

Aus der Klinik für Anästhesiologie und Operative Intensivmedizin

(Direktor: Prof. Dr. med. Markus Steinfath)

im Universitätsklinikum Schleswig-Holstein, Campus Kiel

an der Christian-Albrechts-Universität zu Kiel

**EFFECTS OF PROPOFOL ON HYPOXIA/REOXYGENATION  
INDUCED NEURONAL CELL DAMAGE IN VITRO**

Inauguraldissertation

zur

Erlangung der Doktorwürde

der Medizinischen Fakultät

der Christian-Albrechts-Universität zu Kiel

vorgelegt von

**Ying Huang**

aus Wuhan, Hubei, Volksrepublik China

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## Abbreviations

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APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
Bcl-2	B-cell lymphoma 2
Bax	Bcl-2-associated X protein
cDNA	Complementary DNA
CM-H <sub>2</sub> DCFDA	6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CAT	Catalase
DCF	Dichlorofluorescein
DD water	Double-distilled water
DEPC water	Diethylpyrocarbonate water
DMEM	Dulbecco's modified eagle medium
DMEM/F12	Dulbecco's modified eagle's medium/ nutrient mixture F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleotide acid
dNTP	Deoxy-ribonucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia
ELISA	Enzyme linked immunosorbent assay
ECL	Enhanced chemiluminescence
FCS	Fetal calf serum
For	Forward
GPX	Glutathione peroxidase
GABA <sub>A</sub>	Gamma-aminobutyric acid receptor A
GSH	Glutathione
GSSG	Glutathione disulfide
HCl	Hydrogen chloride
H <sub>2</sub> DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HRP	Horseradish peroxidase
IMR-32	Human neuroblastoma cells
IgG	Immunoglobulin G
L	Liter
LDH	Lactate dehydrogenase

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M	Mol
mM	Millimol
min	Minute
ml	Milliliter
mRNA	Messenger RNA
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP-40	Tergitol-type NP-40
ng	Nanogram
nm	Nanometer
O <sub>2</sub>	Oxygen
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PKB	Protein kinase B
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PVDF membrane	Polyvinylidene-fluoride membrane
Rev	Reverse
RIPA buffer	Radio-immunoprecipitation assay buffer
RNA	Ribonucleic acid
RNase inhibitor	Ribonuclease inhibitor
RPMI-1640	Roswell park memorial institute-1640
RT-PCR	Reverse transcription polymerase chain reaction
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TEMED	Tetramethylethylenediamine
TBST buffer	Tris-buffered saline and tween 20 buffer
Tween20	Polyoxyethylene (20) sorbitan monolaurate
$\mu$ g	Microgram
$\mu$ l	Microliter
$\mu$ M	Micromol

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## **1. Introduction**

### **1.1. Hypoxia/reoxygenation**

#### **1.1.1. Cell damage**

Hypoxia/reoxygenation is a common physiological stress which widely occurs under various clinical conditions such as circulatory shock, myocardial ischemia, stroke, transplantation of organs (Levinson et al. 1986, McCord 1985) and brain injury (Cazevieuille et al. 1993). Hypoxia/reoxygenation via different pathological pathways causes cell damage. Typical pathways include induction of inflammation primarily mediated by the NF- $\kappa$ B (Fabiano et al. 2008), loss of mitochondrial cytochrome C, activation of various apoptosis-associated factors such as bcl-2, bax, pro-caspase-3, active-caspase-3 and generation of reactive oxygen species which is one of the most important triggers of hypoxia/reoxygenation induced cell damage (Torre-Bouscoulet et al. 2008).

Reactive oxygen species generated during hypoxia/reoxygenation can induce cell death via necrosis or apoptosis (Tan et al. 1998). Recently, antioxidants have been reported to inhibit DNA fragmentation and PARP cleavage, which implies that the inhibition of reactive oxygen species by antioxidants might play a crucial role in the attenuation of hypoxia/reoxygenation induced cell damage (Mizukami et al. 2000).

In the brain, hypoxia/reoxygenation events can result in various pathological processes including oxidative and inflammatory stress (Lim et al. 2010) and often lead to severe cellular damage which causes neurological dysfunction and poor clinical outcome (Hamm et al. 1990, Obrenovitch et al. 1988, Schlaepfer 1987, Semenza 2000). Cerebral neurons can tolerate at least 20 min of ischemic hypoxia under normothermic condition. However, in the clinic, cerebral recovery is often counteracted by complex secondary dysfunctions of multiple organ systems after reoxygenation (Safar 1986). The underlying mechanisms of hypoxia/reoxygenation injury in the brain are multifactorial and complex, but recent studies point towards a crucial role of reactive oxygen species which are generated during the process of hypoxia/reoxygenation (Wang et al. 2010) and may damage cellular macromolecules by lipid peroxidation, oxidation of proteins as well as inhibition of specific enzymes by oxidation of co-factors (Elahi et al. 2009).

#### **1.1.2. Two-enzyme hypoxia model**



The investigation of intracellular pathways and mechanisms during hypoxia/reoxygenation induced cell damage in vivo is of clinical importance, but technically difficult. Cellular models of hypoxia/reoxygenation are considered as an effective method to study the mechanisms of reactive oxygen species mediated cell damage in hypoxia/reoxygenation injury (Watkins et al. 1995). In the present study, we employed an in vitro two-enzyme hypoxia model in combination with human neuronal cells (IMR-32) to mimic hypoxia/reoxygenation (Zitta et al. 2010a, Zitta et al. 2010b). This model is based on two enzymatic reactions (Baumann et al. 2008) (Fig.1). In reaction 1, glucose oxidase catalyzes the oxidation of glucose consuming the oxygen in the culture medium. In reaction 2, catalase converts the produced hydrogen peroxide from reaction 1 to water and oxygen.

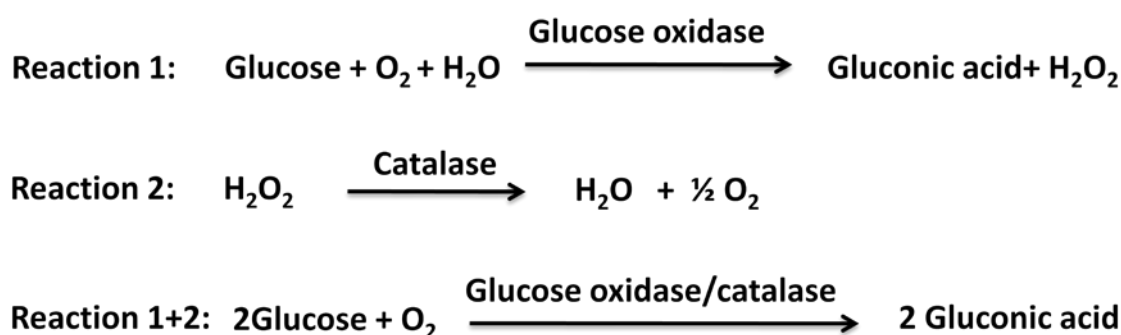


Figure 1: Generation of hypoxia using the two-enzyme system.

The two-enzyme hypoxia system offers a unique opportunity to control oxygen levels in cell culture. Moreover, the reactants involved in the enzymatic system are derived from either physiological products (water, glucose) or physiological intermediate products (gluconic acid), which are common components in all cell culture medium (Mueller et al. 2009).

## 1.2. Reactive oxygen species

Reactive oxygen species are intracellular factors which are associated with the immune system and cell signaling. However, excessive reactive oxygen species are destructive and lead to cell damage and are also involved in various diseases such as heart disease, Alzheimer's disease, Parkinson's disease and tumors (Tabner et al. 2001).

Generation of reactive oxygen species is a crucial event in hypoxia/reoxygenation induced neurologic damage. Reactive oxygen species such as the peroxide ( $\cdot\text{O}_2^{-2}$ ), the superoxide anion ( $\cdot\text{O}_2^-$ ), and the hydroxyl radical ( $\cdot\text{OH}$ ) are oxidants which structures include unpaired electrons

(Jambunathan 2010) (Fig.2). This unstable structure can accept electrons from other molecules by oxidation.

Reactive oxygen species are involved in reduction and oxidation reactions between cellular and sub-cellular components and induce damage to membranes, proteins and DNA (Mohsenzadegan et al. 2012). Lipids are the major target of intracellular reactive oxygen species, which cause an oxidation of polyunsaturated fatty acids in cellular membranes and initiate lipid peroxidation (Humphries et al. 1998). In addition, intracellular reactive oxygen species also directly attack both the base and the sugar moieties of DNA molecules resulting in DNA damage (Sies 1993) and induce deleterious modifications in proteins such as oxidation of sulfhydryl groups and amino acid (Fucci et al. 1983, Grune et al. 1997).

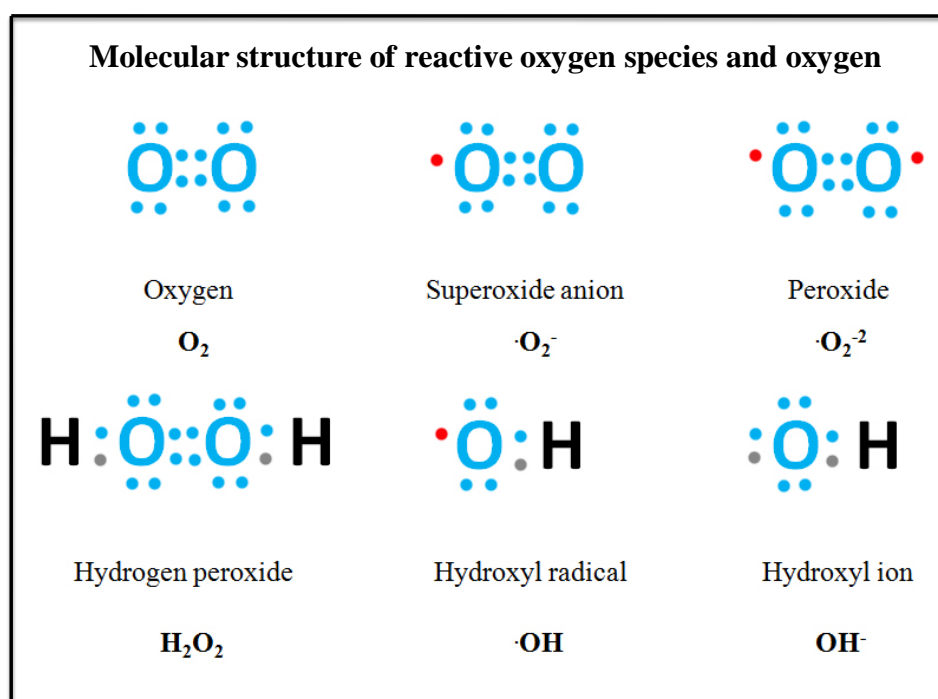


Figure 2: Molecular structures of reactive oxygen species and oxygen. Red points denote unpaired electrons.

### 1.3. Antioxidant and neuroprotective effects of propofol

Reactive oxygen species mediated oxidative stress plays an important part in hypoxia/reoxygenation induced cell damage. The intravenous anaesthetic, propofol, has been shown to protect different cells from oxidative damage (Vasileiou et al. 2009). Numerous studies have shown antioxidant effects of propofol which are possibly mediated via a phenolic hydroxyl

group leading to structural similarities between propofol and the natural antioxidant vitamin E (Ansley et al. 1998) (Fig.3).

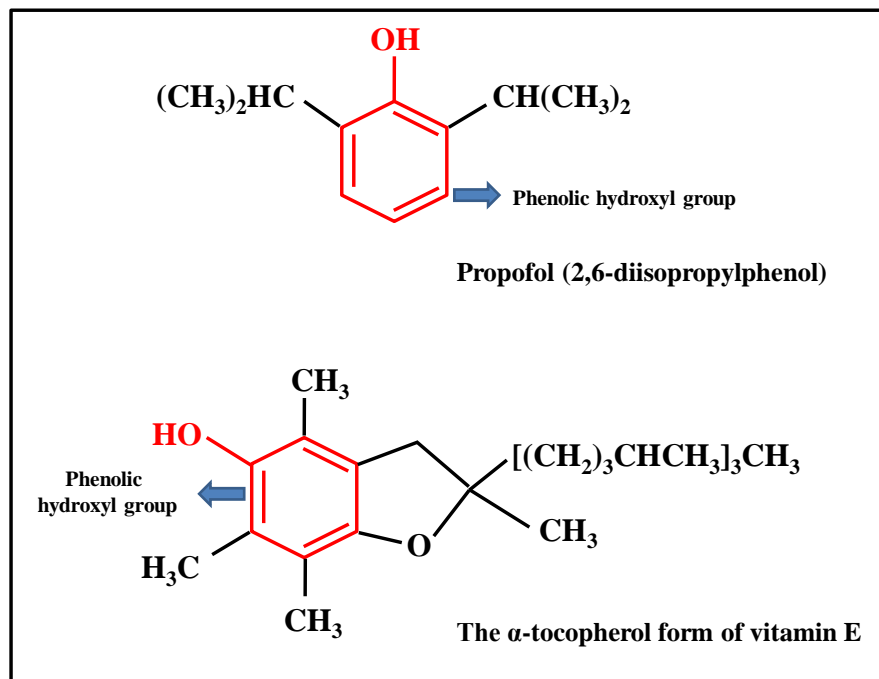


Figure 3: Chemical structure of propofol and the α-tocopherol form of vitamin E.

Propofol has been shown to protect neuronal cells from hypoxia/reoxygenation induced damage as well (Vasileiou et al. 2009). Yamaguchi et al. reported that propofol protects CA1 pyramidal cells from death by attenuating the generation of malondialdehyde which is a marker of lipid peroxidation (Yamaguchi et al. 2000). Other studies showed that propofol reduces the brain electric activity and consumption of ATP by activating GABA<sub>A</sub> receptors (Concas et al. 1991, Orser et al. 1994, Williams et al. 2002).

Nevertheless, the detailed antioxidative and neuroprotective mechanisms of propofol are a wide spectrum of opinions and still under discussion.

#### 1.4. Cellular antioxidant mechanisms

The increase of reactive oxygen species is usually accompanied by an activation of the antioxidant defense system (Droge 2002, Ferreira et al. 2004, Vassalle et al. 2008). Cellular peroxidases such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) are the most important components of the cellular antioxidant system (Fig.4).

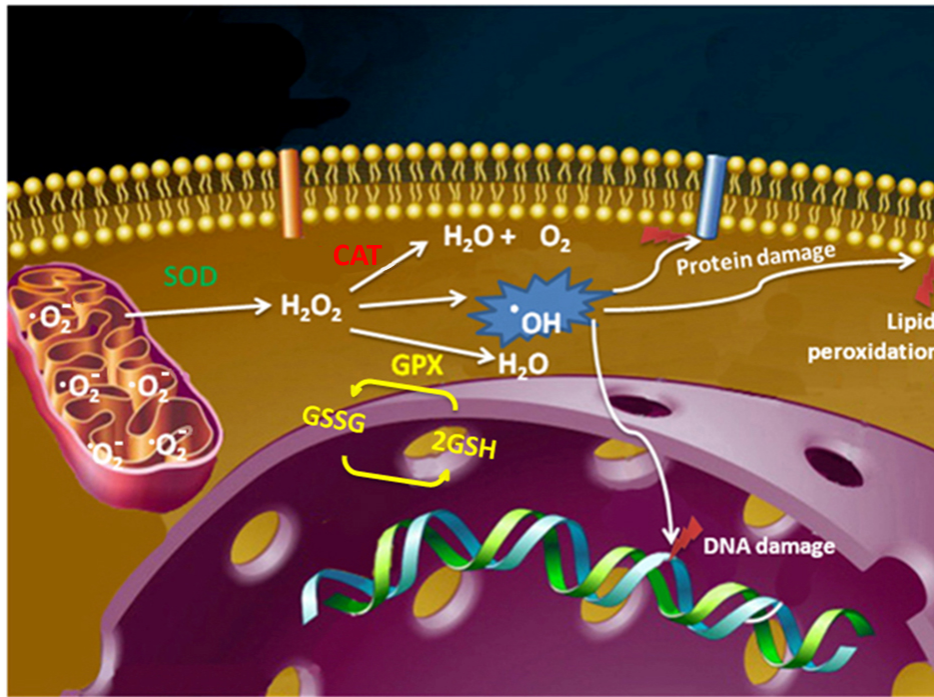


Figure 4: Cellular enzymatic antioxidant system: the superoxide anion ( $\cdot\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the hydroxyl radical ( $\cdot\text{OH}$ ) cause damage to membranes, proteins and DNA. Dismutation of superoxide anion generated in mitochondria is catalyzed by superoxide dismutase (SOD). The produced hydrogen peroxide is converted to water and oxygen by glutathione peroxidase (GPX) and catalase (CAT). Taken and modified from van Golen et al. 2012.

Superoxide dismutase is one of the major intracellular antioxidant enzymes in animals, plants and in the human system. Superoxide dismutase is regarded as the scavenger of intracellular reactive oxygen species. According to the metal cofactors, superoxide dismutase can be classified into three groups: Cu/Zn-SOD, Mn-SOD and Fe-SOD (Mandryk et al. 2007).

Glutathione peroxidase is an enzyme with peroxidase activity, which also plays an important role in protecting cells from oxidative stress. Glutathione peroxidase catalyzes the conversion of glutathione (GSH) to glutathione disulfide (GSSG) and converts toxic peroxides into non-toxic hydroxyl compounds simultaneously (Williams et al. 1998).

Catalase accounts for 40% of the total amount of peroxidases and is one of the most efficient enzymes described so far. One molecule of catalase catalyzes the conversion of millions of molecules of hydrogen peroxide to water and oxygen per second. (Putnam et al. 2000).

Besides the above mentioned cellular enzymatic antioxidant system, non-enzymatic system also prevents cells from oxidative stress. Members of these natural antioxidants are vitamin A, C, E and bilirubin (Fang et al. 2002).

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## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Oligonucleotides

Oligonucleotides were synthesized by Metabion (Martinsried, Germany)

Oligonucleotide	Sequence	Annealing temperature
Catalase For	5'-AGCTGGTTAATGCAAATGGG-3'	55°C
Catalase Rev	5'-TCTGTTCCCTCATTCAGCAC-3'	
Bax For	5'-GGGCCCTTTTGCTTCAGGGGA-3'	62°C
Bax Rev	5'-CTGGGGGCCTCAGCCCATCT-3'	
Bcl-2 For	5'-AGCACCGCTTGGTGTGGCTC-3'	62°C
Bcl-2 Rev	5'-GGCACTTGTGGCGGCCTGAT-3'	
Caspase-3 For	5'-AGTGCTCGCAGCTCATACCT-3'	55°C
Caspase-3 Rev	5'-TCAACCTTGTCGGCATACTG-3'	
18S rRNA For	5'-GTTGGTGGAGCGATTTGTCTGG-3'	58°C
18S rRNA Rev	5'-AGGGCAGGGACTTAATCAACGC-3'	

#### 2.1.2. Enzymes

Enzyme	Manufacturer
Catalase	Sigma-Aldrich, Schnelldorf, Germany
Glucose oxidase	Sigma-Aldrich, Schnelldorf, Germany
DNA Taq Polymerase	Solis BioDyne, Tartu, Estonia

#### 2.1.3. Antibodies

Antibody	Manufacturer
Anti-catalase IgG (1:15,000)	Abcam, Cambridge, UK
Anti-caspase-3 IgG (1:1,000)	Santa Cruz, Heidelberg, Germany
$\beta$ -actin (1:200)	Santa Cruz, Heidelberg, Germany
Anti-rabbit IgG-HRP (1:10,000)	Dako, Hamburg, Germany
Anti-goat IgG-HRP (1:2,000)	Santa Cruz, Heidelberg, Germany

#### 2.1.4. Kits and assays

Kit or assay	Manufacturer
Cytotoxicity detection kit	Roche, Mannheim, Germany
cDNA reverse transcription kit	Applied Biosystems, California, USA

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Qiagen RNeasy mini kit	Qiagen GmbH, Hilden, Germany
QuantiChrom™ peroxide assay kit	BioAssay Systems, Hayward, USA
Roti®-Quant assay	Carl Roth GmbH, Karlsruhe, Germany
ECL kit	Amersham Pharmacia Biotech, Piscataway, USA

### 2.1.5. Cells

For propagation, human neuroblastoma cells IMR-32 (LGC Standards, Teddington, UK) were cultured in RPMI-1640 medium containing 20% fetal calf serum, 4mM L-glutamine (Seromed, Berlin, Germany), 1% non-essential amino acid and 0.1mg/ml gentamycine. For experiments, cells were cultured in DMEM/F12 medium with 1% fetal calf serum and 0.1mg/ml gentamycine. Cells were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub> atmosphere.

### 2.1.6. Chemicals and reagents

Chemical or reagent	Manufacturer
Agarose	Carl Roth GmbH, Karlsruhe, Germany
APS	Carl Roth GmbH, Karlsruhe, Germany
Acrylamide	Carl Roth GmbH, Karlsruhe, Germany
Acetic acid	Carl Roth GmbH, Karlsruhe, Germany
Bromophenol blue	Sigma-Aldrich, Schnelldorf, Germany
BSA	New England BioLabs, Frankfurt, Germany
Buffer B 10 X	Solis BioDyne, Tartu, Estonia
Collagen A	Biochorm, Berlin, Germany
DMEM/F12 medium	PAA, Coelbe, Germany
DMSO	Carl Roth GmbH, Karlsruhe, Germany
DEPC water	Carl Roth GmbH, Karlsruhe, Germany
DMEM red-free medium	Gibco, Carlsbad, California, USA
dNTPs	Solis BioDyne, Tartu, Estonia
EDTA	Bioproducts, Ingelheim, Germany
FCS	PAA, Coelbe, Germany
Gentamycine	PAA, Coelbe, Germany
Glycerol	Sigma-Aldrich, Schnelldorf, Germany
Glycine	Sigma-Aldrich, Schnelldorf, Germany
HCl	Carl Roth GmbH, Karlsruhe, Germany
L-glutamine	PAA, Coelbe, Germany

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Methanol	Sigma-Aldrich, Schnellendorf, Germany
MgCl <sub>2</sub>	Solis BioDyne, Tartu, Estonia
NP-40	Sigma-Aldrich, Schnellendorf, Germany
Non-essential amino acids	PAA, Coelbe, Germany
PBS	PAA, Coelbe, Germany
Propofol	Sigma-Aldrich, Schnellendorf, Germany
Phosphatase inhibitor cocktail tablets	Roche, Mannheim, Germany
Protease inhibitor cocktail tablets	Roche, Mannheim, Germany
RPMI-1640 medium	PAA, Coelbe, Germany
Roti®-safe gel stain	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride	Sigma-Aldrich, Schnellendorf, Germany
Sodium deoxycholate	Sigma-Aldrich, Schnellendorf, Germany
Sodium azide	Sigma-Aldrich, Schnellendorf, Germany
SDS	Sigma-Aldrich, Schnellendorf, Germany
Starting block™ T20 blocking buffer	Thermo, Rockford, USA
Trypsin/EDTA	Bioproducts, Ingelheim, Germany
Trypan-blue	Sigma-Aldrich, Schnellendorf, Germany
Triton X -100	Sigma-Aldrich, Schnellendorf, Germany
Tris-HCl	Sigma-Aldrich, Schnellendorf, Germany
TEMED	Sigma-Aldrich, Schnellendorf, Germany
Tween 20	Roche, Mannheim, Germany
Tris	Carl Roth GmbH, Karlsruhe, Germany
β-mercaptoethanol	Carl Roth GmbH, Karlsruhe, Germany
2'7'-dichlorodihydrofluorescein diacetate	Sigma-Aldrich, Schnellendorf, Germany
6 X DNA loading dye	Fermentas Inc., Maryland, USA
DNA ladder	Fermentas Inc., Maryland, USA

### 2.1.7. Solutions

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4 X Laemmli buffer: 2.5ml 1M Tris pH 6.8, 0.8g SDS, 2ml β-mercaptoethanol, 4ml glycerol, 0.1ml 1% bromophenol blue, filled up to 10ml with DD water.

Resolving gel buffer: 45.42g Tris, 1g SDS, filled up to 250ml with DD water, pH 8.8.

Stacking gel buffer: 15.14g Tris, 1g SDS, filled up to 250ml with DD water, pH 6.8.

Running buffer: 12.2g Tris, 57.6g glycine, 4g SDS, filled up to 4L with DD water.

Transfer buffer: 28.6g glycine, 24.2g Tris, 0.4L methanol, filled up to 2.4L with DD water.

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TBST buffer: 6.06g Tris, 20.25g sodium chloride, 5ml 0.5M EDTA, 2.5ml Tween 20, filled up to 2.5L with DD water, pH 7.5.

50 X TAE buffer: 242g Tris, 57.1ml acetic acid, 100ml 0.5M EDTA, filled up to 1L with DD water, pH 8.0.

RIPA buffer: 4.38g sodium chloride, 5ml NP-40, 3.03g Tris, 5g sodium deoxycholate, 5ml 10% SDS stock solution, filled up to 500ml with DD water, pH 7.6.

Phosphatase inhibitor solution: 1 tablet of phosphatase inhibitor, 1ml DD water, filtered before use.

Protease inhibitor solution: 1 tablet of protease inhibitor, 2ml DD water, filtered before use.

RIPA complete buffer: 5ml phosphatase inhibitor solution, 4ml protease inhibitor solution, filled up to 50ml with RIPA buffer.

### 2.1.8. Consumables

Cell culture flasks, 96-well plates and falcon tubes were purchased from Greiner bio-one GmbH (Frickenhausen, Germany); eppendorf tubes and pipette tips were purchased from Eppendorf (Cologne, Germany); serological pipettes were purchased from Sarstedt (Nümbrecht, Germany); PVDF membranes and Hyperfilm ECL were purchased from Amersham Pharmacia Biotech (Piscataway, USA); nitrocellulose membranes were purchased from Sigma-Aldrich (Schnellendorf, Germany).

### 2.1.9. Instruments

<u>Instrument</u>	<u>Manufacturer</u>
Thermocycler T Gradient	Biometra, Göttingen, Germany
Darkroom cabinet Evo III	Raytest, Straubenhardt, Germany
Electrophoresis chamber 40-1214	Peqlab, Erlangen, Germany
Incubator Heraeus	Kendro, Hanau, Germany
Tissue culture incubator Hera Cell	Kendro, Hanau, Germany
Orbital Shaking Incubator S150	Bibby Sterilin, Staffordshire, UK
Photometer Genesys	ThermoSpectronic, Germany
Centrifuge Sorvall Super T21	Kendro, Hanau, Germany
Centrifuge Biofuge fresco	Kendro, Hanau, Germany
Rotator Intelli-Mixer	Neo-lab, Heidelberg, Germany
Mini-Trans-BlotCell	Bio-Rad, München, Germany
Tissue oxygen pressure monitor	Integra, Plainsboro, USA
Bright field microscope	Leica, DMIL, Germany



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ELISA reader	Tecan, Crailsheim, Austria
Fluorescence ELISA reader (Genios FL)	Tecan, Crailsheim, Austria
Bandelin SONOREX RK 106S	Schalltec, Mörfelden-Walldorf, Germany
Eppendorf single-channel pipettes	Eppendorf AG, Hamburg, Germany
Handy step repeating pipette	Brand GmbH, Wertheim, Germany
Charge-coupled device camera	Raytest GmbH, Straubenhardt, Germany

### 2.1.10. Software

XnView v1.95.4 was used to take pictures from cells. Images of RT-PCR and western blotting experiments were analyzed with ImageJ (v1.41o, NIH). ELISA reader and fluorescence ELISA reader were combined with the Magellan software v1.1. Statistics were performed using GraphPad Prism version 5.01 for Windows.

## 2.2. Methods

### 2.2.1. Experimental setting

In vitro hypoxia was induced by our recently described enzymatic system (Zitta et al. 2012a, Zitta et al. 2012b, Zitta et al. 2010a, Zitta et al. 2010b) in combination with nitrocellulose membranes (Huang et al. 2012, Zitta et al. 2010b). Membranes were co-incubated with culture medium for 60min. During this time, partial pressure of oxygen ( $pO_2$ ) rapidly decreases to levels lower than 5mmHg which is defined as hypoxia (Wilensky et al. 2005). During hypoxia, culture flasks were additionally filled with nitrogen and closed tightly.  $PO_2$  was measured by a tissue oxygen pressure monitor (LICOX® CMP Oxygen Catheter; Integra) (Fig.5).

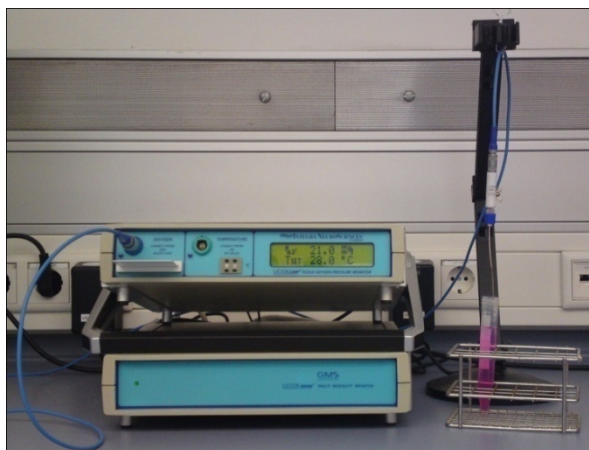


Figure 5:  $PO_2$  was measured by a tissue oxygen pressure monitor LICOX® CMP Oxygen Catheter.

Cells were maintained in hypoxic medium for 2h. Hypoxia was terminated by the addition of fresh oxygenated culture medium. This step represents the reoxygenation phase in vivo. All molecular, biochemical and cellular analyses were performed with samples taken during the reoxygenation phase (Fig.6). Stimulations were performed with anaesthetic concentrations of propofol (30 $\mu$ M) dissolved in DMSO. Briefly, a stock solution I of 250mM propofol was prepared in DMSO and diluted 1:100 with the respective cell culture medium to achieve a 2.5mM propofol stock solution II. The working solution of propofol (30 $\mu$ M) was then prepared by diluting the stock solution II 1:83 with the respective cell culture medium. Therefore, the final concentration of DMSO in the working solution was 0.012%. Controls were performed by adding DMSO at a final concentration of 0.012%, which does not affect gene expression, cell proliferation or apoptosis (Adler et al. 2006, Nishimura et al. 2008, Santos et al. 2003).

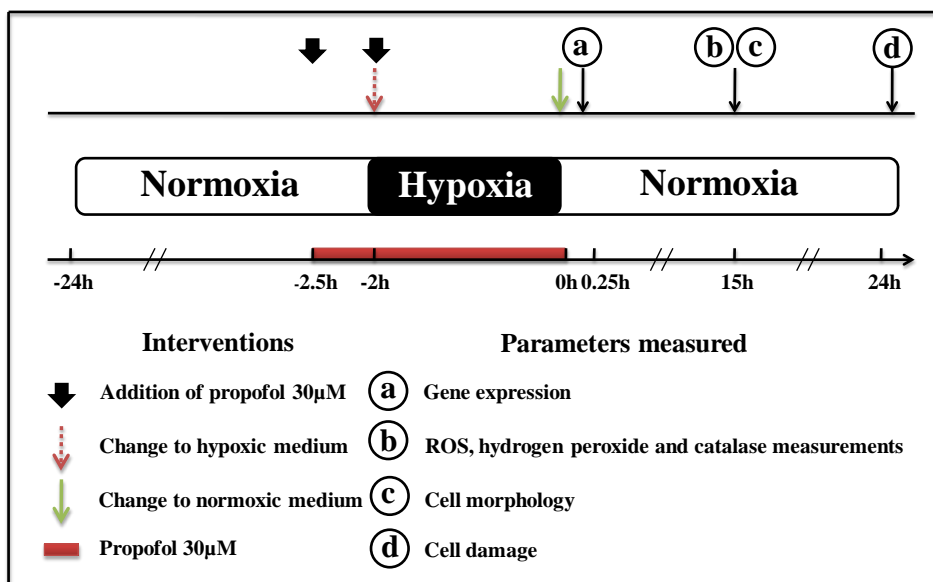


Figure 6: Experimental setting and time frame.

### 2.2.2. Cell culture

IMR-32 neuroblastoma cells were propagated in RPMI-1640 medium supplemented with 20% fetal calf serum, 4mM L-glutamine, 1% non-essential amino acid and 0.1mg/ml gentamycine. As high concentrations of fetal calf serum may affect the LDH and hydrogen peroxide measurements, DMEM/F12 medium containing 1% fetal calf serum was used for all experiments. Cells were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Confluent cells were detached by trypsinization (0.025% trypsin-1mM EDTA) at 37°C. Cell numbers were evaluated

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with a hemocytometer and viability was assayed by the trypan-blue dye exclusion technique. Cell morphology was evaluated by bright field microscopy.

### **2.2.3. Reverse transcription polymerase chain reaction (RT-PCR)**

#### **2.2.3.1. RNA extraction**

Cells were washed once with pre-warmed PBS before RNA isolation, lysed in 600 $\mu$ l RLT buffer and then well mixed with 600 $\mu$ l ethanol. The Qiagen RNeasy mini kit in combination with the manufacturer's protocol was employed to isolate RNA. For measuring the concentration, isolated RNA was diluted 100 times in DD water.

#### **2.2.3.2. Reverse transcription**

A spectrophotometer was used to measure the RNA concentrations. The purity of the isolated RNA was evaluated by the ratio of 260/280nm. 200ng RNA diluted in 10 $\mu$ l DEPC water was transcribed to cDNA using a reverse transcription kit with random hexamer primers. 20 $\mu$ l reaction mix was prepared as follows: 2 $\mu$ l RT buffer 10 X, 0.8 $\mu$ l 100mM dNTPs Mix, 2 $\mu$ l random primers 10 X, 1 $\mu$ l 50U/ $\mu$ l reverse transcriptase, 1 $\mu$ l 20U/ $\mu$ l RNase inhibitor, 3.2 $\mu$ l DEPC water, 200ng RNA diluted in 10 $\mu$ l DEPC water.

The reverse transcription program was set as follows:

10min	25 °C
2h	37 °C
5s	85 °C
30s	72 °C
$\infty$	4 °C

Transcribed samples were stored at -20 °C until use.

#### **2.2.3.3. Polymerase chain reaction**

2 $\mu$ l cDNA was used for the polymerase-chain reaction. 1 $\mu$ l of forward and reverse primers were used for specific fragment amplification. 20 $\mu$ l reaction mixture was prepared as follows: 2 $\mu$ l Buffer B 10 X, 1 $\mu$ l 16mM dNTPs Mix, 3 $\mu$ l 5mg/ml BSA, 1.6 $\mu$ l 25mM MgCl<sub>2</sub>, 0.5 $\mu$ l 5U/ $\mu$ l Taq Polymerase, 7.9 $\mu$ l DEPC water, 2 $\mu$ l cDNA, 1 $\mu$ l forward primer and 1 $\mu$ l reverse primer.

The amplification was performed in a thermocycler using the following program:

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	15min	95 °C
40 cycles:	30s	95 °C
	30s	55-62 °C
	30s	72 °C
	10min	72 °C
	∞	4 °C

The PCR amplification products and DNA ladder were diluted in 6 X loading buffer and then separated on 2.5% agarose gels containing 0.005% Roti®-Safe gel stain. Bands were visualized using UV-transillumination. Images were taken with a charge-coupled device camera in combination with the Diana v1.6 program and analyzed with the ImageJ (v1.41o, NIH) software.

#### **2.2.4. Cytotoxicity assay**

LDH released into the medium was measured by a colorimetric cytotoxicity detection assay kit. Samples were prepared following the manufacturer's protocol. The culture medium was collected 24h after hypoxia and stored at -20°C. For evaluation of the total LDH, cells were lysed with 2% Triton X -100 and incubated for 15min at 37°C. LDH activity in culture medium without contacting to cells was used as background control. 100µl of each sample was transferred into 96-well plates and mixed with 100µl detection solution. Samples were measured in 96-well plates at 492nm using an ELISA reader in combination with the Magellan software v1.1.

#### **2.2.5. Western blotting**

##### **2.2.5.1. Total protein extraction and quantification**

300µl ice cold RIPA complete buffer was used to lyse the cells. The mixture was then sonicated for 15min on ice and centrifuged at 10,000 rpm for 10min at 4°C. The supernatant was collected for protein quantification using Roti®-Quant assays. 200µl prepared Roti®-Quant detection solution was mixed with the 50µl supernatant and incubated at room temperature for 5min. Samples were measured at 620nm using an ELISA reader. Absorbance was converted to protein concentration using Roti®-Quant assay standard curve (see appendix 7.1.).

##### **2.2.5.2. Electrophoresis**

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30µg total protein diluted in 7.5µl RIPA complete buffer was mixed with 2.5µl 4 X Laemmli buffer and heated for 3min at 95°C before loading. The mixture was then separated by 10% SDS-PAGE at constant 120V for 1.5h. SDS-Polyacrylamide gel (10%) preparation was performed as follows:

Stacking gel: 2.6ml stacking gel buffer, 1.13ml acrylamid, 6.19ml DD water, 60µl 10% APS, 30µl TEMED.

Resolving gel: 2.6ml resolving gel buffer, 2.5ml acrylamid, 4.81ml DD water, 60µl 10% APS, 30µl TEMED.

### **2.2.5.3. Transfer**

The proteins in the gels were transferred at constant 400mA for 1.5h to PVDF membranes. For activation, the PVDF membranes were maintained in methanol for 15s, rinsed in DD water and equilibrated in transfer buffer for at least 10min before use.

### **2.2.5.4. Blocking and antibody incubation**

After transfer, the PVDF membranes were incubated in blocking solution for 1h at room temperature, and then co-incubated with the appropriate dilutions of primary antibody over night at 4°C. Primary antibodies were directed against catalase (Abcam; 1:15,000), caspase-3 (Santa Cruz; 1:200) and β-actin (Santa Cruz; 1:1,000). Membranes were then washed in TBST buffer 3 times for 10min and incubated for 1h with the secondary HRP coupled- antibody, anti-rabbit (IgG-HRP) or rabbit anti-goat (IgG-HRP). After washing in TBST buffer, the membranes were finally incubated with ECL detection reagent for 5 min. For chemiluminescent detection, the membranes were exposed to autoradiography films in the dark. Relative intensities of the protein bands were analyzed by ImageJ (v1.410, NIH).

### **2.2.6. H<sub>2</sub>DCFDA assay**

10,000 cells per well were seeded into collagen-coated 96-well black plates. After being cultured for 72h, cells were washed with pre-warmed PBS and maintained in a phenol red-free medium with 1% fetal calf serum until 15h after hypoxia/reoxygenation. Intracellular reactive oxygen species were measured by adding 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) which is oxidized to fluorescent dichlorofluorescein (DCF) by intracellular reactive oxygen species at a final concentration of 10µM. DCF-loaded cells were then kept in the dark and were immediately placed in a fluorescence ELISA reader at 37°C. Fluorescence was measured at an excitation

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wavelength of 485 nm and an emission wavelength of 535nm. Data were obtained at 0min and 30min. The increase of fluorescence per well was calculated by the formula:  $(Ft30 - Ft0)/Ft0 * 100$ , where Ft30= fluorescence at 30 min and Ft0= fluorescence at 0 min (Wang et al. 1999).

### **2.2.7. Hydrogen peroxide assay**

Hydrogen peroxide levels were measured with a QuantiChrom™ peroxide assay kit. Supernatant from culture medium was collected 15h after hypoxia and stored at -20°C. Samples (20µl) were prepared in 96-well plates following the manufacturer's protocol. The culture medium that was not in contact with cells was used as background control. Absorbance was measured at 610nm using an ELISA reader in combination with the Magellan software v1.1. The hydrogen peroxide concentration standard curve is shown in the appendix (7.2.).

### **2.2.8. Catalase activity assay**

Cells were detached by a rubber policeman and then centrifuged at 2,000rpm for 10 minutes at room temperature. The cell pellet was homogenized on ice in 300µl cold PBS containing 1mM EDTA and then centrifuged at 10,000rpm for 15min at 4°C. The supernatant was removed and the protein concentration was quantified with a Roti®-Quant assay. Samples were stored at -80°C. Catalase activity was estimated with the QuantiChrom™ peroxide assay kit by measuring the hydrogen peroxide left over in each sample. Briefly, catalase was dissolved in water and the following standards were prepared: 50 Units/ml, 25 Units/ml, 12.5 Units/ml, 6.25 Units/ml, 3.125 Units/ml, 1.563 Units/ml and 0 Units/ml. 20µl of each sample containing 1.25µg protein and catalase standards were transferred to 96-well plates for measuring. To start the reaction, 20µl of hydrogen peroxide (150µM) solution was added to each well followed by a 10min incubation at room temperature. For the detection of catalase activity, 200µl detection reagent (prepared as described by the manufacturer's protocol) was added to each well. Plates were incubated at room temperature for 30min and absorbance was read at 610nm using an ELISA reader (Tecan). Catalase activity in the samples was calculated from the standard curve (see appendix 7.3.).

### **2.2.9. Statistics**

All experiments were independently performed 3-4 times using at least duplicated samples. Statistics were performed with the statistic software GraphPad Prism version 5.01 for Windows. For normalization, all data were transformed using  $\arcsin\sqrt{X}$  to allow the subsequent use of parametric test. Data were then analysed by one-way analysis of variance, and in cases of

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significant differences, adjusted for multiple comparisons (Tukey). Variables are expressed as means  $\pm$  S.D.

### 3. Results

#### 3.1. Induction of in vitro hypoxia

Employing the two-enzyme hypoxia system and the previously described experimental setting, partial pressure of oxygen ( $pO_2$ ) in the culture medium was reduced to levels below 5 mmHg within 10 min and could be maintained at steady-state levels for up to at least 4 h (Fig.7).

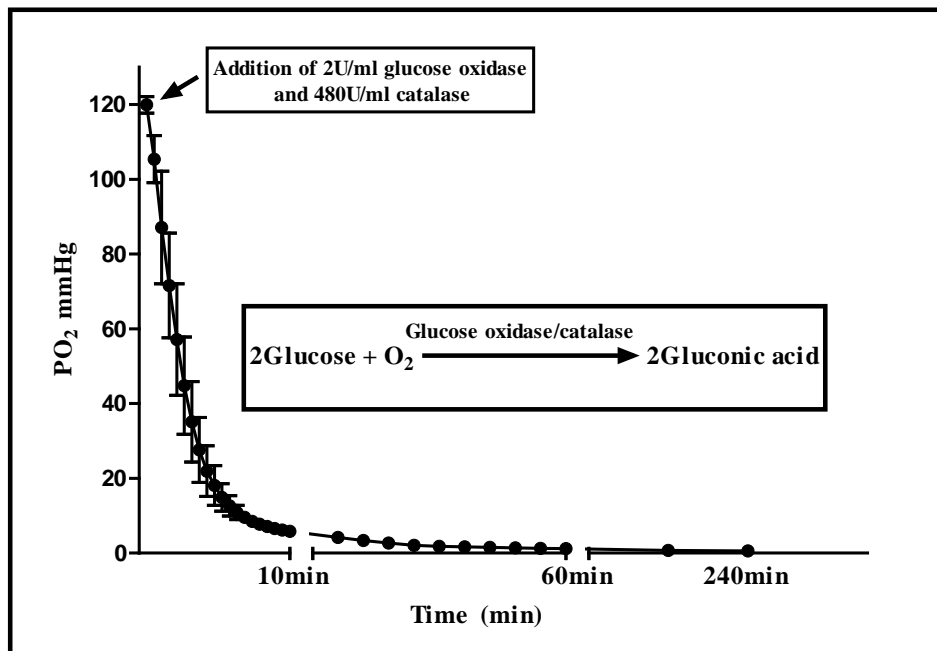


Figure 7: Induction of in vitro hypoxia. Partial pressure of oxygen ( $pO_2$ ) is rapidly reduced in the culture medium utilizing the two-enzyme hypoxia system and kept at a steady-state level for up to 4 h. Bars denote S.D.

#### 3.2. Effects of propofol on hypoxia induced cell damage

RT-PCR results showed that addition of propofol significantly reduced the mRNA expression of caspase-3 (hypoxia:  $0.23 \pm 0.08$  a.u.; hypoxia + propofol:  $0.04 \pm 0.05$  a.u.;  $P < 0.05$ ) whereas the expression of bax and bcl-2 was not significantly influenced by the addition of propofol (Fig.8 A). Although gene expression of caspase-3 was regulated by propofol, evaluation of protein levels of inactive pro-caspase-3 and active caspase-3 did not show any differences among the groups (pro-caspase-3 hypoxia:  $1.42 \pm 0.04$  a.u., pro-caspase-3 hypoxia + propofol:  $1.51 \pm 0.12$  a.u. pro-caspase-3 normoxia control:  $1.47 \pm 0.14$  a.u.; active-caspase-3 hypoxia:  $0.64 \pm 0.08$  a.u., active-caspase-3 hypoxia + propofol:  $0.64 \pm 0.05$  a.u., active-caspase-3 normoxia control:  $0.57 \pm 0.04$  a.u.;  $P > 0.05$ ; Fig.8 B). Nevertheless, morphologically, IMR-32 cells that received propofol before and during hypoxia showed reduced signs of cell damage such as cell rounding and detachment of the growth surface (Fig.8 C). This observation was also confirmed by the evaluation of released LDH as a marker for cell damage. Hypoxia increased the release of LDH



(hypoxia:  $0.78 \pm 0.21$  a.u., normoxia control:  $0.39 \pm 0.07$  a.u.;  $P < 0.05$ ) and addition of propofol reduced the hypoxia-mediated cell damage (hypoxia + propofol:  $0.44 \pm 0.17$  a.u.;  $P < 0.05$  vs hypoxia; Fig.8 D).

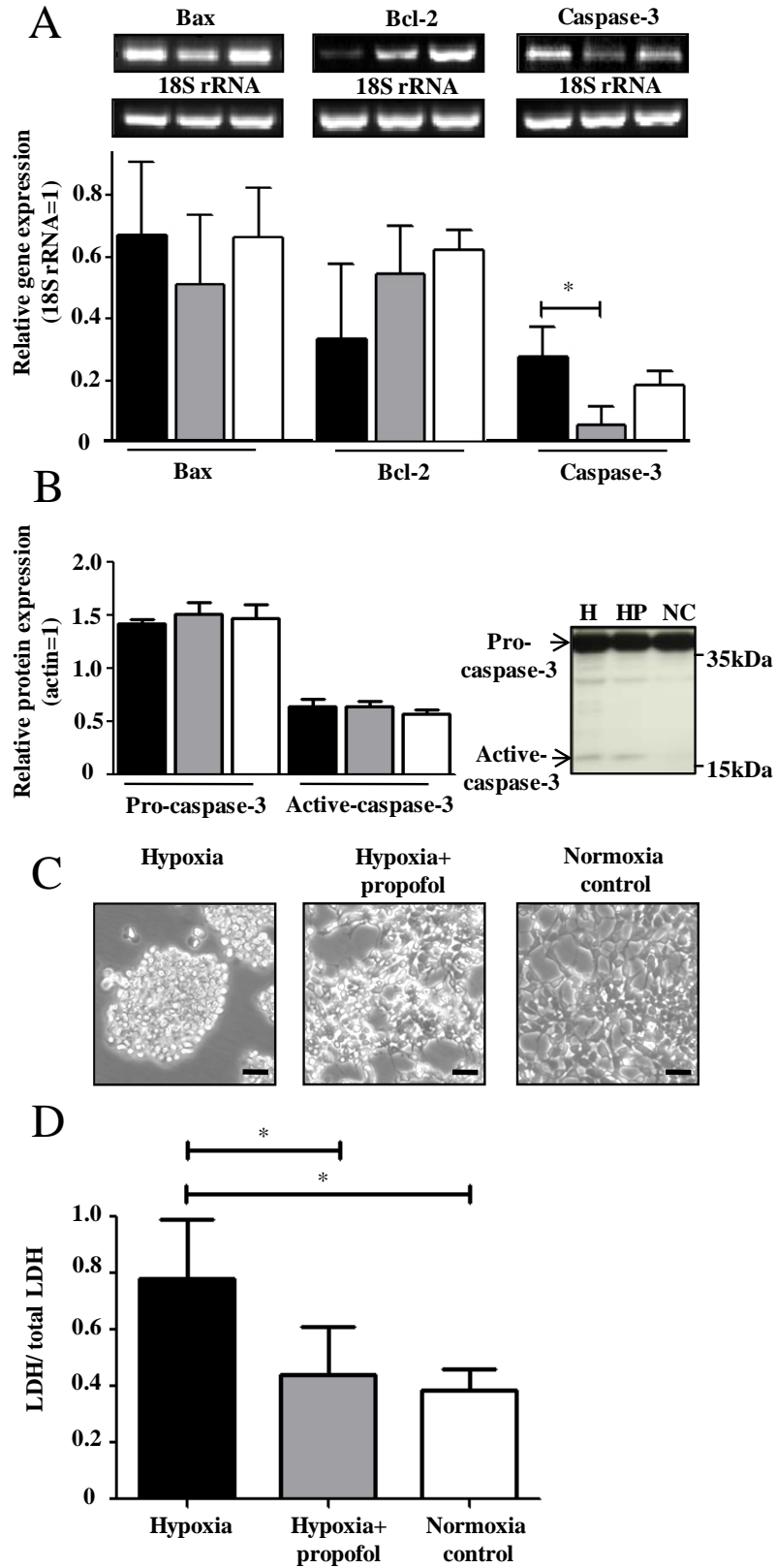


Figure 8: Effects of hypoxia and propofol on gene expression and cell damage. (A) Gene expression of bax, bcl-2 and caspase-3. Images of one representative PCR experiment are shown above the columns. (B) Protein expression of pro-caspase-3 and active-caspase-3. No significant differences are observed among the groups. One representative western blot is shown on the right. (C) Morphological signs of cell damage (e.g. cell shrinking, rounding and detachment from the growth surface) are observed 15h after hypoxia and reduced by the addition of propofol. (D) Lactate dehydrogenase (LDH) assays show that cell damage is increased by hypoxia and attenuated after addition of propofol. Bars in the photomicrographs denote 50 $\mu$ m. Columns show the mean of 3 independent experiments. Bars denote S.D.; \*P<0.05; H,  $\blacksquare$  = hypoxia; HP,  $\blacksquare$  = hypoxia+propofol; NC,  $\square$  = normoxia control. Taken from: Huang et al., 2012.

### 3.3. Effects of propofol on hypoxia induced generation of reactive oxygen species

H<sub>2</sub>DCFDA assays were performed to evaluate the influence of hypoxia and propofol on the generation of intracellular reactive oxygen species. Hypoxia increased intracellular reactive oxygen species (hypoxia: 267.70 $\pm$ 28.91 a.u., normoxia control: 206.60 $\pm$ 27.99 a.u.; P<0.01) and addition of propofol significantly reduced the generation of reactive oxygen species (hypoxia + propofol: 234.00 $\pm$ 33.39 a.u.; P<0.05 vs hypoxia Fig.9).

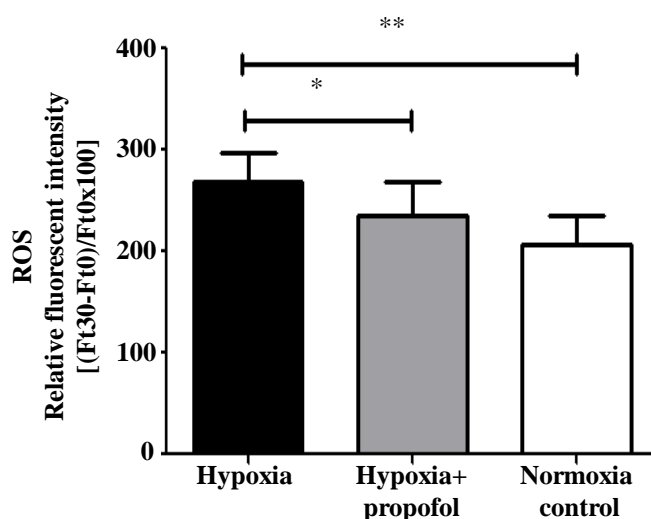


Figure 9: Production of reactive oxygen species. Intracellular reactive oxygen species were measured using 2',7'-dichlorodihydrofluorescein diacetate which is cleaved by esterases and is oxidized to fluorescent dichlorofluorescein (DCF). Propofol reduces the hypoxia induced increase of reactive oxygen species. Ft0 = fluorescence at 0min, Ft30 = fluorescence at 30min. Columns show the mean of 4 independent experiments. Bars denote S.D.; \* P<0.05, \*\* P<0.01. Taken from: Huang et al., 2012.

### 3.4. Effects of propofol on hypoxia induced generation of hydrogen peroxide

Hydrogen peroxide assays were used to measure the release of hydrogen peroxide into the culture medium. The concentrations of hydrogen peroxide in the culture medium were significantly increased 15h after hypoxia (hypoxia: 17.38 $\pm$ 2.70  $\mu$ M, normoxia control: 1.68 $\pm$ 0.14

$\mu\text{M}$ ;  $P < 0.001$ ) while addition of propofol resulted in diminished levels of hydrogen peroxide (hypoxia + propofol:  $13.22 \pm 1.29 \mu\text{M}$ ;  $P < 0.05$  vs hypoxia; Fig.10).

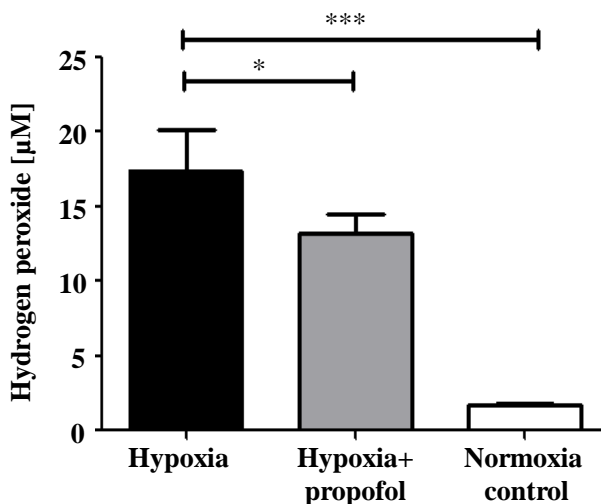


Figure 10: Release of hydrogen peroxide into the culture medium. Propofol attenuates the hypoxia induced increase of hydrogen peroxide. Columns show the mean of 3 independent experiments. Bars denote S.D.; \*  $P < 0.05$ , \*\*\*  $P < 0.001$ . Taken from: Huang et al., 2012.

### 3.5. Effects of propofol on hypoxia regulated expression and activity of catalase

Catalase regulates the decomposition of hydrogen peroxide to water and oxygen and therefore plays a central role in hydrogen peroxide metabolism. Gene expression of catalase was slightly reduced by the addition of propofol (hypoxia:  $0.20 \pm 0.04$  a.u., hypoxia+propofol:  $0.11 \pm 0.12$  a.u.;  $P > 0.05$ ; Fig.11 A). Western blotting results showed increased amounts of catalase under hypoxic conditions (hypoxia:  $1.29 \pm 0.04$  a.u., normoxia control:  $0.92 \pm 0.04$  a.u.;  $P < 0.001$ ) and addition of propofol decreased the amount of catalase protein (hypoxia + propofol:  $1.04 \pm 0.03$  a.u.;  $P < 0.01$  vs hypoxia; Fig.11 B). Interestingly, measurements of catalase activity revealed a statistically significant increase in the enzymatic activity after addition of propofol (hypoxia:  $2.45 \pm 0.46$  U/ml, hypoxia+propofol:  $5.17 \pm 1.68$  U/ml;  $P < 0.05$ ; Fig.11 C).

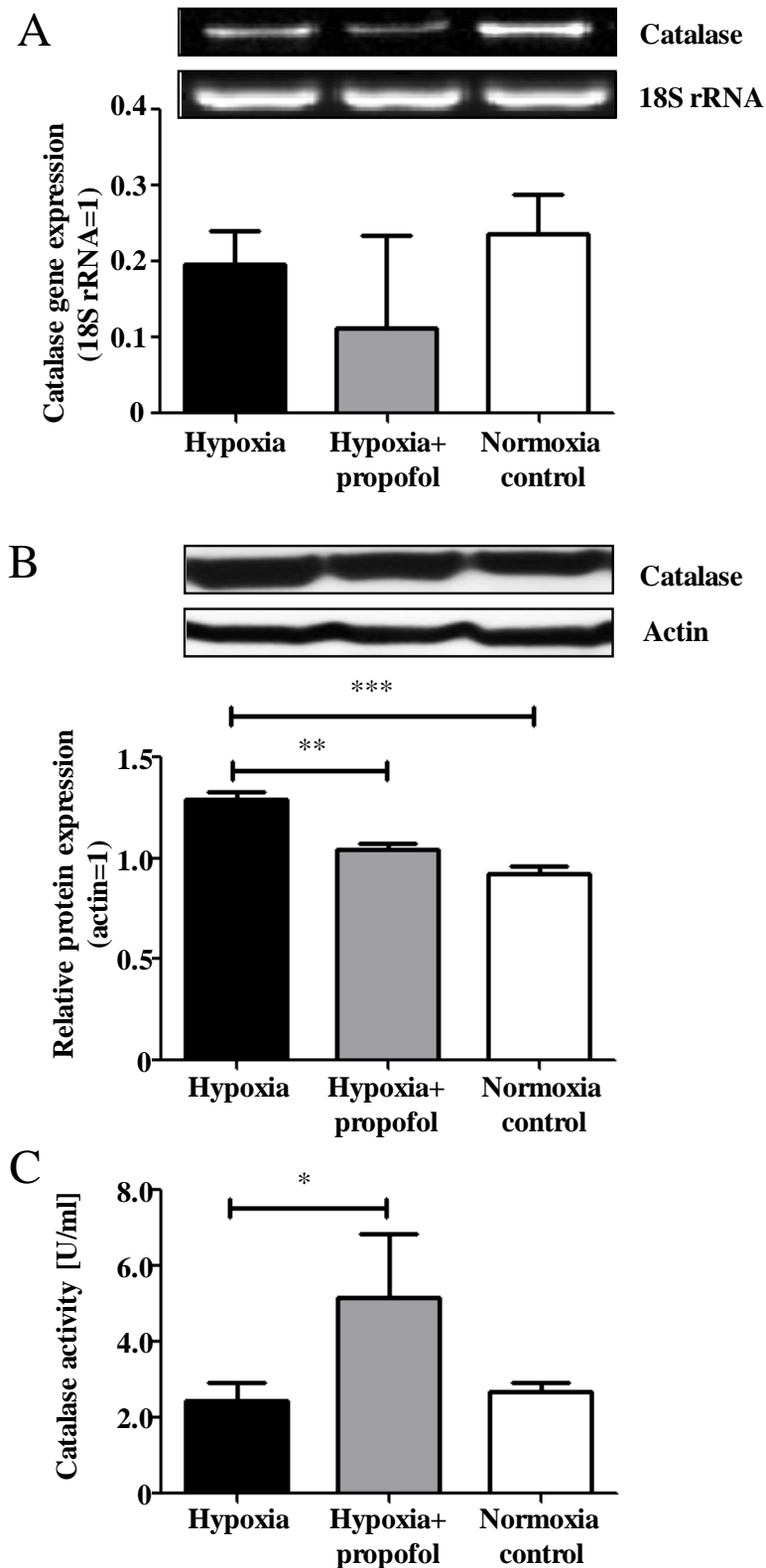


Figure 11: Gene expression, protein expression and activity of catalase. (A) Catalase gene expression was evaluated by RT-PCR. (B) Catalase protein levels were quantified by western blotting 15h after hypoxia and normoxia, respectively. Compared to hypoxia, the amount of catalase protein is reduced after addition of propofol. (C) Intracellular catalase activity was evaluated by catalase assays. Addition of propofol increases the enzymatic activity of catalase in IMR-32 cells. Columns show the mean of 3 independent experiments. Bars denote S.D.; \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . Taken from: Huang et al., 2012.

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## **4. Discussion**

### **4.1. Induction of in vitro hypoxia**

Cellular models have been proven to be an efficient way to study hypoxia/reoxygenation injury (Watkins et al. 1995). There are several ways to induce oxygen deficiency in cell culture systems. Some researchers utilized sodium dithionite (Huang et al. 2009, Zheng et al. 2003) to reduce the levels of dissolved oxygen in culture medium. However, addition of sodium dithionite causes an exothermic reaction which generates sodium sulfite and toxic sulfur dioxide resulting in serious side effects during the induction of hypoxia. Cobalt chloride was used to induce hypoxia in cell culture as well (Law et al. 2012, Saxena et al. 2012). However, cobalt is a heavy metal and therefore predisposes tissue and cells to poisoning. One of the most commonly used systems for reducing oxygen levels in cell culture is the hypoxic chamber which utilizes a sealed chamber with an adjustable gas mixture system composed mostly of 95% N<sub>2</sub> and 5% CO<sub>2</sub> (Lievre et al. 2000). Nevertheless, a disadvantage of this hypoxic chamber is that the generation of hypoxia is a very slow process which can last up to 24h (Allen et al. 2001). Moreover, as the hypoxic chamber is a sealed system, interventions are difficult to perform.

In contrast to the hypoxic chamber, in the enzymatic system employed here, stable hypoxia is established rapidly within minutes, and terminated by the addition of fresh oxygenated culture medium ((Zitta et al. 2012a, Zitta et al. 2012b, Zitta et al. 2010a, Zitta et al. 2010b). Moreover, glucose is gradually and physiologically depleted from the medium, which leads to glucose deficiency as it is also observed under ischemic conditions in vivo. Therefore, the two-enzyme hypoxia system is an ideal model to investigate mechanisms that are associated with clinical hypoxia/reoxygenation injury such as stroke, myocardial infarction and cerebral or intestinal ischemia.

### **4.2. Effects of propofol on hypoxia induced cell damage**

Hypoxia and reoxygenation are a crucial events in physiological as well as pathophysiological processes and numerous clinical conditions are associated with hypoxia /reoxygenation (Li et al. 2002). Especially in the brain, intra- or peri-operative ischemia can lead to hypoxia/reoxygenation injury which is associated with severe neuronal cell damage (Dominguez et al. 2007, Gazzolo et al. 2009). Propofol, a short-acting intravenous anaesthetic, has been shown to protect neuronal cells from hypoxia/reoxygenation injury. Nevertheless, the detailed cellular and molecular mechanisms of the protective effects of propofol are still under

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discussion.

In the study presented, hypoxia significantly increased the damage of IMR-32 cells (LDH measurements, cell morphology), but did not change the activity of caspase-3 which is a key effector molecule in apoptosis (Riedl et al. 2004). Anaesthetic doses of propofol (30 $\mu$ M; Spelina et al. 1986, Turtle et al. 1987) decreased the hypoxia /reoxygenation induced cell damage without attenuating caspase-3 activity. Similar results have been obtained by Tu et al. who investigated the effects of propofol on neuropathogenesis in newborn rats under hypoxic conditions and showed that neither propofol nor hypoxia did influence apoptosis (Tu et al. 2011). Employing the neuroblastoma cell line SH-SY5Y, Wu et al. demonstrated that 10 $\mu$ M propofol improved cell proliferation and inhibited dynorphin A induced cytotoxicity (Wu et al. 2011), an observation that is in concordance with our in vitro results obtained with 30 $\mu$ M propofol. Also other authors have shown protective effects of propofol in different experimental systems (Acquaviva et al. 2004, Ishii et al. 2002, Scapagnini et al. 2002, Wang et al. 2002).

#### **4.3. Effects of propofol on hypoxia induced generation of reactive oxygen species and hydrogen peroxide**

Generation of reactive oxygen species is a significant component in hypoxia/reoxygenation induced cerebral damage (Olmez et al. 2012). According to the previous studies, propofol has been shown to be not only an efficient neuroprotective reagent but also a potent antioxidant.

Several studies propose that the rise in intracellular reactive oxygen species is due to a loss of the mitochondrial membrane potential ( $\Psi_m$ ) by the absence of oxygen. Under physiological conditions, the mitochondrial membrane is polarized and has a  $\Psi_m$ , which is maintained by ATP and proton gradients across the membrane. These gradients are in turn dependent on oxygen availability. Maintenance of  $\Psi_m$  keeps proteins such as cytochrome C and reactive oxygen species within the confines of the mitochondria. Periods of hypoxia on the other hand disturb the proton gradients and have the potential to reduce  $\Psi_m$  which in turn results in an increase of reactive oxygen species in the cytoplasmic compartment (Li et al. 2002, Millar et al. 2007).

Hypoxia/reoxygenation injury is associated with a generation of reactive oxygen species which have various mostly deleterious effects on the affected tissue (Chi et al. 2010, Razorenova 2012, Tan et al. 1999). Several authors suggested that cerebral hypoxia/reoxygenation leads to cytotoxic damage and that these events can be attenuated by the antioxidant activity of propofol (Budic et al. 2010, De La Cruz et al. 1998). In our study, we showed that compared to normoxic

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conditions, reactive oxygen species and hydrogen peroxide concentrations are increased under hypoxia and that this effect is significantly attenuated by the addition of propofol. Similar results were shown by Hsing et al. who used CM-H<sub>2</sub>DCFDA assays to investigate intracellular reactive oxygen species and demonstrated that anaesthetic doses of propofol had anti-inflammatory effects by regulating reactive oxygen species mediated PKB and NF- $\kappa$ B signaling in vitro and in vivo (Hsing et al. 2011).

#### **4.4. Effects of propofol on hypoxia regulated expression and activity of catalase**

Although our findings confirm the above-mentioned studies suggesting antioxidant properties of propofol being at least partly responsible for the observed neuroprotective effects, they do not verify whether direct antioxidant effects of propofol mediated by its phenolic chemical structure, indirect actions of propofol (e.g. induction of an antioxidant response in the target cells) or a combination of both are responsible for the antioxidant activity of the anaesthetic.

Under physiological conditions, cytoplasmic reactive oxygen species are scavenged by SOD, GPX, CAT as well as other small molecular antioxidants, including glutathione, ascorbic acid and  $\alpha$ -tocopherol. These endogenous antioxidants are unable to cope with the excess of reactive oxygen species generated during hypoxia/reoxygenation, resulting in an increased oxidation of macromolecules (e.g. lipids, proteins and nucleic acids) and cell damage (Sugawara et al. 2003).

Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen and may therefore be a potential candidate for indirect target cell mediated cytoprotective effects of propofol (Giorgio et al. 2007). Our western blotting experiments showed a reduction of the amount of catalase protein in IMR-32 cells after the addition of propofol under hypoxic conditions. Nevertheless, the activity measurements revealed an increased catalase activity in the cells. These results point towards a possible augmentation of the catalytic function by direct interactions of the anaesthetic with the catalase molecule.

Another explanation for our findings might be based on the fact that the catalase activity assay employed in this study uses hydrogen peroxide as substrate and may therefore also detect other hydrogen peroxide degrading peroxidases. Propofol may indeed increase the activities of other hydrogen peroxide degrading enzymes. This has also been demonstrated by groups that detected elevated activities of glutathione peroxidase in serum and plasma of pigs and adult patients during propofol anaesthesia (Abou-Elenain 2010, Allaouchiche et al. 2001).

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## 5. Summary

**Background:** Propofol, a widely used intravenous anaesthetic agent, has been shown to protect neuronal cells from hypoxia/reoxygenation injury. However, the underlying mechanisms of these neuroprotective effects are still unclear but may be related to reactive oxygen species which are produced during hypoxia/reoxygenation induced injury. Aim of the study was to investigate the molecular and cellular effects of propofol on hypoxia-induced cell-damage employing the neuronal cell line IMR-32.

**Results:** Hypoxia led to morphological signs of cell damage and increased the release of LDH (hypoxia:  $0.78 \pm 0.21$  a.u., normoxia:  $0.39 \pm 0.07$  a.u.;  $P < 0.05$ ). Addition of propofol significantly reduced the hypoxia induced LDH release (hypoxia + propofol:  $0.44 \pm 0.17$  a.u.;  $P < 0.05$  vs hypoxia), diminished morphological signs of cell damage but did not influence protein levels of caspase-3. Reactive oxygen species were increased under hypoxic conditions and significantly reduced by the addition of propofol (hypoxia:  $267.70 \pm 28.91$  a.u., normoxia:  $206.60 \pm 27.99$  a.u.;  $P < 0.01$ ; hypoxia + propofol:  $234.00 \pm 33.39$  a.u.;  $P < 0.05$  vs hypoxia). Addition of propofol also decreased hydrogen peroxide levels (hypoxia:  $17.38 \pm 2.70$   $\mu$ M, hypoxia + propofol:  $13.22 \pm 1.29$   $\mu$ M;  $P < 0.05$ ). Western blotting experiments showed increased amounts of catalase under hypoxic conditions (hypoxia:  $1.29 \pm 0.04$  a.u., normoxia:  $0.92 \pm 0.04$  a.u.;  $P < 0.001$ ). Addition of propofol decreased the amount of catalase (hypoxia + propofol:  $1.04 \pm 0.03$  a.u.;  $P < 0.01$  vs hypoxia) but increased enzymatic activity of the protein (hypoxia:  $2.45 \pm 0.46$  U/ml, hypoxia + propofol  $5.17 \pm 1.68$  U/ml;  $P < 0.05$ ).

**Conclusion:** We suggest that propofol protects neuronal cells from hypoxia/reoxygenation injury and that the associated events may besides direct antioxidant actions of propofol also be due to the capability of the anaesthetic to induce cellular antioxidant mechanisms.



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## 6. References

- Abou-Elenain K. (2010) Study of the systemic and pulmonary oxidative stress status during exposure to propofol and sevoflurane anaesthesia during thoracic surgery. *Eur J Anaesthesiol.* Jun;27(6):566-71.
- Acquaviva R, Campisi A, Murabito P, Raciti G, Avola R, Mangiameli S, Musumeci I, Barcellona ML, Vanella A, Li Volti G. (2004) Propofol attenuates peroxynitrite-mediated DNA damage and apoptosis in cultured astrocytes: an alternative protective mechanism. *Anesthesiology.* Dec;101(6):1363-71.
- Adler S, Pellizzer C, Paparella M, Hartung T, Bremer S. (2006) The effects of solvents on embryonic stem cell differentiation. *Toxicol In Vitro.* Apr;20(3):265-71.
- Allaouchiche B, Debon R, Goudable J, Chassard D, Duflo F. (2001) Oxidative stress status during exposure to propofol, sevoflurane and desflurane. *Anesth Analg.* Oct;93(4):981-5.
- Allen CB, Schneider BK, White CW. (2001) Limitations to oxygen diffusion and equilibration in in vitro cell exposure systems in hyperoxia and hypoxia. *Am J Physiol Lung Cell Mol Physiol.* Oct;281(4):L1021-7.
- Ansley DM, Lee J, Godin DV, Garnett ME, Qayumi AK. (1998) Propofol enhances red cell antioxidant capacity in swine and humans. *Can J Anaesth.* Mar;45(3):233-9.
- Baumann RP, Penketh PG, Seow HA, Shyam K, Sartorelli AC. (2008) Generation of oxygen deficiency in cell culture using a two-enzyme system to evaluate agents targeting hypoxic tumor cells. *Radiat Res.* Nov;170(5):651-60.
- Budic I, Pavlovic D, Cvetkovic T, Djordjevic N, Simic D, Milojevic I, Stojanovic M. (2010) The effects of different anesthesia techniques on free radical production after tourniquet-induced ischemia-reperfusion injury at children's age. *Vojnosanit Pregl.* Aug;67(8):659-64.
- Cazevieille C, Muller A, Meynier F, Bonne C. (1993) Superoxide and nitric oxide cooperation in hypoxia/reoxygenation-induced neuron injury. *Free Radic Biol Med.* Apr;14(4):389-95.
- Chi AY, Waypa GB, Mungai PT, Schumacker PT. (2010) Prolonged hypoxia increases ROS signaling and RhoA activation in pulmonary artery smooth muscle and endothelial cells. *Antioxid Redox Signal.* Mar 1;12(5):603-10.
- Concas A, Santoro G, Serra M, Sanna E, Biggio G. (1991) Neurochemical action of the general anaesthetic propofol on the chloride ion channel coupled with GABAA receptors. *Brain Res.* Mar 1;542(2):225-32.
- De La Cruz JP, Villalobos MA, Seden G, Sanchez De La Cuesta F. (1998) Effect of propofol on oxidative stress in an in vitro model of anoxia-reoxygenation in the rat brain. *Brain Res.* Jul 27;800(1):136-44.
- Dominguez TE, Wernovsky G, Gaynor JW. (2007) Cause and prevention of central nervous system injury in neonates undergoing cardiac surgery. *Semin Thorac Cardiovasc Surg.* Fall;19(3):269-77.

---

Droge W. (2002) Free radicals in the physiological control of cell function. *Physiol Rev.* Jan;82(1):47-95.

Elahi MM, Kong YX, Matata BM. (2009) Oxidative stress as a mediator of cardiovascular disease. *OxidMedCellLongev.* Nov-Dec;2(5):259-69.

Fabiano G, Pezzolla A, Filograna MA, Ferrarese F. (2008) [Traumatic shock--physiopathologic aspects]. *G Chir.* Jan-Feb;29(1-2):51-7.

Fang YZ, Yang S, Wu G. (2002) Free radicals, antioxidants, and nutrition. *Nutrition.* Oct;18(10):872-9.

Ferreira SM, Lerner SF, Brunzini R, Evelson PA, Llesuy SF. (2004) Oxidative stress markers in aqueous humor of glaucoma patients. *Am J Ophthalmol.* Jan;137(1):62-9.

Fucci L, Oliver CN, Coon MJ, Stadtman ER. (1983) Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implication in protein turnover and ageing. *Proc Natl Acad Sci U S A.* Mar;80(6):1521-5.

Gazzolo D, Abella R, Marinoni E, Di Iorio R, Li Volti G, Galvano F, Pongiglione G, Frigiola A, Bertino E, Florio P. (2009) Circulating biochemical markers of brain damage in infants complicated by ischemia reperfusion injury. *Cardiovasc Hematol Agents Med Chem.* Apr;7(2):108-26.

Giorgio M, Trinei M, Migliaccio E, Pelicci PG. (2007) Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat Rev Mol Cell Biol.* Sep;8(9):722-8.

Grune T, Reinheckel T, Davies KJ. (1997) Degradation of oxidized proteins in mammalian cells. *FASEB J.* Jun;11(7):526-34.

Hamm TF, Dorman RV. (1990) Arachidonic acid metabolism in gerbil cerebra: effects of ischemia and pentobarbital. *J Neurosci Res.* Aug;26(4):488-94.

Hsing CH, Lin MC, Choi PC, Huang WC, Kai JI, Tsai CC, Cheng YL, Hsieh CY, Wang CY, Chang YP, Chen YH, Chen CL, Lin CF. (2011) Anesthetic propofol reduces endotoxic inflammation by inhibiting reactive oxygen species-regulated Akt/IKKbeta/NF-kappaB signaling. *PLoS One.*6(3):e17598.

Huang L, Li Q, Li H, He Z, Cheng Z, Chen J, Guo L. (2009) Inhibition of intracellular Ca<sup>2+</sup> release by a Rho-kinase inhibitor for the treatment of ischemic damage in primary cultured rat hippocampal neurons. *Eur J Pharmacol.* Jan 14;602(2-3):238-44.

Huang Y, Zitta K, Bein B, Scholz J, Steinfath M, Albrecht M. (2012) Effect of propofol on hypoxia re-oxygenation induced neuronal cell damage in vitro\*. *Anaesthesia.* Oct 22. doi: 10.1111/j.1365-2044.2012.07336.x. [Epub ahead of print]

Humphries KM, Szweda LI. (1998) Selective inactivation of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase: reaction of lipoic acid with 4-hydroxy-2-nonenal. *Biochemistry.* Nov 10;37(45):15835-41.

Ishii H, Arai T, Segawa H, Morikawa S, Inubushi T, Fukuda K. (2002) Effects of propofol on lactate accumulation and oedema formation in focal cerebral ischaemia in hyperglycaemic rats.

---

Br J Anaesth. Mar;88(3):412-7.

Jambunathan N. (2010) Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. *Methods Mol Biol.*639:292-8.

Law PC, Auyeung KK, Chan LY, Ko JK. (2012) Astragalus saponins downregulate vascular endothelial growth factor under cobalt chloride-stimulated hypoxia in colon cancer cells. *BMC Complement Altern Med.* Sep 19;12(1):160.

Levinson RM, Shure D, Moser KM. (1986) Reperfusion pulmonary edema after pulmonary artery thromboendarterectomy. *Am Rev Respir Dis.* Dec;134(6):1241-5.

Li C, Jackson RM. (2002) Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *American journal of physiology.* Feb;282(2):C227-41.

Lievre V, Becuwe P, Bianchi A, Koziel V, Franck P, Schroeder H, Nabet P, Dauca M, Daval JL. (2000) Free radical production and changes in superoxide dismutases associated with hypoxia/reoxygenation-induced apoptosis of embryonic rat forebrain neurons in culture. *Free Radic Biol Med.* Dec 15;29(12):1291-301.

Lim DC, Veasey SC. (2010) Neural injury in sleep apnea. *Curr Neurol Neurosci Rep.* Jan;10(1):47-52.

Mandryk S, Lushchak OV, Semchyshyn HM, Lushchak VI. (2007) [Role of Cu, Zn- and Mn-containing superoxide dismutases during the yeast *Saccharomyces cerevisiae* growing on ethanol and glycerol]. *Mikrobiol Z.* Mar-Apr;69(2):35-42.

McCord JM. (1985) Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med.* Jan 17;312(3):159-63.

Millar TM, Phan V, Tibbles LA. (2007) ROS generation in endothelial hypoxia and reoxygenation stimulates MAP kinase signaling and kinase-dependent neutrophil recruitment. *Free radical biology & medicine.* Apr 15;42(8):1165-77.

Mizukami Y, Kobayashi S, Uberall F, Hellbert K, Kobayashi N, Yoshida K. (2000) Nuclear mitogen-activated protein kinase activation by protein kinase  $\zeta$  during reoxygenation after ischemic hypoxia. *J Biol Chem.* Jun 30;275(26):19921-7.

Mohsenzadegan M, Mirshafiey A. (2012) The immunopathogenic role of reactive oxygen species in Alzheimer disease. *Iran J Allergy Asthma Immunol.* Sep;11(3):203-16.

Mueller S, Millonig G, Waite GN. (2009) The GOX/CAT system: a novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture. *Adv Med Sci.*54(2):121-35.

Nishimura M, Nikawa T, Kawano Y, Nakayama M, Ikeda M. (2008) Effects of dimethyl sulfoxide and dexamethasone on mRNA expression of housekeeping genes in cultures of C2C12 myotubes. *Biochemical and biophysical research communications.* Mar 14;367(3):603-8.

Obrenovitch TP, Garofalo O, Harris RJ, Bordi L, Ono M, Momma F, Bachelard HS, Symon L. (1988) Brain tissue concentrations of ATP, phosphocreatine, lactate, and tissue pH in relation to reduced cerebral blood flow following experimental acute middle cerebral artery occlusion. *J*

---

Cereb Blood Flow Metab. Dec;8(6):866-74.

Olmez I, Ozyurt H. (2012) Reactive oxygen species and ischemic cerebrovascular disease. *Neurochem Int.* Jan;60(2):208-12.

Orser BA, Wang LY, Pennefather PS, MacDonald JF. (1994) Propofol modulates activation and desensitization of GABAA receptors in cultured murine hippocampal neurons. *J Neurosci.* Dec;14(12):7747-60.

Putnam CD, Arvai AS, Bourne Y, Tainer JA. (2000) Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. *J Mol Biol.* Feb 11;296(1):295-309.

Razorenova OV. (2012) Brain and muscle ARNT-like protein BMAL1 regulates ROS homeostasis and senescence: A possible link to hypoxia-inducible factor-mediated pathway. *Cell Cycle.* Jan 15;11(2):213.

Riedl SJ, Shi Y. (2004) Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol.* Nov;5(11):897-907.

Safar P. (1986) Cerebral resuscitation after cardiac arrest: a review. *Circulation.* Dec;74(6 Pt 2):IV138-53.

Santos NC, Figueira-Coelho J, Martins-Silva J, Saldanha C. (2003) Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochemical pharmacology.* Apr 1;65(7):1035-41.

Saxena S, Shukla D, Bansal A. (2012) Augmentation of aerobic respiration and mitochondrial biogenesis in skeletal muscle by hypoxia preconditioning with cobalt chloride. *Toxicol Appl Pharmacol.* Sep 8.

Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. (2002) Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. *Mol Pharmacol.* Mar;61(3):554-61.

Schlaepfer WW. (1987) Neurofilaments: structure, metabolism and implications in disease. *J Neuropathol Exp Neurol.* Mar;46(2):117-29.

Semenza GL. (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol.* Apr;88(4):1474-80.

Sies H. (1993) Damage to plasmid DNA by singlet oxygen and its protection. *Mutat Res.* May;299(3-4):183-91.

Spelina KR, Coates DP, Monk CR, Prys-Roberts C, Norley I, Turtle MJ. (1986) Dose requirements of propofol by infusion during nitrous oxide anaesthesia in man. I: Patients premedicated with morphine sulphate. *Br J Anaesth.* Oct;58(10):1080-4.

Sugawara T, Chan PH. (2003) Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. *Antioxidants & redox signaling.* Oct;5(5):597-607.

Tabner BJ, Turnbull S, El-Agnaf O, Allsop D. (2001) Production of reactive oxygen species

---

from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr Top Med Chem*. Dec;1(6):507-17.

Tan S, Wood M, Maher P. (1998) Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J Neurochem*. Jul;71(1):95-105.

Tan S, Zhou F, Nielsen VG, Wang Z, Gladson CL, Parks DA. (1999) Increased injury following intermittent fetal hypoxia-reoxygenation is associated with increased free radical production in fetal rabbit brain. *J Neuropathol Exp Neurol*. Sep;58(9):972-81.

Torre-Bouscoulet L, Castorena-Maldonado A, Sada-Ovalle I, Meza-Vargas MS. (2008) [Mechanisms of cardiovascular damage in obstructive sleep apnea]. *Rev Invest Clin*. Nov-Dec;60(6):502-16.

Tu S, Wang X, Yang F, Chen B, Wu S, He W, Yuan X, Zhang H, Chen P, Wei G. (2011) Propofol induces neuronal apoptosis in infant rat brain under hypoxic conditions. *Brain Res Bull*. Aug 10;86(1-2):29-35.

Turtle MJ, Cullen P, Prys-Roberts C, Coates D, Monk CR, Faroqui MH. (1987) Dose requirements of propofol by infusion during nitrous oxide anaesthesia in man. II: Patients premedicated with lorazepam. *Br J Anaesth*. Mar;59(3):283-7.

van Golen RF, van Gulik TM, Heger M. (2012) Mechanistic overview of reactive species-induced degradation of the endothelial glycocalyx during hepatic ischemia/reperfusion injury. *Free Radic Biol Med*. Apr 15;52(8):1382-402.

Vasileiou I, Xanthos T, Koudouna E, Perrea D, Klonaris C, Katsargyris A, Papadimitriou L. (2009) Propofol: a review of its non-anaesthetic effects. *Eur J Pharmacol*. Mar 1;605(1-3):1-8.

Vassalle C, Pratali L, Boni C, Mercuri A, Ndreu R. (2008) An oxidative stress score as a combined measure of the pro-oxidant and anti-oxidant counterparts in patients with coronary artery disease. *Clin Biochem*. Oct;41(14-15):1162-7.

Wang H, Joseph JA. (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med*. Sep;27(5-6):612-6.

Wang J, Yang X, Camporesi CV, Yang Z, Bosco G, Chen C, Camporesi EM. (2002) Propofol reduces infarct size and striatal dopamine accumulation following transient middle cerebral artery occlusion: a microdialysis study. *Eur J Pharmacol*. Oct 11;452(3):303-8.

Wang Y, Zhang SX, Gozal D. (2010) Reactive oxygen species and the brain in sleep apnea. *Respir Physiol Neurobiol*. Dec 31;174(3):307-16.

Watkins MT, Haudenschild CC, al-Badawi H, Velazquez FR, Larson DM. (1995) Immediate responses of endothelial cells to hypoxia and reoxygenation: an in vitro model of cellular dysfunction. *Am J Physiol*. Feb;268(2 Pt 2):H749-58.

Wilensky EM, Bloom S, Leichter D, Verdiramo AM, Ledwith M, Stiefel M, LeRoux P, Grady MS. (2005) Brain tissue oxygen practice guidelines using the LICOX CMP monitoring system. *J Neurosci Nurs*. Oct;37(5):278-88.

Williams DB, Akabas MH. (2002) Structural evidence that propofol stabilizes different

---

GABA(A) receptor states at potentiating and activating concentrations. *J Neurosci.* Sep 1;22(17):7417-24.

Williams K, Frayne J, Hall L. (1998) Expression of extracellular glutathione peroxidase type 5 (GPX5) in the rat male reproductive tract. *Mol Hum Reprod.* Sep;4(9):841-8.

Wu GJ, Chen WF, Hung HC, Jean YH, Sung CS, Chakraborty C, Lee HP, Chen NF, Wen ZH. (2011) Effects of propofol on proliferation and anti-apoptosis of neuroblastoma SH-SY5Y cell line: new insights into neuroprotection. *Brain Res.* Apr 12;1384:42-50.

Yamaguchi S, Hamaguchi S, Mishio M, Okuda Y, Kitajima T. (2000) Propofol prevents lipid peroxidation following transient forebrain ischemia in gerbils. *Can J Anaesth.* Oct;47(10):1025-30.

Zheng S, Zuo Z. (2003) Isoflurane preconditioning reduces purkinje cell death in an in vitro model of rat cerebellar ischemia. *Neuroscience.* 118(1):99-106.

Zitta K, Meybohm P, Bein B, Heinrich C, Renner J, Cremer J, Steinfath M, Scholz J, Albrecht M. (2012a) Serum from patients undergoing remote ischemic preconditioning protects cultured human intestinal cells from hypoxia-induced damage: involvement of matrixmetalloproteinase-2 and -9. *Mol Med.* 18:29-37.

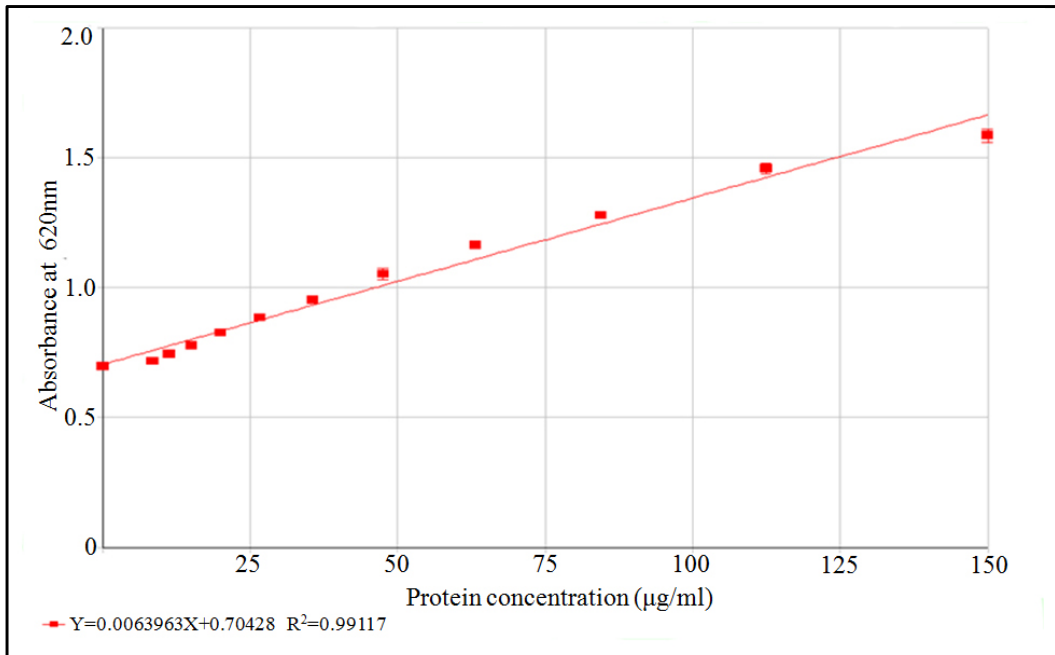
Zitta K, Meybohm P, Bein B, Huang Y, Heinrich C, Scholz J, Steinfath M, Albrecht M. (2012b) Salicylic acid induces apoptosis in colon carcinoma cells grown in-vitro: influence of oxygen and salicylic acid concentration. *Exp Cell Res.* Apr 15;318(7):828-34.

Zitta K, Meybohm P, Bein B, Ohnesorge H, Steinfath M, Scholz J, Albrecht M. (2010a) Cytoprotective effects of the volatile anesthetic sevoflurane are highly dependent on timing and duration of sevoflurane conditioning: findings from a human, in-vitro hypoxia model. *Eur J Pharmacol.* Oct 25;645(1-3):39-46.

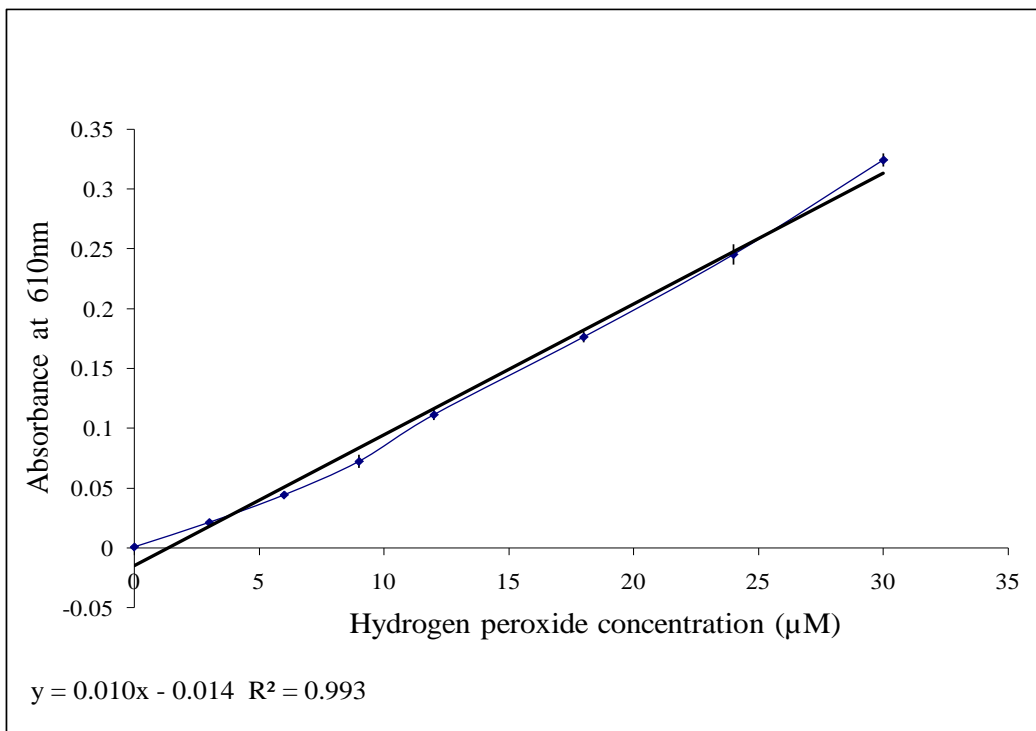
Zitta K, Meybohm P, Bein B, Rodde C, Steinfath M, Scholz J, Albrecht M. (2010b) Hypoxia-induced cell damage is reduced by mild hypothermia and postconditioning with catalase in-vitro: application of an enzyme based oxygen deficiency system. *Eur J Pharmacol.* Feb 25;628(1-3):11-8.

## 7. Appendix

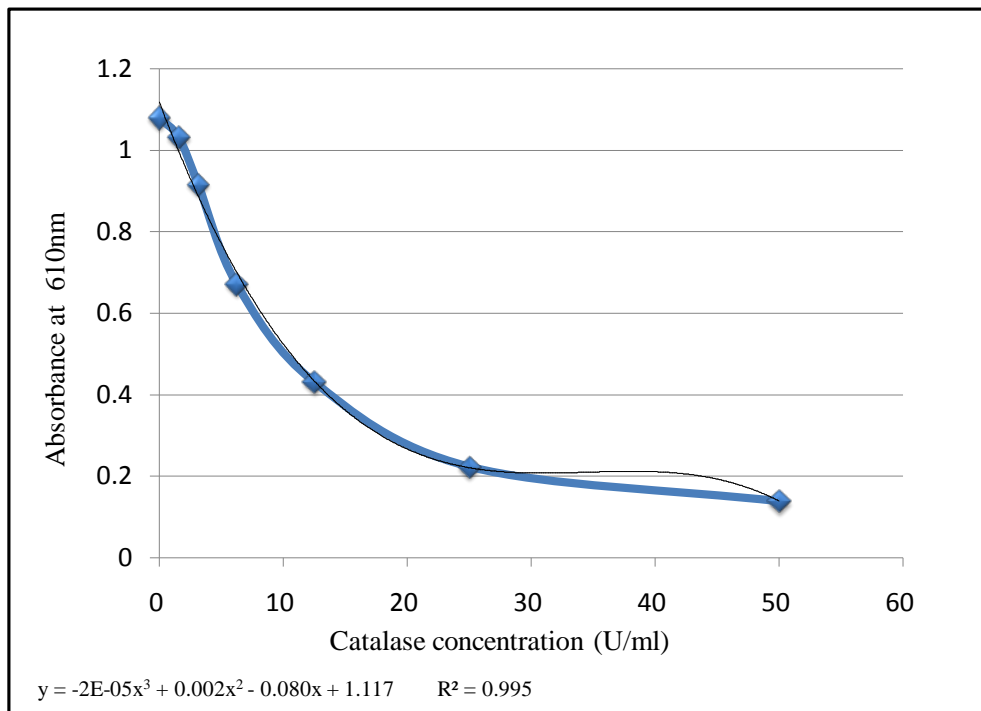
### 7.1. Standard curve of Roti®-Quant assays



### 7.2. Standard curve of hydrogen peroxide assays



7.3. *Standard curve of catalase activity assays*





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## 9. Curriculum vitae

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January 2006-July 2007	General internship, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan City, Hubei Province, PRC
July 2007-July 2009	Major internship, Department of Anaesthesiology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan City, Hubei Province, PRC
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## Publications

**Huang Y**, Zitta K, Bein B, Scholz J, Steinfath M, Albrecht M. (2012) Effects of propofol on hypoxia re-oxygenation induced neuronal cell damage in vitro. *Anaesthesia*. Oct 22. doi: 10.1111/j.1365-2044.2012.07336.x. [Epub ahead of print]

Zitta K, Meybohm P, Bein B, **Huang Y**, Heinrich C, Scholz J, Steinfath M, Albrecht M. (2012) Salicylic acid induces apoptosis in colon carcinoma cells grown in vitro: influence of oxygen and salicylic acid concentration. *Exp Cell Res*. Apr 15;318(7):828-34.

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