EFFECT OF ISCHAEMIA-REPERFUSION ON RABBIT KIDNEY AND HUMAN BRAIN

by

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Abstract

Effect of Ischaemia-Reperfusion on Rabbit Kidney and Human Brain
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Free radicals are produced in various organs at ischaemia-reperfusion. The final stage in radical damage is lipid peroxidation. We have demonstrated previously that a lipid-soluble antioxidant improves restoration of bioenergetics in rabbit kidneys after ischaemia, as reflected in ^{31}P spectrometry. Radical production in the brain during surgery for carotid artery stenosis can be measured using an *ex vivo* spin trap method.

Aims of the present study: 1. To examine whether pretreatment with a combination of a lipid-soluble and a water-soluble antioxidant causes improved restoration of bioenergetics in rabbit kidneys after ischaemia compared to single treatment with a lipid soluble antioxidant. 2. To examine whether pretreatment with allopurinol or acetylcysteine influences radical production in conjunction with surgery for carotid artery stenosis. 3. To study the relationship between various markers for arteriosclerosis and the production of free radicals in conjunction with surgery for carotid artery stenosis.

Methods: New Zealand white rabbits were used for the NMR experiments. Volume-selective ^{31}P spectrometry was used to determine changes in bioenergetics during and after ischaemia following various pretreatments. An *ex vivo* spin trap method was used to measure radical production in the brain during carotid endarterectomy in control patients as well as patients pretreated with allopurinol or acetylcysteine. ICAM-1, MCP-9, MMP-1 and oxLDL serum levels were determined in the control patients.

Results: Pretreatment with a combination of a lipid-soluble and a water-soluble antioxidant resulted in improved restoration in cell bioenergetics after ischaemia compared to single treatment with a lipid-soluble antioxidant. Production of radicals can be measured reproducibly using the *ex vivo* spin trap method. Pretreatment with allopurinol eliminated the strong correlation between e.g. degree of stenosis and leucocyte counts and radical production, which might indicate a beneficial effect of pretreatment with a xanthine oxidase inhibitor. Pretreatment with acetylcysteine on the other hand appeared to increase radical production. High levels of MMP-1 and low levels of ICAM-1 were associated with high radical production.

Conclusion: A combination of a lipid-soluble and a water-soluble antioxidant is most effective in improving cell bioenergetics after ischaemia in rabbit kidneys. Allopurinol appears to have a beneficial effect in conjunction with carotid endarterectomy while acetylcysteine appears to increase radical production. MMP-1 is associated with increased radical production.

Keywords: Free radicals, ischaemia-reperfusion, cell bioenergetics, NMR, ESR, carotid artery stenosis, endarterectomy, allopurinol, acetylcysteine, MMP-1, ICAM-1, MCP-9, oxLDL.

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List of Appended Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Enhanced post-ischaemic recovery in rabbit kidney after pretreatment with an indeno-indole compound and ascorbate monitored *in vivo* by ^{31}P magnetic resonance spectroscopy.
Olof Jonsson, Ann Lindgård, Anita Fae, Jagdish Gondalia, Anders Åneman, Bassam Soussi.
Scand J Urol Nephrol 2003; 37:450-455.

II. Effects of pretreatment with a xanthine oxidase inhibitor on free radical levels during carotid endarterectomy.
Susanna Waters, Anita Fae, Jagdish Gondalia, Jan Holm, Lars Karlström, Ulf Nilsson, Olof Jonsson.
Free Radic Res 2004; 38:283-293.

III. Effect of pretreatment with N-acetylcysteine on free radical levels during carotid endarterectomy.
Jagdish Gondalia, Anita Fae, Jan Holm, Lars Karlström, Ulf Nilsson, Susanna Waters, Olof Jonsson.
In manuscript.

IV. Relationships between free radical levels during carotid endarterectomy and markers of arteriosclerotic disease.
Jagdish Gondalia, Björn Fagerberg, Johannes Hulthe, Lars Karlström, Ulf Nilsson, Susanna Waters, Olof Jonsson.
Int J Med Sci 2007; 4:124-130.

CONTENTS

ABBREVIATIONS

ADP	Adenosine di-Phosphate
AMP	Adenosine mono-Phosphate
α-SMA	Alpha Smooth Muscle Acting
ATP	Adenosine tri-Phosphate
ESR	Electron Spin Resonance
GSH	Glutathione Stimulating Hormone
H290/51	Lipid Peroxidation Inhibitor
ICAM-1	Intracellular Adhesion Molecule-1
MCP-1	Monocyte Chemoattractant Protein-1
MMP-9	Matrix-degrading Metalloproteinase-9
NAC	N-Acetylcysteine
NMR	Nuclear Magnetic Resonance
OFR	Oxygen Free Radical
OXANOH	Spin Trap
OxLDL	Oxidised Low Density Lipoprotein
PCA	Principle Component Analysis
PCr	Phosphocreatinine
PDE	Phosphodiesters
Pi	Inorganic Phosphate
PLSR	Partial Least Squares Regression
PME	Phosphomonoesters
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SOD	Superoxide Dismutase
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase

"The art of medicine consists in
amusing the patient
while nature cures the disease"
Voltaire (1694-1778)

INTRODUCTION

Ischaemia-Reperfusion

Ischaemia is a Greek term derived from the word "Iskhaimos", which means stopping of blood. Although the mechanism behind ischaemia is complex, the major cause of ischaemia is atherosclerosis, which leads to rupture of a lesion, thrombosis and blockage of an essential coronary or cerebral artery. Ischaemia and hypoxia are in fact the major causes of death in western society and are used interchangeably in the literature (Halliwell and Gutteridge, 2000).

Historical Notes

The systematic, experimental study of the influence of interrupting coronary circulation can be said to have begun more than 150 years ago with Erichsen (1841), followed by the work of Panum (1862), who embolised the coronary arteries of a young dog with a mixture of tallow, wax, oil and lamp black (Gasser et al, 1994). Bezold and Boyemann (1862) were the first to produce ventricular fibrillation by clamping a rabbit's left coronary artery ("coronaria magna"). Samuelson (1881) demonstrated the first "successful reperfusion" after a 4-min ligation of a coronary artery. Conheim and von Schulthess-Rechenberg (1881) introduced the first experimental model, designed to produce "ischaemia-induced arrhythmias" (Gasser et al, 1994).

However, several models have been introduced, based on *in vivo* and *in vitro* studies, with the aim of examining ischaemia cell injury. In 1956, de Baker performed an *in vivo* study involving interrupting the blood flow to the whole organ, such as the liver (de Baker, 1956). Similarly, in 1982, Hansson studied *in vivo* models for the kidney (Hansson et al, 1982). The *in vitro* studies, however, were performed by including

isolated perfused organs such as the heart (de Jong et al, 1982) or the liver (Dawkins, 1959). Farber and Young conducted a few studies on isolated cells such as hepatocytes (Farber and Young, 1981). The advent of technology and subsequent research changed this view and a great deal of enthusiasm regarding ischaemia–reperfusion was generated.

The hallmark functional observation during ischaemia is restriction of blood flow leading to deprivation of oxygen and substrates to tissue and the exposure of accumulated and potentially toxic metabolites, while during the reperfusion phase it restores the supply of oxygen and substrate and removes metabolic products. Certain alternatives, such as ATP depletion and cytoskeleton dearrangements, are rapidly induced by ischaemia/ hypoxia but may resolve quickly during reperfusion providing the ischaemia/hypoxia phase is not too severe (Weinberg, 1991; Bonventre, 1993; Edelstein et al, 1997). However, Parks, Granger, McCord and their colleagues in USA showed in the early 1980s that re-introduction of O_2 to an ischaemic or hypoxic tissue could cause an additional insult to the tissue (Granger et al, 1981; Parks et al, 1982). In other words, reperfusion itself may introduce and amplify the mechanism for, for example, reactive oxygen species and a leukocytic-dependent mechanism, leading to cell injuries. The relative importance of re-oxygenation injury thus depends on the time of hypoxia and the type of tissue.

In the case of prolonged ischaemia, reoxygenation injury can have an irreversible effect on the tissue. The reperfusion of dead tissue *in vivo* releases potentially toxic agents, such as xanthine oxidase and catalytic transition metal ions, into the systematic circulation, causing problems to the other body tissue (Bandyopadhyay et al, 1999). This contention is largely based on the observation that allopurinol, an inhibitor of xanthine oxidase, is as effective as oxygen radical scavengers in attenuating the tissue injury associated with ischaemia reperfusion. Xanthine oxidase exists in various tissues as the NAD-reducing xanthine dehydrogenase, which is converted into oxygen radical-producing XO during ischaemia (Grisham et al, 1986; McKelvey et al, 1988).

Concomitantly, cellular adenosine tri-phosphate (ATP) is catabolised to hypoxanthine, which may then be released into plasma. Hypoxanthine levels in plasma

have been found to be elevated (-25 μm) during tissue hypoxia (Saugstad, 1975). Upon reperfusion (reoxygenation), XO can react with purine substrates (hypoxanthine or xanthine) and molecular oxygen to generate highly reactive oxygen metabolites, superoxide, hydrogen peroxide and, indirectly, hydroxyl radical (Fig. 1).

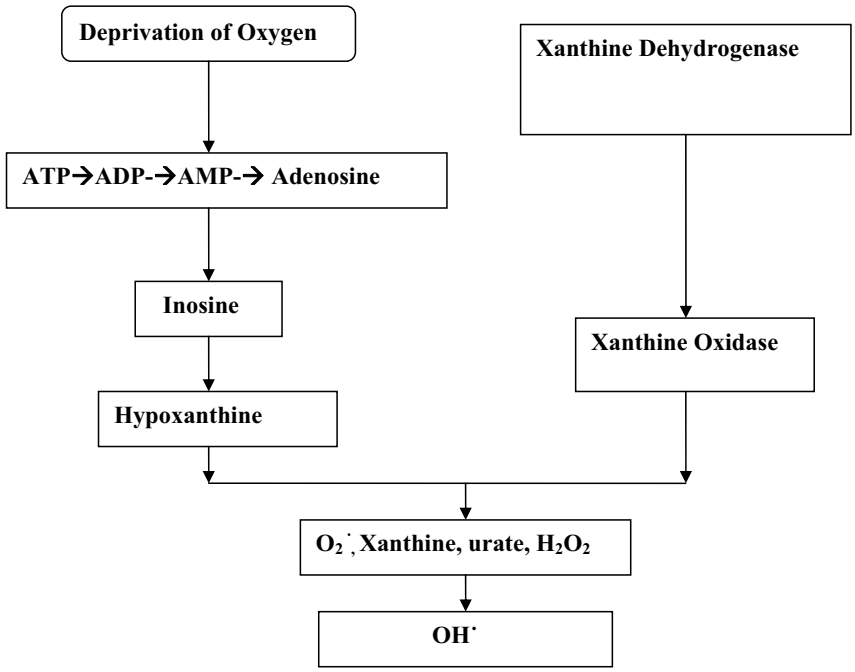


Fig. 1. A suggested mechanism for tissue injury upon reoxygenation of ischaemic tissue.

The proposed mechanism came from the Granger study, which is a cornerstone in this field. Partial occlusion of the artery supplying blood to a segment of cat small intestine (hypoxia), followed by reperfusion, causes gross, histologically observable damage to the tissue and increases intestinal vascular permeability. Intravenous administration of SOD, or oral administration of allopurinol to the animals before removal of arterial occlusion, decreases damage. Infusion of a mixture of hypoxanthine and xanthine oxidase into the

arterial supply of a segment of normal cat intestine increased vascular permeability, an effect that was decreased by the presence of SOD or dimethylsulphoxide in the infusion (Parks et al, 1982).

In later years the beginning of a new field of exploration in free radical research has been initiated. This has resulted in a considerable increase in knowledge in this area although the role of free radicals in ischaemia-reperfusion syndrome is still not clear. In 1992, the Haraldsson study showed the production of oxygen radicals during recirculation in ischaemic kidneys (Haraldsson et al, 1992). The final step of these oxygen radicals is lipid peroxidation (Paller et al, 1984). The reaction continues as a branched chain reaction and produces lipid-derived radicals. This has continued to attract major attention and numerous reports have been published on its effects in a number of pathological conditions believed to be associated with free radical generation, including studies of renal ischaemia and reperfusion (Hansson, 1983; Paller et al, 1984; Baker et al 1985; Koyama et al, 1985). There are numerous studies of renal ischaemia-reperfusion in which the effects of SOD on, for example, post-ischaemic blood circulation and post-ischaemic kidney function have been investigated (Fridovich, 1975; Chang et al, 1995).

At the same time, however, a number of fundamental scientific issues need to be resolved. Methods for free radical detection are still relatively primitive and indirect measures are often employed. It was thus important to combine radical detection techniques with the use of agents that disturb the process. In the earlier studies of our group it was demonstrated that pretreatment of animals with a potent new lipid peroxidation inhibitor, a vitamin E analogue, indeno-indole derivative (Code name H290/51) improved the restoration of cell bioenergetics after ischaemia–reperfusion (Sørensen et al, 1996). Subsequently, we studied the combined effect of the lipid-soluble H290/51 and a water-soluble antioxidant ascorbate to improve the recovery of cell bioenergetics at reperfusion after ischaemia. Together, these present new information and insight into the role of vitamin E and ascorbate in the recovery of ischaemia.

Because of the evanescent nature of free radicals, it was difficult to perform clinical studies. So, we applied a unique technique where we measure radical production using an

ex vivo spin trap method. The method was based on the fact that the radical produciton takes place in regional venous blood sample. As the technique was complicated, we applied advance stastitical mehtods like PCA and PLS to obtain the results. The reproducibility of correlation between radical production and degree of stenosis supported the techniques. As we wanted to carry out the investigations directly on patients, we investigated whether preoperative xanthine oxidase inhibitor and acetylcysteine effects free radicals levels in conjuctions with suregery for carotid stenosis. Also, it was equally important that a drug is not harmful and is readily available. Because of these characteristics, the protective effects of NAC on radio contrast-induced renal damage were found in some studies (Tepel et al, 2000).

There were concerns regarding the relationship between the production of free radicals during and after surgery for stenosis of the carotid artery and the blood levels of ICAM-1, MMP-9, MCP-1, and oxLDL. We therefore studied the relationship between markers for lesion progression in arteriosclerosis, production of radicals and clinical characteristics.

Enzymatic Antioxidants

Generally, three groups of enzymes play a significant role in protecting cells from oxidant stress (Diplock, 1991).

Superoxide dismutases: Superoxide dismutases are enzymes that catalyse the conversion of two superoxides into hydrogen peroxide and oxygen. The main advantage is that hydrogen peroxide is substantially less toxic than superoxide. SOD accelerates this detoxification reaction roughly 10,000-fold over the non-catalysed reaction.

$$O_2^{\cdot -} + O_2^{\cdot -} \rightarrow O_2 + H_2O_2 \rightarrow OH + OH + O_2$$

SOD is metal containing enzymes and depends on the bound manganese, copper or zinc for its antioxidant activity. The manganese-containing enzyme is found in abundance in mitochondria of mammals while the zinc or copper forms predominantly in cytoplasm.

Catalase: These are found in peroxisomes of eukaryotic cells. Catalase degrades hydrogen peroxidase into water and oxygen and thus helps to detoxify the reaction started by SOD.

Glutathione peroxidase: Glutathione peroxidase is a group of enzymes that contains selenium. Similar to catalase, these enzymes degrade hydrogen peroxide and also reduce organic peroxide into alcohols. It thus provides a route for eliminating toxic oxidants.

Several other enzymes, such as glutathione transferase, ceruloplasmin and hemoxygenase, may participate in the enzymatic control of oxygen radicals and their products.

Non-Enzymatic Antioxidants

Three non-enzymatic antioxidants are particularly important.

Vitamin E: Vitamin E acts as a major lipid-soluble antioxidant and plays a vital role in protecting membranes from oxidative damage. Its primary role is to trap peroxy radicals in cellular membranes (Burton and Ingold, 1989).

H290/51: H290/51 (cis-5,5a,6,106-tetrahydro-8-methoxy-6-methylindeno (2,1-6) indole) has been synthesised by Astra Hässle, Mölndal, Sweden. It is a representative member of a series of indeno-indole compounds that terminate the lipid peroxidation chain reaction (Sjöqvist et al, 1994). H290/51 has the capacity to recycle with ascorbate (Björquist et al, 1996), as is the case with vitamin E.

Vitamin C: Vitamin C, commonly referred as ascorbic acid, is a water-soluble antioxidant. It can reduce radicals from various sources and also participate in the recycling of vitamin E radicals. One of the important features of vitamin C is that it can also function as a pro-oxidant under certain circumstances (Podmore et al, 1998).

Glutathione: Glutathione is the most important intracellular defence against the reactive oxygen species. It is a tri-peptide (glutamyl-cysteinyl-glycine). The cysteine provides an exposed free sulphydryl group (SH), which is very reactive and thus produces an abundant, target-free radical attack. Reaction with radicals oxidises glutathione,

although in reduced form it is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH (Toborek et al, 1995).

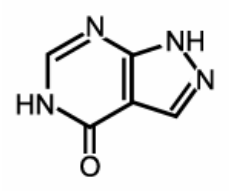
There are numerous small molecules which also act as antioxidants. These include bilirubin, uric acid, flavonoids and carotenoids.

N-Acetylcysteine (NAC)

The antioxidant supplement, **N-acetylcysteine**, is a sulphur-based amino acid which has been in clinical use as a mucolytic drug for more than 30 years and is considered a potential therapeutic agent for oxidant-associated diseases. NAC showed repressed α-SMA expression associated with attenuated activity of the CArG box element (Zafarullah et al, 2003). α-SMA is known to be a crucial marker for activation and dedifferentiation of mesangial cells (Aruoma et al, 1989). Expression of α-SMA is absent or only faintly detectable in normal glomeruli, whereas expression is markedly upregulated in proliferative mesangial cells in experimental and human glomerular diseases (Arstall et al 1995). In this context, the antioxidant NAC may have the ability to shift the cellular phenotype of mesangial cells toward deactivation and differentiation. This effect, at least in part, occurs via the three-dimensional cyto-organisation. NAC may therefore affect the expression of α-SMA directly by modulating the function of redox-sensitive signalling pathways (Kelly, 1998).

In the kidney, for example, administration of NAC ameliorates ischaemic renal failure and cisplatin-induced renal injury (Liu et al, 1999). The findings presented here raise the possibility that *in vivo* administration of NAC could be therapeutic in glomerular diseases, with NAC scavenging pathogenic oxidants and facilitating subsidence of glomerular cells.

Allopurinol



Allopurinol (4-hydroxypyrazolo (3, 4-d) pyrimidine is a natural purine in the body and is a potent inhibitor of xanthine oxidase. The efficacy of a potential antioxidant agent in post-ischaemia therapy, especially with respect to allopurinol, and as an inhibitor of xanthine oxidase, has attracted widespread interest. Many studies have shown that it is effective in preventing damage arising from ischaemia-reperfusion in vital organs (Palmer et al, 1990; van Bel et al, 1998; Clancy et al, 2001).

Previous Studies Concerning Ischaemia-Reperfusion of the Kidney

Previously, four thesis have been published through the Department of Urology, Göteborg University, concerning the importance of oxygen radicals on kidney damage caused by ischaemia-reperfusion. It was shown in the thesis by Roland Hansson (Hansson, 1983) that hypoxanthine is accumulated in the renal tissue during ischaemia. During recirculation there was a rapid but transient increase in the hypoxanthine concentration and a slight increase in the xanthine concentration in the renal venous blood. The oxidation of hypoxanthine into xanthine was blocked completely by allopurinol pretreatment. The erythrostasis found in the outer strip of the medulla after ischaemia-reperfusion could be prevented by pretreatment, either with SOD, catalase or allopurinol. The morphological lesions found in the rabbit kidney observed showed that ischaemia-reperfusion was attenuated after pretreatment with either allopurinol, superoxide dismutase or catalyse. When rabbits were exposed to one hour of renal ischaemia and contralateral nephrectomy, pretreatment with radical scavengers significantly reduced the post-ischaemic interest in serum creatine values.

In 1990, Stefan Bratell demonstrated in his thesis that the albumin leakages in the renal cortex observed after ischaemia-reperfusion could be reduced by combined pre-treatment with SOD catalyse and nifedipine or mannitol alone (Bratell, 1990). In a series of experiments using a different species of rabbits (French Loop Rabbits), the mortality caused by 60 min of renal ischaemia and contralateral nephrectomy without pretreatment was much higher than in previous series using New Zealand white rabbits. The high mortality rate in the control group could only be explained in part by kidney dysfunction. Pretreatment with a combination of SOD and catalyse, lidoflazine or a mixture containing hydroxyl radical scavengers and magnesium, significantly reduced the mortality caused by 60 min of renal ischaemia and contralateral nephrectomy in French Loop Rabbits. Release of a cardiomyodepressant factor into the renal venous blood during reperfusion after ischaemia was observed. This release was not influenced by pretreatment with SOD and catalyse improved the filterability after ischaemia.

The thesis of Gudjon Haraldsson (Haraldsson, 1993), described a technique for studying radical production after ischaemia-reperfusion in rabbit kidneys by using a spin trap technique. A reduced spin label (OXANOH) with the ability to read with the radicals and form a stable secondary radicals (OXANO) was infused in the renal artery. Under conditions where the arterial concentration was at equilibrium with the tissue concentration, venous samples were collected for subsequent analysis of the secondary radical using the electron spin resonance technique (ESR). At recirculation after 60 min of ischaemia, increased amounts of radicals were observed for at least one hour. Several different scavengers were found to be effective in reducing the production of radicals.

Significant contributions to the understanding of the role of oxygen radicals in the ischaemia-reperfusion syndrome in kidneys have been made by other authors, e.g. Paller et al (1984), Wolgast et al (1991) and Defraigne et al (1994).

In the previous studies of our group on the role of oxygen radicals for ischaemia-reperfusion damage, we mainly focused our interest on specific or relatively specific antioxidant therapies. However, to obtain a more general protective effect on radical damage, irrespective of where the radical originates, Viggo Sørensen (1998) studied the

end-stage of radical damage, i.e. lipid peroxidation (Sørensen, 1998). As discussed above, vitamin E is of particular importance in this respect although an indeno-indole compound with properties similar to vitamin E (code name H290/51), which in initial experiments turned out to be one hundred times more efficient as an antioxidant than tocopherol, has attracted our interest. Furthermore, the fact that transitional ions escalate the radical damage has focused our interest on the chelation of iron.

Most of the experiments on ischaemia–reperfusion damage have been performed on animal models. Since differences exist between species with regard to enzyme systems (e.g. xanthine oxidase and superoxide dismutase), it is not possible to extend results directly from animal experiments to humans (Muxfeldt and Schaper, 1987; Southard et al, 1987; Weinberg, 1991). The number of clinical studies performed is very limited. However, there are studies on humans where the production of radicals or lipid peroxidation in conjunction with reperfusion of the kidneys (Pincemail et al, 1993; Rabl et al, 1993; Davenport et al, 1995; Hower et al, 1996), the liver (Risby et al, 1994) and the heart (Menasche et al, 1987) has been reported. In some studies, pretreatment with scavengers or antioxidants has reduced these markers of radical activity (Rabl et al, 1993; Hower et al, 1996). So far, a positive clinical effect of such pretreatment has been observed in very few studies. Intravenous infusion of a multivitamin solution, including vitamin C, to renal transplantation recipients improved kidney function (Rabl et al, 1993) and more impressive pretreatment with rh-SOD increased graft survival in a prospective randomised double-blind trial (Land et al, 1994).

The present series of experiments presents new information and insight into the role of vitamin E and ascorbate in the recovery of ischaemia and are the first reports of direct measurements of radical production in human beings.

AIMS

1. To investigate whether pretreatment with a combination of a lipid-soluble vitamin E-analogue, indeno-indole derivative, code name H290/51, and water-soluble antioxidant ascorbate could improve the recovery of cell bioenergetics at reperfusion after ischaemia better than single treatment with H290/51.

2. To analyse the importance of pretreatment with a xanthine oxidase inhibitor on free radical production during ischaemia-reperfusion in conjunction with carotid endarterectomy.

3. To investigate the importance of pretreatment with acetylcysteine on free radical production during ischaemia–reperfusion in conjunction with carotid endarterectomy.

4. To explore whether there are any associations between the markers for tissue damage in arteriosclerosis (MMP-9, ICAM-1, MCP-1, oxLDL) and free radicals during ischaemia-reperfusion in carotid surgery.

MATERIALS AND METHODS

Animal Study

Thirteen white New Zealand rabbits with an average weight of 2-4 kg were used in the studies. A standard diet (EWOS brood stock feed for rabbits and guinea pigs K_1), composed of crude protein 19.5%, carbohydrates 46%, crude fat 3.5%, fibre 14.5%, crude ash 6.5% and moisture 10%, was fed to the rabbits. Prior to surgery the rabbits were deprived of food for 12 hours but had free access to water.

Pretreatment

H290/51 used in pretreatment has been synthesised by Astra Hässle, Mölndal, Sweden. It is a representative member of a series of indeno-indole compounds that breaks the lipid peroxidation chain reaction. As is the case with vitamin E, H290/51 has the capacity to recycle along with ascorbate (Björquist et al, 1996). In six rabbits, H290/51 along with polyethylene glycol at a dosage of 20 U $^{mol\text{-}kg\text{-}1}$ was injected into an ear vein 10 min before ischaemia.

In seven rabbits, H290/51 along with PEG and ascorbate 1 g/kg was injected into an ear vein 10 min before ischaemia. The same dose of ascorbate was given 10 min before reperfusion.

Anaesthesia

Anaesthesia was induced with a 0.5 ml/kg i.m. injection of a mixture containing phentanylcitrate (0.3 g/ml) and fluanision (10 mg/ml; Hypnorm Vet; Leo, Sweden). Anaesthesia was maintained by means of repeated injections of 0.5 ml of the same mixture every 30 min.

At the end of the preparation, midazolam (1 mg/kg; Dormicum, Roche, Switzerland) was administered subcutaneously. The same dosage of midazolam was administered 2-3 h after the first injection. An indwelling catheter in the right common iliac artery monitored systemic blood pressure. The rabbits were placed on heating pads.

The body temperature was measured using a rectal thermal probe. The animals were tracheotomised. The animals breathed spontaneously a gas mixture consisting of 50% oxygen and 50% nitrogen. The arterial blood acid-base balance was analysed three times during each experiment. The animals were hydrated with a continuous intra-arterial infusion of isotonic sodium chloride containing 0.5 mg sodium bicarbonate per ml. The infusion rate was 25 ml/kg/h.

Operating Procedures

All operating procedures on animals were performed under aseptic conditions. The abdominal cavity was opened with a mid-line incision and the abdominal aorta between the right and left renal arteries was dissected free. The dissected aorta was enclosed by a fenestrated plastic tube (length = 6 mm) between the more cranial right renal artery and the more caudal left renal artery. The tip of a Fogarty catheter was introduced through the plastic tube outside the aorta. Care was taken to adjust the position of the plastic tube so that the balloon of the Fogarty catheter remained inside the plastic tube and with inflation compressed the aorta between the renal arteries. The left kidney was then moved from the abdominal cavity into a subcutaneous pocket. A plastic tube containing MnCl was wrapped around the left kidney. The blood pressure in the right common iliac artery was recorded (Fig. 2).

The rabbit was placed on its left side on a plastic plate and its position adjusted so that the left kidney was just above the surface coil (diameter 5 cm). The plastic plate containing the rabbit was introduced into the central hole of the magnet. The length of the central hole was 1.2 m and the diameter 20 cm. The head of the rabbit was situated at the outer end of the central hole, facilitating i.v. injection via an ear vein. The body of the animal was kept inside the central hole. The animal was maintained in this position for the rest of the experiment (~ 5 h).

Initially, a ^{1}H NMR image of the kidney was acquired. The MnCl-filled plastic tube around the kidney was visible in this image, facilitating further analysis. A 3.5-ml tube, comprising the cortex and outer medulla, was selected for ^{31}P NMR spectra. Two

control spectra were recorded for 2 h before ischaemia. The aorta between the right and left renal arteries was then compressed by inflating the balloon of the Fogarty catheter. A third ^{31}P NMR spectra was recorded for a period of 1 h of ischaemia. The balloon was then deflated and two more ^{31}P NMR spectra recorded during the following 2 h of reperfusion. PCr, Pi, phosphomonoesters (PME), α-, β-, γ-ATP peaks were identified from their chemical shifts. After the experiments the animals were killed by means of an i.v. injection of pentobarbital sodium (110 g/100 ml) and ethanol (290 g/100 ml).

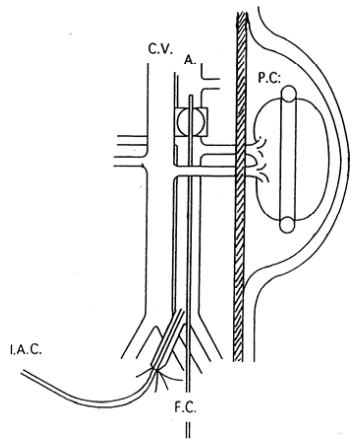


Fig. 2. Schematic illustration of the experimental set-up in the NMR experiments. C.V. = caval vein, A. = aorta, P.C. = plastic catheter, F.C. = Fogarty catheter, I.A.C. = iliac artery catheter.

NMR

Atomic nuclei are electrically charged. In some, nuclei rotation about their axes produces a magnetic dipole moment. This is the case with ^{1}H, ^{31}P and ^{13}C. When these nuclei are placed in a magnetic field, they align themselves in the direction of the field, either parallel or counter-parallel. The energy level in the counter-parallel orientation is higher than in the parallel one. The energy difference between these two energy levels is proportional to the magnitude of the magnetic field applied. When a radio frequency pulse is applied, more nuclei are switched to the high energy level. The return of the

nuclei to their equilibrium values releases energy which can be registered by a receiver coil. The recorded spectrum from a certain nucleus is influenced by the chemical milieu around the nucleus. Analyses of the ^{31}P NMR spectra thus allow calculation of the relative distribution of different phosphorous-containing compounds.

Human Studies

Allopurinol study: Between 19 May 1999 and 23 November 2000, 25 patients were randomised to either the control group or the treatment group. 300 mg x 3 of allopurinol were given perorally to the treatment group the day before surgery. Important clinical data for the patients are given in Table I. No difference in gender, age, diabetes, hypertension, cardiac disease, or smoking habits was observed between the groups. Twelve operations in the control group and 13 in the treatment group were performed by Associate Professor Lars Karlström, who had a CVA complication rate of less than 3%.

Table I. Total number of patients, gender, age at operation, prevalence of diabetes, hypertension, cardiac disease and smoking habits in control patients and patients pretreated with allopurinol or acetylcysteine. Mean ± SEM. ***** $p < 0.05$ versus control group.

	Total	F/M	Age years	Diabetes	Hyper-tension	Cardiac Disease	Smoking
Control group (Allopurinol)	13	4/9	72 (58-81)	4/13	7/13	5/13	6/13
Treatment group (Allopurinol)	12	3/9	72 (54-77)	3/12	7/12	5/12	4/12
Control group (Acetylcysteine)	17	5/12	71 ± 2	4/17	10/17	4/17	5/17
Treatment group (Acetylcysteine)	10	4/6	64 * ± 3	3/10	7/10	3/10	4/10

Acetylcysteine study: From 6 March 2001 to 3 December 2003, 41 patients were included in the study. The patients were randomised to either the control group or the treatment group, where 600 mg acetylcysteine were given perorally the day before surgery. The same dose was given during the morning of the day of the operation. No placebo was given to the control group. Due to the fact that a shunt had to be used in two patients and because of technical difficulties, 12 patients were excluded from further analysis. Data concerning the remaining 27 patients are presented in the above table.

The mean age was seven years higher in the control group ($p = 0.041$). There were no differences concerning gender, diabetes, hypertension or cardiac disease between the groups. Neither were there any differences concerning serum creatinine, smoking habits, side of operation or degree of contralateral stenosis between the groups. The operating procedure and sampling procedure were the same as in Study II.

Operating Procedure

The operation was performed in cases where carotid stenosis was more than 70%. During the operation cerebral oxygen saturation was monitored continuously using cerebral oximetry. An incision along the anterior border of the sternocleidomastoid muscle exposed the carotid artery. A 1-mm catheter was passed into the jugular vein through the facial vein and advanced up to 12-15 cm, just above the skull base, until resistance. The catheter was withdrawn 1 cm until blood could be aspired without resistance. The common carotid and internal carotid arteries were isolated separately without touching carotid bifurcation and the external carotid artery was then isolated.

The common and external carotid arteries were clamped and stump pressure was measured using an arterial pressure monitor via a needle inserted into the internal carotid artery distal to the stenotic lesion. By means of longitudinal arteriotomy the internal and common carotid arteries were opened and plaque was removed. In order to prevent dissection, the remaining intimal layer was secured distally using intimal sutures when considered appropriate. The arteriotomy was closed using 6-0 prolene continuous sutures.

Sampling Procedure

The clamping time was 41.5 ± 4.3min for right-side stenosis and 38.7± 2.1 for left-side stenosis. After declamping, the blood flow was monitored using a sterile doppler probe and a flow meter. A total of 4 cm^3 of blood sample were drawn from the jugular vein in three phases. In the first phase, three blood samples were drawn at five-minute intervals before clamping the carotid artery. In the second phase, one sample was drawn 1 min after clamping and one 3 min before declamping. In the third phase, another four samples were drawn at 1, 5, 10 and 15 min after declamping.

Measurement of Radical Production

The 4-ml blood samples were divided into two 1-ml portions (one sample and one blank) after heparinisation. OXANOH was added to both tubes. To distinguish the part of the ESR signal attributed to superoxide or hydroxyl radicals, superoxide dismutase, catalase and desferrioxamine were added to the blank tube to a final concentration of 0.1 mg ml^{-1}, 16,000 units ml^{-1} and 0.4 mg ml^{-1}, respectively. The same volume of isotonic sodium chloride as used to solute the scavenger substances was added to the sample tube. Subtraction of the ESR signal seen in the samples treated with an antioxidant cocktail from that of the saline samples yields the part of the signal that can be attributed to superoxide and/or hydroxyl radicals or any secondary radicals dependent on these. The tubes were shaken and centrifuged at 14,000 rpm for 1 min. The plasma was removed immediately and frozen in liquid nitrogen and the time from sampling to freezing was thus less than 2 min.

In this study, 2-ethyl-3-hydroxy-2,4,4-trimethyloxazolidine (OXANOH) was used as a spin trap.

The molecular weight of OXANOH is 158 and the lipid solubility over water solubility for OXANOH is 3. The low molecular weight combined with high solubility in both lipid and water means that the spin trap rapidly equilibrates across cellular membrane. Consequently, OXANOH easily detects the formation of radicals, both intracellularly and extracellularly. Although OXANOH equilibrates in the total tissue

water it will only trap a fraction of the radicals actually formed in the system. If the amount of a spin trap substance administered is low, the radicals trapped should be proportional to the amount actually produced. An important characteristic of OXANOH is that it is not acutely toxic (Nilsson, 1989).

Electron Spin Resonance

As electron spin resonance (ESR) allows selective and sensitive detection of unpaired electrons in complex samples such as blood or tissue and it can be used to measure the stable radical OXANO concentration in the venous blood. Due to the unpaired electron in their outermost orbital, radicals act like tiny magnets, whereas the molecules with paired electrons in the outermost shell are magnetically neutral. If a radical is exposed to the external magnetic field, the unpaired electrons can have their magnetic moment oriented either parallel or antiparallel to the external magnetic field. These orientations are called spin states. The parallel orientation is associated with slightly lower energy than the antiparallel. The strength of the external magnetic field acting on the electron is proportional to the energy difference. When electromagnetic radiation is applied to the system, transitions between the two energy levels occur, which leads to absorption of energy from the microwave radiation. This energy absorption can be recorded as an ESR spectrum.

Measurement of MCP-1, ICAM-1, MMP-9 and oxLDL

Blood samples were withdrawn for the analysis of markers of arteriosclerosis together with OXANO samples 1, 5 and 9, i.e. before clamping, just before declamping and 15 minutes after the start of reperfusion. The blood samples were heparinised and centrifuged and the plasma was then frozen in liquid nitrogen for later analysis. MCP-1, ICAM-1 and MMP-9 were measured using commercially available ELISA kits from R&D systems, UK. Circulating levels of oxLDL were measured using a commercially available ELISA kit from Mercodia, Uppsala, Sweden.

Statistics and Ethics

In the NMR and in the MCP-1, ICAM-1, MMP-9 and oxLDL studies the significance of difference was assessed using ANOVA multiple measures.

In studies II and III the observations and variables contain much more information and hence an adequate multivariate characterisation is necessary. Multivariate data accurately measure intelligently selected observations and variables and play an important role in intellectual and practical terms. The data generated must be expressed in a comprehensible way and for this purpose two data analytical tools, PCA and PLSR, were used.

Principle Component Analysis (PCA)

Principal component analysis provides a method for finding a new set of axes for the data. In simple terms it rotates the data into a new set of axes in such a way that the first few axes reflect most of the variations within the data. By plotting the data on these axes, major underlying structures can be spotted automatically. The value of each point, when rotated to a given axis, is called the principal component value.

The first principal components show the major variation within the two X variables, this being the one that is related to the Y variable. The second component contains the small noise factor, which is responsible for the differences between the X variables. In this study, PCA explored the relationships among both variables and observations. As presentation of the modelling was graphically oriented many diagnostics and parameters are available for model interpretation and validation.

All variables were scaled to zero mean and unit variance. A variance/covariance matrix is calculated on the basis of scaled variance. Each succeeding principal component accounts for as much as possible of the variation unaccounted for by preceding principal components. These are calculated as the eigen-vectors of this matrix, yielding the variable loadings. Due to simultaneous analysis of several variables, PCA reduces noise. Results generated using the PCA method are thus more robust than corresponding univariate

descriptors or bivariate correlation analysis and are hence used on the total data set of the study.

Partial Least Squares Regression (PLSR)

Partial Least Squares Regression (PLSR) is an algorithm which is used to examine both X and Y data and extracts components (factors), which are directly relevant to both sets of variables. These are extracted in decreasing order of relevance. As partial least squares regression is the least restrictive of the various multivariate extensions of the multiple linear regression model it is used in situations where the use of traditional multivariate methods is severely limited, such as when there are fewer observations than predictor variables. Furthermore, it can be used as an exploratory analysis tool to select suitable predictor variables and to identify outliers before classical linear regression. To form a model, correct numbers of factors must be extracted in order to reveal underlying effects.

Partial least squares (PLS) was therefore applied in subsequent analysis. For the regression coefficients yielded in PLS analyses, standard errors were estimated using the jackknife procedure. This procedure is used because it is a general principle for the estimation of errors in various estimates, it is suitable for PLS regression coefficients and it is also non-parametric. All PCA and PLS models, as well as graphics and standard error estimates, were generated using the Simca-P 8.0 Software (Umetrics, Inc.).

RESULTS

1. Cell Bioenergetics

Aim: To investigate whether pretreatment with a combination of a lipid-soluble vitamin E-analogue, indeno-indole derivative, code name H0290/51, and water-soluble antioxidant ascorbate could improve the recovery of cell bioenergetics at reperfusion after ischaemia better than single treatment with H290/51.

Tables II and III show the blood pressure values and details of blood gas analysis. All parameter values were found to be normal prior to operation. The distal aortic blood pressure fell to ~70mm Hg during clamping of the aorta in both groups. It was normalised after reperfusion. In the blood gas analysis hypoxia, hypercapnia and acidosis were observed during ischaemia and these changes were partly restored after two hours of reperfusion. No difference was found in any parameter between the two groups.

Table II. Arterial blood pressure, arterial pO_2 and arterial oxygen saturation preoperatively, after 60 min of unilateral renal ischaemia and after 120 min of reperfusion in rabbits pretreated with H290/51 (n = 6) or H290/51 and ascorbate (n = 7). Mean ± SEM.

* p<0.05, ** p<0.01, *** p<0.001 compared with preoperative values.

	Blood Pressure (mmHg)		**pO_2 (kPa)**		**Oxygen Saturation (%)**	
	H290/51	**H290/51 + ascorbate**	**H290/51**	**H290/51 + ascorbate**	**H290/51**	**H290/51 + ascorbate**
Preoperatively	115±7	113±4	21.5±4.5	26.6±3.9	100.0±1.3	100.6±1.1
After 60 min of ischaemia	70±4**	73±10**	14.8±3.7**	12.6±3.4*	89.2±5.2	92.7±3.7
After 120 min of reperfusion	113±5	111±6	21.6±4.2	18.4±4.7*	94.7±4.8	88.0±6.3

Table III. pH, pCO_2 and standard bicarbonate in arterial samples preoperatively, after 60 min of unilateral renal ischaemia and after 120 min of reperfusion in rabbits pretreated with H290/51. Mean ± SEM.

* p<0.05, ** p<0.01, *** p<0.001 compared with preoperative values.

	pH		**pCO_2 (kPa)**		**Standard bicarbonate (mmol/l)**	
	H290/51	**H290/51 + ascorbate**	**H290/51**	**H290/51 + ascorbate**	**H290/51**	**H290/51 + ascorbate**
Preoperatively	7.38±0.02	7.39±0.01	5.69±0.54**	5.35±0.26	24.1±0.84	24.2±0.5
After 60 min of ischaemia	7.17±0.03***	7.21±0.03**	14.8±1.36**	12.13±1.11***	28.2±0.94	29.3±1.3
After 120 min of reperfusion	7.21±0.04*	7.21±0.04**	13.51±1.75**	13.71±1.77**	30.2±1.5	30.7±1.8

The peaks representing the different phosphorous metabolites (PME, Pi, PDE and α-, β-, γ-ATP) were analysed before, during and after ischaemia in both groups. During ischaemia the peaks representing ATP and PME decreased and the peak representing Pi increased to a similar extent in both groups. During reperfusion the peaks normalised to some extent. In order to be able to compare the two groups, ratios β-ATP over Pi were calculated for peaks recorded before, during and after ischaemia and expressed as a percentage of preischaemic values (Fig. 3). The β-ATP/Pi decreased to approximately the same levels in both the H290/51 and combined H290/51 groups as well as in an untreated group treated in the same way and reported in a previous article (Sørensen, 1998). However, at reperfusion the β-ATP-Pi ratio improved significantly better than in the H290/51 ascorbate group compared to the other groups.

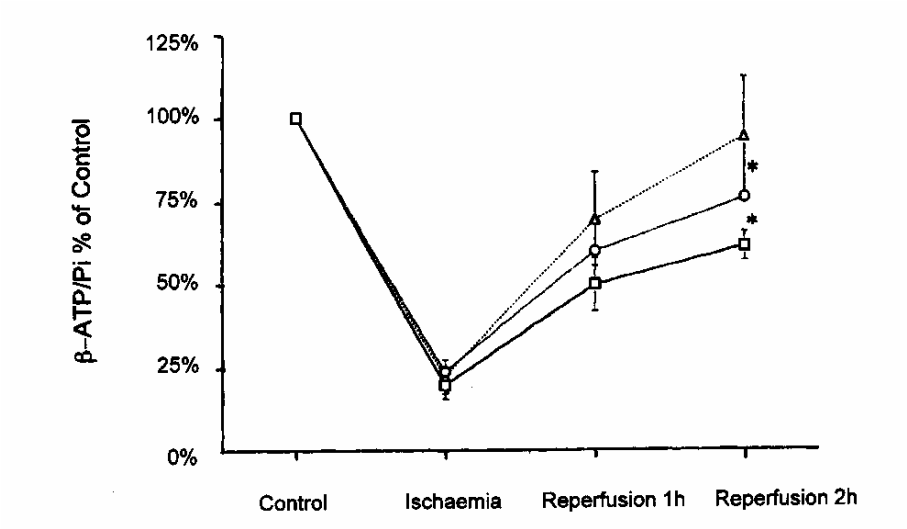


Fig. 3. Percentage changes in β-ATP/Pi during ischaemia-reperfusion. The squares denote untreated animals. The circles denote animals pretreated with H290/51 before ischaemia (n = 6). The triangles denote animals pretreated with H290/51 + ascorbate before ischaemia and with ascorbate before reperfusion (n = 7). The asterisks denote significant differences between the H290/51 + ascorbate group and the other two groups.

Comments

In a previous study of our group we demonstrated that pretreatment with H290/51 increased survival and tubular function after 60 minutes of kidney ischaemia and contralateral nephrectomy in survival experiments (Sørensen et al, 1996). The great advantage of the present technique using ^{31}P volume selective spectrometry is that it allows quantitative analysis of changes in kidney bioenergetics *in vivo* after different experimental conditions and different pretreatments without using survival experiments.

The drop in blood pressure during ischaemia and the restoration after reperfusion, as well as the changes in blood gas values, were the same in the two groups, indicating that the beneficial effect of combined pretreatment was not due to better general condition of the animals in this group but to a direct preventive effect on the kidneys. The fall in blood pressure during clamping in the present series of experiments was not as pronounced as in our previous study (Sørensen et al, 1998). The reason for this difference is not known.

The drop in blood pressure was nevertheless similar in both groups. This observation suggests that the kidneys in the present study were exposed to severe hypoperfusion rather than complete ischaemia. The changes in arterial blood gas values indicate that one-side kidney ischaemia as well as ischaemia of the whole lower part of the body initiates an acidosis that is mainly of the respiratory type. This acid base imbalance is partly restored at reperfusion.

The fact, that the β-ATP/Pi ratio was almost neutralised after 2 h in the combined group indicates that wash-out of the adenine pool at reperfusion does not occur to any major extent under these experimental conditions.

The result confirms the powerful antioxidant effect of vitamin E analogue and ascorbate when combined together. One explanation for this beneficial effect may be that mitochondrial oxidative phosphorylation is enhanced when cardiolipin, which is required for maximal cytochrome c oxidase activity, is protected from oxidative attack by ascorbate and the vitamin E analogue. Accordingly, the importance of protecting this phospholipid, as reported in a previous paper (Lagerwall et al, 1997) is emphasised.

The main finding in the present study is that pretreatment with a combination of a lipid-soluble and water-soluble antioxidant causes almost complete restoration of kidney bioenergetics as reflected in the β-ATP-Pi ratio after severe prolonged hypoperfusion/ischaemia. This kind of pretreatment should be considered in clinical practice when the kidney is subjected to ischaemia-reperfusion, as is the case in renal transplantation and resection of kidney tumours during arterial clamping.

2. Effect of Allopurinol

Aim: To analyse the importance of pretreatment with a xanthine oxidase inhibitor on free radical production during ischaemia-reperfusion in conjunction with carotid endarterectomy.

The data collected in this study were investigated using multivariate techniques in order to derive maximum advantage from the large number of variables in combination with a small sample size. To detect outliers among the patients or variables, a PCA was

performed on a complete data set. The PCA generated two significant components, describing 35% of the information contained in the 42 variables (R^2 X_{cum} =0.35), Q^2_{cum} = 0.046. The outcome of the PCA of the complete data set is shown in Figure 4.

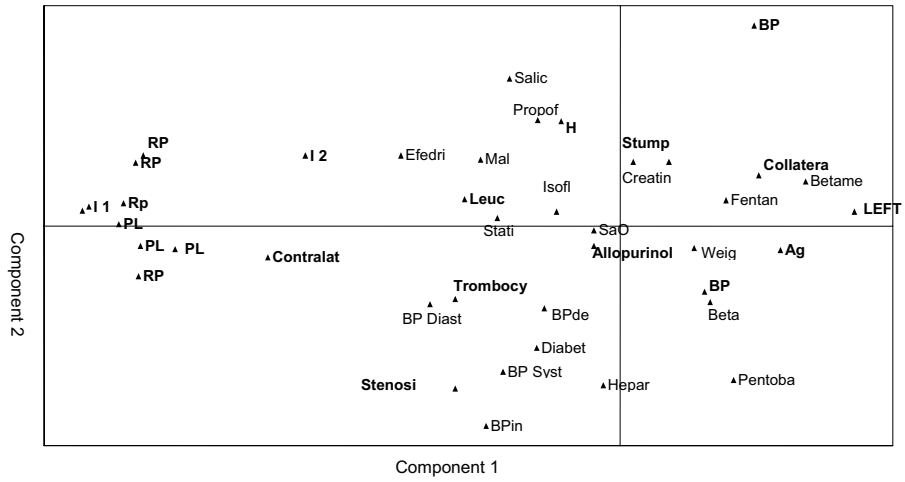


Fig. 4. Variable loadings derived from a PCA of the full data set, including controls and allopurinol-treated patients. The position of each variable in the loading plot indicates its relationship to other variables. Strongly correlated variables are located close to each other. Abbreviations: PL1, PL2, PL3, OXANO baseline values; I1 etc, OXANO levels after 1 min during clamping etc; RP1 etc, OXANO levels 1 min after start of reperfusion etc; SaO_2, O_2 saturation during clamping; BP rest, resting systolic blood pressure; BP decr, peroperative BP decrease; BP inc, Peroperative BP increase; BP Stab, BP stable peroperatively; BP diast, Diastolic pressure during clamping; BP syst cl, systolic pressure during clamping; collateral, degree of collateral circulation; contralat stenosis, degree of contralateral stenosis; leuc, white blood cell count. Variables discussed specifically in the results section are highlighted (bold).

PCA of all subjects: variable loading plot

The OXANO radicals level (PI, I, RP) measured at different time points all show a strong positive correlation, which is apparent from their location in a cluster at the far left of the X-axis. The random order within the cluster reflects the lack of an obvious time course in the OXANO levels measured during operation. At origin is the variable “Allopurinol”, indicating a lack of correlation between allopurinol treatment and any of the other variables. Collateral circulation, left-side surgery, age and betamethasone are all located in the opposite direction to the OXANO cluster, which suggests a negative correlation between these variables and the general level of radical production in these patients. Contralateral stenosis on the other hand appears to be positively correlated to radical production.

PLS regression analysis

As there was a correlation between OXANO and several clinical variables, PLS regression models were calculated separately for controls and allopurinol-treated groups. In the control group, the PLS regression analysis showed a statistically significant model, relating the various clinical measures to the radical production (two components, R^2X_{cum}= 0.271, R^2Y_{cum} = 0.982, Q^2_{cum} = 0.57) while in the allopurinol group the PLS model came out as non-significant. The regression coefficients for the control group and allopurinol group are shown in Figures 5a, 6a and 5b, 6b, respectively.

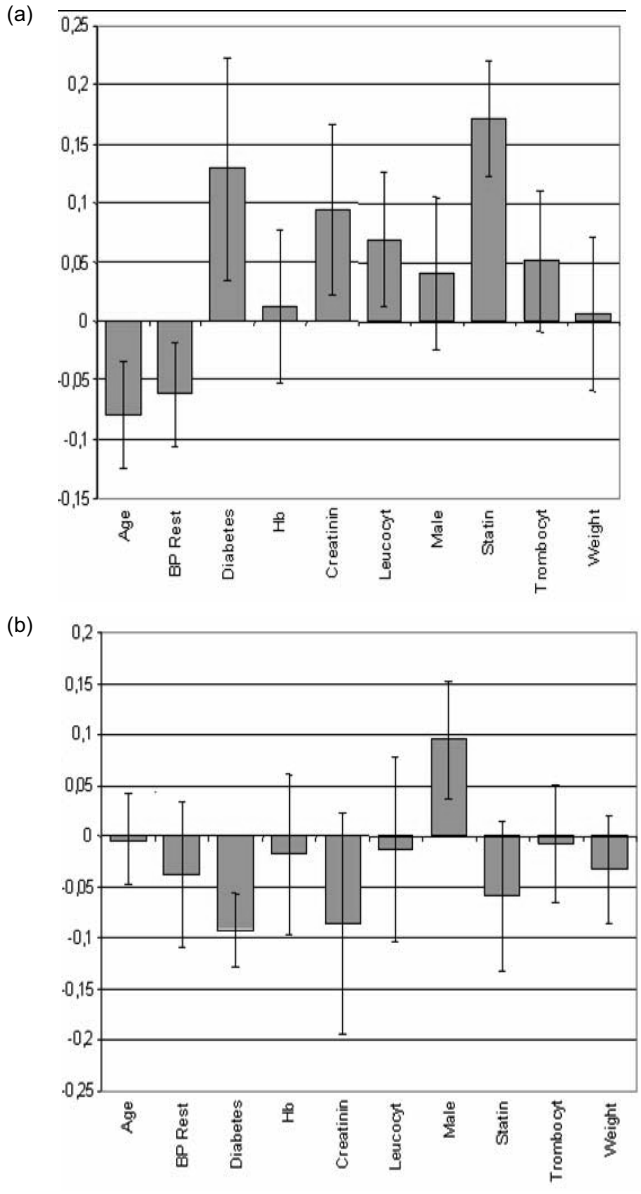


Fig. 5. Regression coefficients in a PLS model, relating radical production to clinical variables in (a) control patients and (b) allopurinol-pretreated patients. Shown are scaled and centred regression coefficients with standard errors estimated using the jackknife procedure. A positive coefficient indicates a positive relationship to radical production and vice versa.

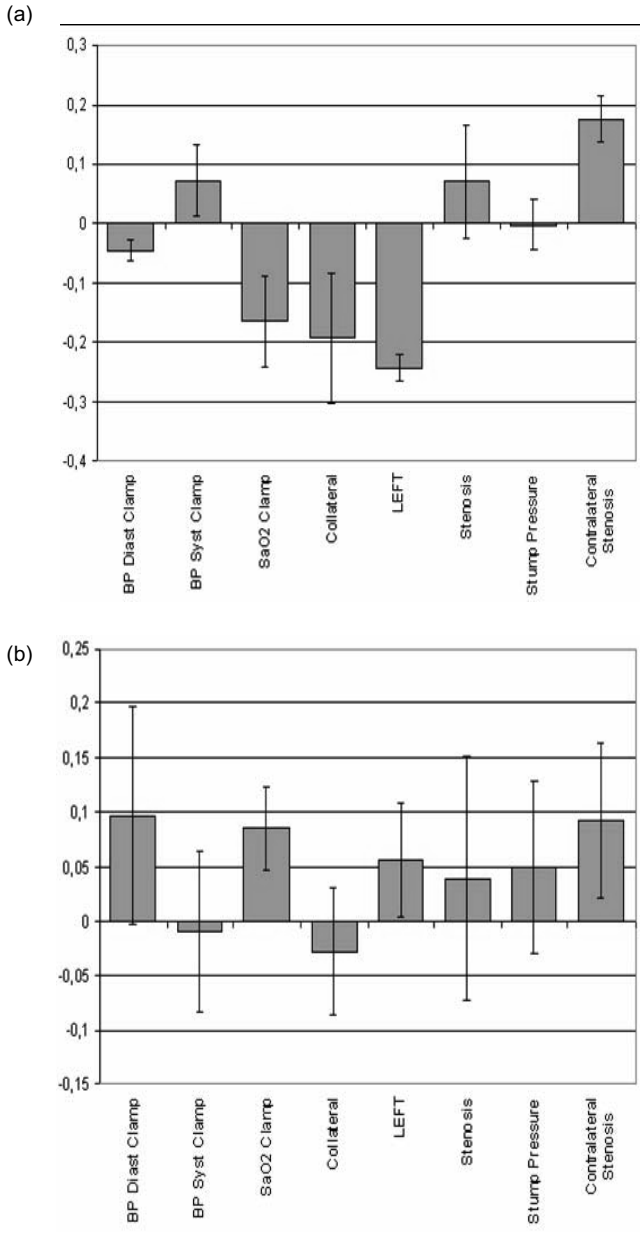


Fig. 6. Regression coefficients in a PLS model, relating radical production to clinical variables in (a) control patients and (b) allopurinol-pretreated patients. Shown are scaled and centred regression coefficients with standard errors estimated using the jackknife procedure. A positive coefficient indicates a positive relationship to radical production and vice versa.

In the control group, the radical production increased with diabetes, higher leucocyte counts, higher creatinine and the occurrence of contralateral stenosis and decreased with higher age, blood pressure, higher arterial oxygen saturation and the occurrence of collaterals. In addition, operation for left-side stenosis resulted in lower radical production. After pretreatment with allopurinol, several of the relationships noted in the control group were lacking, causing this part of the model to emerge as insignificant. Notably, no correlations with leukocyte count, side of operation or collateral circulation were seen after allopurinol pretreatment.

Comments

The data collected in this study were investigated using multivariate techniques to take maximum advantage of the large number of variables in combination with a small sample size. The basic idea is that in the case where several variables are collected in a sample, information can be gained by looking at variable patterns, rather than examining one variable at a time. PCA and PLS, which are closely related, generate principal components, which are composite variables representing the full data set in an optimal fashion. The results from a PCA or PLS are most conveniently examined in "object score plots", or "variable loading plots". The score plot shows the scores of each object with respect to the principal components. Objects that are similar overall appear near each other in a score plot. The score plot is thus useful to detect clusters and outliers among objects. The loading plot shows the pattern of correlations in the data set. Closely correlated variables show up near each other, while independent variables appear in orthogonal directions. Such plots give an overview of how the variables are interrelated.

A significant pattern of relationships between the clinical and perioperative variables was found in a PCA. Part of this pattern reflects predictable relationships in this patient population, such as the covariance between age, weight, and resting systolic blood pressure. Another reasonable feature is the observed negative correlation between contralateral stenosis and collateral circulation/stump pressure, where the latter two co-vary. Some of these relationships have been reported in our previous study (Holm et al,

2001). It is conceivable that a large degree of contralateral stenosis contributes to a low degree of collateral circulation and low stump pressure, and good collateral circulation increases stump pressure. Furthermore, a large degree of contralateral stenosis is likely to be related to stenosis at other locations, e.g. ipsilaterally. Consequently, contralateral and ipsilateral stenosis are to some extent positively correlated, both variables located to the left along component 1.

Apart from the haemodynamic interactions, it is worth noting that the degree of contralateral stenosis appears to be the variable that is most strongly related to radical production as reflected in *ex vivo* spin trap measurements. This finding is entirely reasonable. The question arises why the degree of ipsilateral stenosis is not even more important. The explanation appears to be the selection of patients. A major criterion for carotid endarterectomy is a degree of ipsilateral stenosis between 70 and 95%, which means that the spread in this variable is rather small compared with the spread in contralateral stenosis, which ranges from 0 to 90% among these patients.

The allopurinol symbol is located close to origo, indicating a lack of correlation with any variable in this general overview. However, this does not exclude the fact that allopurinol pretreatment influences the relationship between single variables and radical production.

When the data set was divided into subsets consisting of either controls or allopurinol-treated patients, clear effects of allopurinol were disclosed. Although the general level of each variable was unaffected by allopurinol, the pattern of relationships between the clinical variables versus radical production is disrupted distinctly by allopurinol treatment. In the control group, the OXANO radical levels were related significantly to the other variables. This relationship pattern appears to have been extinguished in the allopurinol group.

Comparison between radical production in the control and allopurinol groups revealed higher production in both groups when contralateral stenosis prevailed. This factor appears to be strongly correlated to radical production and is not preventable with a blockade of the xanthine oxidase system. However, the positive correlation between

leucocyte number and radicals found in the control group disappeared after allopurinol pretreatment, which suggests that this pretreatment interfered with leucocyte radical formation.

In the control group, radical production is more pronounced in patients operated on for right-side carotid stenosis. This could be due to the fact that the right jugular vein drains both hemispheres while the left jugular vein more selectively drains the ipsilateral hemisphere (Hafferl, 1957). This side difference disappeared after allopurinol pretreatment, which could be due to lowered radical production in the right-side stenosis group.

The present study shows that radical production can be determined in conjunction with surgery for carotid artery stenosis using an *ex vivo* spin trap technique with OXANOH as the spin trap. The major finding is that allopurinol pretreatment affects the relationships between the clinical variables seen in controls, which disappear after pretreatment. This might indicate a beneficial effect as the enhanced radical production in, for example, diabetes and at elevated leukocyte counts is lost after pretreatment.

3. Effect of Acetylcysteine

Aim: To investigate the importance of pretreatment with acetylcysteine on free radical production during ischaemia-reperfusion in conjunction with carotid endarterectomy.

One patient in each group died during the first post-operative year for reasons not associated with the arteriosclerotic disease. After one year, the carotid arterial blood flow in 38 patients was up to a satisfactory level. Information was not available for one patient belonging to the control group.

A PLS analysis of the relationship between radical data from the control group and clinical variables revealed in accordance with previous results increased radical production at e.g. advanced stenosis and high leucocyte and platelet counts (Waters et al, 2004). These relationships were not found in the acetylcysteine treatment group.

A PCA of the complete data set is shown in Figure 7.

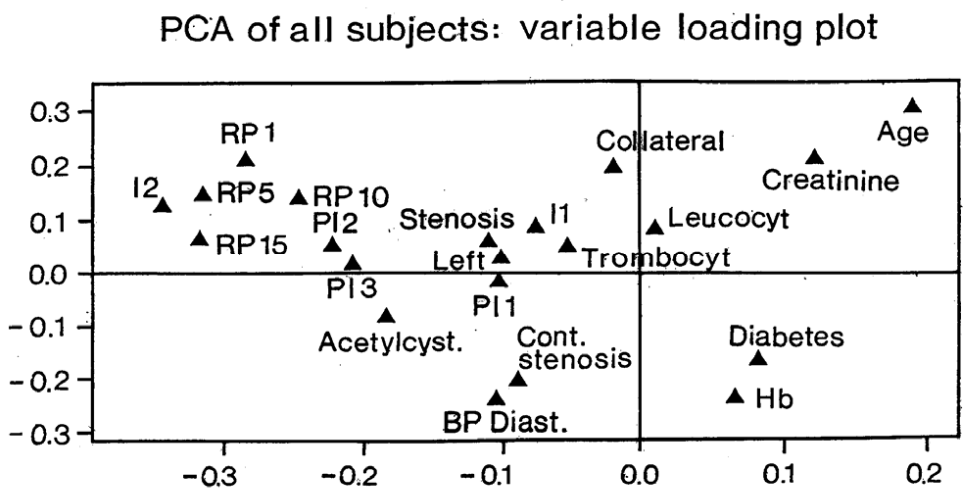


Fig. 7. Variable loading derived from a PCA of the full data set, including control and acetylcysteine-treated patients. Radical production data are expressed in absolute values. PL1, PL2, PL3, OXANO baseline values, I1, I2, OXANO values during clamping, RP1, RP5, RP10, RP15, OXANO values 1-15 minutes after the start of reperfusion. Acetylcyst – patients treated with N-acetylcysteine. Cont. stenosis – degree of contralateral stenosis; Left – left-side stenosis; Age – age at operation; Stenosis – degree of stenosis on operated side; BP. Diast – diastolic pressure during clamping; Thrombocyte – platelet counts; Leucocyte – white blood cell counts; Hb – haemoglobin.

The OXANO radical levels expressed in absolute values measured at different time points all show a positive correlation, which is apparent from their location in the left-hand part of the X-axis. The variable acetylcysteine is located in the left-hand part of the figure, indicating a positive correlation between this variable and the general level of radical production. It thus appears as if NAC pretreatment increases radical production in conjunction with this kind of surgery. A similar conclusion can be drawn from the PLS regression model presented in Figure 8, where the relationships between acetylcysteine pretreatment and the mean radical production during and after clamping as well as various clinical variables are described. The symbols reflecting radical production and

NAC pretreatment are located close together in the right-hand part of the diagram, indicating a positive correlation. Again, N-acetylcysteine pretreatment appears to increase rather than decrease radical production (Fig. 8).

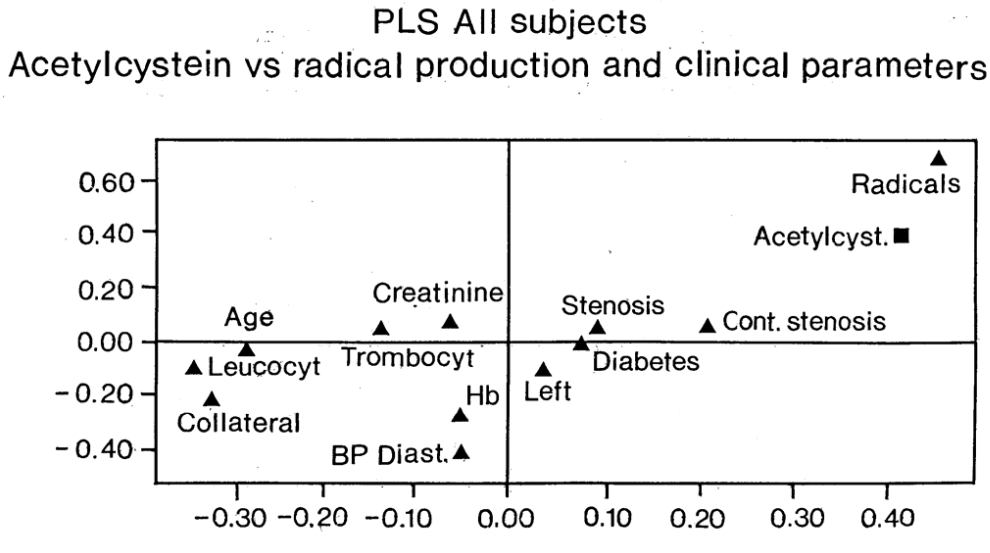


Fig. 8. Variable loading plot in a PLS model relating acetylcysteine treatment (squares) to mean values for OXANO levels during and after clamping (Radicals) as well as to various clinical variables. Symbols as in Figure 2.

Comments

The study confirms the results of earlier studies concerning the relationship between various clinical parameters and the production of free radicals in conjunction with surgery for carotid artery stenosis. The degree of stenosis, right-side stenosis, diabetes and haemoglobin is associated with high radical production while, for example, high age appears to decrease radical production although pre-treatment of patients with acetylcysteine eradicated these relations. PCA and PLS analyses of our results suggest that acetylcysteine pretreatment increases radical production although this interpretation is jeopardised by the age difference between the groups. At least it can be concluded that

our results do not support the hypothesis that acetylcysteine decreases radical production. Previous results concerning the protective effect of acetylcysteine on ischaemia-reperfusion damage are conflicting. In experiments on rats a preventive effect of acetylcysteine on ischaemia-induced renal damage was reported in survival experiments but not in acute experiments (Nitescu et al, 2006a; Nitescu et al, 2006b). Considerably higher dosages of NAC than used in the present study improve heart function in conjunction with acute myocardial infarction (Yesilbursa et al, 2006). Nevertheless, we have not been able to demonstrate any beneficial effect of pretreatment with NAC with regard to the production of free radicals in conjunction with surgery for carotid artery stenosis.

4. Relationship Markers for Arteriosclerosis and Free Radicals

Aim: To explore whether there are any associations between the markers for tissue damage in arteriosclerosis (MMP-9, ICAM-1, MCP-1, oxLDL) and free radicals during ischaemia-reperfusion in carotid surgery.

The values for the arteriosclerosis markers determined before, during and after clamping are given in Figure 9.

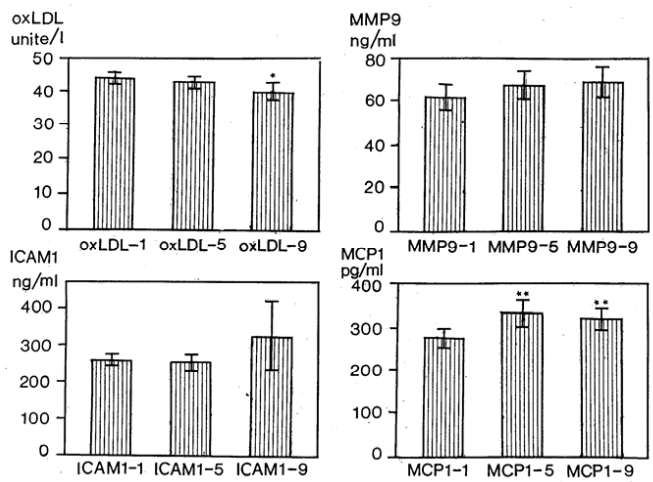


Fig 9. Mean values (SEM) of oxLDL, MMP-9, ICAM-1 and MCP-1 determined before clamping (1), during clamping (5) and 15 minutes after declamping (9).
Stars denote significant differences compared to preclamp values.
* $p < 0.05$, ** $p < 0.01$.

MCP-1 increased significantly from the first time point to the second and third time points, while oxLDL tended to decrease. No statistically significant change was observed regarding MMP-9 and ICAM-1.

The outcome of the PCA of the radical data, data concerning the degree of atherosclerotic disease and some relevant clinical data, are shown in Figure 10. The OXANO radical levels are located in a cluster at the far left of the X-axis together with the MMP-9 values, indicating a positive correlation between these variables. ICAM-1 values are located in the opposite direction, indicating a negative correlation with OXANO levels as well as MMP values. MCP-1 and oxLDL values are close to origin, indicating a lack of correlation between these values and any of the other variables.

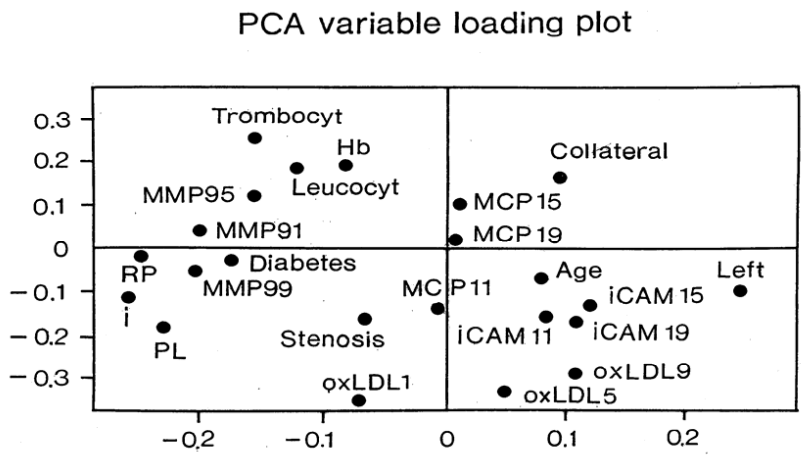


Fig. 10. Variable loadings derived from a PCA of some of the recorded data. The position of each variable in the loading plot indicates its relationship to other variables. Strongly correlated variables are located close to each other.

Abbreviations: PL, I and RP OXANO levels at baseline, clamp and reperfusion respectively. MCP11, MCP15, MCP19; MCP levels before, during and after clamping, ICAM11, ICAM15, ICAM19; ICAM levels before, during and after clamping, MMP91, MMP95, MMP99; MMP9 levels before, during and after clamping, oxLDL1, oxLDL5, oxLDL9; oxLDL levels before, during and after clamping, Collateral; degree of collateral circulation, Stenosis; degree of ipsilateral stenosis, Left; operation for left-side stenosis, Diabetes; occurrence of diabetes, Leucocyte; white blood cell count, Hb; haemoglobulin concentration, Thrombocyte; thrombocyte count.

As indicated in the PLS analysis presented in Figure 11, the positive correlation between MMP-9 and radicals and the negative correlation between ICAM-1 and radicals are statistically significant.

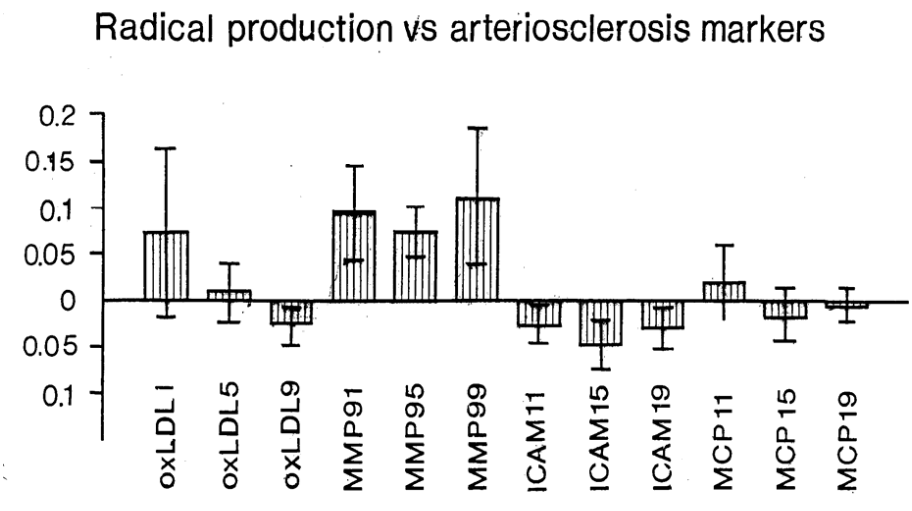


Fig. 11. Regression coefficients in a PLS model relating radical production to oxLDL, MMP9, ICAM1 and MCP1 determined before clamping (1), during clamping (5) and 15 minutes after declamping (9). Shown are scaled and centred regression coefficients with standard errors estimated using the jackknife procedure. If the bars do not include the zero line a significant relationship prevails. A positive coefficient indicates a positive relationship to radical production and vice versa.

For this reason the relationship between these markers for arteriosclerosis and some clinical parameters has been analysed in more detail. The results of these PLS analyses are given in Figures 12 and 13.

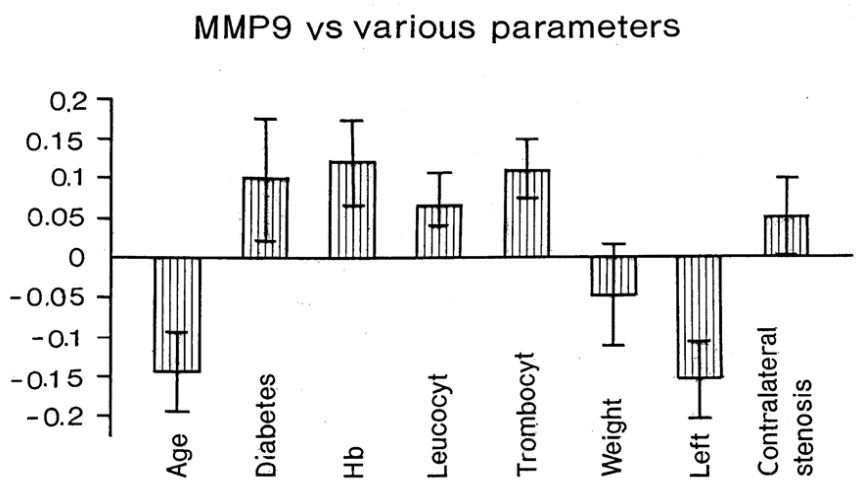


Fig. 12. Regression coefficients in a PLS model relating MMP9 levels to various clinical variables. Shown are scaled and centred regression coefficients with standard errors estimated using the jackknife procedure. If the bars do not include the zero line a significant relationship prevails. A positive coefficient indicates a positive relationship to radical production and vice versa.

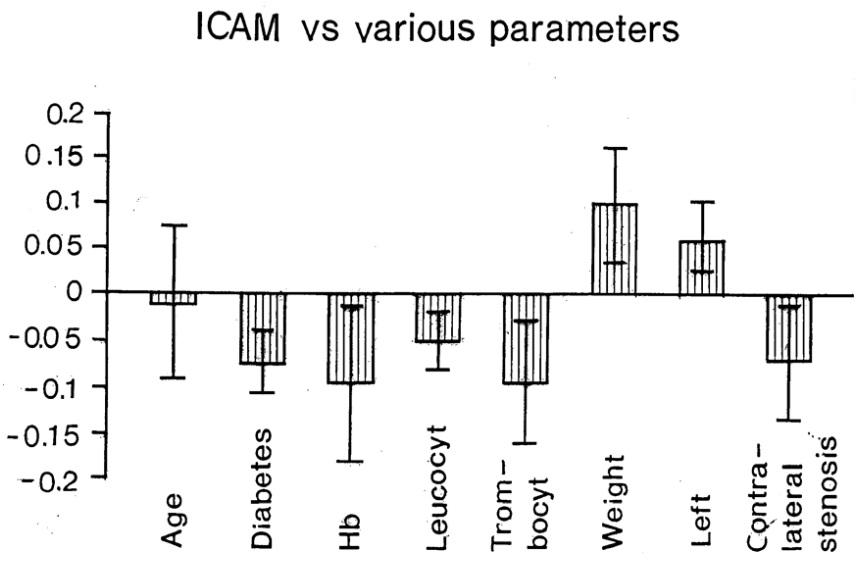


Fig. 13. Regression coefficients in a PLS model relating ICAM-1 levels to various clinical variables. Shown are scaled and centred regression coefficients with standard errors estimated using the jackknife procedure. If the bars do not include the zero line a significant relationship prevails. A positive coefficient indicates a positive relationship to radical production and vice versa.

Increased MMP-9 values are found in conjunction with diabetes, high haemoglobin, leucocyte and platelet values and contralateral stenosis while low values are found in conjunction with high age, high blood pressure, operation for left-side stenosis and high stump pressure (Fig. 12).

As regards ICAM-1, high values are found in conjunction with heavy weight, operation for left-side stenosis and high stump pressure. Low values are found in conjunction with diabetes, high haemoglobin, leukocyte and thrombocyte values and when a contralateral stenosis is present (Fig. 13).

Comments

The most important finding in the present study is the strong positive correlation between MMP and radical production. MMPs are a group of zinc-dependent enzymes that degrade the molecules of the extracellular matrix. It has been shown that matrix degradation caused by these proteinases occurs during progression of atherosclerosis (Galis et al, 1994). Furthermore, an increase in MMP activity occurs after stroke, which contributes to ischaemic brain injury, including infiltration of leucocytes in the damaged tissue (Weiss and Peppin, 1986; Matsuo et al 1994; Rosenberg et al, 1996). In animal experiments it has been demonstrated that inhibition of MMP-9 reduces brain injury after stroke (Romanic et al, 1998). MMP-9 levels are increased in internal jugular venous blood after traumatic brain injury in patients (Suehiro et al, 2004). These studies indicate that for various reasons MMP-9 is involved in the pathogenesis of brain damage. Our results of a positive correlation between levels of free radicals and MMP-9 in conjunction with surgery for carotid artery stenosis further support this role of metalloproteinases in brain tissue damage. The positive correlation between MMP-9 and leukocyte counts also appears logical since neutrophils can also produce these enzymes (Weiss and Peppin, 1986). Our finding of a positive association between MMP-9 and diabetes fits in well with previous observations (Cipollone et al, 2003).

The negative correlation between ICAM-1 values and radicals is somewhat unexpected. The plasma concentration of ICAM-1 as well as other adhesion molecules is increased in conjunction with transient ischaemic attacks, indicating a central nervous system inflammatory reaction (Selakovic et al, 2003). The lack of a positive correlation between radicals and ICAM-1, as well as between clinical parameters known to be associated with increased radical production, indicates that the possible hypoperfusion that occurs during carotid endarterectomy, and which is responsible for radical production, is not enhanced enough to cause cerebral inflammation, resulting in increased ICAM-1 values.

Another possible explanation for the lack of a positive correlation between ICAM-1 and radical production in the present series is that our patients suffered from

late-stage atherosclerosis. In previous studies it has been shown that plasma levels of ICAM-1 are associated with sub-clinical femoral atherosclerosis in clinically healthy middle-aged men and also with endothelial function in healthy young subjects (Holmlund et al, 2002; Hulthe et al, 2002). Various stages of atherosclerosis in different patient populations might explain why various authors report different results regarding ICAM-1 and the risk of cerebrovascular complications. Elevated concentrations of ICAM-1 were reported to be associated with an increased risk of stroke (Tanne et al, 2002) whereas no correlation was found between the expression of ICAM-1 and the severity of symptomatic carotid disease (Nuotio et al, 2003).

The major finding in the present study was that MMP-9 in plasma was related to the production of free radicals during carotid endarterectomy and also other variables known to covariate with MMP-9, such as leucocyte count, diabetes and more extensive atherosclerosis.

GENERAL DISCUSSION

The NMR experiments reflect short survival experiments where the animals are not awakened. Suffering for the animals is thus reduced and data reflecting kidney activity after reperfusion are still obtained. Combined pretreatment is more effective. Although these experiments entail a sophisticated methodology, the data obtained are still from animal experiments. The question is to what extent these animal data reflect the clinical situation. Clinical experiments are few due to the fact that an abundance of techniques to study radical production has hitherto been lacking. Our approach, involving measurements of radical production using an *ex vivo* spin trap method, is rather unique. The method is based on the fact that radical production takes place in venous samples *ex vivo*. This might be the effect of active leukocytes or oxidation of hypoxanthine by xanthine oxidase. Although previous studies by our group have been performed on kidneys for practical reasons, the clinical studies have been performed in conjunction with surgery for carotid artery stenosis. The latter operations are more common and measurements of radical production can be performed without disturbing the operation. Initially, experiments were performed on humans in conjunction with kidney tumour resection under ischaemia. However, the measurements could not be performed simultaneously with surgical activity. Furthermore, placement of the catheter in the renal vein was sometimes insecure due to the short vessel. The method for radical production in kidneys and brains is rather similar and in both cases is based on oxidation of accumulated hypoxanthine and on activity of polymorph nuclear leucocytes.

Advanced statistical methods include PCA. PLS has also been used since radical production is influenced by a large number of variables. Analyses of mean values of radicals before, during and after ischaemia provide no information. Although the technique used appears complicated, basically the same results are obtained in three different groups of control patients. Strong correlations thus exist between radical production and degree of stenosis, left-side stenosis etc. This reproducibility supports the

technique. In some illustrations the absolute radicals have been presented and in some the ratio between radicals during and after ischaemia versus before ischaemia has been presented. Although there appears to be a numeral difference between radical levels during and after ischaemia, basically the levels before, during and after clamping are associated quite closely with each other. The interpretation of this is that we do not study exclusively the influence of carotid artery stenosis surgery on radical production but more the combined effect of anaesthesia, peroperative procedures and surgery for carotid artery stenosis. We are not able to separate the individual effects of each of these components.

Our studies support the hypothesis that a combination of a lipid-soluble and water-soluble antioxidant is superior in preserving kidney metabolism in conjunction with ischaemia-reperfusion. This hypothesis should be tested in a clinical situation. Pretreatment of patients who are about to undergo surgery for carotid artery stenosis with allopurinol might reduce the production of free radicals while pretreatment with acetylcysteine appears to have the opposite effect, although alternative interpretations of the results can be made. MMP-9 appears to be related to the production of free radicals during carotid endarterectomy while ICAM-1 was negatively correlated.

CONCLUSIONS

1. Pretreatment of rabbits with a combination of H290/51 and ascorbate causes more rapid normalisation of mitochondrial function at reperfusion after ischaemia compared to pretreatment with H290/51 alone.

2. Production of free radicals in conjunction with carotid endarterectomy can be reproducibly measured using an *ex vivo* spin trap method. Pretreatment of the patients with a xanthine oxidase inhibitor attenuates strong correlations with various clinical parameters and radical production found in the control group. This might indicate a positive effect of pretreatment with a xanthine oxidase inhibitor.

3. Pretreatment of patients with acetylcysteine appears to increase radical production in conjunction with carotid endarterectomy.

4. There is a positive/negative correlation between radical production in conjunction with carotid endarterectomy and MMP-9/ICAM-1. These results support the hypothesis that MMP-9 could cause brain tissue damage in conjunction with cerebral ischaemia-reperfusion.

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