

Effect of Ala16Val Genetic Polymorphism of *MnSOD* on Antioxidant Capacity and Inflammatory Response in Open Heart Surgery

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Abstract. *Background:* Ischemia-reperfusion injury and inflammation in cardiac surgical patients involves complex humoral and cellular interactions. We investigated the effect of genetic polymorphism of manganese superoxide dismutase (*MnSOD*) a natural antioxidant, on cytokine release and manganese superoxide dismutase (*MnSOD*) were measured by enzyme linked immunoabsorbent assay (ELISA). *Results:* Baseline IL-6 did not differ between patients with different *MnSOD* genotypes. Postoperatively IL-6 levels were significantly higher in all patients but more significantly in V(VV+AV) carriers ($p=0.003$). The wild-type AA genotype had the highest preoperative ($p<0.05$) and postoperative IL-6 level. The *MnSOD* VV genotype was associated with significantly lower preoperative *MnSOD* levels compared to the AA carriers ($p<0.05$). *Conclusion:* These data demonstrate that *MnSOD* Ala16Val polymorphism influences IL-6 production and baseline *MnSOD* activity, suggesting that preoperative *MnSOD* concentration plays a role in cytokine release.

Open heart surgery has become a routine procedure worldwide. Despite many therapeutic strategies, the incidence of postoperative myocardial dysfunction and

related complications are still unacceptably high. The etiology of these side-effects is multifactorial and mostly related to ischemia reperfusion (IR) injury and systemic inflammatory response with secretion of cytokines and activation of leukocytes during cardiopulmonary bypass (CPB). These oxidative events result in the depletion of plasma antioxidants and increased lipid peroxidation. Superoxide dismutase (SOD) is a major enzymatic defense against oxidants formed in body. In humans, SOD is present in three different forms: cytosolic copper/zinc-SOD (Cu/Zn-SOD), mitochondrial manganese-SOD (*MnSOD*) and extracellular superoxide dismutase (EC-SOD). Of these, Mn-SOD plays a major role in the oxidant resistance of vital organs.

MnSOD is a nuclear-encoded protein that is transported, after translation, into mitochondria via an N-terminal signal sequence (1). The signal sequence is essential for correct transport activity of proteins by mitochondria (2). A polymorphism on the second exon of the *MnSOD* gene results in a alanine-to-valine change at amino acid position 16 (Ala16Val), also described as the -9 position of the *MnSOD*'s signal sequence (3). This polymorphism may change the structural conformation and mitochondrial transport of *MnSOD* (3,4); the alanine-containing protein showed normal transport and generated 30-40% more active enzyme than did the valine form of the enzyme (5).

Evidence is accumulating that genetic variations, or polymorphisms, can significantly affect an individual's response to adverse effects. To date many studies have demonstrated the time course of formation and nature of the reactive oxygen species (ROS) in cardiac surgery. As yet, no functional studies of the genotype polymorphisms have been performed in myocardial ischemia-reperfusion injury. Because of the evidence implicating *MnSOD* in the pathogenesis of oxidative stress, we investigated whether the polymorphisms in the "MnSOD" are related to the severity of ischemia reperfusion injury and cytokine release in patients undergoing CPB.

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Patients and Methods

Patient selection. After approval by the local Ethics Committee, 42 consecutive patients undergoing coronary artery bypass grafting (CABG) with CPB were enrolled in the study. Informed consent was obtained from each patient. Patients with recent myocardial infarction (less than 6 weeks), congestive heart failure, chronic renal failure and history of infection were excluded from the study. The preoperative and postoperative data were collected and the mean Euroscore were calculated. Euroscore is a method of calculating predicted operative mortality for patients undergoing cardiac surgery (6). The anesthetic and surgical technique used was in accordance with standard practice. Midazolam was used for premedication and the anesthetic agent consisted of a combination of fentanyl, midazolam and pancuronium. Anesthesia was maintained with midazolam and vecuronium infusion and with inhaled sevoflurane. Median sternotomy was used in all patients. CPB was performed with a Jostra roller pump system and Jostra Quadrox hollow-fiber oxygenator (Jostra GmbH, Hirrlingen Germany). Moderate hypothermia (32-34°C) and a pulsatile flow of 2.2 to 2.4 L/m² were used. Cold blood cardioplegia was used for myocardial protection with an initial dose of 15 mL/kg and repeated infusions of 300 mL every 20 minutes. Rewarming to a nasopharyngeal temperature greater than 34°C was achieved with a heat-exchange oxygenator and rewarming blanket. The left internal mammary artery and saphenous vein were preferred as bypass conduits in all patients. Aprotinin and steroids were not used.

For each patient, peripheral venous blood samples were drawn before the induction of general anesthesia (T1), during cardiopulmonary bypass after declamping the aorta (T2) and 24 hours after the operation (T3).

DNA isolation. Blood specimens were collected in tubes containing EDTA and DNA samples were extracted from whole blood with a salting-out procedure (7).

MnSOD Ala16Val genotyping. For amplification of the "MnSOD" Ala16Val polymorphism, the following primers (MBI Fermentas, Lithuania) were used: 5'-ACC AGC AGG CAG CTG GC GCC GG-3'; 5'- GCG TTG ATG TGA GGT TCC AG -3'.

For detection of "MnSOD" Ala16Val, 50-100 ng genomic DNA was amplified with 1x reaction buffer, 3 mM MgCl₂, 0.2 mM each dNTP, 0.2 μM each primer and Taq polymerase (MBI Fermentas) in a 25 μl reaction volume. The PCR conditions were: initial denaturation step at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 61°C for 1 min, 72°C for 2 min and 72°C for 7 min. PCR products were digested with PdiI restriction enzyme (MBI Fermentas) at 37°C overnight and electrophoresed on 3% agarose gels stained with ethidium bromide. Genotypes were determined as VV (107 bp), AV (18, 89, 107 bp) or AA (18, 89 bp) for the polymorphism (8).

Assay of plasma MnSOD and IL-6 levels. Blood samples drawn before surgery, during CPB and after CPB were immediately centrifuged (3000 rpm for 5 minutes) and plasma was separated and frozen at -20°C until plasma MnSOD (Chemicon Int, USA, Cat No: APT290) and IL-6 (Biosource Int, USA, Cat No: KHC0062) levels were determined by enzyme linked immunoassay. **Statistical analyses.** Statistical analyses were performed with the SPSS

for Windows 7.5 version (SPSS, Chicago, IL, USA). The Chi-square test was used to analyze the relationship between categorical data. Student's *t*-test was used to compare IL-6 and MnSOD levels and different outcomes between groups. The association between two continuous variables was determined using paired samples *t*-test. Continuous variables are given as mean±standard deviation of the mean (SD). Expected and observed frequencies of genotypes were compared with Chi-square test. On obtaining cell frequencies of less than 5, frequency analyses were performed with Fisher's exact test. A two-sided *p*-value less than 0.05 was considered as significant.

Results

The details of the study group and surgery are given in Table I. There were no significant differences between the patients concerning age, duration of CPB and aortic cross clamping. The comparison of the Euroscore within "A(AA+AV)" carriers and "V (VV+AV)" carriers were 3.85±84 versus 3.82±81 (*p*<0.05). No serious complications were observed in any of the included patients. Postoperative clinical data were similar in all patients except for the postoperative ventilation time and length of intensive care unit stay which were higher in "MnSOD" V allele carriers (*p*<0.001 and *p*<0.05, respectively).

Of the total 42 patients, the distribution of "MnSOD" Ala16Val genotypes was as follows: 7 AA (16.7%), 28 AV (66.7%) and 7 VV (16.7%), which corresponds to allele frequencies of 50 for A and 50 for V.

The difference between preoperative and postoperative IL-6 and MnSOD levels according to MnSOD polymorphisms are given in Figures 1 and 2. All *p* values were obtained after controlling for age, sex and body mass index.

Postoperative IL-6 (*p*=0.003) levels and MnSOD (*p*=0.04) levels in patients with the V allele (mutant type) were significantly higher compared to their preoperative levels (Figure 1A and 1B). Furthermore we observed that the "MnSOD" VV genotype was associated with significantly lower preoperative "MnSOD" levels compared to the AA genotype (*p*<0.05) (Figure 2A). Contrary to this, patients who have wild-type AA genotype had the highest preoperative (1.02±0.19 U (unit) in AA; 0.76±0.38 U in VV, *p*<0.05) and postoperative MnSOD (0.96±0.17 U in AA; 0.85±0.29 U in VV) level (Figure 2A and 2B).

These results indicate a reduced antioxidant capacity and severe inflammatory response in patients with MnSOD mutant "VV" genotype carriers.

Discussion

Ischemia-reperfusion injury is associated with an acute inflammatory response after cardiac surgery (9). Thrombin and tissue factors generated after such injury can contribute to activation of proteases which can also stimulate cytokine release. The influence of genetic variants affecting inflammatory response after cardiac surgery has been

Table I. Clinical parameters in patients according to "MnSOD" genotype.

Characteristics	MnSOD Ala16Val genotypes			
	AA+AV (n:35)	AA (n:7)	VV+AV (n:35)	VV (n:7)
Age (year)	59.91±9.38	66±10.61	58.69±8.86	59.86±10.55
Gender (female/male) (n)	7/28	3/4	6/29	2/5
BMI (kg/m ²)	28.55±3.85	28.66±3.66	28.24±4.13	27.12±4.90
Smoking (n/%)	23/65.7	6/85.7	20/57.1	3/42.9
Hypertension (n/%)	26/74.3	6/85.7	25/71.4	5/71.4
IABP (n/%)	2/5.7	-	2/5.7	-
CPB time (min)	90.06±30.49	87.14±23.68	90.86±31.12	91.14±28.08
ACC time (min)	49.83±24.08	52±17.54	48.43±24.42	45±19.74
Ventilation time (h)	6.81±1.79	5.78±0.80	7.62±2.21*	9.85±2.11**
Blood loss, 24 hours (ml)	968.51±374.75	935.43±337.85	969.14±388.58	938.57±417.38
Need for inotropic agents (n/%)	2/5.7	-	3/8.6	1/14.3
ICU stay (h)	38.84±29.14	27±9.91	40.34±29.45*	34.29±18.69

n: number of individuals; the results are shown as means± SD, *p<0.05, **p<0.001.

previously identified (10-12). The effect of genetics on ischemia reperfusion has not been established previously.

SODs are the most important antioxidant enzyme type for defence against ROS (13, 14). In particular, MnSOD, which is only available in the mitochondria, plays an important role in protecting cells from oxidative stress. It catalyzes the superoxide radicals in mitochondria to hydrogen peroxide and oxygen. The inhibition of MnSOD causes the accumulation of superoxide radicals and leads to damage of mitochondrial membranes.

The Val to Ala single amino acid change due to the single base transition (T-C) at the 16th position of the mitochondrial signal sequence of MnSOD results in a conformational change in the structure of the protein (3, 4). This change in the mitochondrial signal sequence of MnSOD may have an impact on the targeting (transportation) of this enzyme to the mitochondria (3). Previous studies on MnSOD polymorphisms showed that the presence of the Val allele affects the cellular distribution of MnSOD and results in inefficient function in mitochondria, which would decrease defense against superoxide radicals.

This study showed that the Ala16Val polymorphism in the MnSOD gene is related to a decrease in antioxidant capacity and an increase in cytokine release in patients after CPB. In our patients, no significant differences could be demonstrated in the clinical outcome except for the length of ventilation, which was significantly higher in V carriers. In this study, we showed that "MnSOD" Ala16Val polymorphism was associated with preoperative and postoperative MnSOD levels, which decreased in the order of the AA<AV<VV genotype in our study (Figure 2A and 2B). Patients with the wild-type AA genotype had the highest preoperative and postoperative MnSOD levels. Furthermore, it was observed

that patients with the MnSOD VV genotype had lower preoperative- and postoperative-MnSOD levels compared with MnSOD AA genotype, by 25,9% and 11,45% respectively. We observed that the MnSOD VV genotype was associated with significantly lower preoperative MnSOD levels compared to AA carriers (p<0.05).

This shows that the, antioxidant capacity in patients with the Val allele carriers is preoperatively reduced.

CPB causes a degree of lung injury, which is usually mild and not significant (15-17). Although this can be related to an increased cytokine release in those patients it is hard to prove a clear relation between the cytokine levels and clinical outcome. However, the human lung consists of an elaborate antioxidant system; lower levels of MnSOD activity, which reflects a decrease in antioxidant capacity, might be responsible for the prolonged ventilation found in our study. It is well known that the increased lipid peroxidation and substantial decrease in primary antioxidants during CPB results in increased pulmonary vascular permeability in postcardiopulmonary bypass patients.

Due to the small number of patients and their being relatively young and healthy, we did not observe any serious morbidity or mortality in our study group. However, the primary aim of the present study was not to address this issue. It would be beneficial to increase the number of patients and incorporate other inflammatory and anti-inflammatory cytokines into the study.

Perioperative care and risk profiling remains an important component of modern cardiac surgery. Patient responses to surgical stress and hemodynamic changes vary. Individual genetic variability in specific biological pathways during and after surgery can contribute to the development of postoperative adverse events. The identification of pre-

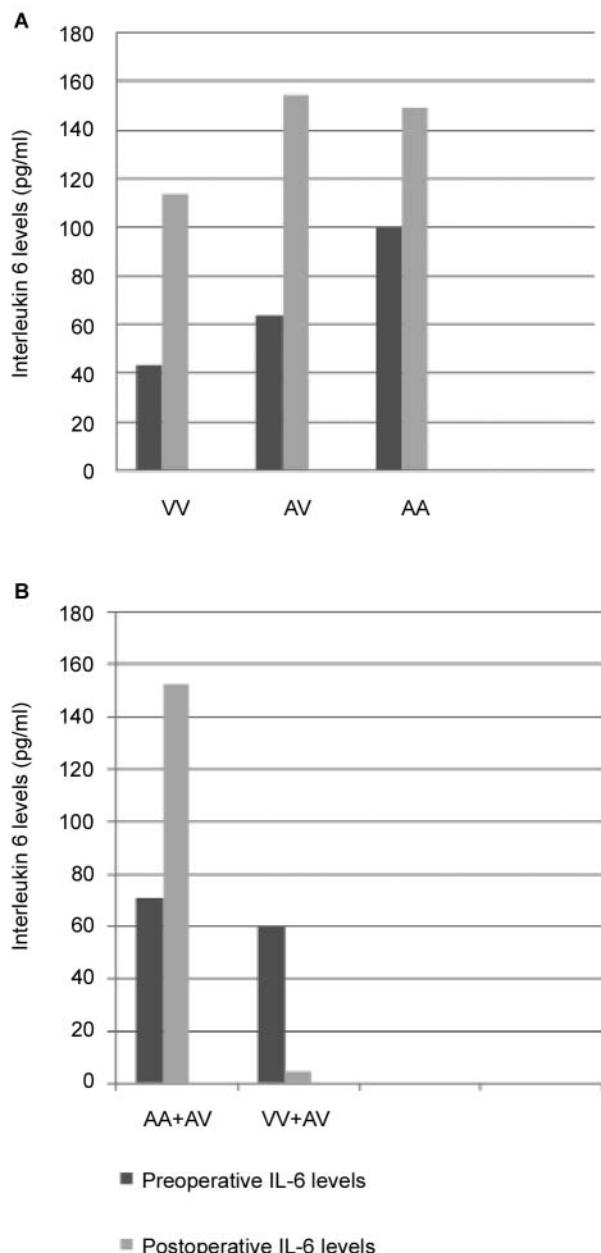


Figure 1. A) Preoperative and postoperative IL-6 levels according to "MnSOD" genotypes in patients. B) Preoperative and postoperative IL-6 levels according to "MnSOD" alleles in patients. All the values are expressed as means.

operatively vulnerable patients based on genetic polymorphisms will become an important tool in daily practice.

In conclusion, preoperative "MnSOD" Ala16Val genotyping may be useful in determining patients who are genetic susceptible to increased cytokine release and oxidative stress. Furthermore, these patients can benefit from off-pump surgery as an alternative to CPB.

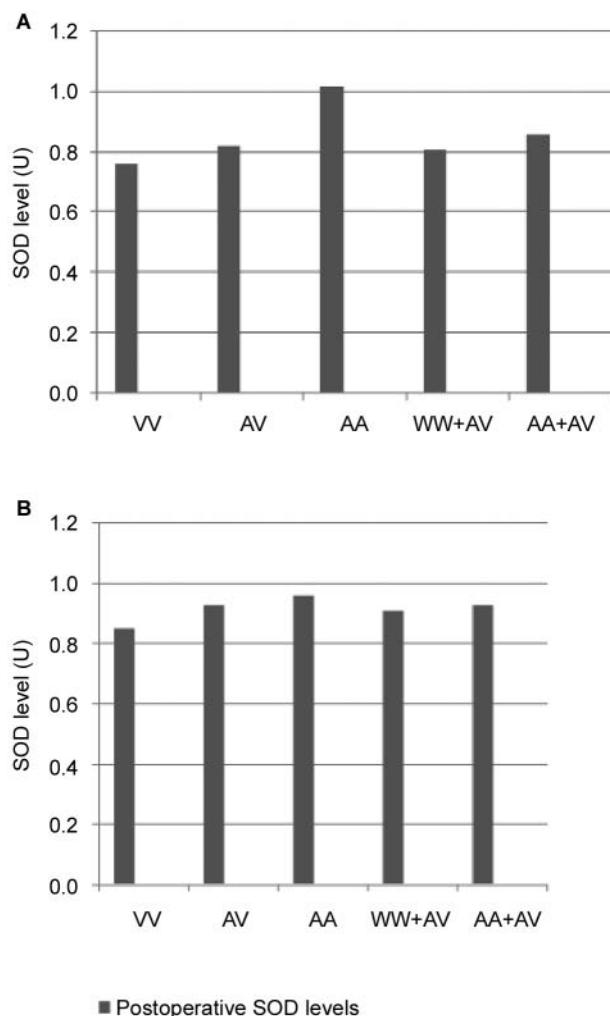


Figure 2. A) Preoperative MnSOD levels according to "MnSOD" genotype in patients. B) Postoperative MnSOD levels according to "MnSOD" genotype in patients. All the values are expressed as means.

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