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Original Article

Superoxide Dismutase (SOD) Enzyme Activity Assay in *Fasciola* spp. Parasites and Liver Tissue Extract

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(Received 22 Nov 2010; accepted 24 Sep 2011)

ABSTRACT

Background: The purpose of this comparative study was to detect superoxide dismutase (SOD) activities in *Fasciola hepatica, F. gigantica* parasites, infected and healthy liver tissues in order to determine of species effects and liver infection on SODs activity level.
Methods: *Fasciola* spp. parasites and sheep liver tissues (healthy and infected liver tissues), 10 samples for each, were collected, homogenized and investigated for protein measurement, protein detection and SOD enzyme activity assay. Protein concentration was measured by Bradford method and SODs band protein was detected on SDS-PAGE. SODs activity was determined by iodonitrotetrazolium chloride, INT, and xanthine substrates. Independent samples *t*-test was conducted for analysis of SODs activities difference.
Results: Protein concentration means were detected for *F. hepatica* 1.3 mg/ ml, *F. gigantica* 2.9 mg/ml, healthy liver tissue 5.5 mg/ml and infected liver tissue 1.6 mg/ml (with similar weight sample mass). Specific enzyme activities in the samples were obtained 0.58, 0.57, 0.51, 1.43 U/mg for *F. hepatica*, *F. gigantica*, healthy liver and infected liver respectively. Gel electrophoresis of *Fasciola* spp. and sheep liver tissue extracts revealed a band protein with MW of 60 kDa. The statistical analysis revealed significant difference between SOD activities of Fasciola species and also between SOD activity of liver tissues (P<.05).
Conclusion: *Fasciola* species and liver infection are effective causes on SOD enzyme activity level.

Keywords: Superoxide Dismutases, *Fasciola hepatica*, *F. gigantica*, liver

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Introduction

Fascioliasis is an important animal and human disease caused by trematodes (*Fasciola hepatica, F. gigantica*). These flukes causing pathological lesions such as fibrosis and cirrhosis, which result from the parasites passage through the liver parenchyma. Acute and chronic fascioliasis is observed primarily in sheep, goats, and cattle, causing important economic losses due to liver injuries (1).

The production of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen, is dependent on oxygen utilization and can cause cellular damage by lipid and protein peroxidation (2,3). Oxidative stress and lipid peroxidation have been related with several types of liver injuries (4). It has been demonstrated that in the cells of hosts infected with parasite, the quantity of ROS which cause lipid peroxidation are increased, thus causing cell and tissue damage (5). Products of lipid peroxidation created in different biochemical reactions are normally removed by antioxidants. Antioxidants are compounds that are involved in effective scavenging of free radicals and in suppressing the actions of reactive oxygen substances. Antioxidant barriers are extensively distributed and include both enzymatic and nonenzymatic systems. The most important enzymatic antioxidants are superoxide dismutase, glutathione peroxidase and catalase. Nonenzymatic factors that may function as antioxidants are reduced glutathione, vitamin C, vitamin E, β-carotene, ceruloplasmin and bilirubin (6). SOD enzymes catalyze the dismutation of superoxide radical into hydrogen peroxide (H2O2) and molecular oxygen (O_2) and consequently present an important defense mechanism against superoxide radical toxicity(7). Cell antioxidants could not protect the proteins against peroxyl radicals (3).

There is no study which shows the sod antioxidant status in the fasciola parasites and liver tissue in Iran. Therefore we designed this study to compare SOD enzyme activity in the sheep liver tissue, healthy and infected, and *Fasciola* spp. parasites in order to evaluate their effects on sod enzyme activity level.

Materials and Methods

Parasite and liver tissue extracts

The mature parasites of *F. hepatica*, *F. gigantica* and sheep liver tissues (healthy and infected liver tissue) were collected from sheep slaughtered at a local abattoir (Saman, Tehran, Iran).The samples (10 samples for each) were washed for three times in PBS, pH 7.4, to remove host material and stored at -20°C. After thawing samples were homogenized in homogenizing buffer (2 ml), PBS 7.2 by a glass homogenizer. Then suspension was centrifuged (10000g for 30 min at 4°C) and supernatant was stored at -20°C.

Protein concentration and electrophoresis

Protein concentration was measured by Bradford method with BSA standard solutions as duplicate. Standards were prepared as a range of 5 to 100 micrograms protein (Bovine Serum Albumin, Merk Product) in 100 µl volume. Diluted samples were obtained between 5 and 100 µg protein in assay tubes containing 100 µl samples. Dye reagent, 5 ml, was added on tubes, incubated 5 minute and measured the absorbance at 595 nm (8).

In order to separate of SOD band proteins and to confirm their existence, samples were added to each well at 15 mA per SDS-

PAGE gel, 15%, for 6 hours and finally the gel stained by comassie blue staining (8).

SOD enzymes Activity assay

SOD enzyme activity was determined using RANSOD kit (Randox Labs, Crumlin, UK), which was based on the method of McCord and Fridovich (9). Xanthine and xanthine oxidase were used to generate superoxide anion radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride quantitatively to form a red formazan dye. SOD inhibits the reaction by converting the superoxide radical to oxygen. Homogenized liver tissues, *Fasciola* spp. parasites and standard solutions were used for the assay of SOD. Absorbance was measured at 505 nm on a Cecil 1021 UV / visible spectrophotometer (Cecil Instruments Ltd Milton Technical Centre Cambridge ENGLAND) for 30s after the addition of xanthine oxidase as start reagent and 3 minute after reaction as duplicate samples (Table 1).

Inhibited percents of standards and samples were calculated by following formula. $100-(\Delta AStd/min\times100)/(\Delta AS1/min) =$ %inhibition (Where S1 is ΔA2-A1/3 uninhibited tube and ΔStd equal A2-A1/3 of inhibited tubes). A standard curve was prepared by using the standard provided in the kit and the SOD activity value for each sample was read from this curve. The SOD activity was expressed as U/ml reagent. One unit is the amount of SOD that inhibits the rate of formazan dye formation by 50%.

To detect the statistical difference between specific SOD activities of samples, two-sample independent *t*-test was conducted.

Results

Protein concentration for *F. hepatica* 1.3 mg/ml, *F. gigantica* 2.9 mg/ml, healthy liver tissues 5.5 mg/ml and infected liver tissue 1.6 mg/ml were detected respectively (with same weight sample mass). Absorbances measures, calculated inhibition percent, total activity and specific activities of SOD enzyme in sample solutions are presented at the table 2 (mean of 10 samples for each). Sample solutions showed one bands protein MW of 60 kDa in SDS-PAGE gel (Fig. 1).

Table 1: Superoxide Dismutase Activity Assay of Samples (*Fasciola* spp & liver tissues) and Standard SOD Solutions using RANSOD kit

Solutions	Uninhibited tube*	Inhibited tubes	Standards (St_{1-5}) tubes
Substrates	850 (µl)	850 (µl)	850 (µl)
Phosphate buffer,7.0	25 (µl)	0	0
Xanthine oxidase	125 (ul)	125 (ul)	125 (ul)
Samples (*F. hepatica, F.gigantica*, healthy and infected liver)	0	25 (µl)	0
Standard solutions (Superoxide Dismutase)	0	0	25 (µl) 0.2, 0.5, 1.0, 2, 4 (U/mg)

*Uninhibited tube or negative control includes all components except SOD or samples

Table 2: Absorbances measures, percentage inhibition calculated, total activity and specific activity of SOD enzyme in sample solutions (mean of 10 samples for each)

Sample tubes	Absorbance 1	Absor-bance2	A2-A1	A2-A1/3	Calcu-lated inhi-bition per-cent*	Total activ-ity of SOD(U/ml)**	Specific activity of SOD(U/mgl)
solutionUn inhibited	0.118	0.204	0.086	0.028	0	0	0
F. hepatica extract	0.135	0.202	0.67	0.022	21.42	0.76	0.58
F. gigantica extract	0.144	0.183	0.039	0.013	53.5	1.58	0.55
extractHealthy liver	0.162	0.174	0.012	0.004	85.7	2.84	0.51
Infected liver extract	0.155	0.178	0.23	0.007	75	2.28	1.43

*The percent inhibition of the test samples correlates with SOD activity using a SOD standard curve.
**One unit of SOD inhibits the rate of increase in absorbance at 550 nm by 50% under the conditions of the assay.
*** Specific activity is total activity of SOD per milligram of total protein.

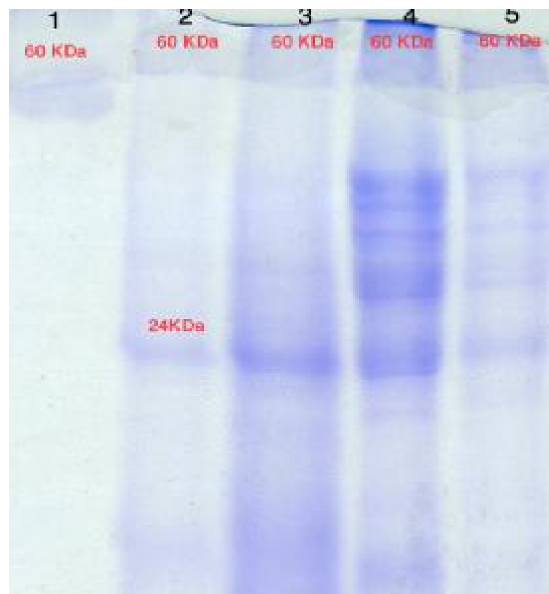

Fig. 1: SDS-PAGE analysis of *Fasciola* spp. parasites and liver tissue extracts. The proteins were analyzed on 15% gel. Lane 1 , superoxide dismutase marker , Lane 2, somatic extract of *Fasciola hepatica* ; lane 3, somatic extract of *Fasciola gigantica* , lane 4, crude extract of healthy liver tissue , lane 5 , crude extract of infected liver

Discussion

SODs has been demonstrated from various helminthes of different species such as *Schistosoma mansoni*, *Onchocerca volvulus*, *Dirofilaria immitis*, *Brugia pahangi*, and *Fasciola hepatica* (10-14).
Although the adult worms have a generally anaerobic metabolism and live in the bile duct, where the oxygen pressure is relatively low, oxygen is necessary for other functions such as egg generation, which generate reactive oxygen species (ROS). In addition to this normal endogenous oxidative tension, the parasite is exposed to reactive oxygen species produced by host responding cells such as macrophages, eosinophils, neutrophils, and platelets. To protect themselves against oxidative stress mechanisms of hosts, parasites have developed antioxidant enzyme systems (14). It has been suggested that antioxidant inhibition of host oxidative stress may play a protective role in the parasite life cycle (15).
At the present work, detected specific enzyme activity in *F. hepatica* was greater than *F. gigantica*. In this regard, researchers have suggested a possible role for this enzyme in the resistance of *F. hepatica* to superoxide-mediated killing (16). Recently, the investigators have showed, over-expression of SOD can reduce oxidative damage and extend life span (17). The higher antioxidant level in *F. gigantica* may help to increase of parasite life span, however further studies are needed for this conclusion.
In this research, the specific enzyme activity of infected liver was more than in the healthy liver. Antioxidant enzyme has a cellular protective role against oxidative stress resulting in liver tissue damage as a result of parasitic invasion (18). The somatic extract of *F.* spp. and liver tissues exhibited 1 band of 60 kDa on SDS-PAGE gel. This protein band has reported from excretory-secretory products of *F. hepatica* (19).

The statistical analysis revealed significant difference between SOD activity of F. *hepatica* and *F. gigantica* ($P<.05$). Statistical results also showed significant difference between healthy and infected liver tissues SOD enzyme activity ($P<0.05$). Therefore, briefly, parasite species and liver infection could be considered as effective causes on SOD enzyme activity level (20).

Acknowledgments

This work was supported by the Research grant 86-03-27-7755 (2008 to 2010) from the Tehran University of Medical Sciences. We are deeply grateful to M.B. Molaei Rad for his technical assistance on SDS-page and to Dr. M. Madah and H. A. Rahmi for collecting infected liver in the abattoirs of Tehran and Ilam cities. The authors declare that there is no conflict of interests.

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