTOCOL REVIEW

All research projects and all laboratory teaching exercises using warm or cold-blooded vertebrate animals must be reviewed by the Animal Investigation Committee, hereby known as the “Research Advisory Committee” (RAC).

For general reference, the publication which details the standards to which the Medical Center attempts to conform is the Animal Welfare Act (9 CFR Part 3) and NIH Guide for the Care and Use of Laboratory Animals. Departure from these published guidelines requires written scientific justification in the animal use protocol form. Other regulations and guidelines may be applicable such as standards published by the professional societies of mammalogy, ornithology, ichthyology, herpetology, etc., and the 1986 report of the AVMA Panel on Euthanasia.
Borgess Medical Center

ASSURANCE OF COMPLIANCE WITH
ANIMAL WELFARE ACT (9 CFR PART 3, USDA)
AND PUBLIC HEALTH SERVICE (PHS) POLICY
ON HUMANE CARE AND USE OF LABORATORY ANIMALS

Borgess Medical Center, hereinafter referred to as the “Institution,” hereby gives assurance that it will comply with the Animal Welfare Act (9 CFR Part 3, USDA) as well as Public Health Service Policy on Humane Care and Use of Laboratory Animals.

I. APPLICABILITY

This assurance is applicable to all research, research training, experimentation, and biological testing and related activities, hereinafter referred to as “Activities,” involving live, vertebrate animals, conducted at this Institution or at another institution as a consequence of the subgranting or subcontracting of a PHS-conducted or supported activity by this Institution. This will include all activities which are supported by the Public Health Service (PHS) and/or inspected by PHS and USDA at this Institution; “Institution” includes the branches and major components of the Borgess Medical Center.

II. INSTITUTIONAL POLICY

A. This Institution will comply with all applicable provisions of the Animal Welfare Act and other federal statutes and regulations relating to animals.

B. This Institution is guided by the “U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.”

C. This Institution acknowledges and accepts responsibility for the care and use of animals involved in activities covered by this assurance. As partial fulfillment of this responsibility, this Institution will make a reasonable effort to ensure that all individuals involved in the care and use of laboratory animals understand their individual and collective responsibilities for compliance with this assurance as well as all other applicable laws and regulations pertaining to animal care and use.

D. This Institution has established and will maintain a program for activities involving animals in accordance with the Animal Welfare Act (9 CFR Part 3) and the Guide for the Care and Use of Laboratory Animals (PHS 86-23).

III. INSTITUTIONAL PROGRAM FOR ANIMAL CARE AND USE
A. This Institution has established a Research Advisory Committee (RAC) [Institutional Animal Care and Use Committee (IACUC)] which is qualified through the experience and expertise of its members to oversee the Institution's animal program, facilities, and procedures.

B. The RAC will:

1. Review at least once every six months the Institution's program for humane care and use of animals, using the Guide for the Care and Use of Laboratory Animals (86) and the Animal Welfare Act (USDA 9 CFR Part 3) as a basis for evaluation.

2. Inspect at least once every six months all of the Institution's animal facilities (including satellite facilities) using the Guide for the Care and Use of Laboratory Animals (86-23) and the Animal Welfare Act (Title 9, Chapter I, Part 3: Subchapter A) as a basis for evaluation.

3. Prepare reports of the RAC evaluations as set forth in the PHS Policy at IV.B.3. as well as Animal Welfare Act (Title 9, Chapter I, Subchapter C) and submit the reports to the Research Director.

4. Review concerns involving the care and use of animals at the Institution.

5. Make written recommendations to the Research Director regarding any aspect of the Institution's animal program, facilities, or personnel training.

6. Review and approve, require modifications in (to secure approval) or withhold approval of those activities related to the care and use of animals as set forth in the PHS Policy in IV.C. and the Animal Welfare Act (Title 9, Chapter I, Subchapter C).

7. Review and approve, require modifications in (to secure approval) or withhold approval of proposed significant changes regarding the use of animals in ongoing activities as set forth in the PHS Policy at IV.C. and the Animal Welfare Act (Title 9, Chapter I, Subchapter C).

8. Notify investigators and the Institution in writing of its decision to approve or withhold approval of those activities related to the care and use of
animals, or of modifications required to secure IACUC approval as set forth in the PHS Policy in IV.C.4 and the Animal Welfare Act (Title 9, Chapter I, Subchapter C).

9. Be authorized to suspend an activity involving animals as set forth in the PHS Policy in IV.C.6 and the Animal Welfare Act (Title 9, Chapter I, Subchapter C).

C. The training or instruction available to scientists, animal technicians, and other personnel involved in animal care, treatment, or use are:

1. As part of the Borgess Medical Center's program for animal care and use, this Institution will provide training or instruction to scientists, animal technicians and other personnel involved in animal care, treatment or use.

2. The Institution will inform the scientists, animal technicians and other personnel involved in animal care, treatment or use of any policies regarding health screening or tests, including the frequency of tuberculosis tests or any required medical examinations.

IV. INSTITUTIONAL STATUS

All of this Institution's programs and facilities (including satellite facilities) for activities involving animals have been and will be inspected by the USDA representatives at least once a year and have been and will be evaluated by the RAC members at least once every six months.

V. RECORDKEEPING REQUIREMENTS

A. This Institution will maintain for at least three years:

1. A copy of this assurance and any modifications thereto as approved by the PHS and USDA.

2. Minutes of RAC meetings, including records of attendance, activities of the committee, and committee deliberations.

3. Records of applications, proposals, and significant changes in the care and use of animals and whether RAC approval was given or withheld.

4. Records of semi-annual RAC reports and recommendations as forwarded to the Research Director.
B. This Institution will maintain records that relate directly to applications, proposals, and proposed changes in ongoing activities reviewed and approved by the RAC for the duration of the activity and for an additional three years after completion of the activity.

C. All records shall be accessible for inspection and copying by authorized USDA or other PHS representatives at reasonable times and in a reasonable manner.

VI. REPORTING REQUIREMENTS

A. At least once every 12 months, the RAC, through the Institutional Official, will report in writing to the Office for Protection from Research Risks (OPPR):

1. Any change in the status of the Institution (i.e., if the Institution becomes accredited by AAALAC or AAALAC accreditation is revoked), any change in the description of the Institution's program for animal care and use as described in this assurance, or any change in IACUC (RAC) membership. If there are no changes to report, this Institution will submit a letter to OPPR stating that there are no changes.

2. Notification of the dates that the RAC conducted its semi-annual evaluations of the Institution's program and facilities (including satellite facilities) and submitted evaluations to the Research Director.

B. The RAC, through the Institutional Official, will provide the OPPR promptly with a full explanation of the circumstances and actions taken with respect to:

1. Any serious or continuing noncompliance with the PHS Policy.

2. Any serious deviations from the provisions of the Guide for The Care and Use of Animal Laboratory.

3. Any suspension of an activity by the RAC.

C. Reports filed under VI.A.2 and VI.B. above shall include any minority views filed by members of the RAC.
Appendix D
Animal Care
Borgess Medical Center

ANIMAL CARE

I. INTRODUCTION

Humane care and the use of laboratory animals are the responsibilities of the Principal Investigator.

The following principles are necessary in order to provide optimal animal care:

1. Animal quarters specifically for housing of animals should be provided.

2. All experimental animals used must be properly cared for. Animal quarters should be made comfortable by provision for sanitation, protection from the elements, and have sufficient space for normal behavioral and postural requirements of the species. The living quarters shall have surfaces that may be easily cleaned, good ventilation and lighting, well-regulated temperatures, and cages of sufficient size to prevent over-crowding. Animals must be protected from direct sunlight or other environmental factors which may disturb the well-being of the animals.

3. Food should be palatable (unless otherwise instructed) of sufficient quantity and balance to maintain a good standard of nutrition. Clean drinking water shall be available at all times. Containers for food and water should be of a design made specifically for that purpose.

4. All animals must be disposed of in a humane manner. If euthanasia has to be carried out, an approved humane method must be used and carried out by an investigator experienced in the use of such procedures.

5. The use of animals must comply with existing local, state, or federal legislation.

II. LAB AND ANIMAL HUSBANDRY PROCEDURES

A. Daily Care

1. Rats:
   (a) Change the bedding as instructed.
   (b) Empty and refill bottles with fresh water.
   (c) Feed the animals unless otherwise instructed.
   (d) Check lights. Light cycle should be on 12/12 from 8:00 a.m.- 8:00 p.m. unless otherwise instructed.
(e) Check the temperature of the room. It should be 68-75°F.

(f) Clean surgical tables and instruments after done with the experiment or surgery.

(g) Report immediately any abnormality observed in animals to your supervisor and/or notify the research investigator.

2. Dogs

(a) Wash each primary enclosure with hot water every other day. The animals have to be removed from their runs prior to hosing (please refer to Sanitation — Dogs).

(b) Flush the drain once every day.

(c) Wash the food receptacle with hot water and dry clean with a clean towel prior to filling.

(d) Feed the animals unless otherwise instructed.

(e) Check lights. Light cycle should be on 12/12 from 8:00 a.m.- 8:00 p.m. unless otherwise instructed.

(f) Check the temperature of the room. It should be 68-75°F.

(g) Clean the surgical tables and instruments after surgery. (Housekeeping Department will be notified by Department Secretary after every dog surgery that the surgical suite will need special attention that evening to return it to surgical standards of cleanliness.)

(h) Report any abnormality observed in animals immediately to your supervisor and/or notify the research investigator.

B. Weekly Care

1. Dogs

Wash each run with high-pressured hot water and bleach and flush floor drains at least once every other week or more if necessary.

2. Rats

(a) Change rat cages at least once a week or more if necessary.
(b) Wash rat water bottles at least once a week.

3. Dogs and Rats

Report immediately any abnormality observed in animals to your supervisor and/or notify the research investigator.

III. LAB ENTRY PROCEDURES

The procedures described below must be followed by anyone entering the animal room.

1. Hang personal lab coats outside the room on the hooks.

2. While in the room wear:
   - Gloves
   - Surgical Gowns/Lab Coats
   - Face Mask
   - Shoe Covers (Optional)
   - Hair Bonnet (Optional)
   - Eye Protection

3. Animals taken from the holding room must not be returned to the holding room unless instructed otherwise.

4. Only clean equipment that has not been exposed to any other animals may be brought into the room.

5. Equipment taken from this room may not be taken to other animal rooms.

6. Eating, drinking, or storage of food for human consumption is prohibited in the animal and surgery rooms.

IV. WATERING/FEEDING

A. Rodent - General (excluding guinea pigs)

1. Water shall be available at all times unless written instructions specify otherwise.

2. Empty and refill bottles with fresh water each day. Return each bottle to the cage from which it was taken. Be sure the spout is below any other part of the bottle, and check with finger for vapor lock.

3. Observe animals for signs of dehydration such as the absence of urine or feces, or animals with hunched posture or rough coat. Feed the preferred lab chow free choice unless instructed otherwise. Check feeders for food blockages. Uneaten food should not be left in hoppers longer than one day. Remove powdered food
pellets as needed. Maintain a reasonable (3 or 4 days) supply of food in each lab but not in the same room in which the animals are housed.

4. The research personnel are responsible for watering the animals. Water bottles need to be emptied and refilled with fresh water every day.

5. The rat food (Teklad 6% M/R Diet) is purchased through Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The cages need to be checked for food at least once every other day unless written instructions specify otherwise.

6. Open supplies of food should be stored in leakproof containers with tightly fitting lids to protect the supplies from spoilage and contamination. Containers should be clearly marked indicating contents.

7. Unopened supplies of food should be stored off the floor, away from the wall in order to allow cleaning around and underneath.

**B. Dogs**

1. Water is available at all times unless written instructions specify otherwise.

2. Palatable food, proportionate to the weight and condition of the dog, should be provided every 24 hours. Puppies less than six months of age should be fed every 12 hours. Good commercial diets are available that provide all the required nutrients and are easy to feed. A complete diet should contain all of the proteins (amino acids), minerals, vitamins, fats (fatty acids), and carbohydrates needed by dogs during growth, gestation, lactation, and normal maintenance as outlined in the National Research Council's *Nutrient Requirements of Dogs* (Dogs: Standards and Guidelines for the Breeding, Care, and Management of Laboratory Animals, p. 10; 38, 1973).

3. Good commercial diets that provide all the required nutrients and are easy to feed can be purchased from any store in town.

4. Open supplies of food should be stored off the floor in leakproof containers with tightly fitting lids to protect the supplies from spoilage and contamination. The containers should be clearly marked indicating the contents.

5. Unopened supplies of food should be stored off the floor and away from the wall, in order to allow cleaning around and underneath.
A. Rodents

1. Keep all surfaces clean, neat and free of clutter. This includes walls, floors, countertops, ledges, vents, sinks, etc.

2. The research facility where the rats are held needs to be swept on a daily basis and damp-mopped on a weekly basis. This will be done by the Housekeeping Department.

3. The waste baskets need to be emptied on a daily basis. This will be done by the Housekeeping Department.

B. Dogs

1. All surfaces need to be kept clean, neat and free of clutter. This includes walls, floors, countertops, ledges, vents, sinks, etc.

2. The runs where the animals are housed need to be washed with high-pressured water at least every two weeks or more often if necessary. A detergent should be used to avoid any possible odors and bacterial spread in the room.

3. Excreta and food waste must be removed from the primary enclosure on a daily basis.

4. When steam-hosing is used to clean, the animals must be removed from the primary enclosure to avoid any distress or possible injury. The animals may be kept in the open area outside of the runs. Runs may be cleaned one at a time.

5. Used primary enclosure and food receptacles must be cleaned using hot water and detergent before they can be used again to house or feed another dog.

VI. ANIMAL OBSERVATION

Report abnormalities as defined below to the Director of Research (388-6895):

1. Sneezing, noisy breathing, coughing

2. Dirty or bloody nostrils

3. Diarrhea or bloody stool

4. Discoloration or wetness near muzzle, anal, urinary, or genital openings

5. Mattering around nose or eyes

6. Pale, anemic look around nose and paws
7. Sores, infections, excessive scratching
8. Animals not eating or drinking
9. Expired animals
Appendix E

Approval From the Institutional Animal Care and Use Committee
February 9, 1996

Dr. Karim Essani
Professor of Biological Sciences
McCraiken Hall
Western Michigan University
Kalamazoo, MI 49008-1028

Dear Dr. Essani:

The protocol, *Practical Training of Vascular and Bladder Anastomoses as They Pertain to Kidney Transplantation*, which was used by Dr. Fernando Lopez-Neblina, was approved by the Research Advisory Committee on April 7, 1995. I believe the enclosed copy of the Minutes will verify this statement.

Sincerely,

[Signature]

Luis H. Toledo, MD, PhD.

Enclosure


