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CLINICAL AND EXPERIMENTAL INVESTIGATION:

relationship of ischaemia/reperfusion injury with oxidative stress in abdominal aortic aneurysm repair and in extracranial brain artery endarterectomy and possibilities of protection against ischaemia using a glutathione analogue in a rat model of global brain ischaemia

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PRIIT PÕDER

TARTU UNIVERSITY
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Department of Vascular Surgery and Biochemistry, University of Tartu, Tartu, Estonia

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Opponent: Arkadiusz Jawien, M.D., Ph.D., Professor of General Surgery, Vascular Surgery and Angiology, Chairman of the Department of Surgery, Collegium Medicum in Bydgoszcz, University of Nicolai Copernicus

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To my family

Some are influenced by the love of wealth
while others are blindly led on the mad fever for
power and domination, but the finest type of man gives
himself up to discovering the meaning and purpose of life itself.
He seeks to uncover the secrets of nature.
This is the man I call philosopher...

Pythagoras

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I P. Põder, A. Aavik, J. Kals, K. Zilmer, T. Kullisaar, A. Pulges, M. Zilmer "Is there high grade oxidative stress during elective abdominal aortic aneurysm repair?" Scandinavian Journal of Surgery 2003; 92: 206–209

II P. Põder, M. Zilmer, J. Starkopf, J. Kals, A. Talonpoika, A. Pulges, Ü. Langel, T. Kullisaar, S. Viirlaid, R. Mahlapuu, A. Zarkovsky, A. Arend, U. Soomets "An antioxidant tetrapeptide UPF1 in rats has a neuroprotective effect in transient global brain ischaemia" Neuroscience Letters 2004, 370: 45–50

III P. Põder, A. Pulges, A. Aavik, K. Zilmer, J. Kals, T. Kullisaar, C. Kairane, M. Zilmer "Time-course of oxidative stress during carotid artery endarterectomy" Journal of Angiology and Vascular Surgery 2006, 12: 111–117

ABBREVATIONS

AAAR	abdominal aortic aneurysm repair
ATP	adenosine triphosphate
BHT	bytylated hydroxytoluene
CAE	carotid endarterectomy
CAT	catalase
CHD	coronary heart disease
DC	diene conjugates
GCL	glutamate-cysteine lipase
GGT	γ-glutamyltranspeptidase
GR	glutathione reductase
GSH	reduced glutathione
GSHPx	glutathione peroxidase
GSSG	oxidized glutathione
GSSG/GSH	glutathione redox ratio
GST	glutathione S-transferase
IRI	ischaemia/reperfusion injury
LA	linolenic acid
LP	lipid peroxidation
NAC	N-acetyl-L-cysteine
NAD	nicotine adenosine dinucleotide
NADH	nicotinamide dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
OxS	oxidative stress
PAD	peripheral arterial disease
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SOD	superoxide dismutase
TAC	total antioxidative capacity
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TGSH	total glutathione
THA	terephthalic acid

INTRODUCTION

Ischaemia occurs clinically with loss of arterial flow to an end-organ as seen when an embolus travels, e.g. to the brain, the intestine or the lower extremities. This knowledge is already over 150 years old since Dr. Rudolf Ludwing Karl Virchow described the phenomena that he called "embolism" and "thrombosis"; since then the understanding of stroke and as well as of the origin of ischaemia has changed (Safavi-Abbasi, Reis et al. 2006).

Despite the long period of time, the issue of ischaemia and restoration of blood flow after ischaemia, i.e. reperfusion, are still addressed within important research fields. Especially during the last three decades, vascular surgeons and scientists have been deeply involved, both in clinical work and in basic research, with the phenomenon of ischaemia/reperfusion (I/R) injury (IRI). They are intensively seeking the knowledge and explanation of IRI and its harmful distal-organ consequences. Every vascular surgeon understands that an episode of clamping during different vascular reconstructive operations is unavoidable, which induces ischaemia and reperfusion event. Such clamping situations are inevitable during ruptured or elective abdominal aortic repair and during carotid artery endarterectomy.

Further, high-grade (severe) oxidative stress (OxS) due to ischaemia, followed by reperfusion, cause significant skeletal or brain damage and dysfunction (Karapanayiotides, Meuli et al. 2005), (Pattwell, McArdle et al. 2001). It is widely accepted that although OxS play a major role in the pathological cascade of I/R injury (Lee, Zipfel et al. 1999), (Lindsay, Walker et al. 1988), the phenomenon of OxS does not yet cover all aspects of IRI. Regardless of this knowledge, recent scientific literature contains innovative information about OxS and scientific results about the possibilities of protection against IRI, associated with the "glutathione world" (Westman, Johansson et al. 2006), (Anderson, Nilsson et al. 2004). Furthermore, some glutathione precursors like N-acetylcysteine have actually shown a tendency of protection against IRI of the heart, liver and intestine (Koramaz, Pulathan et al. 2006), (Akgun, Caliskan et al. 2005), (Caglikulekci, Dirlik et al. 2006), (Weinbroum 2006). Use of glutathione analogues is also believed to be a promising tool against IRI and high-grade OxS (Anderson, Nilsson et al. 2004), (Yamoto M 1993).

One part of the current PhD thesis deals with the *in vitro* and *in vivo* investigation of a novel glutathione analogue, named UPF1, using a well-known and widely recognised rat model of four-vessel occlusion. Another part addresses the not yet fully elucidated issue of grade of OxS and I/R injury (both in cellular and systemic antioxidant systems) during abdominal aortic aneurysm resection and carotid artery endarterectomy.

We set the hypothesis of the possible occurrence of cellular or systemic high-grade OxS during elective abdominal aortic aneurysm resection and carotid artery endarterectomy, which might induce harmful consequences due to

IRI. We investigated also an original glutathione analogue to diminish the harmful disorders caused by IRI.

We believe that more efforts should be made to study potent protective agents as glutathione analogues and to simultaneously explore more thoroughly the mechanisms of IRI using both *in vitro* and *in vivo* methods. We hope that the current PhD thesis contains some "drops" of scientific knowledge feeding the huge and heterogenic "science sea" and offering some clues in this research field.

REVIEW OF THE LITERATURE

1. Ischaemia/reperfusion injury of the skeletal muscle and elective abdominal aortic aneurysm (AAA) repair

Re-establishment of blood flow to previously ischaemic organ/tissues is known as reperfusion syndrome (which is itself consisting the term of the ischaemia/ reperfusion injury) (Ihnken, Beyersdorf et al. 1995). It should be noted that scientific literature contains many synonyms of revascularisation syndrome, which are mainly classified according to the cause of acute arterial occlusion, e.g. postischaemic syndrome, revascularisation syndrome or Haimovici-Lagrain-Cornier syndrome, declamping syndrome (directly related to clamping and declamping of the aorta), tourniquet syndrome and crush syndrome (caused by compression) (Beyersdorf 1991). This list can be supplemented with the ischaemia/reperfusion injury (IRI) syndrome of the skeletal muscle.

Reperfusion syndrome was first identified in patients in whom blood flow was re-established to ischaemic extremities. Evidently based on literature data, the first description of an ischaemic lower limb was published in 1881 by Volkmann who found that the ischaemic muscle is related to ischaemic contracture following fractures (Von Volkmann 1881). Since then, there have been several observations of complications of the ischaemic muscle or of lower torso ischaemia. For the first time in 1964, Patman, Poulos and Shires used the decompression technique, fasciotomy, in the treatment of ischaemic muscle swelling (oedema) (Patman, Poulos et al. 1964). Haimovichi first documented the risk of arterial revascularisation of ischaemic extremities in 1960 (Haimovici 1960). After restoration of blood flow (by embolectomy or/and endarterectomy) in an ischaemic limb there may occur, depending of the degree of ischaemia, reperfusion syndrome / revascularisation syndrome first named "myonephropathic-metabolic syndrome" by Haimovici. He described renal failure after the revascularisation of an ischaemic muscle. In 1969 Blaidsell et al reviewed the results of revascularisation in patients with ischaemic extremities and reported 85% mortality in patients who had paralysis and bluish mottling of the lower limb prior to re-establishment of blood flow. Furthermore, they found that the reasons for mortality were cardiopulmonary but not mainly renal failure as was reported by Haimovici (Stallone, Blaisdell et al. 1969).

The skeletal muscle of the limb is the tissue most vulnerable to ischaemia. Because the muscle represents the bulk of the tissue mass in the extremity, damage to the muscle is the most critical aspect of limb reperfusion (re-vascularisation syndrome). In general, the skeletal muscle appears to tolerate ischaemia for up to 4–6 h (Blaisdell 2002).

The liability to develop the ischaemia/reperfusion syndrome of ischaemic limbs increases mainly with (i) the degree of ischaemia (i.e. complete/ in-complete), (ii) duration of ischaemia, (iii) volume of the reperfused tissue.

The degree of ischaemia is the most important single determinant of development of the IRI syndrome of the skeletal muscle. Complete ischaemia is associated with significantly higher incidence of IRI syndrome compared with incomplete ischaemia. Furthermore, acute occlusion of the artery (caused by embolus or by clamping during reconstruction vascular operations), without collateral circulation, carries higher risk for IRI than clinical situations with acute thrombosis where incomplete ischaemia prevails (Dale 1984).

Duration of ischaemia is among the causes of development of the IRI of the skeletal muscle. The latter may occur already within 1 hour of acute complete occlusion, whereas several hours of incomplete ischaemia may not produce local or systemic alterations after reperfusion. According to literature data and our experience, the abdominal aortic clamping time is approximately 1 hour, which may lead to IRI (Thompson, Nasim et al. 1996).

The volume of the reperfused tissue is of major importance for development of IRI leading to systemic complications and death. The highest mortality rates (40–63%) are seen after occlusion of the aorta causing simultaneously ischaemia in both lower limbs (Dale 1984), (Littooy and Baker 1986).

It should be emphasised that reperfusion after an ischaemic period in the lower limbs always results in some local and systemic alterations. Already as early as the 80's it was noted that the metabolic activity of the skeletal muscle of patients undergoing periods of aortic cross-clamping during aorto-iliac surgery did not return to normal levels for up to several hours after release of the clamp (Eklof, Neglen et al. 1981), (Neglen, Carlsson et al. 1980), (Eklof, Neglen et al. 1981), (Neglen, Jabs et al. 1989), (Luo, Hammarqvist et al. 1998).

2. Elective abdominal aortic aneurysm repair and ischaemia/reperfusion injury

Even today AAA repair is a substantial challenge for vascular surgeons, especially ruptured AAA, which result in significant morbidity and mortality. However, the positive (good) outcome of AAA resection depends on many factors including IRI (Murphy, Kolvenbach et al. 1992). During AAA repair, abdominal aortic cross clamp application induces lower limb ischaemia/ reperfusion event. This situation is unavoidable during aortic surgery. The main problem seems to be the duration of the ischaemic event, which may last long enough, with the following reperfusion period (after declamping of the aorta), which causes IRI affecting tissue/organs (Ar'Rajab, Dawidson et al. 1996; Lindsay, Luo et al. 1999).

During aortic cross clamping, lack of oxygen leads to depletion of the energy reserve due to lack of ATP production via aerobic metabolism. Deficiency of the metabolic energy requirements in the skeletal muscle tissue causes leakage of the membrane, failure in Na-pump function, and cellular excess of sodium and calcium, resulting in cell oedema.

At the same time, excessive production of reactive species (including reactive oxygen species, ROS) occurs, accompanied with elevated degradation of ATP as well as other purines, forming more hypoxanthine. Normally, hypoxanthine is oxidized into xanthine by xanthine dehydrogenase, but in ischaemic conditions, at elevated cytosolic calcium, xanthine dehydrogenase is converted into xanthine oxidase (Schaffer, Roy et al. 1983; Russell, Roth et al. 1989; Pang, Forrest et al. 1993). Unlike xanthine dehydrogenase, which uses NAD as a substrate, xanthine oxidase uses oxygen. Therefore, restoration of oxygen supplementation during reperfusion leads to excessive production of ROS by the xanthine/xanthine oxidase system, as well as by mitochondrial electron transport chain leakage and by activated neutrophils (Collard and Gelman 2001). ROS as superoxide, hydroxyl radical, hydrogen peroxide and hypochloric acid attack the biomembrane of polyunsaturated fatty acids, resulting in rapidly elevated lipid peroxidation. Lipid peroxidation of cellular membranes leads to a number of harmful alterations, ranging from decreased fluidity to loss of selective permeability and signal transduction. Because of the high reactivity and short half-life of ROS, their direct quantification is technically very complicated. The only technique capable of direct analysis of ROS *in vivo* is electron spin resonance (ESR) spectroscopy, which is used in conjunction with appropriate ROS spin trapping (i.e. secondary radical stabilising) chemical agent. A number of investigations have described the application of ESR spectroscopy for analysis of ROS generated in various animal model systems, e.g. canine gracilis muscle model for studying ischaemia/reperfusion phenomena (associated with oxygen free radicals) in the skeletal muscle (Choudhury, Sakaguchi et al. 1991). This situation allows the spin trapping agent to be introduced via injection or infusion prior to initiation of ROS formation, thereby enabling immediate and quantitative complexation of ROS generated with the spin trapping agent for subsequent analysis. Use of ESR spectroscopy in analysis of ROS in patients is limited since it is not possible to introduce spin traps into the human body because of ethical considerations (Brown, Dugdill et al. 1998). The ESR procedure cannot be applied in routine clinical work and, besides, it is also very costly.

Furthermore, IRI generates high-grade OxS mediated by reactive species and leading to several levels of tissue and organ damage. The human body compromises an antioxidant network, including enzymatic (e.g. superoxide dismutase and catalase) and nonenzymatic antioxidants (e.g. vitamin E and C and crucial cellular antioxidant glutathione), to withstand high grade damage caused by OxS. To get a real picture of OxS associated with IRI, it is necessary to assess both generalized (systemic) or cellular OxS using an appropriate set of a number of biochemical indices as total antioxidative capacity (TAC), glutathione content and its redox ratio, indices of lipid peroxidation (diene conjugates, DC thiobarbituric acid reactive substances, TBARS) and activity/ amount of antioxidative enzymes (CAT, GSHPx, SOD).

3. Carotid artery endarterectomy as a "clinical model" of focal ischaemia/reperfusion injury

Since 1954, when carotid artery endarterectomy (removal of atherosclerotic plaque) was first performed in St Mary's Hospital in London by Pickering and Rob (Eastcott, Pickering et al. 1954), this operation has been used to prevent brain stroke in patients with symptomatic or asymptomatic stenosis of the carotid arteries. Nowadays carotid artery endarterectomy (CAE) is a widely used and accepted method with appropriate guidelines and indications with reasonable benefits (owing to enormous work involving several large randomized clinical trials in the last two decades). Unfortunately, CAE itself poses a risk of perioperative stroke and hyperperfusion syndrome (which is believed and associated partly with transient focal ischaemia/reperfusion event during surgery) (Rubio, Martinez-Yelamos et al. 2005), (Karapanayiotides, Meuli et al. 2005). In recent years huge efforts have been made in refining both surgical and interventional techniques to reduce periprocedural stroke risk. Actually, the incidence of stroke during CAE has been reduced by use of perioperative aspirin and intraoperative heparin, encephalogram, and transcranial Doppler monitoring and shunting when it is necessary to prevent hemispheric ischaemia (Calligaro and Dougherty 2005). Despite these efforts in the recent decade, brain IRI as a phenomenon has been intensively studied to explore protection strategies against the IRI of the brain more thoroughly.

Carotid artery endarterectomy presents a discrete episode of focal cerebral ischaemia followed by reperfusion. In the scientific community it is believed that CAE is a relevant clinical model for studying transient focal IRI. Carotid artery endarterectomy as a model of brain IRI has both merits and drawbacks. The major disadvantage, in experimental terms, is lack of severity of infarction, since the short period of ischaemia, presence of collateral circulation, and use of shunts during surgery all combine to reduce the extent of injury, and clinically significant neuronal injury is rare (1991). However, major advantages of the model include its clinical substrate, controllability, and ability to estimate the severity of ischaemic insult with multimodality monitoring based on, e.g. transcranial Doppler, retrograde stump pressure, jugular venous saturation, and electrophysiological detection of the consequences of cerebral ischaemia (Kirkpatrick, Smielewski et al. 1995). Furthermore, recently it has been possible to monitor consciousness of the patient during CAE performed under local anaesthesia.

It is well known that the brain is at high risk from reactive species mediated injury due to its intensive aerobic metabolism, high content of PUFAs, quite large iron stores and relatively limited potency of antioxidant defences (Lee, Zipfel et al. 1999). Consequently, OxS has been implicated in cerebral IRI (Lipton 1999) and the latter has been shown to be prevented or ameliorated by an adequate and sufficient antioxidant administration in animal models

(Sakamoto, Ohnishi et al. 1991; Yu, McCowan et al. 1992). The human body comprises an antioxidant network including enzymatic (e.g. superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic antioxidants (e.g. vitamin E, vitamin C and glutathione), to combat disorders caused by high-grade OxS. Under physiological conditions, this antioxidant system enables to control OxS, however, when these protective mechanisms are overwhelmed, injury of the surrounding tissues may occur (Starkopf, Tamme et al. 1997).

At present, despite previous studies of the products of lipid peroxidation (e.g. malondialdehyde, diene conjugates), information about the grade of OxS and as well as about the changes occurring on the systemic and cellular levels of OxS during carotid artery endarterectomy (CAE) is still limited. The expression of OxS (generalised/systemic or cellular) and the grade of OxS can be evaluated using an assay of appropriate biochemical indices.

4. Global brain ischaemia

Cerebral ischaemia-caused diseases and health conditions are a leading problem in Western countries. The latter can be exactly related to stroke, global brain ischaemia caused by cardiac arrest and temporary or permanent neurological disorders. During the last decades enormous efforts have been made to understand and discover the pathogenic cascade and mechanisms of cerebral ischaemia. Moreover, several agents and pro-drugs have been studied with the purpose to protect brain ischaemic event and reperfusion. Despite many clinical trials with different agents like NOS inhibitor, Na^+ channel blockers, NMDA receptor antagonists, Ca^{2+} channel antagonist, etc. up to now no efficient agent has been discovered (Keynes and Garthwaite 2004), (Horn and Limburg 2001), (Moro, Cardenas et al. 2004), (Muir and Lees 2003).

4.1. Incidence of brain ischaemia

Stroke is the third leading cause of death and of serious long-term disability in a majority of industrialised countries, with an incidence of ~350 per 100,000 population aged 45–89 years (Warlow 1998). More than 80% of strokes are the consequence of permanent or prolonged occlusion, caused either by the thrombosis or embolism of one of the main or secondary cerebral arteries (Mohr, Caplan et al. 1978). In addition, cardiopulmonary resuscitation in patients of cardiac arrest, both within and outside hospital, succeeds in restoring of spontaneous circulation in about 70,000 patients per year in the United States. At least 60% of these patients subsequently die in hospital as a result of extensive brain damage, and only 3–10% of resuscitated patients are finally able to resume their former lifestyles (Krause, Kumar et al. 1986). Thus, brain injury

by transient complete global brain ischaemia (cardiac arrest) and regional incomplete ischaemia (ischaemic stroke) afflicts a very large number of patients with death or permanent disability.

4.2. Cerebral injury during aortic arch surgery

There are two basic mechanisms that lead to ischaemic cerebral injury during aortic arch surgery. First, global ischaemia due to interrupted flow leads to temporary neurological dysfunction. This clinical situation is commonly believed to be self-limited and benign, but it may have subtle permanent sequealae, which have been noted recently using detailed neuropsychometric testing that has shown alterations in the memory function. Second, macroembolic injury leads to strokes, which are reported by most investigators as the only neurological consequences of these operations (Griepp and Griepp 1992), (Juvonen, Anttila et al. 2000).

4.3. Requirements and vulnerability of the brain

Compared with the other organs, the brain appears to be especially endangered with regard to generation and detoxification of ROS (reactive oxygen species), which are generated continuously during oxidative metabolism. The cells of the human brain utilize 20% of the oxygen consumed by the body, the brain's energy requirement is more than seven times higher than that of the rest of the body, although this organ comprises only 2% of body mass (Vespa 2006). Different regions of the brain utilize energy at different rates, and therefore some regions are more vulnerable to ischaemic injury than others. The earliest signs of histological ischaemic injury are seen in the hippocampus (Petito, Feldmann et al. 1987). The main source of neuronal energy is generation of ATP by aerobic metabolism of glucose. In the absence of glucose and glycogen stores, the high rate of oxygen utilization requires a steady blood supply to provide the substrate as well as oxygen at a constant rate. High oxygen requirement of the brain indicates generation of a large quantity of ROS during oxidative phosporylation. Further, high iron content has been reported for some brain areas (Gerlach, Ben-Shachar et al. 1994), (Haacke, Cheng et al. 2005), which is able to catalyze generation of ROS. In addition, the brain might be especially vulnerable to ROS because it is rich in lipids with polyunsaturated acids (PUFA), the targets of lipid peroxidation. Moreover, the brain has only low to moderate superoxide dismutase, catalase and glutathione peroxidase activity compared with the kidney or the liver (Cooper and Kristal 1997).

4.4. Brief description of cerebral ischaemia

Cerebral ischaemia results in a time-depend cascade of molecular events, including (i) rapid depletion of the intracellular ATP stores, (ii) anaerobic glycolysis, lactic acidosis, and membrane depolarization, (iii) glutamate excitotoxicity, (iv) entry of calcium, sodium, and water into cells, resulting in cell swelling, (v) activation of calcium-stimulated enzymes, mitochondrial dysfunction, reactive species overproducation, (vi) activation of the immune system, (vii) overexpression of particular genes, and (viii) increased neuronal death (Lerouet, Beray-Berthat et al. 2002), (Kato and Kogure 1999; Lee, Grabb et al. 2000). Furthermore, delay in recirculation during the reperfusion period increase the damage of the ischaemia-injured arterial wall; developing cerebral inflammatory response and excessive reactive species injury lead to damage of the brain tissue (Aronowski, Strong et al. 1997), (Scott and Gray 2000).

4.5. Cerebral ischaemia/reperfusion injury and glutathione system

Ischaemia/reperfusion injury is an important problem in clinical practice and it is associated with several different disorders like myocardial infarction, acute lower limb ischaemia, renal and hepatic transplantation, and ischaemic event, i.e. a clamping episode during reconstructive surgery of the arteries and cerebral infarction. It is essential to have excellent knowledge of IRI to understand, interpret and solve complicated clinical situations (related to IRI) as well to provide high quality medical management and treatment.

Evidence has been accumulated over the last two decades supporting the idea that reactive oxidative species (ROS) as the hydroxyl radical, superoxide anion and hydrogen peroxide play a principal role in brain IRI (Cao, Carney et al. 1988), (Chan 1996), (Muralikrishna Adibhatla and Hatcher 2006). Simultaneously, it has been reported that glutathione (GSH), a crucial cellular antioxidant, depletion occurs both during focal brain ischaemia and after transient global brain ischaemia (Anderson, Nilsson et al. 2004), (Candelario-Jalil, Mhadu et al. 2001). Candelario-Jalil et al reported the results of a time course study of OxS in different regions during and after transient global brain ischaemia in gerbils. They found that after 5 min of bilateral carotid artery occlusion in gerbils, hippocampal GSH content significantly decreased in the early reperfusion phase and maximum reduction of hippocampal GSH was detected 48 and 72h after ischaemic event. Moreover, in the corpus striatum, GSH levels remained constant at all time points tested. Nor were there changes in GSH or GSSG during the ischaemic phase in the cortex, striatum and hippocampus. Further, GSHPx activity decreased significantly in the hippocampus 48h and 72h after reperfusion. Yet no statistically significant differences in GSH-related enzyme activities were observed at any time point in the other brain regions (Candelario-Jalil, Mhadu et al. 2001). Additionally, Ahlemeyer et

al reported that GSH depletion by BSO (buthionine sulfoximine), an inhibitor of glutathione synthesis, under conditions of serum deprivation (which itself increased mitochondrial ROS production (Atabay, Cagnoli et al. 1996), induced the increase in apoptotic neurons in cultured neurons (Ahlemeyer and Krieglstein 2000). These findings suggest that high GSH content is especially needed for survival of neurons under damaging conditions such as ischaemia/ reperfusion injury.

5. Glutathione, its biofunctions and analogues

Glutathione is a water-soluble tripeptide composed of the amino acids glutamine, cysteine and glycine (L-γ-Glu-L-Cys-Gly, MW 307, reduced glutathione, GSH). Reduced glutathione is a major low-molecular thiol compound in animals and plants, present in the millimolar range in mammalian cells (Meister and Anderson 1983); (Anderson 1997). In humans the highest levels of GSH are found in the RBCs, liver, pancreas, kidneys, spleen, eyes, lungs and intestinal cells. Molecules containing cysteine residue (the sulfhydryl group) readily participate in thiol-disulfide exchange. GSH is oxidized to glutathione disulfide (GSSG). The latter is maintained at less than 1% of total glutathione (Brigelius, Muckel et al. 1983); (Dickinson and Forman 2002) *via* rapid reduction back to GSH by a concerted action of NADPH and flavoenzyme glutathione reductase (GR). Hence, GSH and GSSG are the most typical representatives of the "glutathione world" in mammalian cells, with GSH being the principal player.

The isopeptide nature of the γ-glutamyl linkage renders GSH resistant to most peptidases. The electronic structure of the sulfur atom underlies the high reactivity of the thiol group to nucleophilic addition, redox reactions (e.g. *via* radical mechanism) and metal chelation. Due to these two special structural features, GSH is able to fulfil an impressive number and variety of biofunctions:

- GSH as the major cellular non-enzymatic antioxidant eliminates reactive species like hydroxyl radical, peroxynitrite, peroxides and N_2O_3 and plays a principal role in cellular defence against high-grade oxidative and nitrosative stress, mainly *via* co-operation with GSHPx, whereas rapid reduction of GSSG back to GSH by NADPH and glutathione reductase (GR) is needed (Anderson, Underwood et al. 1989; Meister 1994; Lucente, Luisi et al. 1998; Griffith and Mulcahy 1999);
- GSH is involved in restoration of the thiol groups of proteins (maintenance of haemoglobin, other proteins and enzymes in active forms) and coenzyme A. It is required for stabilization of cell membranes and for synthesis of proteins, nucleic acids, leukotrienes and prostaglandins (Anderson 1997; Valencia, Hardy et al. 2001; Pastore, Federici et al. 2003).

- GSH is used for detoxification of several xenobiotics by GSTs, involved in the transport of nitric oxide, and in the modulation (glutathionylation) of several key-enzymes and proteins (GAPDH, phosphorylase, creatinine kinase, Ras) (Anderson 1997), (Karelson, Mahlapuu et al. 2002; Townsend, Tew et al. 2003);
- GSH is the cell redox regulator and the glutathione redox ratio (GSSG/GSH) as a redox buffer modulates the action of numerous redox-sensitive proteins (including a number of transcription factors) (Schafer and Buettner 2001), (Filomeni, Rotilio et al. 2002), (Huang and Huang 2002).

GSH plays a crucial role in OxS process; it provides a first line defence against ROS as a scavenger of free radicals and reducer of H_2O_2. The second line of defence against OxS, related to GSH, is associated with the glutathione-dependent enzymes — GSHPx and GR. GSHPx catalyses the conversion of H_2O_2, produced by SOD, with the dismutation of the superoxide anion (O_2^-), to H_2O_2. The GR contains flavin adenine dinucleotide and uses reducing equivalents from NADPH to convert GSSG back to 2GSH (Pastore, Federici et al. 2003).

An impressive spectrum of the biofunctionality of GSH and depletion of cellular GSH during infection, intestinal lesions, major trauma, hypovolemic shock, and sepsis and ischaemia/reperfusion injury explains why pharmaco-clinical strategies have begun to use the glutathione system as the target. These strategies that maintain or replenish the depleted stores of GSH may minimize tissue injury. There are several ways to maintain or restore cellular GSH levels in the body, for example, parenterally, intraarterially, however despite high concentrations of GSH used, the results in clinical practice have remained modest. Thus, administration of GSH to guarantee its necessarily very high (mM) physiological cellular concentration has substantial limitations like rapid extracellular degradation in the gut and circulation, poor penetration of the blood-brain barrier, limited direct uptake into all cell types or its absence. The bioavailability of cysteine is a principal limitation for the de novo synthesis of cellular GSH and markedly elevated administration of cysteine cannot restore/maintain GSH levels. Cysteine is autooxidized being toxic for cultured cells and newborn mice (Anderson 1998). Many studies have been published showing beneficial effect of cysteine precursor like N-acetyl-L-cysteine (NAC) against ischaemic brain damage in gerbils and in rats (Cuzzocrea, Mazzon et al. 2000). (Sekhon, Sekhon et al. 2003). Further, NAC-enriched cold-blood cardioplegia diminished myocardial injury during the coronary artery bypass surgery (Koramaz, Pulathan et al. 2006).

The esters of GSH are readily taken up by cells and are de-esterified in cells to provide GSH. These esters have a protective effect against cerebral brain ischaemia in rats (Anderson, Nilsson et al. 2004), (Yamoto M 1993).

However, it seems that one perspective approache might be designing and studying analogues of GSH, which are promising candidates for increasing

GSH levels to improve or maintain cellular defences related to GSH system. It would be of significant pharmaco-clinical interest to design analogues of GSH with higher hydrophobicity (to improve penetration through biomembranes), which are also able to mimic or even exceed the functionality (including the antioxidative potency) of GSH itself and which cannot be used by glutathione S-transferase. Such GSH-like new compounds would expand the possibility to support some parts of the glutathione system and may have an impact, as an adjuvant therapeutic factor, for instance, in the case of high-grade OxS when the production of the potent pro-oxidant GSSG is powerful.

On the basis of mentioned above understandings a novel glutathione analogue, named UPF1, 4-MeO-Tyr-γ-Glu-Cys-Gly; MW 483.5, (assignee Vulpes Ltd., no. 110035500, PCT/SE01/01351) is a water soluble acidic peptide has been designed at Department of Biochemistry of Tartu University. To improve the antioxidativity of GSH, we added additional nonproteinogenic amino acid 4-methoxy-phenylalanine to the GSH trimer N-terminus, increasing in this way the hydrophobicity of GSH as well as introducing the methoxy group. Consequently, one part of the current thesis characterises this novel glutathione analogue and explores its effect on global brain ischaemia in rats.

6. Animal models of brain ischaemia

For studies of brain ischaemia many different animal models have been used during the last two decades. These models can be predominately divided into models of global and focal brain ischaemia. Models of global brain ischaemia can be associated with cardiac arrest or with aortic arch reconstructive surgery (which may lead to selective neuronal necrosis). At the same time, models of focal brain ischaemia are considered to be of greater relevance in acute ischaemic stroke due to brain infarction (Macrae, Robinson et al. 1993). Focal brain ischaemia can be roughly categorized into two types: permanent and reversible. Permanent focal ischaemia results in the appearance of a region of ischaemic damage (the core), while degenerative changes are seen to spread from this region. The aim is therefore to attempt to reduce the spread of damage by protecting the penumbral area at risk. It is questionable whether total and permanent cessation of blood flow in a brain region because of thrombus disintegration and endogenous thrombolysis occurs clinically in a majority of strokes. Therefore, reversible occlusion models have also been developed and, in this situation, damage will be the result of both ischaemia and the consequences of reperfusion (Mohr 1993).

6.1. Models of focal brain ischaemia

The techniques of focal brain ischaemia are available for both permanent and reversible occlusion. The model of middle cerebral artery occlusion is applicable in several species. Variation in technique allows some selection regarding which cerebral areas are affected. This model has been used in hypertensive rat strains. Occlusion of the artery can be induced by techniques including cauterization, clips and threads (filaments), endothelin 1 administration, or photochemically by induced thrombolytic occlusion (Karpiak, Tagliavia et al. 1989) (Ginsberg and Busto 1989) (Macrae, Robinson et al. 1993),(Kuge, Minematsu et al. 1995),(Longa, Weinstein et al. 1989).

6.2. Models of global brain ischaemia

There are basically three different models of global brain ischaemia: gerbil bilateral carotid occlusion, two-vessel rat occlusion with simultaneously induced hypotension and, finally, four-vessel rat occlusion. It should be emphasized that the literature offers several modifications of the above mentioned models. Therefore, different scientific centres and communities have obtained similar or mostly similar results using, for instance, the rat model of four-vessel occlusion, while animal experiments have been performed with minor or major modifications. These modifications of animal models produce variation of results and mixing up of many scientific conclusions that are unfortunately not comparable to each other.

The model of gerbil bilateral carotid occlusion has met wide popularity because of the relative ease of the surgical techniques involved. Also this model is highly suitable for screening purposes. By the way, the gerbil (*Meriones unguiculatus*) does not have connecting arteries between the arterial system of the internal carotid arteries and the vertebral arteries. The rat model of global brain ischaemia includes both two- and four-vessel occlusion models, the former requiring induction of hypotension and the latter involving, according to the original method described by Pulsinelli, a two-stage procedure (Pulsinelli and Brierley 1979; Pulsinelli, Brierley et al. 1982). In the current thesis a model of global brain ischaemia was developed and adapted to a one-stage procedure. Yet it should be emphasized that all global brain ischaemia methods, as the name implies, result in ischaemia over a large proportion of the brain (commonly the forebrain) and involve a variable period of transient ischaemia (Pulsinelli, Levy et al. 1983).

Unfortunately, all of the above described models of brain ischaemia have drawbacks. For instance, the gerbil bilateral carotid artery occlusion model has variable outcome due to variations in cerebral circulation. Also, it is difficult to perform physiological measurements in these small animals; and the rat two-vessel occlusion model plus the rat hypotension model requires inducement of

hypotension, post-ischaemic seizures are quite often observed, etc. (Ginsberg and Busto 1989). The main drawback, high mortality rate in spontaneously breathing animals, can be noted and observed when implementing the rat model of four-vessel occlusion model. The mortality rates published in the original description of the model of global cerebral ischaemia were 10% and 40% after 20 min and 30 min of bilateral carotid occlusion, respectively (Pulsinelli, Brierley et al. 1982). The corresponding rates were up to 75% higher in other laboratories where a similar model was used (Rosenbaum, Grotta et al. 1990), (Vered, Bar-Joseph et al. 1994). In a study by Herguido et al., mortality rate for 20 min of global brain ischaemia was 34% in the perioperative period and 50% in the postoperative period (Herguido, Carceller et al. 1999). Moreover, it has to be emphasized that in our animal experiments (10 min of global brain ischaemia) general mortality rate was 38% (the lowest in the UPF1 reperfusion group and the highest in the vehicle group, 11% and 45%, respectively). Further, to achieve correctly interpretable data, using the rat four-vessel occlusion model, it is most important to adhere to exclusion criteria. Rats that remain unresponsive throughout the ischaemic period, or rats whose body temperature drop below 36.0C° and rises above 37.5C°, or rats that develop epileptic seizures, or rats that require assistant pulmonary ventilation perioperatively are excluded from the study group. Consequently, when mortality rate is high and the model requires several a priori postulated exclusion criteria, the survived animals and the included animals belong to a particularly ischaemia-resistant subpopulation. Therefore much effort is needed to reduce animal mortality to as minimum level as possible and this not only for ethical and practical reasons but also in order to eliminate this source of bias.

Moreover, during animal experiments of brain ischaemia it is mandatory to keep the body temperature of the animal in the physiological range and to maitain this condition during the postoperative period. Thus, the deleterious effect of hyperthermia on the outcome of stroke in both rodents and humans has long been recognised (Thornhill and Corbett 2001). In contrast, the beneficial effect of hypothermia is well established in animal models, but there is still limited evidence of its effectiveness in clinical trial settings (Schwab, Georgiadis et al. 2001).

Furthermore, on the basis of our experience and according to literature data, to achieve well reproducible animal experiments of brain ischaemia, it is obligatory to observe very carefully clinical conditions of the animal (breathing pattern, temperature, blood pressure, blood gases). Moreover, in critical life threatening situations during animal experiments one must quickly undertake necessary actions (treatment possibilities in correct order) to ensure survival of animals after brain ischaemia.

Finally, we would like to emphasize that elaboration of an animal model like a rat model of four-vessel occlusion is a very time consuming and difficult task. Unfortunately, it is almost impossible to merely take a scientific description of a complicated animal experiment from some publication and to develop, during a

short period of time, a well reproducible animal model. Therefore, according to the experience obtained from this work we believe that it is mandatory to perform well organized multistage pilot studies. Moreover, we suggest that for high quality animal experiments it is obligatory to have dedicated and well-oriented team members and who have possibilities of consulting specialists in the same scientific area from other research centers.

AIMS OF THE STUDY

The general purpose of this study was to explore ischaemia/reperfusion injury associated with oxidative stress during different vascular reconstructive operations and to examine the novel glutathione analogue UPF1 *in vitro*, in primary cell cultures and in a rat model of global brain ischaemia.

The goal of the current study was divided into specific aims as follows:

1. Elective abdominal aortic aneurysm repair — **to study the time course of oxidative stress – both the manifestation (systemic, cellular) and nature (low or high grade) of oxidative stress**

2. Endarterectomy of the carotid artery — **to study** the **time course of oxidative stress — both the manifestation (systemic, cellular) and nature (low or high grade) of oxidative stress**

3. **Using in vitro and in vivo methods, to clarify the effects of a potent antioxidant, the glutathione analogue UPF1 — its antioxidative nature and possible neurotoxicity — and to establish its possible protective character against IRI using a rat model of global brain ischaemia.**

MATERIAL AND METHODS

7. Time-course measurement of oxidative stress during vascular reconstructions

7.1. Presurgical and surgical procedures

7.1.1. Elective abdominal aortic aneurysm repair

Presurgical procedures were performed as follows: one hour before surgery, morphine and dehydrobenzperidol (Droperidol) were given intramuscularly as premedication. Arterial and Swan-Ganz pulmonary arterial catheters were inserted in all patients. Induction of anesthesia was performed with atropine, etomidate and phentanyl. Endotracheal intubation was carried out with succinylcholine. Muscle relaxation was achieved with pipecuronium and analgesia was performed with continuous infusion using fentanyl 3–4 μg/kg/h. The lungs were ventilated with isoflurane up to 1.5 vol. % with oxygen and N_20. During AAA surgery, the central hemodynamic parameters were checked and measured with a Swan-Ganz catheter at three time points: before surgical manipulation, after aortic cross clamping and at the end of the operation. Before reperfusion, mannitol (0.5 g/kg) was administered regularly. We performed elective AAAR as follows: a collagen impregnated woven prosthesis was inserted; the proximal anastomosis was done end-to-end to the abdominal aorta below the renal arteries and the distal anastomoses were done end-to-side to the common femoral arteries or end-to-end to the common iliac arteries. Each patient received intravenously 3000 units of heparin before the cross clamping of the abdominal aorta.

7.1.2. Carotid artery endarterectomy

After premedication with midazolam, anaesthesia was induced with atropine, etomidate and fentanyl. Endothracheal intubation was carried out with succinylcholine. Muscle relaxation was achieved with pipercuronium and analgesia was performed with continuous infusion using fentanyl 3–4 μg/kg/h. The lungs were ventilated with isoflurane up to 1.5 vol. % with oxygen and N_2O. All patients were mechanically ventilated to maintain normocapnia with $PaCO_2$ of 38 to 41 mmHg. In all patients an arterial catheter was inserted, ECG, end-tidal capnometry, and changes in arterial blood pressure were continuously recorded. A neurological examination was performed immediately after the patient awakened, 1 hour later, and then daily until the patient was discharged. During carotid surgery, we did not use transcranial Doppler ultrasound, or the monitoring of somatosensory evoked potentials, or electroencephalography. Each

patient received 3000 units of heparin before cross clamping. During endarterectomy blood pressure was raised and maintained pharmacologically (at least at 10 mmHg of mean blood pressure) with phenylephrine above individual blood pressure baseline values in each patient (Calligaro and Dougherty 2005).

7.2. Blood sampling and time points during vascular reconstructions

7.2.1. Elective abdominal aortic aneurysm repair

Samples were obtained from the peripheral arterial line (radial artery) at four time points during elective AAAR: (i) before induction of anaesthesia; (ii) 5 min after infrarenal abdominal aortic cross-clamping; (iii) 5 min after the removal of the infrarenal abdominal aortic cross-clamp and (iv) 30 min after the removal of the infrarenal abdominal aortic cross-clamp.

7.2.2. Carotid artery endarterectomy

Blood samples were collected regularly from the peripheral arterial line (the radial artery) at four time points: (i) before anaesthesia, (ii) 5 min after carotid artery clamping; (iii) 5 min and (iv) 30 min after declamping during CAE.

Blood was taken into 7 ml sterile lavender vacutainer tubes and promptly transported to the laboratory for analysis. The serum was separated by centrifugation (4 x 600g by discarding the plasma and the top layer cells) and kept in plastic tubes at -20°C until analysis.

7.3. Assessment of oxidative stress indices

All measurements of oxidative stress markers were performed in triplicate within 4 h of blood sampling. The means were calculated and used for statistical analysis. The haemolysed samples were excluded from analysis. All reagents were purchased from Sigma (St. Louis, Missouri, USA).

7.3.1. Lipid peroxidation

Lipid peroxidation (LP) products were detected in serum to avoid the possible influence of the substances required for plasma preparation (Annuk, Zilmer et al. 2001). LP samples were treated with antioxidant butylated hydroxytoluene (BHT) twice, immediately after obtaining and before adding the test reagents to suppress artefact changes during handling and assay procedures (Annuk, Zilmer et al. 2001).

At present, both serum and plasma were used as routine test samples to check LP in the body, to investigate free radical injury, or to establish the diagnosis of LP-mediated diseases. In the process of LP (i.e., free radical conversion of PUFAs residues), the first stage, consisting of the molecular rearrangement of double bonds in PUFAs residues of lipids, leads to formation of DC. Conversion of DC yielded secondary stable products (aldehydes, alkenals) of LP. The latter were measured as thiobarbituric acid reactive products (TBARS).

Serum levels of DC were measured according to the methods described previously (Regnstrom, Strom et al. 1993), with some minor modifications. Briefly, the samples (150 µl) + (150 µl) of 0.9% NaCl (reagent blank contains only isotonic saline) were incubated at 37°C for 25 min, 0.25% BHT (150 µl) was added and the lipids were extracted by heptane/isopropanol (1:1). Then the samples were acidified by 5 mol/l hydrochloric acid and extracted by cold heptane (1600 µl). After centrifugation (for 5 min at 3000 rpm) and absorbance of the heptane fraction, they were measured spectrophotometrically at an absorbance maximum between 220 and 250 nm. To correct the results for hemodilutation, protein content in the samples was determined according to (Lowry OH 1951).

Serum levels of TBARS were measured according to the method of Ohkawa et al. (Ohkawa, Ohishi et al. 1979) with a few minor modifications (Annuk, Zilmer et al. 2001). Briefly, the samples were incubated with 475 µM Fe^{2+} at 37°C for 30 min. After incubation, BHT (0.25%) was added to the samples (250 µl), which were then treated with an acetate buffer (pH 3.5) and heated with the presents of the TBA solution (1% 80°C, 70 min). Then the samples were cooled and acidified (5 mol/l hydrochloric acid). After extraction with cold butanol, the samples were centrifuged and the absorbance of butanol was measured at 534 nm.

7.3.2. Total antioxidative capacity

It is suggested that the blood antioxidative system is able to control the level of reactive oxygen species. Among the serum antioxidants, one particular component can not fully reflect the protective potency of whole blood. Hence, when assessing serum TAC, GSH, GSSH, SOD, GSHPx and CAT, complex measurements of serum antioxidant potency were performed.

Serum TAC was measured through the ability of the serum samples to inhibit linolenic acid (LA) peroxidation. In preliminary experiments we established that this procedure shows good accordance with the total antioxidant activity method (Annuk, Fellstrom et al. 2001). Briefly, standard LA in isotonic saline (400 µl), sodium dodecyl sulphate (15 µl), and serum (30 µl 1:3:3 in isotonic saline) were incubated in the presence of 200 µmol/iron at 37°C for 60 min. BHT (0.25%) was added, then the samples were treated with an acetate

buffer (pH 3.5), heated with the presents of the TBA solution (1%, 80°C, 35 min) and assessed for TBARS. The results were expressed as the percentage of inhibition of LA peroxidation, induced by the serum samples.

SOD and GSHPx were measured according to special protocols using commercially available kits (Randox Laboratories Ltd, Ardmore, UK) (1998). CAT was measured using TBARS according to a method described in the literature (Goth 1991). Total glutathione (TGSH) and oxidized glutathione (GSSG) were measured by the enzymatic method of Tietze (Tietze 1969), which was slightly modified for the present study (Annuk, Fellstrom et al. 2001). The content of GSH was calculated as the difference between the total amounts of glutathione and GSSG (TGH-GSSG).

8. Glutathione and UPF1 as protective molecules

First, we tested the possible antioxidativity of UPF1 to verify the hydroxyl radical scavenging effects, and second, we compared the antioxidativity of UPF1 to that of GSH. Briefly, we determined the antioxidativity of UPF1 as described by Barreto et al. (Barreto, Smith et al. 1995), using terephthalic acid (THA) as the chemical dosimeter for hydroxyl radicals. The THA dosimeter solution contained 10 mM THA in a 14.75 mM sodium phosphate buffer at pH 7.5 which was used as the control. Hydroxyl radicals were generated via the Fenton reaction by adding $CuSO_4$ and H_2O_2 to the dosimeter solution in order to achieve the final concentration of 10 μM and 1 mM, respectively. The effect of suppression of hydroxyl radicals was measured by a spectrofluorometer (Perkin Elmer LS5) at 312 nm excitation and at 426 nm emission (Pahkla, Zilmer et al. 1998). The IC_{50} values were determined by sigmoid dose-response (viable slope) analysis using the GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA).

9. Primary cell cultures — cerebellar granular cell culture and GSH and UPF1

Primary cultures of cerebellar granule cells were prepared according to the method described by Gallo et al, with minor modifications (Gallo, Ciotti et al. 1982). Briefly, the cerebelli were dissected from 8-day-old Wistar rats and the cells were dissociated by mild trypsinization followed by trituration in a 0.004% DNAse solution containing 0.05% soybean trypsin inhibitor. The cells were resuspended in Basal Medium Eagle with Earle's Salts containing 10% heat-inactivated foetal bovine serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamycin. The cell suspension was seeded into 40-mm, dishes pre-coated with 5 μg/ml of poly-L-lysine with a density of $1.3–1.4 \times 10^6$ cells/ml. The cell

cultures were grown for 8 days in a humidified 5 % CO2/95% O_2 atmosphere at 37°C. After 24 hours of incubation 10 μM cytosine-β-arabino-furanoside was added to the culture medium to prevent proliferation of nonneuronal cells. Seven- or 8-day-old cultures were used in the current experiment.

We treated each well with 2 μl of either GSH (as the negative control) or UPF1, both mixed with 50 μl of the medium in final concentrations 0.01, 0.1, 10 and 100 μM. After 24 hours of incubation, cell viability was assessed by a trypan blue assay. The cultures were washed with phosphate buffered saline and were then incubated with a 0.4% Trypan blue solution at room temperature for 10 min. Only dead neurons were stained with Trypan blue (Tymianski, Charlton et al. 1993). The number of Trypan blue positive cells and viable non-stained cells were counted. Approximately 1000 cells per three fields in each well were counted to determine the viability of the cell cultures. For each concentration, three wells were used.

10. Rat model of global brain ischaemia

Animal experiments were performed using a rat model of four-vessel occlusion. The Ethics Committee of the University of Tartu approved the experimental protocol. Male Wistar rats weighing 270-350g were used. The four-vessel occlusion model of forebrain ischaemia, described by Pulsinelli and Brierly, was employed with some modification (Pulsinelli and Brierley 1979; Pulsinelli and Buchan 1988).

Briefly, the rats were fasted overnight; anaesthesia was induced with 3% halothane in oxygen (at a rate of 0.9 l/min), followed by orotracheal intubation, and the animals were respirated during the surgical procedure with a respirator (TSE Animal Respirator "Advanced") (Jou, Tsai et al. 2000). A rectal temperature probe was inserted and body temperature was monitored and maintained at 37.0°C using a heating pad. First, the rats were mounted on a head holder and tilted downward by approximately 30°. The vertebral arteries were coagulated through both *alar foramina*'s of the first cervical vertebra. The bilateral common carotid arteries were exposed via a ventral midline incision and transient global forebrain ischaemia was induced for 10 minutes by clamping both common carotid arteries with microvascular clamps.

During ischaemia the animals were monitored for body temperature, respiration pattern, loss of righting reflex, unresponsiveness, corneal reflexes, as well as for fixed and dilated pupils. Restoration of blood flow in the carotid arteries was confirmed by careful visualisation. In the sham-operated group of rats, both vertebral arteries were cauterised and both common carotid arteries were exposed but not clamped.

The rats which remained unresponsive throughout the ischaemic period, those whose temperature dropped below 36.0°C, those which developed epileptic seizures and those which required persistent assisted pulmonary ventilation postoperatively were excluded from the study.

10.1. Experimental protocol of global brain ischaemia

The animals were assigned randomly into one of the following groups: sham group; vehicle group of four-vessel-occlusion (4VO) rats, which were given isotonic saline (up to 0.5 ml) into the external jugular vein; 4VO rats, which were given UPF1 into the external jugular vein 20 minutes before inducing global brain ischaemia (UPF1 preischaemic); 4VO rats, which received UPF1 immediately before reperfusion (UPF1 reperfusion).

The UPF1 was injected in a dose of 0.9 mg/kg dissolved in a saline solution up to 0.5 ml into the jugular vein as a bolus dose during 30 seconds. This dose was selected proceeding from both the calculation derived from the measurements of the scavenging potency of UPF1 and from the analysis of some previously published reports (Marin, Cornet et al. 2000), (Chabrier, Auguet et al. 1999).

11. Histological evaluation of the pyramidal cells of the dorsal hippocampus

Seventy-two hours following ischaemia, the animals were killed by an intraperitoneal injection of fentanyl-fluanisone and midazolam for perfusion fixation of the brain with phosphate-buffered paraformaldehyde (4%) at pH 7.4 after rinsing with 0.9% saline by cannulating the ascending aorta transcardially.

The representative coronal sections (7 μm) of the dorsal hippocampus of the paraffin-embedded brains were sliced at 200 μm intervals and were stained with cresyl-violet. The number of surviving neurons in the CA1 area of the dorsal hippocampus was counted to quantify the extent of ischaemic neuronal injury. To minimize bias in counting, all slices were assigned randomly and counted separately by three technicians blinded to the experimental protocol. To standardize the counting procedures, a virtual frame lying at the middle portion of the dorsal hippocampus was used. The size of the virtual frame was 0.4 × 1 mm and its major axis proceeded along the pyramidal cell layer as in Bae et al. (Bae, Lee et al. 2000) (Figure 1). All viable neurons within six frames in the left and right CA1 regions of the three coronal sections of the dorsal hippocampus for each animal were counted under the criteria proposed by Kirino (Fortuna, Pestalozza et al. 1997) at 832-fold magnification using an image analysis software package (AnalySIS 3.0; Soft Imaging System, Munster, Germany). Both sides of the dorsal hippocampus were counted separately and the average number of viable pyramidal cells in each coronal section of the brain was selected as the representative value.

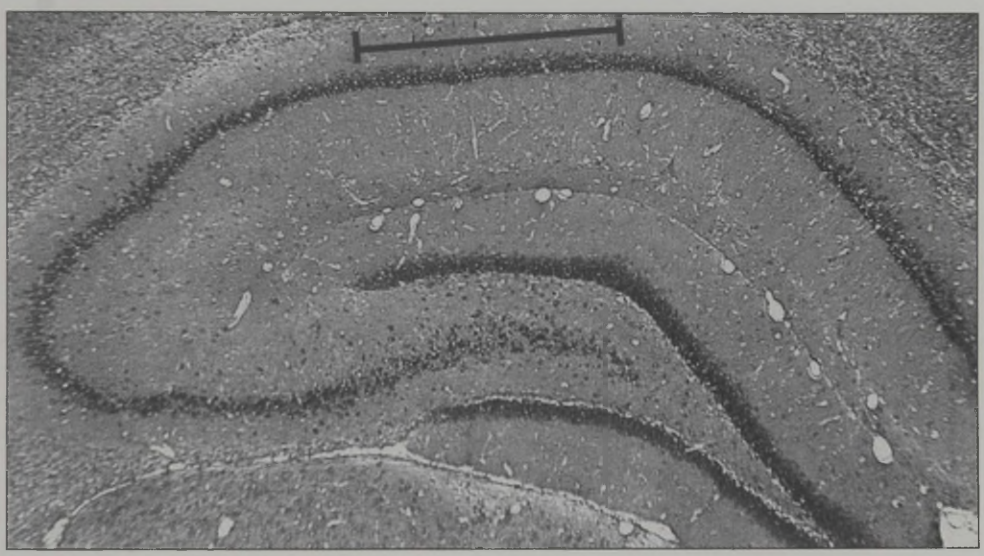

Figure 1. Low-power (x4.5) light micrograph of a 7 μm coronal section stained by cresyl violet, showing the localisation of the counting frame at the middle part of the region of CA1. The size of the virtual frame was 0.4 × 1 mm and its major axis was along the pyramidal cell layer (scale bar = 1000 μm).

12. Statistical analysis

The data were recorded and used for statistical analysis with the GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA). For repeated measurements over the time course of elective carotid artery endarterectomy, the non-parametric Friedman test was used for comparison of the preoperative value with the values for the other time points, correcting multiple comparison with the Dunn test. The results are presented as the mean ± standard error of the mean (SEM) in the text and in the tables. The limit of a statistically significant P value was set at 0.05.

All experimental data are shown as mean ± SEM. A normal distribution of the number of viable neurons was established using the Shapiro-Wilk test ($p > 0.05$). The mean number of viable pyramidal cells in the UPF1 treated groups was compared with the number of viable pyramidal cells in the vehicle-treated group by the one-way ANOVA test with the Dunnett correction using the statistical program GraphPad Prism version 3.0. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

13. Oxidative stress during elective aortic aneurysm repair

This study examined the time-course of the indices of systemic and cellular OxS in 18 patients undergoing elective AAAR (Table 1). There were no complications associated with surgery, or death cases during the study period. Average perioperative blood loss was 2033 ml and blood transfusion was 965 ml. A cell-saver was used during two abdominal aortic aneurysm resections. However, two patients died during the postoperative course, one from recurrent myocardial infarction and the other from multiple organ failure resulting from aspirated bilateral bronchopneumonia.

Table 1. Main clinical characteristics of the study group

Female/male	3/15
Mean age, y	67.0, range 53–77
Abdominal aortic cross-clamping time (min)	61.3±3.9
Mean transversal diameter of abdominal aortic aneurysm (cm) [1]	7.8, range 5.8–9.7
Operating time (min)	229.6±11.1
Smokers	5 of 18 (28%)
Preoperatively existing diseases	
PAD	10 of 18 (56 %)
Hypertension	11 of 18 (61%)
Symptomatic myocardial ischemia	9 of 18 (50%)
Chronic respiratory disease	1 of 18 (6%)
Diabetes	0 of 18 (0%)

PAD, peripheral arterial diseases

[1] The diameter of AAA was determined preoperatively by Doppler ultrasound measurement

Arterial CAT revealed a significant elevation (96.0 ± 11.7) 30 min after aortic clamp removal in comparison with the preclamping value (56.9 ± 7.3) and 5 min after the application of the aortic crossclamp (63.7 ± 4.6). Simultaneously, changes were observed in GSHPx 30 min after the removal of the abdominal aortic clamp. The GSHPx was significantly elevated (51, 5%) and TAC was decreased (31.4%) in comparison with the corresponding value at the preclamping time point. The markers of lipid peroxidation, DC and TBARS, as well as the glutathione redox status and the antioxidant enzyme, SOD, did not show any significant changes during AAA surgery (Table 2).

Table 2. Comparative dynamics of the systemic and cellular of OS indices during elective AAAR (n = 12–18).

Time points during AAAR	A	B	C	D
TBARS, nmol/ml	1.4±0.1	1.3±0.1	1.5±0.1	1.4±0.1
DC, μmol/ml	24.1±1.3	23.7±1.5	23.0±1.4	21.1±1.2
TAC, %	36.5±1.4*	35.2±1.3	33.5±1.1	31.4±1.2
CAT, U/l	56.9±7.3*	63.7±4.6**	67.9±5.8	96.0±11.7
SOD, U/gHb	719.2±35.1	648.4±41.4	670.9±34.3	748.3±46.8
$GSHP_x$, U/gHb	39.9±3.3*	41.2±3.5	46.2±5.9	51.5±3.9
GSSG/GSH	0.3±0.03	0.3±0.03	0.4±0.07	0.3±0.04

*$p < 0.05$ for differences between the values of time points A and C
** $p < 0.05$ for differences between the values of time points B and C
A. before induction of anesthesia
B. 5 min after infrarenal abdominal aortic cross-clamping
C. 5 min after removal of infrarenal abdominal aortic cross-clamp
D. 30 min after removal of infrarenal abdominal aortic cross-clamp

14. Oxidative stress during carotid artery endarterectomy

The time-course of the indices of systemic and cellular OxS was studied in 24 patients undergoing elective CAE diagnosed symptomatic carotid artery stenosis. There were no fatal complications associated with surgery. However, two patients had postoperative permanent neurological deficit. One patient became hemiparetic and the other patient had apalic syndrome. Both patients had had cerebral infarction preoperatively, which had been identified with CT, and these patients had minor neurological deficit before surgery. The indications for CAE are shown in Table 3. No vascular shunt was used during CAE. Seventeen patients of 24 took aspirin (Lannacher, Austria) as antiplatelet medication, but none of the patients took antioxidants.

Arterial TBARS revealed a statistically significant increase (1.7±0.1) 5 min after carotid clamp removal in comparison with the preclamping value (1.5±0.1). At the same time, an elevation in CAT and GSHPx (89±9 and 38±3, respectively) was observed 5 min after declamping in comparison with the corresponding value at the preclamping time point. The other markers as DC, SOD and TAC did not show any statistically significant changes during CAE. Simultaneously, the glutathione redox ratio did not reveal any changes during CAE, either (Table 4).

Table 3. Main clinical characteristics of the study group

Female/male	5/19
Mean age, y	68.0, range 44–84
Clamping time of the carotid artery (min)	18±1.1
Standard endarterectomy versus eversion endarterectomy	13 vs 11
Indication for carotid endarterectomy	
Symptomatic carotid artery stenosis (trombembologenic plaque)[1] 60–70%	6 of 24 (25%)
Symptomatic carotid artery stenosis (≥ 70%)	18 of 24 (75%)
Diseases existing preoperatively	
Cerebral microinfarcts	2 of 24 (8%)
PAD	13 of 24 (54 %)
Hypertension	10 of 24 (42 %)
CHD	3 of 24 (13 %)
Diabetes	1 of 24 (4 %)

PAD, peripheral arterial disease
CHD, coronary heart disease
[1] Detected by Doppler ultrasound measurement

Table 4. Comparative dynamics of the indices of systemic and cellular of OxS during CAE (n = 17–24)

Time points during CAE	A	B	C	D
TBARS, nmol/ml	1.5±0.1	1.6±0.1	1.7±0.1*	1.6±0.1
DC, μmol/ml	29.7±2.1	30.6±2.1	28.5±1.9	27.9±1.9
TAC, %	36±1	35±1	35±1	35±1
CAT, U/l	65±6	84±7	89±9*	85±13
SOD, U/gHb	674±31	686±39	649±43	655±44
$GSHP_x$, U/gHb	36±3	37±3	38±3*	37±3
GSSG/GSH	0.9±0.17	1.0±0.19	0.9±0.19	1.0±0.23

*$p < 0.05$ for the differences between the values of time points A and C
A. before induction of anesthesia
B. 5 min after carotid artery clamping
C. 5 min after removal of the carotid artery clamp
D. 30 min after removal of the carotid artery clamp

15. Glutathione and UPF1 — their antioxidative effect

We found that UPF1 possesses an evident hydroxyl radical scavenging nature *in vitro*. It means that linking of hydrophobic moiety with an attached methoxy group to the N-terminus of GSH increased dramatically the antioxidative properties of peptide.

The IC_{50} of UPF1 (20.5±2.3 μM) was about 60 times lower compared with the corresponding value of GSH (IC_{50}=1254.1±382.2μM) (Figure 2).

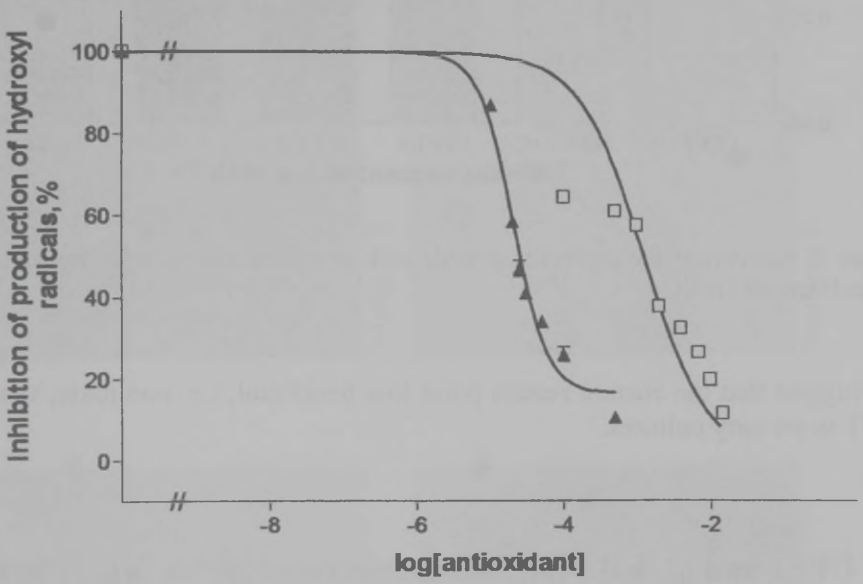

Figure 2. Comparison of antioxidative potency of UPF1 (filled triangles) and GSH (empty squares).

16. Common characteristics of glutathione and UPF1 in primary cell cultures

The viability of the cerebellar granule cells treated with UPF1 (ranging from 90% to 87%) was similar to that of the non-treated and GSH-treated controls (ranging from 92% to 90%) at each concentration used (even at a concentration of 100 μM the survival data were similar to the corresponding data for the control group) (Figure 3).

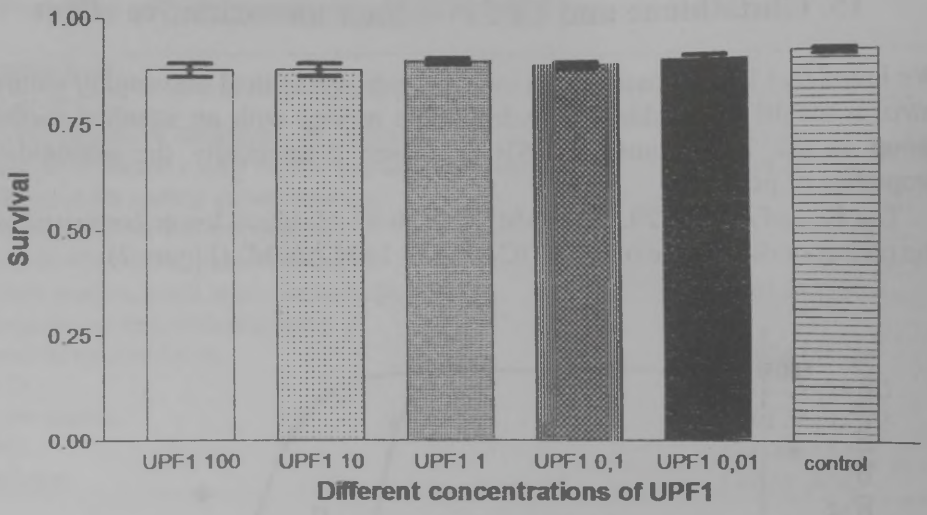

Figure 3. Survival of the cerebellar granule cells at different concentrations of UPF1. Control: utreated cells.

We suggest that the current results point to a beneficial, i.e. non-toxic, trend of UPF1 in primary cultures.

17. UPF1 and global brain ischaemia/reperfusion injury in rats

Sixty-three rats were used in the animal experiments; 24 animals died and 16 rats, which remained unresponsive throughout the ischaemic period, those whose temperature dropped below 36.0 °C, those which developed epileptic seizures and those which required persistent assisted pulmonary ventilation postoperatively were excluded from the study. Five rats in the sham group, 8 rats in the vehicle group, 5 rats in the UPF1 preischaemic group, and 5 rats in the UPF1 reperfusion group completed the study.

The representative sections, obtained 72 hours after ischaemia and stained with cresyl violet, are shown in Figure 4. The number of intact neurons was 152±2.0 in the sham group, 66±7.2 in the vehicle group, 126±6.0 in the UPF1 preischaemic group, and 102±6.4 in the UPF1 reperfusion group (Figure 5).

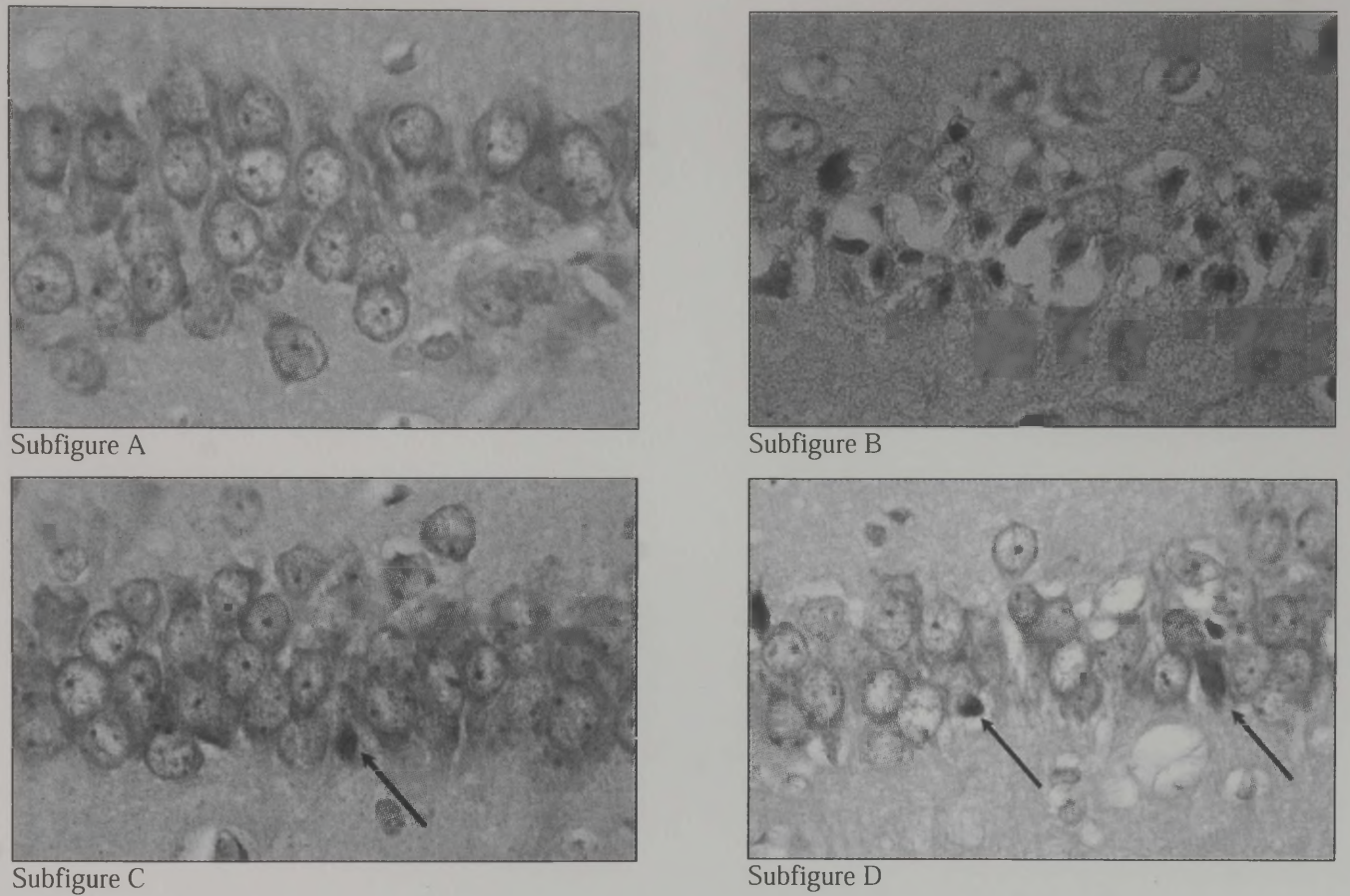

Figure 4. Photomicrographs of the hippocampal CA1 region of rats, obtained from (A) the sham-operated, (B) the saline-treated, (C) the UPF1-treated 20 min before ischemia, and (D) the UPF1-treated immediately before reperfusion at x 1579 magnification (scale bar = 20 µm). Note no cell necrosis in (A), almost no intact pyramidal neurons in (B), and a few necroses (indicated by arrows) in a large number of intact pyramidal neurons in (C) and (D).

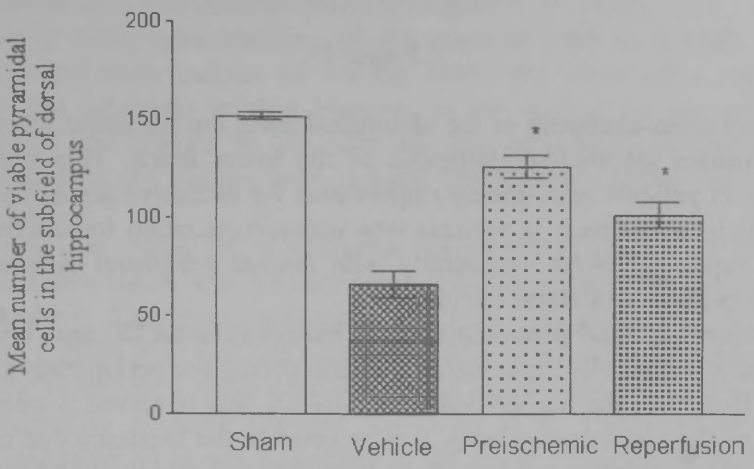

Figure 5. The mean number of viable cells over the 1000 μm length of the CA1 layer. Vehicle — saline-treated rats; preischemic — UPF1-treated 20 min before ischemia; reperfusion — UPF1-treated immediately before reperfusion. Bars show S.E.M.
* $P < 0.05$ in comparison with the vehicle group.

DISCUSSION

Paper I

Temporary cross-clamping of the abdominal aorta during aortic reconstructive surgery causes incomplete ischaemia of the lower limbs. This ischaemia is moderate in patients who undergo operations for occlusive aorto-iliac disease. Ischaemia is more severe in patients who undergo operation for elective AAAR or even ruptured AAAR (especially with normal peripheral circulation, e.g. without developed collateral circulation).

We found that lipid peroxidation (LP) indices (plasma DC and TBARS) did not reveal a statistically significant alteration during the perioperative period of AAAR. However, the highest value of TBARS was observed 5 min after the removal of the aortic cross-clamp. It is reported in the literature that changes in blood lipid peroxidation products have been detected during open-heart surgery involving cardiopulmonary bypass (Starkopf, Zilmer et al. 1995) and in a canine model of skeletal muscle ischaemia/reperfusion injury (Lindsay, Walker et al. 1988). However, findings similar to those of our study have been described when measuring the specific marker of lipid peroxidation, F_2-isoprostane, which remained unchanged in the perioperative course of elective AAA repair (Lindsay, Luo et al. 1999). Another study reported that the measured LP marker, lipid hydroperoxide (harvested from the radial arterial line), showed a significant sharp increase immediately on reperfusion in patients undergoing elective thoracoabdominal aortic aneurysm repair but not in patients undergoing elective AAA repair (Hafez, Berwanger et al. 2000).

Another marker of systemic OxS, serum TAC, reflects the potency of total antioxidative protection in plasma. We demonstrated that TAC decreased significantly 30 min after the ischaemia of the lower limbs. In another study, Khaira et al observed that total antioxidant activity decreased significantly during the ischaemia of the lower torso and immediately after declamping of the abdominal aorta in comparison with the preclamping levels in AAA resection and grafting (Khaira, Maxwell et al. 1996). It has to be emphasised that the marked decrease in TAC during the clamping of the abdominal aorta is an unexpected result before the generation of ROS during the reperfusion phase. It is probably caused by partial ischaemia resulting from the collateral supply of the lower torso and gastrointestinal organs during aortic clamping. Furthermore, Murphy et al have shown that antioxidant consumption occurs during aortic clamping whereas LP (the result of ROS damage) is more marked during reperfusion (Murphy, Kolvenbach et al. 1992). Another study performed by Hafez et al showed a decrease in the levels of TAC immediately after reperfusion lasting up to 1 hour in patients treated for thoracoabdominal aortic aneurysm and abdominal aortic aneurysm (Hafez, Berwanger et al. 2000). Moreover, controversial results were published by Brown et al who found no changes in the

levels of TAC during AAAR; however, the samples were obtained from the femoral vein of only four patients (Brown, Dugdill et al. 1998).

To have a better understanding of the grade of OxS in AAAR, we also investigated the main indices of cellular OxS. We observed a statistically significant but relatively modest increase in the activity of the antioxidant enzymes CAT and GSHPx 30 min after the removal of the aortic cross-clamp. At the same time, we did not note changes in the antioxidative enzyme SOD during AAAR.

Moreover, the principal well-known intracellular marker of OxS, the glutathione redox status, was not significantly changed during the intraoperative period of AAAR. This fact speaks against cellular high-grade OxS. It has been reported by Pedrini et al that the muscular GSH/GSSG ratio decreased significantly in the reperfusion phase after 3 hours of acute abdominal aortic balloon-catheter occlusion in sheep (Pedrini, Guarnieri et al. 1993). These findings could be explained by severe ischaemia of the lower limbs, while it should be noted that a decrease was observed in the glutathione redox ratio in the skeletal muscle. Furthermore, Pedrini and the co-workers described a marked elevation of glutathione measured from the venous blood of an ischaemic site in patients suffering from acute lower limb ischaemia. Moreover, they observed a rapid increase of glutathione five minutes after the revascularisation of an acute ischaemic limb (Pedrini, Guarnieri et al. 1993), (Pedrini 1999). It is worth noting that release of GSH from the ischaemic tissue should cause elevation of the levels of systemic GSH. Additionally, Sirsjo et al have reported depletion of skeletal muscle glutathione after severe hind limb ischaemia for 4 hours in rats during the reperfusion phase (Sirsjo, Kagedal et al. 1996).

Further, in a recent publication Westman et al have noted that the muscle level of reduced glutathione after AAAR was decreased, but there occurred no changes in the glutathione redox status. These results indicate that OxS elicited by elective AAAR is outbalanced by compensated GSH metabolism, not allowing increase oxidized GSH (Westman, Johansson et al. 2006), (Westman, Johansson et al. 2006).

It should be added that administration of heparin as a widely known agent with possible antioxidativity helps withstand ischaemia/reperfusion injury during this type of operations (Li, Cooley et al. 1995). Furthermore, regular administration of mannitol during elective AAA repair should be considered. Being a hyperosmolar sugar and a hydroxyl radical scavenger, mannitol is not a sufficiently strong antioxidant to act significantly in the human body (Oredsson, Plate et al. 1994). However, neither heparin nor mannitol influenced the OxS markers measured by us. Consequently, we eliminated theoretical considerations concerning administration of heparin and mannitol to solve the high grade of OxS or mimicking the moderate grade of OxS in patients during elective AAA resections.

Finally, we analysed and compared different subgroups (patients without collateral peripheral circulation/ patients with peripheral circulation) in our study group and found no statistically significant changes or alterations in the

OxS markers during elective AAAR (data not shown). It should be noted that the patients' number was not sufficient for valid comparison.

Paper III

Carotid endarterectomy for removing atherosclerotic plaque restores blood flow and reduces the incidence of ispilateral infarction (symptomatic and asymptomatic carotid stenosis being ≥70%) (Rothwell and Warlow 1999), (Biller, Feinberg et al. 1998). At the same time, CAE itself possesses the risk for cerebral ischaemia and reperfusion injury.

We found changes in different markers of OxS, assessed from the peripheral arterial line, only during reperfusion (5 min after carotid clamp removal) in comparison with the preoperative values but not during ischaemia or later (at time point 30 min) during reperfusion.

A marker of lipid peroxidation, TBARS, showed the highest value 5 min after the beginning of reperfusion. At the same time, another more specific marker of lipid peroxidation, DC, did not reveal statistically significant changes during CAE. Soong et al. have described an elevation of the malondialdehyde and diene conjugates obtained from the jugular vein after the removal of the Benner shunt and closure of arteriotomy with an average duration of 83 seconds (i.e. cross clamping time of the carotid artery) (Soong, Young et al. 1996). However, in comparing our data with those of the above study, it should be emphasized that we collected blood samples from the peripheral arterial line, while Soong et al obtained samples from the jugular vein. Further, a significant increase in malondialdehyde occurred during the reperfusion phase in patients who needed a temporary shunt during CAE (Weigand, Laipple et al. 1999).

A marker of systemic OxS, serum TAC, reflects the potency of total antioxidative protection of the plasma. According to the findings of our study, there occurred no changes in the level of TAC during CAE. Interesting findings were obtained by Weigand et al. who described a significant decrease in TAC during carotid artery clamping (and before shunt insertion) in patients with inadequate collateral blood flow (detected by somatosensory-evoked potentials as the extent of brain ischaemia during CAE). Furthermore, the above authors detected a higher baseline of TAC (measured from jugular venous-arterial blood) in patients who needed a temporary shunt during CAE (Weigand, Michel et al. 1999). Additionally, another study has reported a decrease in TAC after declamping of the carotid artery. We believe that the episode of ischaemia that occurred during CAE was not sufficiently severe or long to cause exhaustion of the potency of such an important system as total antioxidative capacity. Our findings did not show any significant changes in TAC during CAE probably because of the site of blood sampling i.e. radial arterial line. Further, we understand that the antioxidative systems currently and mainly in the lungs may influence the results obtained by us.

In order to better understand the grade of OxS during CAE, we also investigated the main markers of intracellular OxS. We detected a statistically significant but relatively modest increase in the activity of antioxidative enzymes, CAT and GSHPx, 5 min after the removal of the carotid artery clamp. However, there occurred no changes in the activity of the antioxidative enzyme SOD during CAE. Next, we established some elevated OxS in the peroxide level controlling system (e.g. CAT, GSHPx), which is interesting as the function of GSHPx is associated with the glutathione system. Moreover, we detected no changes in a well-known intracellular marker for OxS, the glutathione redox ratio, during this time-course study, while we observed a high level of the glutathione redox ratio at the first time point (preoperative value). In our previous studies we established the glutathione redox ratio from age-matched healthy volunteers and calculated the reference value that ranged from 0.13 to 0.3. We observed a significant difference between the reference value and the preoperative value of the glutathione redox ratio, which indicates a high level of intracellular OxS in these patients. It seems that any correction of the glutathione system using different manipulations (e.g. involving glutamine, N-Acetyl-L-cystein, etc) should be considered for these patients. Furthermore, we emphasise an interesting fact which might be correlated with the high values of the glutathione redox ratio for our study group. We established concomitant peripheral arterial disease in thirteen patients of 24. Recently, Lapenna et al. demonstrated that long-term depletion of GSH is associated with oxidative atherogenic effects (Lapenna, Pierdomenico et al. 2004). It is evident that further investigations should be performed to clarify the association of GSH depletion with PAD.

There are some limitations concerning this study. First, it is problematic to obtain fully reliable results using a relatively small number of patients as was the case with the present study. Despite this, we believe that the study might offer some interesting information about the time-course of OxS during CAE. Secondly, we did not perform transcranial Doppler or measured somatosensory-evoked potentials to detect the potential extent of brain ischaemia during CAE. Additionally, it should be noted that we measured the retrograde stump pressure of ICA (in cases where we suspected insufficient collaterals in the brain). And finally as in our study blood samples were only obtained from the peripheral artery, we emphasise that the changes that occurred in the OxS markers can not be interpreted organ-specifically.

Paper II

Powerful antioxidants as, e.g. carvelidol and its metabolite SB 211475, compromise a methoxy group connected to the benzyl ring. To study the effect of these moieties, we added a non-coded amino acid residue, 4-methoxy-phenylalanine, to

the N-terminus of the GSH trimer to enhance antioxidativity, to increase hydrophobicity and to prolong the half-life of UPF1 in comparison with GSH in the organism. The UPF1 dissolves readily in water and even high concentrations of the peptide can be used in different *in vitro* and *in vivo* studies.

In the present study, UPF1 showed significantly higher antioxidativity compared with GSH, measured by a THA dosimeter. Whether this 60-fold increase in antioxidativity (Figure 2) is caused by the methoxybenzyl group has to be studied further. Moreover, UPF1 did not show neurotoxicity in experiments with the primary cell cultures of cerebellar granule cells in the concentration range 0.01 to 100 μM. However, it might be important to note, that as only three wells were used for each concentration, we suggest the existence of a non-neurotoxicity trend of UPF1 in primary cell cultures.

As the glutathione analogue UPF1 showed high antioxidativity and a trend of non-toxicity in cell cultures, we performed animal experiments using a model of global brain ischaemia (i.e. four-vessel occlusion model). We demonstrated that administration of UPF1 (at a concentration of 0.9 mg/ml with a bolus dose) via the jugular vein, both before ischaemia and immediately before a reperfusion episode of global brain ischaemia, provides definite protection against ischaemia/reperfusion injury. The best treatment result was achieved when UPF1 was used 20 min before global brain ischaemia; however, this protector has also a significant effect when used immediately before reperfusion.

A model of forebrain transient global ischaemia in experimental animals is aimed to mimic human cerebral ischaemia due to transient cardiac arrest, or aortic arch reconstructive operations, or surgery of the extracranial arteries (including either various types of endarterectomies or/and bypass operations). In clinical practice, mainly during previously mentioned operations, it is possible to administer potential protective agents as UPF1 at the preischaemic time point. Additionally, such compounds can be administered during ischaemia, immediately before reperfusion and later during a reperfusion event. As transient cardiac arrest or stroke cannot be predicted, it is not possible to use pre-treatment strategies. Therefore, these cases can only be treated after an ischaemic event, e.g. stroke or transient cardiac arrest. Considering these clinical outcomes, we selected the first time point 20 minutes before ischaemia and then the time point immediately before a reperfusion event.

Moreover, induction of global cerebral ischaemia by a transient occlusion of the common carotid arteries or by a permanent occlusion of the vertebral arteries in rat causes delayed neuronal death in specific areas of the brain. Further, two to four days after transient global ischaemia, cell loss develops in the CA1 region of the hippocampus, evidently due to the damage caused by free oxygen radicals (Kitagawa, Matsumoto et al. 1990), (Kirino 1982).

We propose that UPF1 can be a promising lead compound for designing novel antioxidant drug precursors. However, up to now the exact mechanism of the action of UPF1 is not known. The UPF1 can act as a powerful free radical scavenger, or a modulator of G proteins in frontocortical membrane

preparations (Karelson, Mahlapuu et al. 2002). Furthermore, one of the action mechanisms of UPF1 as a signal molecule can be associated with modulation of or increase in cellular GSH levels. Preliminary experiments have shown that basal GSH level increases about 20% in SH-SY5Y neuroblastoma cells after application of a 10 μM UPF1 solution on the cells (our unpublished data). To study the mechanism of UPF1 more thoroughly, we have designed and synthesised a library of UPF1 analogues (26 different analogues). In the light of our data, we suggest that UPF1 may have a direct protective impact on oxygen free radicals – acting as a scavenger, or acting as a signal molecule modulating the level of GSH itself or the GSH redox ratio.

CONCLUSIONS ACCORDING TO THE PAPERS

Paper I

We demonstrated a decrease in TAC and an increase in the antioxidant enzymes CAT and GSHPx during the reperfusion phase. Neglecting the alterations in TAC, CAT and GSHPx and considering the almost unchangeable character of the other OxS parameters (e.g GSH), we conclude that the OxS observed at the systemic and cellular levels in patients undergoing AAA surgery is not high grade OxS and does not serve as a principal agent for ischaemia/reperfusion injury. Therefore, we suggest that routine administration of exogenous antioxidants is not an argument to be considered for elective AAA repair.

Paper III

We observed an increase in TBARS 5 min after carotid clamp removal; at the same time point the antioxidant enzymes CAT and GSHPx were elevated. Despite these moderate findings, we did not find occurrence of high grade OxS either in systemic or cellular level during CAE. The patients undergoing CAE showed a high baseline of the glutathione redox ratio. The latter phenomenon might support further investigation of the glutathione system with more focus on adjuvant therapy.

Paper II

In conclusion, the evidence presented in our study clearly suggests that UPF1 is a more powerful antioxidant agent compared with GSH *in vitro*, being also a non-neurotoxic and effective potential agent which diminishes neuronal injury in global cerebral ischaemia. Further research is required to increase the time window for using the glutathione analogue UPF1. Main interest should be directed to reperfusion injury protection management, e.g. to post-treatment strategy with the purpose to mimic a clinical situation after transient cardiac arrest or to possible treatment options after stroke.

General conclusions

1. High-grade OxS manifestation was not noted in patients during elective AAAR.
2. High-grade OxS manifestation was not found in patients during CAE.
3. High (i.e. abnormal) baseline of the glutathione redox ratio and simultaneous lack of cellular high-grade OxS in patients undergoing CAE as well as useless of administration of GSH itself lead to the necessity to investigate the effects of glutathione analogues during IRI.
4. The glutathione original analogue UPF1 showed a significant protective character in a rat model of global brain ischaemia.

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SUMMARY IN ESTONIAN

Kliiniline ja eksperimentaalne uurimus: isheemia/reperfusiooni kahjustuse seos oksüdatiivse stressiga kõhuaordi aneurüsmi resektsioon-proteseerimisel ja ekstrakraniaalse ajuarteri endarterektoomial ning isheemia vastase kaitsevõimaluse uurimine glutatiooni analoogi UPF1 abil globaalse ajuisheemia mudelil rotil.

Isheemia ja verevoolu taastamine ehk reperfusioon on aktuaalne teadusuuringute valdkond. Viimase kolme aastakümnendi jooksul on süvenenud vastavateemaliste kliiniliste ja prekliiniliste uurimuste integratsioon. Need uurimused on tihti suunatud isheemia/reperfusiooni kahjustusele ja nende tagajärgedele. Iga veresoontekirurg teab, et arteri klemmimise episood on vältimatu erinevate vaskulaarsete rekonstruktiivste operatsioonide puhul. Arteri klemmimine ja klemmi eemaldamine põhjustab vastavalt isheemia ja reperfusiooni. Selline tegevus on tehniliselt möödapääsmatu ruptureerunud ja elektiivse kõhuaordi aneurüsmi resektsioon-proteseerimisel ja unearteri endarterektoomial. Sügavast isheemiast ja sellele järgnevast reperfusioonist põhjustatud sügav oksüdatiivne stress tekitab olulisi kahjustusi ja sealhulgas düsfunktsiooni vöötlihastes, ajus jt. organites. Käesoleval ajal on laialdaselt aktsepteeritud seisukoht, et sügav oksüdatiivne stress mängib olulist rolli isheemia/reperfusiooni kahjustuse patoloogilises kaskaadis. Uuem teaduskirjandus sisaldab innovaatilist informatsiooni oksüdatiivse stressi ja isheemia/reperfusiooni kahjustuse kaitse võimalustest. Perspektiivikaks peetakse „glutatiooni süsteemi" uuringuid. Mõned glutatiooni prekursorid nagu N-atsetüültsüsteiin omavad kaitset isheemia/ reperfusiooni kahjustuse vastu nii südamelihases, maksas kui ka sooles. Samuti peetakse perspektiivseks glutatiooni analoogide kasutamist isheemia/reperfusiooni kahjustuse ja sügava oksüdatiivse stressi korrigeerimiseks.

Käesolevas töös püstitati järgmised ülesanded:

- Uurida oksüdatiivse stressi loomust (kas rakuline või süsteemne) ja raskusastet (nõrk või sügav) elektiivse kõhuaordi aneurüsmi resektsioon-proteseerimisel
- Uurida oksüdatiivse stressi loomust (kas rakuline või süsteemne) ja raskusastet (nõrk või sügav) unearteri endarterektoomial
- Selgitada glutatiooni analoogi UPF1 antioksüdatiivset loomust ja võimalikku neurotoksilisust ning testida UPF1 kaitsevõimet isheemia/reperfusiooni kahjustuse korral kasutades globaalse ajuisheemia mudelit rotil

Katsealusteks olid isikud, kellel teostati kõhuaordi aneurüsmi resektsioon-proteseerimine ja unearteri endarterektoomia. Neurotoksilisuse katseteks kasutati hiire väikeaju granulaarrakkude primaarkultuure. Globaalse ajuisheemia katsed viidi läbi isastel Wistari liini rottidel.

Põhijäreldused

Käesoleva uurimustöö kokkuvõtteks võib teha järgnevad järeldused:

- Sügav oksüdatiivne stress perioperatiivselt ei kujune patsientidel, kellel teostati kõhuaordi aneurüsmi resektsioon-proteseerimine.
- Sügavat oksüdatiivne stress perioperatiivselt ei kujune patsientidel, kellel teostati unearteri endarterektoomia.
- Mitte-füsioloogiline (väga kõrge) glutatiooni redoks-suhte (GSSG/GSH) basaaltase ja samaaegne sügava rakulise oksüdatiivse stressi puudumine patsientidel, kellel teostati unearteri endarterektoomia ning glutatiooni enda kasutamisvõimaluse limiteeritus viitavad vajadusele uurida glutatiooni analoogide efektiivsust isheemia/reperfusiooni kahjustuse tingimustes.
- Originaalne glutatiooni analoog UPF1 omas olulist kaitseefekti globaalse ajuisheemia mudelil rottidel.

Järeldused vastavalt publikatsioonidele

Artikkel I

Uuringu tulemusena selgus, et keskmiselt 60 minutilise alajäsemete isheemia tingimustes oli tõusnud CAT ja GSHPx väärtuste tase ajamomendil 30 minutit peale kõhuaordi klemmi eemaldamist võrreldes operatsioonieelsete väärtustega. Lisaks oli langenud üldise antioksüdatiivsuse näitaja markeri TAC'i väärtus võrreldes operatsioonieelse väärtusega. Nende tulemuste interpretatsioonil järeldasime, et elektiivse kõhuaordi aneurüsmi operatsiooni korral ei tekki sügavat oksüdatiivset stressi ei rakulisel ega süsteemsel tasandil.

Eelnevast omakorda johtuvalt oli meiepoolne arvamus ja soovitus, et seda tüüpi operatsioonide korral pole tavaliselt manustatud mannitoolile ja hepariinile lisaks vaja kasutada rutiinseid eksogeenseid antioksüdante.

Artikkel III

Uuringu tulemusena selgus, et 5 minutit peale unearterilt klemmi eemaldamist oli tõusnud TBARS'i väärtus võrreldes operatsioonieelse väärtusega. Samal ajamomendil olid ka antioksüdatiivsete ensüümide CAT ja GSHPx väärtused statistiliselt oluliselt kõrgemad võrreldes operatsioonieelsete väärtusega. Samas peab rõhutama fakti, et antud uuringugrupi patsientidel olid GSSG/GSH operatsioonieelsed väärtused tunduvalt kõrgenenud võrreldes GSSG/GSH väärtustega tervetel samaealistel vabatahtlikel. Sügavat süsteemset oksüdatiivset stressi ei tekkinud unearteri endarterektoomial.

Lisaks väidame, et GSSG/GSH kõrgenenud tase võib olla tingitud samaaegselt esinenud alajäsemete kroonilisest isheemiast (ehk alajäseme arterite oblitereeruvast ateroskleroosist), kuid see hüpotees vajab edasisi täpsustavaid uuringuid.

Artikkel II

Uuringu tulemusena selgus, et UPF1 on oluliselt võimsam antioksüdant võrreldes GSH'ga *in vitro*, samuti pole UPF1 rakendavates kontsentratsioonides neurotoksiline. Glutatiooni analoog UPF1 osutus protektiivseks globaalse ajuisheemia kahjustuse korral.

Edasised uuringud peaksid tooma selgust UPF1 ajaliste kasutuspiiride kohta. Erilist tähelepanu peaks pöörama isheemiajärgsetele ravivõimalustele, jäljendades kliinilist situatsiooni südame seiskumise ja vahetus ajuinfarkti järgses olukorras.

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PUBLICATIONS

I

P. Põder, A. Aavik, J. Kals, K. Zilmer, T. Kullisaar, A. Pulges, M. Zilmer
"Is there high grade oxidative stress during elective abdominal aortic aneurysm repair?"
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IS ELECTIVE ABDOMINAL AORTIC ANEURYSM REPAIR ACCOMPANIED BY HIGH GRADE OXIDATIVE STRESS?

P. Põder[1], A. Pulges[1], J. Kals[2], A. Aavik[1], K. Zilmer[2], T. Kullisaar[2], C. Kairane[2], M. Zilmer[2]

[1] Department of Vascular and Thoracic Surgery, University of Tartu, Tartu, Estonia
[2] Institute of Biochemistry, University of Tartu, Tartu, Estonia

ABSTRACT

Background and Aims: During elective abdominal aortic aneurysm repair (AAAR), lower torso ischaemia-reperfusion event is unavoidable. Previous studies on AAAR have reported the importance of oxidative stress (OS) in ischaemia-reperfusion injury, however, the grade of OS has not been adequately clarified up to now. The aim of this study was to perform a complex investigation of the time-course and grade of systemic and cellular OS in patients undergoing AAAR.

Material and Methods: Arterial blood samples were taken from 18 patients undergoing elective AAAR (at four points in time: before anaesthesia, 5 min after aortic clamping and 5 min and 30 min after clamp removal). Diene conjugates (DC), thiobarbituric acid reactive substances (TBARS), total antioxidative capacity (TAC), glutathione redox ratio (GSSG/GSH), and levels of antioxidative enzymes as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) were measured from the radial arterial blood.

Results: 30 min after the removal of the aortic cross-clamp, arterial CAT showed significant elevation (96.0 vs 56.9 U/l, $p < 0.05$); GSHPx was significantly elevated (51.5 vs 39.9 U/g Hgb, $p < 0.05$) and TAC was decreased (31.4 vs 36.5 %, $p < 0.05$) in comparison with preoperative value.

Conclusions: We found limited alterations of several OS parameters, which do not characterize either systemic or cellular high-grade OS during elective AAAR.

Key words: Abdominal aortic aneurysm repair; oxidative stress; ischaemia-reperfusion injury

INTRODUCTION

Abdominal aortic aneurysm (AAA) continues to result in significant morbidity and mortality. Even today elective abdominal aortic aneurysm repair (AAAR) is an important challenge for vascular and general surgeons. However, the positive outcome of AAAR depends on several factors including ischaemia-reperfusion (I/R) damage (1). During elective AAAR, aortic clamp application (average time 1 hr) induces the lower torso I/R event. Simultaneously, prolonged ischaemia, followed by reperfusion, can result in I/R caused tissue/organ injury (2, 3). During aortic cross clampings lack of oxygen leads to a depletion of the energy stores due to malproduction of ATP via aerobic metabolism. Imbalance of the energy requirements in the skeletal muscle tissue causes leakage of the membrane, failure in Na-pump function, and cellular excess of sodium and calcium, resulting in cell edema. At the same time, excessive production of reactive oxygen species (ROS) occurs,

Correspondence
P Põder M D
Department of Vascular and Thoracic Surgery,
University of Tartu
Puusepa 8, Tartu 51014, Estonia
Email: Priit.Poder@kliinikum.ee

accompanied with elevated degradation of ATP, forming hypoxanthine. Normally, hypoxanthine is oxidized to xanthine by xanthine dehydrogenase, but in ischaemic conditions, at elevated cytosolic calcium, xanthine dehydrogenase is converted to xanthine oxidase (4, 5, 6). Unlike xanthine dehydrogenase, which uses NAD as a substrate, xanthine oxidase uses oxygen. Therefore, the restoration of oxygen supplementation during reperfusion leads to excessive production of oxygen free radicals by the xanthine/xanthine oxidase system, as well as by mitochondrial electron transport chain leakage and by activated neutrophils (7). Reactive oxygen species as superoxide, hydroxyl radical, hydrogen peroxide and hypochloric acid attack the biomembrane of polyunsaturated fatty acids resulting in elevated lipid peroxidation.

The human body comprises an antioxidant network, including enzymatic (e.g. superoxide dismutase and catalase) and nonenzymatic antioxidants (e.g. vitamin E and C), to withstand high grade damage caused by OS. Furthermore, OS can be generalized (systemic) or cellular and its grade can be evaluated using different biochemical markers.

Today, despite the existence of previous studies on AAAR, it is evident that there are yet unclear aspects (incomplete relevant data) of the grade of OS during elective AAAR.

The aim of this study was to evaluate the time-course nature of both systemic and cellular levels and grades of OS during elective AAAR by determining superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), the glutathione redox ratio, total antioxidative activity and the lipid peroxidation markers (diene conjugates and thiobarbituric acid reactive substances).

MATERIAL AND METHODS

The study was performed on 18 adults undergoing elective AAAR. The protocol used in this study was approved by the Ethics Committee of the University of Tartu. The main clinical characteristics are given in Table 1.

Presurgical procedures were performed as follows: one hour before surgery, morphine and dehydrobenzperidol (Droperidol) were given intramuscularly for premedication. Arterial and Swan-Ganz pulmonary arterial catheters were inserted in all patients. Induction of anesthesia was performed with atropine, etomidate and phentanyl. Endotracheal intubation was carried out with succinylcholine. Muscle relaxation was achieved with pipecuronium and analgesia was performed with continuous infusion using fentanyl 3-4 μg/kg/h. The lungs were ventilated with isoflurane up to 1.5 vol. % with oxygen and N_20. During AAA surgery, the central hemodynamic parameters were checked and measured with a Swan-Ganz catheter at three time points: before surgical manipulation, after aortic cross clamping and at the end of the operation. At reperfusion, mannitol (0.5 g/kg) was administered regularly. We performed elective AAAR as follows: a collagen impregnated woven prosthesis was inserted; the proximal anastomosis was done end-to-end to the abdominal aorta below the renal arteries and the distal anastomoses were done end-to-side to the common femoral arteries or end-to-end to the common iliac arteries. Each patient received intravenously 3000 units of heparin immediately before the cross clamping of the abdominal aorta.

Samples were obtained from the peripheral arterial line (radial artery) at four time points during elective AAAR (Table 2). Blood was harvested into 7-ml sterile lavender vacutainer tubes and was promptly transported to the laboratory for analysis. The serum was separated by centrifugation and kept in plastic tubes at -4 °C until analysis.

TABLE 1 *Main clinical characteristics of the study group.*

Femae/male	3/15
Mean age, y	67.0, range 53-77
Abdominal aortic cross-clamping time (min)	61.3 ± 3.9
Mean transversal diameter of abdominal aortic aneurysm (cm)[1]	7.8, range 5.8-9.7
Operating time (min)	229.6 ± 11.1
Smokers	5 of 18 (28 %)
Preoperatively existing diseases	
PAD	10 of 18 (56%)
Hypertension	11 of 18 (61 %)
Symptomatic myocardial ischemia	9 of 18 (50 %)
Chronic respiratory disease	1 of 18 (6 %)
Diabetes	Oof 18(0%)

PAD, peripheral arterial diseases

[1] The diameter of AAA was determined preoperatively by Doppler ultrasound measurement

TABLE 2

Comparative dynamics of the systemic and cellular of OS indices during elective AAAR (n - 12-18).

Time points during AAAR	A	B	C	D
TBARS, nmol/ml	1.4 ±0.1	1.3 ±0.1	1.5 ±0.1	1.4±0.1
DC, umol/ml	24.1 ±1.3	23.7 ± 1.5	23.0 ±1.4	21.1 ± 1.2
TAC,%	36.5 + 1.4*	35.2 ±1.3	33.5 ± 1.1	31.4±1.2
CAT,U/l	56.9 ± 7.3*	63.7 ± 4.6**	67.9 ± 5.8	96.0 ±11.7
SOD, U/gHb	7192 ± 35.1	648.4 + 41.4	670.9 ± 34.3	748.3 ± 46.8
GSHP, U/gHb	39.9 + 3.3*	41.2 ±3.5	46.2 ± 5.9	51.5 ±3.9
GSSG/GSH	0.3 ± 0.03	0.3 ± 0.03	0.4 ± 0.07	0.3 ± 0.04

$p < 0.05$ for differences between the values of time points A and D

$p < 0.05$ for differences between the values of time points B and C

A.before induction of anaesthesia

B. 5 min after infrarenal abdominal aortic cross-clamping

C. 5 min after removal of infrarenal abdominal aortic cross-clamp

D. 30 min after removal of infrarenal abdominal aortic cross-clamp

The following markers of systemic and cellular OS were measured: diene conjugates (DC), thiobarbituric acid reactive substances (TBARS), serum total antioxidative capacity (TAC), glutathione redox status (the ratio of oxidized to reduced glutathione or GSSG/GSH), and the level of antioxidative enzymes (superoxide dismutase, SOD, catalase, CAT and glutathione peroxidase, GSHPx). The methods of measurement of oxidative stress markers have been described previously in detail. Briefly, TBARS were measured according to Ohkawa et al (8) with modifications (9). For the study of DC, the serum samples were incubated at 37 °C for 25 min, the reaction was stopped by adding an antioxidant (butylated hydroxytoluene, BHT) and the lipids were extracted. Thereafter the samples were spectrophotometrically analysed at 233 nm (10). To correct the results for hemodilutation, protein content in the samples was determined according to Lowry et al (11). To assess TAC, the ability of the serum to inhibit in vitro linolenic acid peroxidation in the presence of 200 µmol/l Fe^{2+} was evaluated (12). SOD and GSHPx were measured according to special protocols using commercially available kits (Randox Laboratories Ltd, Ardmore, UK) (13). CAT was measured using thiobarbituric acid reactive substances according to the method described in literature (14). Total glutathione (TGSH) and CSSG as the parameters were measured by the enzymatic method of Tietze (15), which we slightly modified for the study (12). The content of GSH was calculated as the difference between the total amount of glutathione and GSSG (TGH-CSSG). All measurements of the lipid peroxidation products and the antioxidant markers were performed in triplicate within 4 h after blood sampling. The means were calculated and used for statistical analysis. The haemolysed samples were excluded from analysis.

The data were analysed with the GraphPad Prism for Windows (version 3.0). For repeated measurments over the time-course of AAAR, the non-parametric Friedman test was used for comparison of the preoperative value with the values for the other time points, correcting multiple comparison with the Dunn test. The results are presented as the mean ± standard error of the mean (SEM) in the text and in the tables. The results were considered statistically significant if the P value was less than 0.05.

RESULTS

This study examined the time-course of the indices of systemic and cellular OS in 18 patients undergoing elective AAAR. There were no complications associated with surgery, or death cases during the study period. Average perioperative blood loss was 2033 ml and blood transfusion was 965 ml. The cell-saver was used during two abdominal aortic aneurysm resections. However/two patients died during the postoperative course, one from recurrent myocardial infarction and the other from multiple organ failure resulting from aspirated bilateral bronchopneumonia.

Arterial CAT revealed a significant elevation (96.0 ± 11.7) 30 min after aortic clamp removal in comparison with the preclamping value (56.9 ± 7.3) and 5 min after the application of the aortic cross-clamp (63.7 ± 4.6). Simultaneously, changes were observed in GSHPx 30 min after the removal of the abdominal aortic clamp. The GSHPx was significantly elevated (51.51 %) and TAC was decreased (31.4 %) in comparison with the corresponding value at the preclamping time point. The markers of lipid peroxidation, DC and TBARS, as well as the glutathione redox status and the antioxidant enzyme, SOD, did not show any significant changes during AAA surgery (Table 2).

DISCUSSION

The following major findings were established in our study. The lipid peroxidation indices (plasma DC and TBARS) did not reveal statistically significant alteration. However, during the perioperative course the highest value of TBARS was observed 5 min after the removal of the aortic cross-clamp. It should be noted that changes in the blood lipid peroxidation products have been detected during open-heart surgery for cardiopulmonary bypass (9) and in a canine model of the skeletal muscle of I/R injury (16). However, findings similar to those of our study have been described when measuring the specific marker, F_2-isoprostane, which remained unchanged in the perioperative course of elective AAA surgery (2).

Another marker of systemic OS, serum TAC, reflects the potency of total antioxidative protection in the plasma. Our observations demonstrated that TAC was significantly decreased 30 rain after the ischemia of the lower limbs. This information indicates the existence of a certain OS-based overload in the plasma during AAAR. In another study, Khaira et al observed that total antioxidant activity decreased significantly during the ischemia of the lower torso and immediately after aortic clamp removal in comparison with the preclamping levels in AAA resection and grafting (17). Controversial results were published by Brown et al who found no changes in the levels of total antioxidant activity during AAAR; however the samples were obtained from femoral vein lines of only four patients (18).

To have a better understanding of the grade of OS in AAAR, we also investigated the main indices of cellular OS. We detected a statistically significant, but relatively modest, increase in the activity of antioxidative enzymes (CAT and GSHPx) 30 min after abdominal aortic declamping. We did not reveal changes in the antioxidative enzyme, SOD, during the operation. Moreover, the principal well-known intracellular marker for OS, glutathione redox status, was not significantly changed during the intraoperative period of AAAR. The latter fact also argues against cellular high-grade OS.

It should be added that administration of heparin as a widely known agent with antioxidative properties helps to withstand ischaemia-reperfusion injury during this type of operations (19). Furthermore, regular administration of mannitol during elective AAAR should be considered. Being a hyperosmolar sugar and a hydroxyl radical scavenger, mannitol is not a sufficiently strong antioxidant to act significantly in the human body (20). However, at the same time, neither heparin nor mannitol influenced the OS markers measured by us. Consequently, we eliminated the theoretical considerations concerning administration of heparin and mannitol - solving the high

grade of OS or mimicking the moderate grade of OS in these patients during elective abdominal aortic aneurysm resections.

CONCLUSIONS

We demonstrated a decrease in TAC and an increase in the antioxidant enzymes CAT and GSHPx during a reperfusion event. Neglecting the alterations in TAC, CAT and GSHPx and considering the almost unchangeable character of the other OS parameters (e.g. GSH), we concluded that the OS observed at systemic or cellular level in the patients undergoing AAA surgery was not high grade OS.

We therefore suggest that the routine administration of exogenous antioxidants is not an argument to be considered for elective AAAR.

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An antioxidant tetrapeptide UPF1 in rats has a neuroprotective effect in transient global brain ischemia

P. Põder[a,b,*], M. Zilmer[a], J. Starkopf[c], J. Kals[a], A. Talonpoika[c], A. Pulges[b], Ü. Langel[d], T. Kullisaar[a], S. Viirlaid[a], R. Mahlapuu[a], A. Zarkovski[e], A. Arend[f], U. Soomets[a]

[a] *Department of Biochemistry, European Centre of Molecular and Clinical Medicine, University of Tartu, Ravila 19, 50411 Tartu, Estonia*
[b] *Clinic of Cardiovascular and Thoracic Surgery, European Centre of Molecular and Clinical Medicine, University of Tartu, Tartu, Estonia*
[c] *Clinic of Anaesthesiology and Intensive Care, University of Tartu, Tartu, Estonia*
[d] *Department of Neurochemistry and Neurotoxicology, Stockholm University, Stockholm, Sweden*
[e] *Department of Pharmacology, University of Tartu, Tartu, Estonia*
[f] *Department of Histology, University of Tartu, Tartu, Estonia*

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Abstract

Different glutathione analogues have potential to maintain or increase tissue glutathione level and to scavenge the reactive oxygen species. We designed and synthesized a novel non-toxic glutathione analogue, named UPF1, which possessed 60-fold higher hydroxyl radical scavenger efficiency in vitro, compared with glutathione itself, and investigated the effects of UPF1 on a four-vessel occlusion model of rats. The UPF1 was administered via the jugular vein in two separate experiments at two time points: 20 min before global brain ischemia and immediately before reperfusion. In both cases the number of pyramidal cells surviving in the subfield of CA1 at the dorsal hippocampus in the UPF1-treated groups of rats was twice as high as in the vehicle group.

Keywords: Four-vessel occlusion model; Global brain ischemia; Glutathione; Hippocampus; Antioxidant

Evidence has been accumulated over the last two decades supporting the idea that reactive oxidative species (ROS) as hydroxyl radical, superoxide anion and hydrogen peroxide play a principal role in brain ischemia-reperfusion injury [5,7]. Simultaneously, it has been reported in several studies that glutathione (GSH) depletion occurs during brain ischemia and reperfusion [1,4]. Furthermore, GSH is present in millimolar concentrations in most tissue cells including the brain. The GSH is known as an abundant non-protein thiol and a principal provider of protection against damage by acting as a scavenger of ROS and as a substrate for glutathione peroxidase [22]. The wide spectrum of the biofunctionality of GSH, including its crucial role in detoxification of electrophiles and in protection against oxidative or nitrosative stress, explains the pharmaco-clinical strategies aimed at maintaining or increasing tissue glutathione level.

The aims of the current study were to design and characterize a novel glutathione analogue, named UPF1 (assignee Vulpes Ltd., no. 110035500, PCT/SE01/01351), and to investigate the effect of UPF1 in a rat model of global brain ischemia.

The glutathione analogue UPF1: 4-methoxy-L-tyrosinyl-γ-L-glutamyl-L-cysteinyl-glycine (4-MeO-Tyr-γ-Glu-Cys-Gly; MW 483.5) was synthesized in accordance with the *t*-Boc-chemistry protocol as described in [16], or manually using Fmoc-chemistry [15]. The molecular mass of the peptide was determined by a MALDI-TOF mass spectrometer (Voyager DE-Pro, Applied Biosystem).

We tested the possible antioxidativity of UPF1 to verify the hydroxyl radical scavenging effects, and also, we compared the antioxidativity of UPF1 to GSH. Briefly, we determined

* Corresponding author.
E-mail address: priit.poder@kliinikum.ee (P. Põder).

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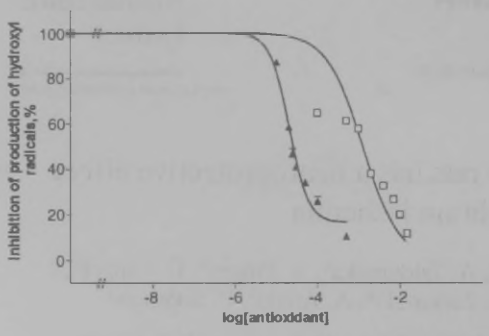

Fig. 1. Comparison of antioxidative potency of UPF1 (filled triangles) and GSH (empty squares).

the antioxidativity of UPF1 as described by Barreto et al. [3], using terephthalic acid (THA) as the chemical dosimeter for hydroxyl radicals. The THA dosimeter solution contained 10 nm THA in a 14.75 mM sodium phosphate buffer at pH 7.5 and this buffer was used as the control. Hydroxyl radicals were generated via Fenton reaction by adding $CuSO_4$ and H_2O_2 to the dosimeter solution in order to achieve the final concentration of 10 μM and 1 mM, respectively. The effect of suppression of hydroxyl radicals was measured by a spectrofluorometer (Perkin-Elmer LS5) at 312 nm excitation and at 426 nm emission [18]. The IC_{50} values were determined by sigmoid dose–response (viable slope) analysis (Fig. 1) with the GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA).

Further, we used primary cultures of cerebellar granule cells for the detection of the possible neurotoxicity of UPF1. The primary cultures of cerebellar granule cells were prepared according to the method described by Gallo et al. with minor modifications [9]. Briefly, cerebelli were dissected from 8-day-old Wistar rats and cells were dissociated by mild trypsinization followed by trituration in a 0.004% DNAse solution containing a 0.05% soybean trypsin inhibitor. The cells were resuspended in Basal Medium Eagle with Earle's Salts containing 10% heat-inactivated foetal bovine serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamycin. The cell suspension was seeded into 40 mm dishes precoated with 5 μg/ml of poly-L-lysine with a density of 1.3–1.4 × 10^6 cells/ml. The cell cultures were grown for 8 days in humidified 5% CO_2/95% O_2 atmosphere at 37 °C. After 24 h of incubation, 10 μM cytosine-β-arabino-furanoside was added to the culture medium to prevent the proliferation of non-neuronal cells. Seven- or 8-day-old cultures were used in the current experiment.

We treated each well with 2 μl of either GSH (as the negative control) or UPF1, both mixed with 50 μl of the medium at final concentrations of 0.01, 0.1, 10 and 100 μM. After 24 h of incubation, cell viability was assessed by a Trypan blue assay. The cultures were washed with phosphate buffered saline and were then incubated with a 0.4% Trypan blue solution at room temperature for 10 min. Only dead neurons were stained with Trypan blue [21]. The number of Trypan blue positive cells and viable non-stained cells was counted. Approximately 1000 cells per three fields in each well were counted to determine the viability of the cell cultures. For each concentration three wells were used. The viability of the cerebellar granular cells was presented as survival rate (e.g. the total number of the cerebellar granule cells minus the number of stained (dead) cells divided by the number of the cerebellar granule cells).

We performed animal experiments using a four-vessel occlusion (4VO) model on rats. The experimental protocol for laboratory animals was approved by the Ethics Committee of the University of Tartu. Male Wistar rats weighing 270–350 g were used. The four-vessel occlusion model of forebrain ischemia, described by Pulsinelli and Brierly, was employed with some modifications [19,20]. Briefly, the rats were fasted overnight; anaesthesia was induced with 3% halothane in oxygen (at a rate of 0.9 l/min), followed by orotracheal intubation and the animals were respirated during the surgical procedure with a respirator (TSE Animal Respirator "Advanced") [11]. A rectal temperature probe was inserted and body temperature was monitored and maintained at 37.0 °C using a heating pad. First, the rats were mounted on a head holder and tilted downward by approximately 30°. The vertebral arteries were coagulated through both *alar foramina*'s of the first cervical vertebra. The bilateral common carotid arteries were exposed via a ventral midline incision and transient global forebrain ischemia was induced for 10 min by clamping both common carotid arteries with microvascular clamps. During ischemia the animals were monitored for body temperature, respiration pattern, loss of righting reflex, unresponsiveness, corneal reflexes, as well as for fixed and dilated pupils. Restoration of blood flow in the carotid arteries was confirmed by careful visualisation. In the sham-operated group of rats, both vertebral arteries were cauterised and both common carotid arteries were exposed but not clamped.

The animals were assigned randomly into one of the following groups: sham group; vehicle group of 4VO rats, which were given isotonic saline (up to 0.5 ml) into the external jugular vein; 4VO rats, which were given UPF1 into the external jugular vein 20 min before inducing global brain ischemia (UPF1 preischemic); 4VO rats, which received UPF1 immediately before reperfusion (UPF1 reperfusion). The UPF1 was injected in a dose of 0.9 mg/kg, dissolved in a saline solution of up to 0.5 ml, into the jugular vein as a bolus dose during 30 s. This dose was selected proceeding from both the calculation derived from the measurements of the scavenging potency of UPF1, and from the analysis of some previously published reports [6,17].

Seventy-two hours following ischemia, the animals were killed by an intraperitoneal injection of fentanyl-fluanisone and midazolam for perfusion fixation of the brain with phosphate-buffered paraformaldehyde (4%) at pH 7.4 after

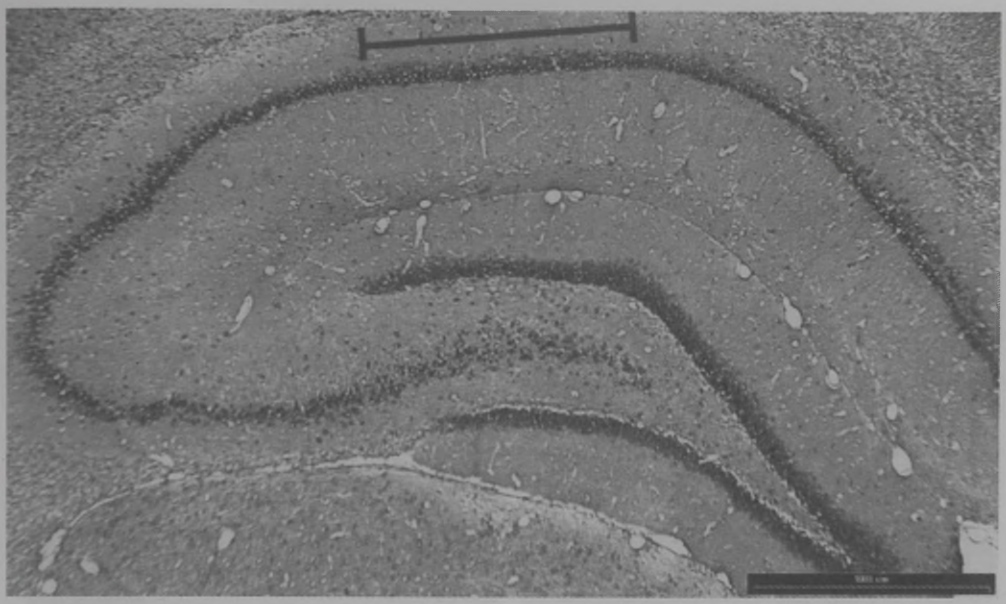

Fig. 2. Low-power (4.5×) light micrograph of a 7 μm coronal section stained by cresyl violet, showing the localisation of the counting frame at the middle part of the region of CA1. The size of the virtual frame was 0.4 mm × 1 mm and its major axis was along the pyramidal cell layer (scale bar = 1000 μm).

rinsing with 0.9% saline by cannulating the ascending aorta transcardially.

The representative coronal sections (7 μm) of the dorsal hippocampus of the paraffin-embedded brains were sliced at 200-μm intervals and were stained with cresyl violet. The number of surviving neurons in the CA1 area of the dorsal hippocampus was counted to quantify the extent of ischemic neuronal injury. To minimize bias in counting, all slices were assigned randomly and counted separately by three technicians blinded to the experimental protocol. To standardize the counting procedures, a virtual frame lying at the middle portion of the dorsal hippocampus was used. The size of the virtual frame was 0.4 mm × 1 mm and its major axis proceeded along the pyramidal cell layer as in Bae et al. [2] (Fig. 2). All viable neurons within six frames in the left and right CA1 regions of the three coronal sections of the dorsal hippocampus for each animal were counted under the criteria proposed by Kirino [8] at 832-fold magnification using an image analysis software package (AnalySIS 3.0; Soft Imaging System, Munster, Germany). Both sides of the dorsal hippocampus were counted separately and the average number of viable pyramidal cells in each coronal section of the brain was selected as a representative value.

All experimental data are shown as mean ± S.E.M. A normal distribution of the number of viable neurons was established using the Shapiro-Wilk test ($P > 0.05$). The mean number of viable pyramidal cells in the UPF1-treated groups was compared with the number of viable pyramidal cells in the vehicle-treated group by one-way ANOVA test with the Dunnett correction using the statistical program GraphPad Prism version 3.0. A value of $P < 0.05$ was considered to indicate statistical significance.

We found that UPF1 possesses a clear hydroxyl radical scavenging nature in vitro. Enhancement of hydrophobic moiety with attached methoxy group to the N-terminus of GSH increased dramatically antioxidative properties of peptide. The IC_{50} of UPF1 (20.5 ± 2.3 μM) was about 60 times lower compared with the corresponding value of GSH (IC_{50} = 1254.1 ± 382.2 μM) (Fig. 1).

The viability of the cerebellar granule cells treated with UPF1 (ranging from 90 to 87%) was similar to that of the non-treated (ranging from 91 to 90%) and GSH-treated controls (ranging from 92 to 90%) at each concentration used (even at the concentration of 100 μM survival was similar to that for the control group, Fig. 3). We suggest that the current results indicate a beneficial, i.e. non-toxic, trend of UPF1 in primary cultures.

Sixty-three rats were used in the animal experiments; 24 animals died; 16 rats, which remained unresponsive throughout the ischemic period, or whose temperature dropped below 36.0 °C, or which developed epileptic seizures, or which required persistent assisted pulmonary ventilation postoperatively, were excluded from the study. Five rats in the sham group, eight rats in the vehicle group, five rats in the UPF1

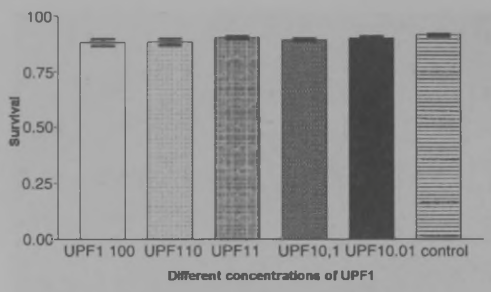

Fig. 3. Survival of the cerebellar granular cells at different concentrations of UPF1. Control: untreated cells.

preischemic group, and five rats in the UPF1 reperfusion group completed the study.

The representative sections obtained 72 h after ischemia and stained with cresyl violet are shown in Fig. 4. The number of intact neurons was 152 ± 2.0 in the sham group, 66 ± 7.2 in the vehicle group, 126 ± 6.0 in the UPF1 preischemic group, and 102 ± 6.4 in the UPF1 reperfusion group (Fig. 5).

Powerful antioxidants as, e.g. carvelidol and its metabolite SB 211475, compromise a methoxy group connected to the benzyl ring. To study the effect of these moieties, we added a non-coded amino acid residue, 4-methoxy-phenylalanine, to the N-terminus of the GSH trimer to enhance antioxidativity, to increase hydrophobicity and to prolong the half-life of UPF1 in comparison with GSH in the organism. The UPF1 dissolves readily in water and even high concentrations of the peptide can be used in different in vitro and in vivo studies.

In the present study UPF1 showed significantly higher antioxidativity compared with GSH, measured by a THA dosimeter. Whether this 60-fold increase in antioxidativity (Fig. 1) is caused by the methoxybenzyl group has to be studied further. Moreover, UPF1 did not show neurotoxicity in the experiments with the primary cell cultures of cerebellar granule cells in the concentration range 0.01–100 μM. However, it might be important to emphasize as only three wells

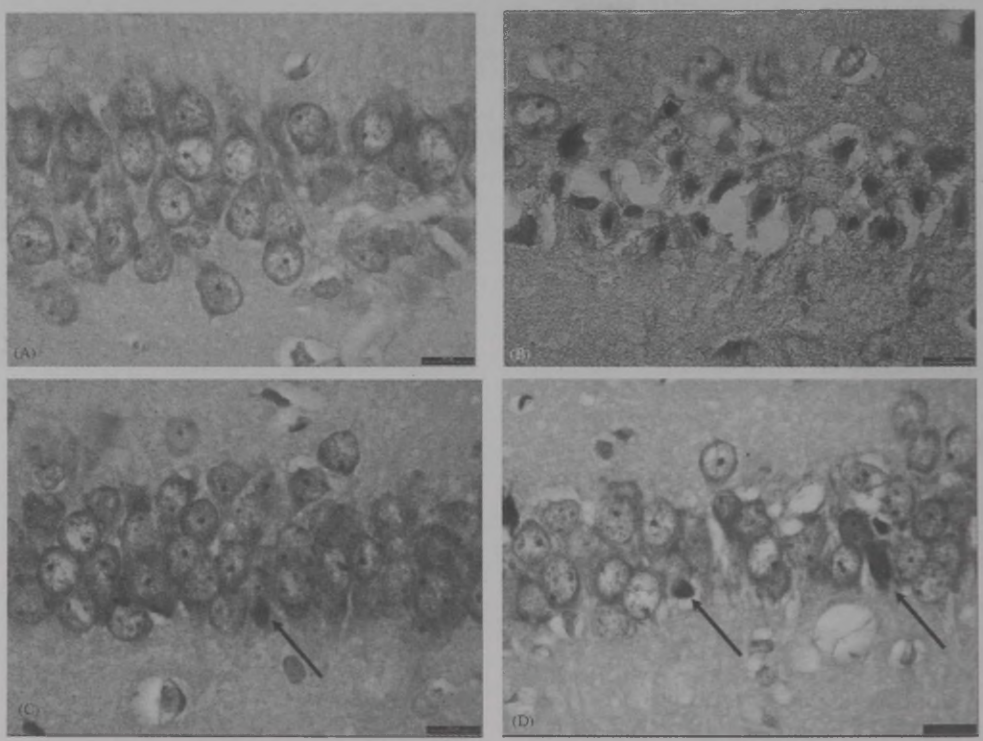

Fig. 4. Photomicrographs of the hippocampal CA1 region of rats, obtained from (A) the sham-operated, (B) the saline-treated, (C) the UPF1-treated 20 min before ischemia, and (D) the UPF1-treated immediately before reperfusion at 1579× magnification (scale bar = 20 μm). Note no cell necrosis in (A), almost no intact pyramidal neurons in (B), and a few necroses (indicated by arrows) in a large number of intact pyramidal neurons in (C) and (D).

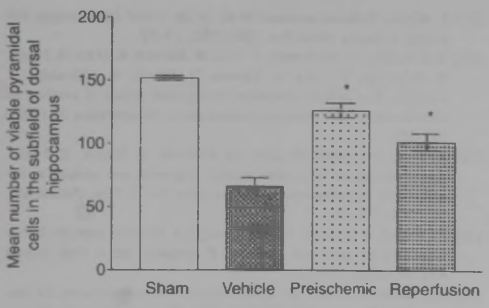

Fig. 5. The mean number of viable cells over the 1000 μm length of the CA1 layer. Vehicle: saline-treated rats; preischemic: UPF1-treated 20 min before ischemia; reperfusion: UPF1-treated immediately before reperfusion. Bars show S.E.M. $^{*}P < 0.05$ in comparison with the vehicle group.

were used for each concentration, we suggest the existence of a non-neurotoxicity trend of UPF1 in primary cell cultures.

As the glutathione analogue UPF1 showed high antioxidativity and a trend of non-toxicity in cell cultures, we performed animal experiments using a global brain ischemia model (i.e. four-vessel occlusion model). We demonstrated that the administration of UPF1 (at a concentration of 0.9 mg/ml with a bolus dose) via the jugular vein, both before ischemia and immediately before a reperfusion episode of global brain ischemia, provides definite protection against ischemia/reperfusion injury. The best treatment result was achieved when UPF1 was used 20 min before global brain ischemia; however, this protector has also a significant effect when used immediately before reperfusion.

Moreover, it has to be emphasized that in our animal experiments (20 min of global brain ischemia) general mortality rate was 38% (the lowest in the UPF1 reperfusion group and the highest in the vehicle group, 11 and 45%, respectively). In studies performed by Herguido et al., mortality rate for 10 min global brain ischemia was 29% in the perioperative period and 29% in the postoperative period [10]. Hence, proceeding from the data of above study, the mortality rate in our study can be considered admissible.

A model of forebrain transient global ischemia in experimental animals is aimed to mimic human cerebral ischemia due to transient cardiac arrest, or aortic arch reconstructive operations, or surgery of the extracranial arteries (including either various types of endarterectomies or/and bypass operations). In clinical practice, mainly during previously mentioned operations, it is possible to administer potential protective agents as UPF1 at the preischemic time point. Additionally, such compounds can be administered during ischemia, immediately before reperfusion and later during a reperfusion event. As the transient cardiac arrest or stroke cannot be predicted, it is not possible to use pre-treatment strategies. Therefore, these cases can only be treated after ischemic event, e.g. stroke or transient cardiac arrest. Considering these clinical outcomes, we selected first time point 20 min before ischemia and then the time point immediately before reperfusion event.

Moreover, induction of global cerebral ischemia by a transient occlusion of the common carotid arteries or by a permanent occlusion of the vertebral arteries in the rat causes delayed neuronal death in specific areas of the brain. Further, 2 to 4 days after transient global ischemia, cell loss develops in the CA1 region of the hippocampus, evidently due to the damage caused by free oxygen radicals [13,14].

We propose that UPF1 can be a promising leading compound for designing novel antioxidant drug precursors. However, up to now the exact mechanism of the action of UPF1 is not known. The UPF1 can act as a powerful free radical scavenger, or a modulator of the G proteins in frontocortical membrane preparations [12]. Furthermore, one of the action mechanisms of UPF1 as a signal molecule can be associated with modulation of or increase in cellular GSH levels. Preliminary experiments have shown that basal GSH level increases about 20% in the SH-SY5Y neuroblastoma cells after application of a 10 μM UPF1 solution on the cells (our unpublished data). To study the mechanism of UPF1 more thoroughly, we have designed and synthesised a library of UPF1 analogues (26 different analogues). In the light of our data, we suggest that UPF1 may have a direct protective impact on oxygen free radicals—acting as a scavenger, or acting as a signal molecule modulating the level of GSH itself or the GSH redox ratio.

In conclusion, the evidence presented in our study clearly suggests that UPF1 is an effective potential agent diminishing neuronal injury in global cerebral ischemia. Further studies are required to increase the time window for using the glutathione analogue, UPF1. Moreover, main interest would be the reperfusion injury protection management, e.g. post-treatment strategy with the purpose to mimic a clinical situation after transient cardiac arrest or possible treatment options after stroke.

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III

P. Põder, A. Pulges, A. Aavik, K. Zilmer, J. Kals, T. Kullisaar, C. Kairane, M. Zilmer
"Time-course of oxidative stress during carotid artery endarterectomy"
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ДИНАМИКА ОКИСЛИТЕЛЬНОГО СТРЕССА ПРИ ВЫПОЛНЕНИИ КАРОТИДНОЙ ЭНДАРТЕРЭКТОМИИ

П.ПОДЕР[1,2], А.ПУЛЖЕ[1], А.ААВИК[1], К.ЗИЛМЕР[2], Я.КАЛЕ[2], Т.КУЛЛИСААР[2], Ч.КАЙРАН[2], М.ЗИЛМЕР[2]

[1] Отделение сердечно-сосудистой и торакальной хирургии, Университет Тарту,
[2] Отделение биохимии, Университет Тарту, Тарту, Эстония

Цель: *Головной мозг особенно уязвим перед травмой, вызванной свободными радикалами кислорода. Ишемически-реперфузионное повреждение после церебральной ишемии связывают с окислительным стрессом. В работе изучалась временная динамика и интенсивность окислительного стресса (ОС) на системном и клеточном уровнях при проведении каротидной эндартерэктомии (КЭАЭ).*

Материал и методы: *Были взяты пробы крови 24 пациентов во время КЭАЭ (в четырех временных точках: перед анестезией, через 5 минут после пережатия сонной артерии, через 5 и 30 минут после удаления зажима с каротидной артерии). Измеряли содержание диеновых коньюгатов (DC), реактивных соединений тиобарбитуровой кислоты (TBARS), общую окислительную активность (ООА), глутатионовый окислительно-восстановительный статус (GSSH/GSH) и уровень антиоксидантных ферментов, таких как супероксиддисмутаза (СОД), каталаза (КАТ) и глутатионовая пероксидаза (GSHPx).*

Результаты: *Через 5 минут после удаления каротидного зажима наблюдалось достоверное повышение уровня TBARS (1,7 против 1,5 нмоль/л, $p<0,05$) и КАТ (89,0 против 64,5 ед/л, $p<0,05$). В той же временной точке достоверно повышалось содержание GSHPx (38,2 против 37,5 ед/гHb, $p<0,05$) по сравнению с предоперационными значениями.*

Выводы: *Во время КЭАЭ окислительный стресс не достигает высокого уровня, как на системном, так и на клеточном уровнях, за исключением умеренных изменений различных маркеров ОС.*

Ключевые слова: ишемически-реперфузионное повреждение, окислительный стресс, каротидная эндартерэктомия, глутатион, общая окислительная перекисное окисление липидов.

ВВЕДЕНИЕ

Процедура каротидной эндартерэктомии (КЭАЭ) сопряжена непродолжительным эпизодом локальной церебральной ишемии с последующей реперфузией. Хорошо известно, что головной

TIME-COURSE OF OXIDATIVE STRESS DURING CAROTID ARTERY ENDARTERECTOMY

P.PODER[1,2], A.PULGES[1], A. AAVIK[1], K.ZILMER[2], J.KALS[2], T.KULLISAAR[2], C.KAIRANE[2], M.ZILMER[2]

[1] Department of Cardiovascular and Thoracic Surgery, University of Tartu,
[2] Department of Biochemistry, University of Tartu, Tartu, Estonia

Purpose: *The brain is at special risk from oxygen free radical mediated injury. Oxidative stress (OxS) has been implicated in cerebral ischemia-reperfusion injury. Study was devised to investigate the time-course nature of both systemic and cellular levels and grades of oxidative stress during carotid endarterectomy (CAE).*

Material and Methods: *Arterial blood samples were taken from 24 patients undergoing CAE (at four time points: before anaesthesia, 5 min after carotid artery clamping, 5 min and 30 min after the removal of the carotid artery clamp). Diene conjugates (DC), thiobarbituric acid reactive substances (TBARS), total antioxidative capacity (TAC), glutathione redox ratio (GSSG/GSH), and levels of antioxidative enzymes as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) were measured.*

Results: *5 min after the removal of the carotid clamp, TBARS showed a significant elevation (1.7 vs 1.5 nmol/l, $p<0.05$), CAT was significantly elevated (89.0 vs 64.5 U/l, $p<0.05$) and at the same time point GSHPx was significantly increased (38.2 vs 37.5 U/gHb, $p<0.05$) in comparison with the preoperative values.*

Conclusions: *During CAE no high grade OxS occurred either at systemic or cellular level measured from peripheral artery, besides moderate changes in different OxS markers.*

Key words: ischemia-reperfusion injury; oxidative stress; carotid artery endarterectomy; glutathione; total antioxidative capacity; lipid peroxidation.

INTRODUCTION

Carotid artery endarterectomy presents a discrete episode of focal cerebral ischemia followed by reperfusion. It is well known that the brain is at high risk from oxygen free radical mediated injury because of its larger iron stores, high levels of polyunsaturated lipids and limited antioxidant defences [1]. Consequently, oxidative stress (OxS) has been implicated in cerebral ischemia-reperfusion injury [2] and such injury in animal models has been shown to be prevented or ameliorated by an adequate and sufficient antioxidant therapy [3, 4]. At present, despite previous studies on the products of lipid peroxidation (e.g. malondialdehyde, diene

мозг подвержен высокому риску поражения, опосредованного свободными радикалами кислорода, из-за больших запасов железа, высокого уровня полиненасыщенных липидов и ограниченной активности антиоксидантной защиты [1]. В результате, считается, что окислительный стресс (ОС) является причиной церебральной ишемически-реперфузионного повреждения [2]. На экспериментальных моделях на животных было показано, что такое повреждение можно предотвратить или ослабить адекватной антиоксидантной терапией [3, 4]. До настоящего времени, помимо некоторых работ о содержании продуктов перекисного окисления липидов (например, малонового диальдегида и диеновых коньюгатов), нет достаточной информации о глубине и динамике ОС на системном и клеточном уровнях при проведении КЭАЭ. Выраженность ОС (генерализованный/системный или клеточный) и его интенсивность можно оценить с помощью различных биохимических маркеров. Более того, человеческий организм имеет систему антиоксидантной защиты, в которой участвуют ферментные (например, супероксиддисмутаза, каталаза и глутатионовая пероксидаза) и неферментные (например, витамины Е и С) антиоксиданты. Они позволяют защитить организм от сильного ОС. В физиологических условиях свободные радикалы нейтрализуются эндогенными антиоксидантами и гасителями свободных радикалов, однако когда эти защитные механизмы иссякают, в окружающих тканях может развиться повреждение [5]. Цель данной работы состояла в изучении временной динамики ОС на системном и клеточном уровнях и интенсивности ОС на организменном уровне при проведении КЭАЭ.

МАТЕРИАЛЫ И МЕТОДЫ

Исследование выполнено на 24 взрослых пациентах, перенесших КЭАЭ. Протокол исследования был одобрен Комиссией по этике Университета Тарту. Основные клинические характеристики пациентов приведены в табл. 1.

2.1. Анестезия

После премедикации мидазоламом (F.Hoffman — La Roche, Швейцария) проводили анестезию атропином (Nycomed Pharma Holding AS, Дания), этомидатом (B. Braun Melsungen AG, Германия) и фентанилом (Gedeon Richter, Словакия).

Эндотрахеальную интубацию проводили под сукцинилхолином (GlaxoSmithKline, Великобритания). Мышечная релаксация достигалась пиперкурониумом (Gedeon Richter, Словакия), а анальгезия — непрерывной инфузией фентанила (3–4 μ/кг/час). Газовая смесь для вентиляции легких содержала изофлуран (Halocarbon Products Corp. США) до 1,5 объемных процентов, кислород и

conjugates), the information on the grade of OxS and on the changes occurring on the systemic and cellular levels of OxS during carotid artery endarterectomy (CAE) is still limited. The expression of OxS (generalised/systemic or cellular) and the grade of OxS can be evaluated using different biochemical markers. Furthermore, the human body comprises an antioxidant network including enzymatic (e.g. superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic antioxidants (e.g. vitamin E and vitamin C), to withstand excessive damage caused by high-grade OxS. Under physiological conditions, free radicals are neutralised by the presence of endogenous antioxidants and free radical scavengers, however, when these protective mechanisms are overwhelmed, injury of the surrounding tissues may occur [5].

The purpose of this study was to investigate the time-course nature of both, systemic and cellular levels of OxS and grades of OxS during the carotid endarterectomy on the organism level.

MATERIAL AND METHODS

The study was performed on 24 adults undergoing elective carotid endarterectomy. The protocol used in this study was approved by the Ethics Committee of the University of Tartu. The main clinical characteristics are given in Table 1.

2.1. Anaesthesia

After premedication with midazolam (F. Hoffmann-La Roche, Switzerland), anaesthesia was induced with atropine (Nycomed Pharma Holding AS, Denmark), etomidate (B. Braun Melsungen AG, Germany) and fentanyl (Gedeon Richter, Slovakia). Endothracheal intubation was carried out with succinylcholine (Glaxo Smith Kline, UK). Muscle relaxation was achieved with pipercuronium (Gedeon Richter, Slovakia) and analgesia was performed with continuous infusion using fentanyl 3–4 μ/kg/h. The lungs were ventilated with isoflurane (Halocarbon Products Corp., SC, USA) up to 1.5 vol. % with oxygen and N_2O. All patients were mechanically ventilated to maintain normocapnia with $PaCO_2$ of 38 to 41 mmHg. In all patients an arterial catheter was inserted, ECG, end-tidal capnometry, and changes in arterial blood pressure were continuously recorded. A neurological examination was performed immediately after the patient awakened, 1 hour later, and then daily until the patient was discharged. During carotid surgery, we did not use transcranial Doppler ultrasound, or the monitoring of somatosensory evoked potentials, or electroencephalography. Each patient received 3000 units of heparin (Leo Pharmaceutical Products, Denmark) before the cross clamping. During the endarterectomy blood pressure was raised and maintained pharmacologically (at least 20 mmHg) with phenylephrine (Sanofi Winthrop, New York, NY, USA) in each patient above individual blood baseline pressure values.

Таблица 1/Table 1

Основные клинические характеристики группы пациентов
Main clinical characteristics of the study group

Пол (ж/м) Female/male	5/19
Средний возраст, лет (интервал) Female/male, y	68.0 range (44-84)
Время пережатия сонной артерии, мин. Clamping time of the carotid artery (min)	18 ± 1,1
Стандартная эндартерэктомия /эверсионная эндартерэктомия Standard endarterectomy versus eversion endarterectomy	13/11
Показания к каротидной эндартерэктомии: Indication for carotid endarterectomy :	
Симптоматический стеноз сонной артерии 60 – 70% (тромбоэмбологенные бляшки)* Symptomatic carotid artery stenosis (trombembologenic plaque) 60 - 70% *	6 из 24 (25%)
Симптоматический стеноз сонной артерии > 70% Symptomatic carotid artery stenosis (>70%)	18 из 24 (75%)
Предшествующие и сопутствующие заболевания: Diseases existing preoperatively:	
Церебральный микроинфаркт Cerebral microinfarcts	2 из 24 (8%)
Заболевание периферических артерий Peripheral arterial disease	13 из 24 (54%)
Гипертония Hypertension	10 из 24 (42%)
Заболевание коронарных сосудов coronary heart disease	3 из 24 (13%)
Диабет Diabetes	1 из 24 (4%)

* — по данным допплерографии
* — Detected by Doppler ultrasound

N_2O. Для всех пациентов применяли искусственную вентиляцию легких для поддержания нормокапнии с $PaCO_2$ от 38 до 41 мм рт.ст. Всем вводили артериальный катетер, непрерывно регистрировали ЭКГ, АД и проводили капнометрию на конце выдоха. Неврологическое обследование осуществлялось сразу после того, как пациент приходил в себя, еще через час и затем ежедневно до выписки пациента из стационара. Во время КЭАЭ мы не использовали транскраниальную допплерографию, регистрацию соматосенсорных вызванных потенциалов или электроэнцефалографию. Каждому пациенту вводили 3 000 ед. гепарина (Leo Pharmaceutical Products, Дания) перед пережатием сонной артерии. Во время эндартерэктомии у каждого пациента вызывали подъем АД не менее, чем на 20 мм рт.ст. относительно исходного уровня, и удерживали повышенное АД с помощью фенилэфрина (Sanofi Withthrop, США).

2.2. Взятие проб крови

Пробы крови отбирали катетером из периферической артерии (лучевой) четырежды, в опреде-

2.2 Blood sampling

Blood samples were collected regularly from the peripheral arterial line (the radial artery) at four time points (before anaesthesia, 5 min after carotid artery clamping, 5 min and 30 min after the declamping) during CAE. The blood was taken into 7-ml sterile lavender vacutainer tubes and promptly transported to the laboratory for analysis. The serum was separated by centrifugation and kfept in plastic tubes at -20°C until analysis.

2.3. Measurement of oxidative stress markers

Several markers of systemic and cellular OxS were measured, as diene conjugates (DC), thiobarbituric acid reactive substances (TBARS), serum total antioxidative capacity (TAC), glutathione redox status (the ratio of oxidized to reduced glutathione or GSSG/GSH), and the level of antioxidative enzymes — superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx). The methods of measurement of the OxS markers have been described in detail previously. Briefly, TBARS were measured according to Ohkawa et al. [6] with modifications [7]. For the study of DC, the

ленное время — перед анестезией, через 5 минут после пережатия сонной артерии, через 5 и 30 минут после удаления зажима с каротидной артерии. Кровь отбирали в стерильные пробирки lavender vacutainer объемом в 7 мл и экстренно направляли в лабораторию для анализа. Плазму отделяли центрифугированием и содержали в пластиковых пробирках при -20°C до проведения анализа.

2.3. Измерение маркеров окислительного стресса

Проводили измерение нескольких маркеров системного и клеточного ОС: диеновых коньюгатов (DC), реактивных соединений тиобарбитуровой кислоты (TBARS), общую окислительную активность плазмы (ООА), глутатионовый окислительно-восстановительный статус (отношение окисленной формы глутатиона [GSSG] к восстановленной форме [GSH]) и уровень антиоксидантных ферментов — супероксиддисмутазы (СОД), каталазы (КАТ) и глутатионовой пероксидазы (GSHPx). Методы измерения маркеров ОС подробно описаны в опубликованных ранее работах. Вкратце, TBARS измеряли по методу Ohkawa et al. [6] в модификации [7]. Для измерения DC пробы плазмы инкубировали при 37°C в течение 25 минут, затем реакцию останавливали добавлением антиоксиданта (бутилированного гидрокситолуена — БГТ) с последующей экстракцией липидов. Затем проводили спектрофотометрический анализ проб при 233 нм [8]. Для поправки результатов на гемодилюцию определяли содержание белка в пробах по методу Lowry et al. [9]. Для оценки ООА измеряли способность плазмы ингибировать in vitro перекисное окисление линоленовой кислоты в присутствии 200 μмоль/л Fe^{+2} [10]. СОД и GSHPx измеряли по специальному протоколу с использованием коммерческих наборов (Randox Laboratories Ltd, Великобритания). КАТ измеряли с помощью реактивных веществ тиобарбитуровой кислоты по описанному ранее методу [12]. Содержание общего глутатиона (TGSH) и его окисленной формы (GSSG) измеряли ферментным методом Tietze [13], несколько модифицированным для нашего исследования [10]. Содержание восстановленной формы глутатиона (GSH) вычисляли как разницу между общим и окисленным глутатионом (TGSH — GSSG). Все измерения продуктов перекисного окисления липидов и маркеров ОС проводили в трех повторах в течение 4 часов после взятия крови. Гемолизированные пробы из анализа исключались.

2.4. Статистический анализ

Регистрацию данные и статистический анализ проводили с помощью программного обеспечения GraphPad Prism 3,0 (GraphPad Software Inc., Сан-Диего, США). Для сравнения предоперационных значений параметров со значениями в других

serum samples were incubated at 37°C for 25 min, the reaction was stopped by adding an antioxidant (butylated hydroxytoluene, BHT) and the lipids were extracted. Thereafter the samples were spectrophotometrically analysed at 233 nm [8]. To correct the results for hemodilution, protein content in the samples was determined according to Lowry et al. [9]. To assess TAC, the ability of the serum to inhibit in vitro linolenic acid peroxidation in the presence of 200 μmol/l Fe^{2+} was evaluated [10]. SOD and GSHPx were measured according to special protocols using commercially available kits (Randox Laboratories Ltd, Ardmore, UK) [11]. CAT was measured using thiobarbituric acid reactive substances according to the method described in literature [12]. Total glutathione (TGSH) and GSSG as the parameters were measured by the enzymatic method of Tietze [13], which was slightly modified for the present study [10]. The content of GSH was calculated as the difference between the total amount of glutathione and GSSG (TGH-GSSG). All measurements of the lipid peroxidation products and the antioxidant markers were performed in triplicate within 4 h after blood sampling. The haemolysed samples were excluded from analysis.

2.4. Statistical analysis

The data were recorded and used for statistical analysis with the GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA). For repeated measurements over the time course of elective carotid artery endarterectomy, the non-parametric Friedman test was used for comparison of the preoperative value with the values for the other time points, correcting multiple comparison with the Dunn test. The results are presented as the mean ± standard error of the mean (SEM) in the text and in the tables. The limit of statistical significance P value was at 0.05.

RESULTS

The time-course of the indices of systemic and cellular OxS was studied in 24 patients undergoing elective CAE. There were no fatal complications associated with surgery. However, two patients had postoperative permanent neurological deficit. One patient became hemiparetic and the other patient had apalic syndrome. Both patients had had cerebral infraction preoperatively, which had been identified with CT and these patients had minor neurological deficit before surgery. The indications for CAE are shown in Table 1. No vascular shunt was used during CAE. Seventeen patients of 24 took aspirin (Lannacher, Austria) as antiplatelet medication, whereas none of the patients took antioxidants. Arterial TBARS revealed a statistically significant increase (1.7 ± 0.1) 5 min after carotid clamp removal in comparison with the preclamping value (1.5 ± 0.1). At the same time, an elevation in CAT and GSHPx (89 ± 9 and 38 ± 3, respectively) was observed 5 min after the declamping in comparison

временных точках применяли непараметрический критерий Фридмана, с поправкой на многократные сравнения с помощью теста Данна. В тексте и таблице результаты представлены как среднее ± стандартная ошибка. Уровень статистической достоверности установлен на 0,05.

РЕЗУЛЬТАТЫ

Временная динамика показателей системного и клеточного ОС изучена на 24 пациентах, перенесших КЭАЭ. Не было зарегистрировано ни одного летального осложнения операции. Однако у двух пациентов после операции развился стойкий неврологический дефицит — у одного гемипарез, у другого апалический синдром. Оба пациента до операции перенесли инфаркт мозга, диагностированный на КТ, и до операции имели небольшой неврологический дефицит. Показания к КЭАЭ представлены в табл. 1. Сосудистые шунты при КЭАЭ не использовались. 17 из 24 пациентов принимали аспирин (Lannacher, Австрия) в качестве антитромбоцитарного средства, ни один из пациентов не принимал антиоксидантов. Содержание TBARS в артериальной крови через 5 минут после прекращения пережатия сонной артерии (1,7 ± 0,1 нмоль/л) достоверно повышалось относительно исходного уровня (1,5 ± 0,1 нмоль/л). Одновременно, то есть через 5 минут после снятия каротидного зажима, наблюдалось повышение уровня КАТ и GSHPx (89 ± 9 ед/л и 38 ± 3 ед/г Hb, соответственно) относительно исходных значений. Для других маркеров ОС (DC, СОД и ООА) не выявлено достоверных изменений после проведения КЭАЭ. Аналогично, глутатионовый окислительно-восстановительный статус остался без изменений (табл. 2).

ОБСУЖДЕНИЕ

Изменения уровня маркеров ОС в периферической артериальной крови относительно исходных значений наблюдались только в период реперфузии (через 5 минут после удаления зажима), но не во время ишемии или в более поздний срок после восстановления кровотока (через 30 минут). Маркер перекисного окисления липидов — TRARS — демонстрировал самые высокие значения через 5 минут после начала реперфузии. Однако в тот же момент with the corresponding value at the preclamping time point. The other markers as DC, SOD and TAC did not show any statistically significant changes during CAE. Simultaneously, the glutathione redox ratio did not reveal any changes during CAE (Table 2).

DISCUSSION

We observed changes in different markers of OxS, assessed from the peripheral arterial line, only during reperfusion (5 min after carotid clamp removal) in comparison with preoperative values, but not during ischemia or later (at time point 30 min) during reperfusion. A marker of lipid peroxidation, TBARS, showed the highest value 5 min after the beginning of reperfusion. At the same time, another more specific marker of lipid peroxidation, DC, did not reveal statistically significant changes during CAE. Previously, Soong et al. obtained similar findings when measuring malondialdehyde and diene conjugates at the time point of 1 min after carotid clamp removal [14]. However, when comparing the results of our study with those of the above mentioned study, it should be noted that we collected the blood samples

Таблица 2/Table 2

Сравнительная динамика показателей системного и клеточного окислительного стресса во время каротидной эндартерэктомии (n = 17-27)

Comparative dynamics of the indices of systemic and cellular OxS during CAE (n = 17-27)

Время измерения Time points during CAE	A	B	C	D
TBARS нмоль/мл TBARS, nmol/ml	1.5±0.1	1.6±0.1	1.7±0.1*	1.6±0.1
DC мкмоль/мл DC, μ,mol/ml	29.7±2.1	30.6±2.1	28.5±1.9	27.9±1.9
ООА, % TAC, %	36±1	35±1	35±1	35±1
КАТ, ед/л CAT, U/l	65±6	84±7	89±9*	85±3
СОД, ед/г Hb SOD, U/gHb	674±31	686±39	649±43	655±44
GSHPx, ед/г Hb GSHPX, U/gHb	36±3	37±3	38±3*	37±3
GSSG/GSH GSSG/GSH	0.9±0.17	1.0±0.19	0.9±0.19	1.0±0.23

* — p<0,05 для разницы между точками A и C;
A — до введения анестезии;
B — через 5 мин после пережатия сонной артерии;
C — через 5 мин после восстановления кровотока в сонной артерии;
D — через 30 мин после восстановления кровотока в сонной артерии.

*p < 0.05 for the differences between the values of time points A and C
A. before induction of anesthesia
B. 5 min after carotid artery clamping
C. 5 min after removal of the carotid artery clamp
D. 30 min after removal of the carotid artery clamp

колебания другого, более специфичного маркера перекисного окисления липидов — DC — не были статистически достоверными. Ранее Soong et al. [14] получили аналогичные результаты, измерив концентрацию малонового диальдегида и диеновых коньюгатов через 1 минуту после восстановления кровотока в сонной артерии. Однако, сравнивая наши результаты с данными этого исследования, следует отметить, что мы отбирали пробы крови из периферической артерии, а Soong et al. — из яремной вены. Другой маркер системного ОС – ООА — отражает общую антиоксидантную способность плазмы. Согласно данным нашего исследования, изменений в ООА при проведении КЭАЭ не было. Мы считаем, что ишемия во время КЭАЭ была недостаточно тяжелой или продолжительной, чтобы истощить резерв такой важной защитной системы, как ООА плазмы крови.

Кроме того, чтобы лучше понять степень ОС при проведении КЭАЭ, мы измерили динамику значений основных маркеров внутриклеточного ОС. Была обнаружена статистически значимое, но довольно небольшое повышение активности антиоксидантных ферментов — КАТ и GSHPx — через 5 минут после удаления зажима сонной артерии. Однако изменений в активности еще одного антиоксидантного фермента — СОД — не выявлено. Кроме того, мы обнаружили интересный факт некоторого повышения ОС в системе, контролирующей уровень перекисного окисления (например, КАТ и GSHPx), поскольку функция GSHPx связана с глутатионовой системой. Более того, не выявлено изменений в хорошо известном внутриклеточном маркере ОС — глутатионовом окислительно-восстановительном статусе (GSSG/GSH) — этот уровень был одинаково высоким, начиная с первого, предоперационного измерения. В наших предшествующих работах мы провели измерение GSSG/GSH у здоровых добровольцев того же возраста и вычислили референтный нормальный интервал значений этого показателя — от 0,13 до 0,3. Таким образом, наблюдалась существенная разница между референтным интервалом и предоперационным уровнем GSSG/GSH, что указывает на значительный внутриклеточный окислительный стресс у наших пациентов. Возможно, для этой категории пациентов целесообразно проводить коррекцию активности глутатионовой системы (например, глютамином, N-ацетил-L-цистеином и др.). Хотелось бы также отметить интересный факт, который может быть связан с чрезвычайно высоким уровнем GSSG/GSH в нашей группе пациентов. У 13 из 24 пациентов диагностирована патология периферических артерий. Недавно Lapenna et al. [15] продемонстрировали, что длительная не-

from the peripheral arterial line, but Soong et al obtained the samples from the jugular vein. Another marker of systemic OxS, serum TAC, reflects the potency of total antioxidative protection in the plasma. According to the findings of our study, there occurred no changes in the level of TAC during CAE. We believe that the episode of ischaemia that occurred during CAE was not sufficiently severe or long to cause exhaustion of potency of such an important system as total antioxidative capacity in the blood plasma.

Furthermore, in order to have a better understanding of the grade of OxS during CAE, we have also investigated the main markers of intracellular OxS. We detected a statistically significant but relatively modest increase in the activity of antioxidative enzymes, CAT and GSHPx, 5 min after the removal of the carotid artery clamp. However, no changes in the activity of the antioxidative enzyme, SOD, during CAE occurred. Furthermore, we established some elevated OxS in peroxide level controlling system (e.g. CAT, GSHPx), which is interesting as GSHPx function is associated with glutathione system. Moreover, we detected no changes in the well-known intracellular marker for OxS, the glutathione redox ratio, during this time-course study, while we observed a high level of the glutathione redox ratio at the first time point (preoperative value). In our previous studies we established the glutathione redox ratio from age-matched healthy volunteers and calculated the reference value that ranged from 0.13 to 0.3. We observed a significant difference between the reference value and the preoperative value of the glutathione redox ratio, which indicates a high level of intracellular OxS in these patients. It seems that any correction of glutathione system using different manipulations (e.g. glutamine, N-Acetyl-L-cystein etc) should be considered for these patients. Furthermore, we emphasise an interesting fact, which might be correlated with the enormously high values of the glutathione redox ratio in our study group. We established concomitant peripheral arterial disease in thirteen patients of 24. Recently, Lapenna et al. demonstrated that long-term depletion of GSH is associated with oxidative atherogenic effects [15]. It is evident that further investigations should be performed to clarify the association of GSH depletion with PAD.

There are several limitations concerning this study. It might be a problem to achieve clearly understandable results with relevant small number of patients like in our study group. However, we believe, that some interesting information about the time-course of OxS during CAE is possible to denote from our study. Moreover, in our study the blood samples were obtained only from peripheral artery and therefore we emphasise that, the changes occurred in OxS markers could not beorgan-specifically interpreted.

хватка восстановленной формы глутатиона (GSH) связана с атерогенным окислительным эффектом. Очевидно, нужны дополнительные исследования для уточнения взаимосвязи между нехваткой GSH и болезнью периферических сосудов.

В нашем исследовании было несколько ограничений. Возможно, на таком небольшом числе пациентов, как в данном случае, трудно сделать однозначные выводы. Однако нам кажется, что эта работа дает некоторую интересную информацию о временной динамике ОС при проведении КЭАЭ. Кроме того, в данном случае пробы крови были получены из периферической артерии. В этой связи необходимо подчеркнуть, что изменения в уровне маркеров ОС нельзя относить на какой-либо отдельный орган.

ВЫВОДЫ

Мы не выявили интенсивного окислительного стресса на системном и клеточном уровнях во время проведения каротидной эндартерэктомии. Кроме того, пациенты имели высокий предоперационный уровень глутатионового окислительно-восстановительного статуса. Последний факт заслуживает дальнейшего исследования и рассмотрения возможности адьювантной терапии.

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Corresponding to:
Priit Poder, MD,
Department of Cardiovascular
and Thoracic Surgery University of Tartu,
Puusepa 8,
Tartu 51014, Estonia
Phone: +372 7 318 295
Fax: +372 7 318 299
E-mail: Priit.Poder@kliinikum.ee

CONCLUSIONS

We observed no high grade OxS either in systemic or cellular level during carotid endarterectomy. Moreover, the patients undergoing CAE had a high baseline of the glutathione redox ratio. The latter phenomenon might support further investigation and tension of adjuvant therapy.

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CURRICULUM VITAE

Priit Põder

Citizenship: Estonian
Born: November 4, 1974 in Tartu, Estonia
E-mail: priit.poder@gmail.com

Education

1981–1990	Tartu, Secondary School No. 12
1990–1993	Hugo Treffner Gymnasium
1993–1999	University of Tartu, Faculty of Medicine
1999–2000	Internship
2000–2004	PhD student in cardiovascular surgery
2004–	Resident in cardiovascular surgery

Special courses

27 May–5 June 2002	Course "Animal, Research & Welfare", St. Petersburg, Russia (certification of C-category in laboratory animal science)
23–25 Sept 2003	International Workshop in Neuroscience "Ischaemia/ Hypoxia in the Brain: Experimental Methods and Basic Mechanisms", Bucharest, Romania
30 Aug-6 Sept 2003	"Cellular Structure to Function" Course at Advanced Light Microscopy, Tartu, Estonia, and Turku, Finland
23 April 2004	"Symposium on microsurgical techniques and applications", Tampere, Finland

Scientific work

Research field: ischaemia/reperfusion injury in the brain and during abdominal aortic aneurysm repair and during carotid artery endarterectomy

Total: 8 scientific publications, 10 presentations at international scientific conferences.

Membership: junior member of the European Society for Vascular and Endovascular Surgery, member of the Scandinavian Society for Laboratory Animal Science, junior member of the European Society for Cardiovascular Surgery

ELULOOKIRJELDUS

Priit Põder

Kodakondsus: Eesti
Sünd. 4 novembril 1974 Tartus
E-mail: priit.poder@gmail.com

Haridus

1981–1990	Tartu 12 Keskool
1990–1993	Hugo Treffneri Gümnaasium
1993–1999	Tartu Ülikool, ravi eriala
1999–2000	Internatuur
2000–2004	Doktorant kardiovaskulaarkirurgia erialal
2004–	Resident kardiovaskulaarkirurgia erialal

Erialane täiendus

27 Mai–5 Juuni 2002	"C category" kursus "Animal, Research & Welfare", St. Petersburg, Venemaa
23–25 Sept 2003	Rahvusvaheline kursus neuroteaduses "Ischaemia/ Hypoxia in the Brain: Experimental Methods and Basic Mechanisms", Bucharest, Rumeenia
30 Aug–6 Sept 2003	Rahvusvaheline kursus "Cellular Structure to Function" Course at Advanced Light Microscopy, Tartu, Eesti ja Turku, Soome
23 Aprill 2004	Rahvusvaheline kursus "Symposium on microsurgical techniques and applications", Tampere, Soome

Teadustegevus

Peamiseks uurimisvaldkonnaks aju ischaemia/reperfusiooni kahjustus, oksüdatiivne stressi uuringud kõhuaordi aneurüsmi ja unearteri endarterektoomia ajal.

Ilmunud 8 publikatsiooni, 10 ettekannet rahvusvahelistel konverentsidel.

Noorliige Euroopa Veresoonte ja Endovaskulaarse kirurgia Seltsis, liige Skandinaavia Katselooma Teaduse Seltsis, noorliige Euroopa Kardiovaskulaarkirurgia Seltsis

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