Antioxidant enzymes expression in lymphocytes of patients undergoing carotid endarterectomy

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ABSTRACT

To remedy carotid artery stenosis and prevent stroke surgical intervention is commonly used, and the gold standard being carotid endarterectomy (CEA). During CEA cerebrovascular hemoglobin oxygen saturation decreases and when this decrease reaches critical levels it leads to cerebral hypoxia that causes neuronal damage. One of the proposed mechanism that affects changes during CEA and contribute to acute brain ischemia (ABI) is oxidative stress. The increased production of reactive oxygen species and reactive nitrogen species during ABI may cause an unregulated inflammatory response and further lead to structural and functional injury of neurons. Antioxidant activity are involved in the protection against neuronal damage after cerebral ischemia. We hypothesized that neuronal injury and poor outcomes in patients undergoing CEA may be results of oxidative stress that disturbed function of antioxidant enzymes and contributed to the DNA damage in lymphocytes.

Background to hypothesis

Acute brain ischemia (ABI) is a neurological condition that most frequently occurs due to sudden carotid artery occlusion that either prevents or dramatically reduces blood flow to the brain [1]. The reduced blood flow lowers the oxygen supply to the brain that leads to cerebral hypoxia and consequently, the death of brain tissue or stroke [2]. To remedy carotid artery stenosis, and prevent stroke surgical intervention is commonly used, the gold standard being carotid endarterectomy (CEA) [3,4]. Despite the great performance of this surgical procedure, tissue injury that can occur as a result of ischemia and reperfusion in patients with carotid artery stenosis may contribute to instances with poor clinical outcome. It was reported that cerebrovascular hemoglobin oxygen saturation (Sco2) decreases in patients undergoing CEA and Sco2 dropping below a critical level leads to hypoxia [5–7]. Thus, CEA affects changes in the underlying molecular mechanisms that facilitate ABI in patients with carotid artery stenosis.

Statement of the hypothesis

Mitochondrial dysfunction and the free radical generation that leads to oxidative stress have been associated with ABI [8]. A few mechanisms have been proposed for generation of free radicals during ABI, such as glutamate stimulation of n-methyl-d-aspartate receptors, activation of inducible nitric oxide (NO) synthase (iNOS), or cyclooxygenase, mitochondrial dysfunction, migration of neutrophils and leukocytes [9–13]. This increased production of reactive oxygen species and reactive nitrogen species during ABI may cause an unregulated inflammatory response and further lead to structural and functional injury of neurons [2]. We recently demonstrated, that amount of NO and iNOS is increased in the lymphocytes of patients that have undergone CEA [14], which supported the notion that inflammatory response may be responsible for the neuronal damage [8]. Also, it was shown that both superoxide dismutase (SOD) and catalase (CAT) are involved in the protection against neuronal damage after cerebral ischemia [15,16]. We hypothesized that loss of SOD and CAT activities may contribute to the DNA damage in lymphocytes caused by CEA.

To build further hypothesis regarding carotid artery occlusion, we
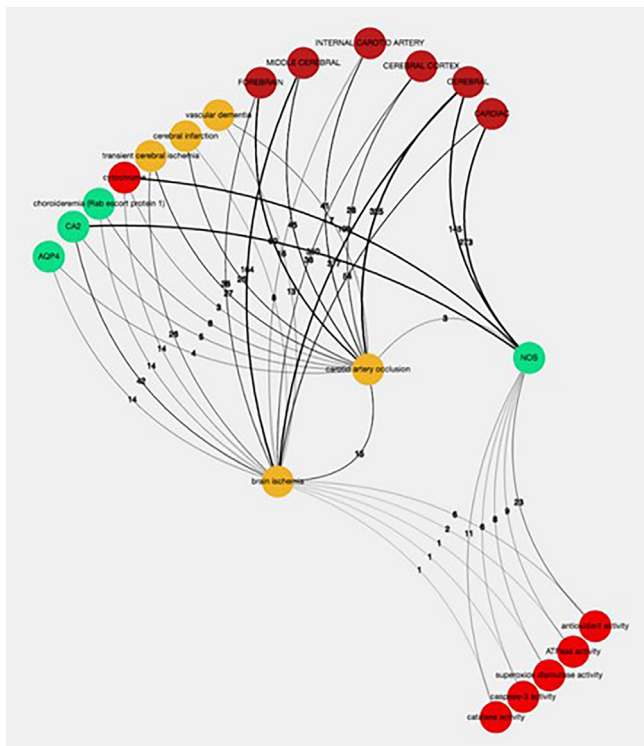

Fig. 1. DES-RedoxVasc network illustrating the relationship between "carotid artery occlusion" and concepts from four different dictionaries (with nodes pruned using a threshold on the connectivity of 1). The red circles denote concepts from the "Molecular Function (GO)" dictionary; the green circles denote concepts from the "Human Genes and Proteins (EntrezGene)" dictionary; the maroon circles denote concepts from the "Human Anatomy" dictionary; and the yellowish circles denote concepts from the "DOID Ontology (Bioportal) Human Disease Ontology" dictionary.

used the topic-specific knowledgebase (KB) named DES-RedoxVasc (https://www.cbrc.kaust.edu.sa/des-rv/) [17], focused on redox control of vascular systems. DES-RedoxVasc associated not only identifies the potential association of NOS that we recently demonstrated was increased in the lymphocytes of patients that have undergone CEA [14], but also antioxidant activity, specifically SOD activity, and CAT activity. This KB uses text-mining and statistical measures to provide concepts of relevance to vascular biology that can be used to generate hypotheses and potentially new knowledge. DES-RedoxVasc was used to expand the concept of interest, "carotid artery occlusion", with molecular function, human anatomy, disease and human genes/protein concepts enriched in vascular biology related literature. The network illustration (Fig. 1) associates concepts such as "antioxidant activity", "ATPase activity", "superoxide dismutase activity", "caspase-3 activity", and "catalase activity". These associations make sense as both "carotid artery occlusion" and "brain ischemia" have been associated with neuronal damage [2] and SOD and CAT activities protect against neuronal damage after cerebral ischemia [15,16]. It was also demonstrated that double-strand DNA breaks were induced by oxidative stress in human carotid arteries with restenosis after carotid angioplasty and stenting (CAS) [18].

Moreover, when CEA is used to treat carotid artery stenosis, CEA may be followed by cognitive dysfunction [19], and neutrophil–lymphocyte ratio can be a predictor of cognitive dysfunction in CEA patients. Specifically, patients with cognitive dysfunction had significantly higher neutrophil–lymphocyte ratio than those without cognitive dysfunction [19]. These reports suggest that low lymphocyte counts may be responsible for the higher neutrophil–lymphocyte ratio, and the low lymphocyte counts may be caused by DNA damage.

Thus we state Hypothesis: CEA increases the number of lymphocytes

with DNA damage through the increased free radicals, but this process is also attributed to by the loss of SOD and/or CAT activities which should be counteracting this effect.

Testing the hypothesis

In order to test the proposed hypothesis, we conducted a pilot study. A group of five patients was admitted in Dedinje Cardiovascular Institute, for surgery. Inclusion criteria for this pilot study was the presence of carotid stenosis higher than 70%. During the surgical procedure, patients were under general anaesthesia, and jugular vein blood was used for further analysis. Collection of blood was performed at three different time points during CEA: one minute prior cross-clamping the carotid artery (C1), one minute during cross-clamping the carotid artery (C2), and one minute post reperfusion (C3).

To assess the effects of hypoxia/ischemia caused by CEA, we measured the levels of specific antioxidant enzymes SOD1, SOD2 and CAT that the DES-RedoxVasc knowledgebase indicated may be involved in this process. Accordingly, the levels of SOD1, SOD2 and CAT in the lymphocytes of patients with carotid stenosis undergoing CEA, were measured using Western blotting analysis as described [14,20]. The mean values of SOD1, SOD2 and CAT for all patients are presented in the Fig. 2A, 2B and 2C, respectively. Each figure indicate the independent mean values of SOD1, SOD2 and CAT levels, at three time points. Similar changes in the levels of SOD1 and CAT were displayed at the three time points. That is, the levels of SOD1 and CAT prior to C1 increased by 38% ($p < 0.05$) and 28% ($p < 0.01$), respectively, during C2, but then decreased post reperfusion to levels almost similar to those obtained prior to cross-clamping (Fig. 2A and 2C). On the other hand, the levels of SOD2 was increased by 31% ($p < 0.05$) during C2 and this increase was maintained post reperfusion (34% ($p < 0.05$)) (Fig. 3B).

We also assessed the percentage of lymphocytes exhibiting DNA damage in the test subjects using Comet assay [21,22]. The mean values that represent the DNA damage in the lymphocytes of all patients are presented in the Fig. 3. The number of lymphocytes with DNA damage prior to cross-clamping increased by approximately 33% ($p < 0.05$) during C2, but this number almost doubled ($p < 0.001$) post reperfusion. The increase in the number of lymphcytes with DNA damage during C2 moment until after reperfusion was also significant ($p < 0.01$).

Discussion – where to go from here

We specifically focused on determining the changes in the levels of antioxidant enzymes in peripheral blood lymphocytes collected from carotid artery stenosis patients undergoing CEA; as a higher than normal neutrophil–lymphocyte ratio, possibly caused by lower lymphocyte counts, indicates cognitive dysfunction in CEA patients [23]. Since inhibition of NO synthesis reduces infarct volume after transient middle cerebral artery occlusion [24], the same lymphocyte samples collected for this study was also used to determine that carotid clamping leads to NO and iNOS overproduction. It was demonstrated that plasma NO before carotid clamping is increased during cross-clamping to more than three times in concentration, and post reperfusion to more than nine times in concentration the initial value post reperfusion [14]. This fact coupled with the knowledge that CEA increases free radicals [25] also suggests a role for antioxidant activity during CEA. Our results support this notion as levels of SOD1, SOD2 and CAT is significantly increased during cross-clamping (C2). Furthermore, SOD enzymes process superoxide radical anions (O_2^-), and in this process produce hydrogen peroxide (H_2O_2) as a by-product, which can be further catalyzed to water and oxygen by CAT or glutathione peroxidase [1]. This shows that SOD1 and CAT act in concert to eradicate free radicals and in this manner, prevents or reduces reactions between NO and the O_2^- that produces the by-product
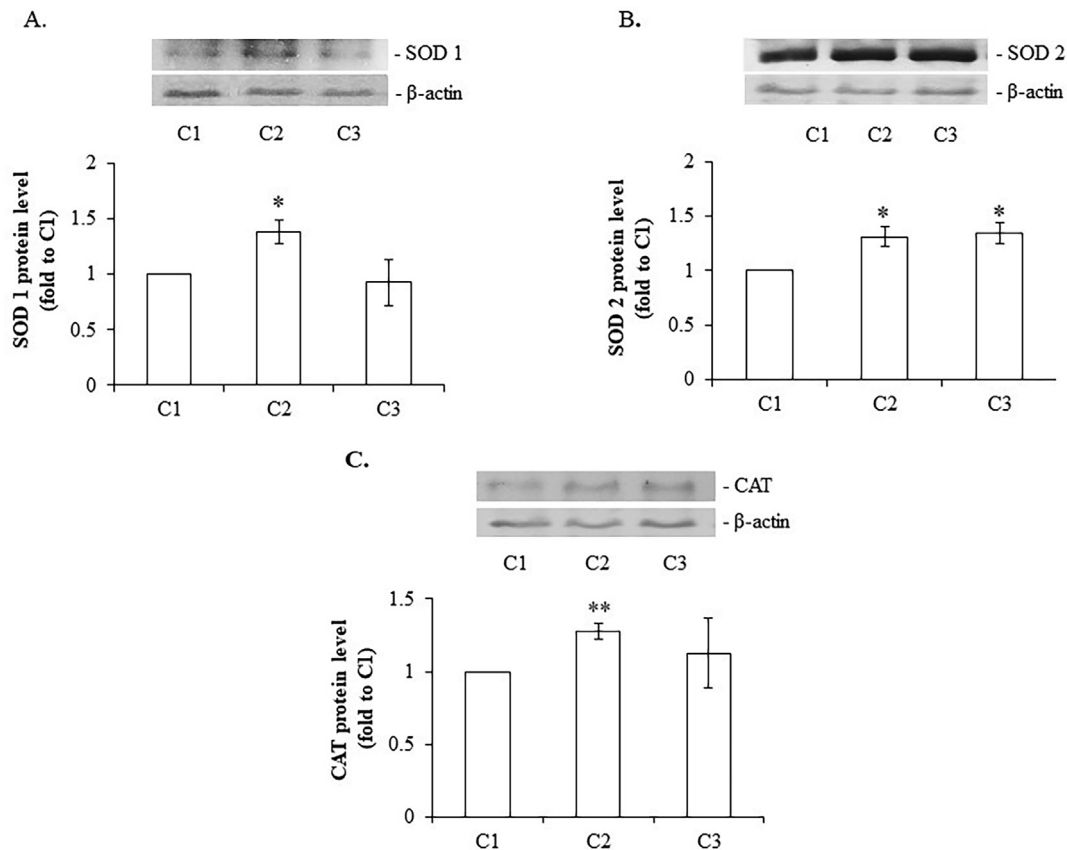
A.

B.

C.

Fig. 2. Levels of SOD1, SOD2, and CAT proteins in the lymphocytes of patients undergoing CEA at three different time points. The western blots depict the levels of (A) SOD1 protein (n = 3), (B) SOD2 protein (n = 3), and (C) CAT protein (n = 3). Results obtained for C2 and C3 are expressed as a percentage of the value obtained for C1 and represent the mean ± SEM (C2, C3 vs. C1; *p < 0.05; **p < 0.01). C1 - one minute prior cross-clamping the carotid artery; C2 – during cross-clamping the carotid artery; C3 - one minute post reperfusion.
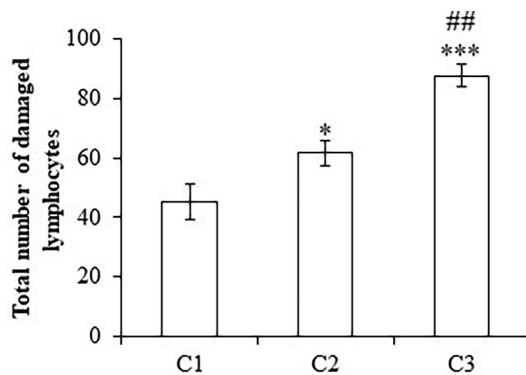

Fig. 3. DNA damage in the lymphocytes of patients undergoing CEA at three different time points. Results are expressed as mean ± SEM (C2, C3 vs. C1 *p < 0.05; *** p < 0.001; C3 vs. C2 ## p < 0.01; n = 5). C1 - one minute prior cross-clamping the carotid artery; C2 – during cross-clamping the carotid artery; C3 - one minute post reperfusion.

peroxynitrite, that has been implicated in the pathogenesis of many diseases [1,26]. Thus, this study does not provide direct evidence but rather suggests that levels of peroxynitrite increased as a result of CEA. Forman et al., [27] has however demonstrated that ischemia produced by bilateral occlusion of the common carotid arteries significantly increases levels of cerebral iNOS, NO, and peroxynitrite, as well as levels of plasma NO in rat models. Also, over-expressed SOD1 in transgenic mice exerts neuroprotective effects in a state of cerebral ischemia [28], while decreased expression of SOD1 causes hypertrophy of cerebral arterioles [29]. Chan et al., [15] also observed that SOD1 achieved

neuroprotective effects, in a state of cerebral ischemia, by reducing the apoptosis of hippocampal CA 1 cells.

Fig. 2A and C further show that the high levels of SOD1 and CAT is not maintained post reperfusion, which further shows that they act in concert and this action that eliminates O_2^- and H_2O_2 is not maintained. Similar to SOD1, CAT was also shown to exert neuroprotective effects. Armogida et al., [16] demonstrated that in the presence of H_2O_2, the hippocampus of a transgenic mouse model over-expressing human CAT exhibits increased resistance against simulated ischemia (oxygen/glucose deprivation), and these mice also exhibited a reduced infarct size after middle cerebral artery occlusion. It was also reported that CAT expression in hypoxic pulmonary arterial smooth muscle is regulated by adenosine monophosphate-activated protein kinase and Forkhead box protein O1 [30]. However, the decrease in the level of CAT in lymphocytes post reperfusion may be a consequence of c-Abl and Arg not only promoting CAT activity but also promoting CAT degradation in the oxidative stress response [31].

The highly inducible SOD2 isoform was also shown to exert neuroprotective effects during cerebral ischemia [32]. That is, SOD2-deficient mice with focal ischemia (induced via intraluminal middle cerebral artery occlusion) exhibited exacerbated cerebral infarction, hemisphere enlargement, and mitochondrial injury [28]. Using the same animal model, it was also demonstrated that deficiency of SOD2 leads to increased mitochondrial cytochrome c release, which is a critical step for apoptosis and subsequent DNA fragmentation in brain cells, after permanent focal cerebral ischemia [33]. In contrast, over-expression of mitochondrial SOD2 in neural cells prevents the accumulation of peroxynitrite and apoptosis induced by Fe^{2+}, amyloid beta-peptide, and NO-generating agents [34]. Also, transgenic mice over-expressing SOD2 exhibited reduced membrane lipid peroxidation,
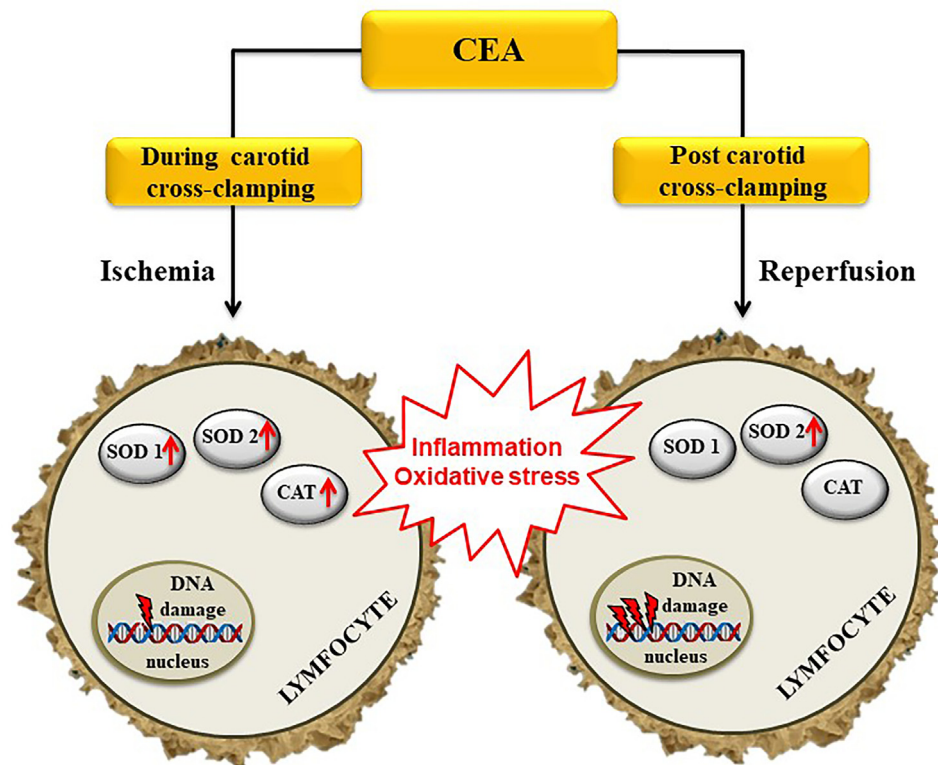
Fig. 4. Changes in lymphocytes isolated from the blood of patients during CEA. CAT - Catalase; CEA - Carotid endarterectomy; DNA - Deoxyribonucleic acid; SOD1 - Superoxide dismutase 1; SOD2 - Superoxide dismutase 2.

protein nitration, and neuronal death after focal cerebral ischemia [34].

Fig. 2 further show that the significantly high levels of SOD2 was maintained post reperfusion. Since the level of SOD1 and SOD2 have been significantly increased in a short time, we assume that these increases were due to the rapid induction of posttranslational modifications of previously synthesized proteins mediated by cytokines, growth factors or effect of free radicals. Although SOD1 and SOD2 are very effective in ameliorating oxidative stress, earlier pharmacokinetic studies in rats have revealed that they have a different half-life, which was 6–10 min for SOD1 and 5–6 h for SOD2 [34]. This may be one of the reasons for the increased level of SOD2, (but not SOD1 isoform) observed post reperfusion in this study.

Damage of DNA in lymphocytes isolated from peripheral blood in humans is often used as an indicator of genotoxicity in various pathological states, including oxidative stress and inflammation [31]. Increased level of free radicals leads to the oxidation of nitrogenous bases and single-strand breaks, while shear stress causes a breakdown of the double helix, chromosomal fragmentation, translocation, and deletion [35]. DNA damage can lead to inactivation of genes that are vital for cell survival, including antioxidant enzymes genes, which makes cell apoptosis virtually inevitable [36]. Fig. 3 shows that the amount of lymphocytes with DNA damage was increased, both during carotid clamping and post reperfusion. Thus, the number of lymphocytes with DNA damage increased despite the increase in the levels of antioxidant enzymes (SOD1, SOD2, and CAT). Nonetheless, SOD and CAT are involved in the protection against neuronal damage after cerebral ischemia [15,16]. Thus, their expression may not be fast enough or high enough to prevent DNA damage in the lymphocytes, or they may not be the antioxidant enzymes required to eliminate the free radicals produces during CEA. Thus, CEA increases the number of lymphocytes with DNA damage through the increased free radicals, but this process is also attributed to by the loss or lack of antioxidant enzymes that SOD should be counteracting this effect. Thus, regulation of these enzymes in blood might be one promising way to protect against the neurological damage

caused by oxidative stress related to ABI in patients undergoing CEA.

Conclusion

This study demonstrates that the levels of SOD1, SOD2, and CAT in lymphocytes, and the number of DNA damaged lymphocytes is increased in patients undergoing CEA. Changes in the level of antioxidant enzymes simultaneously with DNA damage in lymphocytes of patients undergoing CEA (together with our previous work demonstrating overproduction of plasma NO [14]), confirm our assumptions that oxidative stress could be responsible for neuronal injury as well as for poor outcomes in patients undergoing CEA (Fig. 4). Future studies on a larger population size is needed to confirm the proposed hypothesis and to capture the role of antioxidant enzymes during CEA, and to determine why we have patients undergoing CEA with poor outcome despite the action of these antioxidant enzymes. We speculate that this understanding may direct us to a combination of antioxidants that could be used to pretreat patients undergoing CEA to reduce poor outcome cases.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

Acknowledgements

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