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Erythrocyte glutathione peroxidase activity and serum lipid pattern: A comparison between Indian immigrants and Danes

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With 5 tables

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Glutathione peroxidase (GSH-Px, E.C.1.11.1.9) is an enzyme that reduces toxic lipid peroxides to hydroxy acids (1, 2, 3). *Awasthy* et al. (4) have shown selenium to be an essential nutrient for man and they have demonstrated that selenium functions as an integral part of glutathione peroxidase (GSH-Px), thus selenium through this enzyme exerts protection against peroxidative damage as does vitamin E. Analysis of this enzyme therefore provides a valuable index to monitor abnormal levels of enzyme activity related to improper selenium supplementation. Recently we have shown a marked decrease of erythrocyte glutathione peroxidase (GSH-Px) activity in Batten's disease (5) and multiple sclerosis (6) and have used the analysis of glutathione peroxidase (GSH-Px) and blood lipids as a diagnostic marker in these diseases. The purpose of this paper is to present a comparative study of serum cholesterol, serum fatty acid patterns and erythrocyte glutathione peroxidase (GSH-Px) activities between Indian immigrants and Danes.

Materials and Methods

The study comprises 11 Indian immigrants: 9 males and 2 females all over 20 years of age, mean age 31 years, range 25–41 years. The results were compared with 11 Danes, 7 males and 4 females, mean age 28 years, range 20–46 years.

All chemicals were of highest obtainable purity from British Drug Houses, Poole, Dorset, England and enzymes and coenzymes were from Sigma Chemical Company, USA and Boehringer, Mannheim, West Germany. Organic peroxides were supplied as generous gifts from Peroxid-Chemie GmbH, D-8023 Höllkriegelskreuth bei München, West Germany.

Isolation of erythrocytes

10 ml venous blood was bled from a cubital vein into a test tube containing 0.5 ml 0.1 M EDTA (Na_2). After centrifugation at 600 g_{av} (4 °C) for 10 min, the clear plasma and the "buffy" leucocyte layer was removed by aspiration (7) and the erythrocytes were cleaned three times with cold saline (4 °C, 0.15 M) and centrifuged as described above.

Extraction of the source of enzyme

1 vol of cleaned concentrated erythrocytes-approximately 12×10^9 cells/ml were added to 4 vols of distilled water (8). By alternating freezing (−20 °C) and thawing 3 times the soluble GSH-Px enzymes were liberated. After centrifugation in a Sorvall centrifuge (4 °C) at 12.500 g_{av} for 2 hours the stroma was sedimentated and the supernatant was used as source of enzyme.

Pretreatment of hemoglobin

The above mentioned source of enzyme was diluted with an equal volume of distilled water. Then an equal volume of Drabkins solution (0.0016 M KCN, 0.0012 M $K_3Fe(CN)_6$ and 0.0238 M $NaHCO_3$) was added in order to convert all hemoglobin to cyanomethemoglobin. Thereafter 0.1 ml of this modified source of enzyme was used in GSH-Px assay.

Hemoglobin determination

The hemoglobin content of the above mentioned cyanomethemoglobin solution was measured at 540 nm and by using the molar extinction coefficient $\varepsilon = 11.0\ M^{-1}\ cm^{-1}$ and a molar weight Mw = 16,114, the hemoglobin content in the modified source of enzyme was expressed in mg/ml.

Assay of glutathione peroxidase (GSH-Px, E.C.1.11.1.9)

Enzyme activity was measured by a modification of the assay procedure of *Paglia* and *Valentine* (8). 0.1 ml of the above mentioned mixture of the source of enzyme and of Drabkins reagent was added to 2.80 ml cold reaction mixture which consisted of 2.58 ml of 0.05 M sodium phosphate buffer (pH 7.4) containing 0.005 M EDTA (Na_2) and 0.01 ml 1.125 M NaN_3, 0.10 ml 0.15 M GSH (reduced glutathione), 0.01 ml glutathione reductase (Hoechst, Germany, 1 E.U. per mg protein/1 ml) and 0.10 ml 0.0084 M NADPH. The reaction mixture was incubated for 5 min at 22 °C in a

Table 1. Glutathione Peroxidase (GSH-Px) in erythrocytes.
Peroxide: Hydrogen peroxide Unit: Units/min/mg Hgb (540 nm)

Indian immigrants				Danes			
Code	Age	Sex*	GSH-Px	Code	Age	Sex*	GSH-Px
S. H.	35	M	4.1	J. J.	26	M	9.1
S.	33	M	7.9	E. A.	21	M	7.7
J. S. B.	36	M	6.4	T. N.	21	M	8.3
K. C. S.	41	M	5.5	K. A.	22	F	7.6
V. K. S.	29	M	9.4	O. L.	22	M	7.9
R. B.	34	F	7.6	U. W.	20	M	8.6
M. U.	27	F	8.9	H. W.	32	F	8.0
T. A.	25	M	12.0	L. F.	42	F	8.3
C. H.	25	M	7.1	M. N.	37	M	11.2
S. U. R.	30	M	7.7	J. R.	46	M	7.4
S. C. R.	32	M	5.5	M. A.	20	F	8.5
Mean ± SD		7.5 ± 2.2				8.4 ± 1.0	
Range		4.1 − 12.0				7.4 − 11.2	
Median		7.6				8.3	
Decile 10%		5.5				7.6	
Decile 90%		9.4				9.1	

* M: male, F: female

quartz cuvette (1 cm path length) of a Beckman spectrophotometer, Acta C III equipped with an automatic recorder. The initiation of the reaction was followed at 340 nm after the addition of 0.1 ml peroxide solution. Initial studies on the kinitic behaviour of GSH-Px revealed a saturation limit of cumene hydroperoxide at 200 µM and of t-butyl hydroperoxide at 300 µM in the assay system. The concentration of H_2O_2 used (73 µM) was as described by *Paglia* and *Valentine*. When H_2O_2 was used as peroxide donor the reaction (oxidation of NADPH) was linear between 2 to 4 minutes. NADPH oxidation was linear between 4 to 6 min, when the organic peroxides were used. Using organic peroxides the assay system contained 10% (w/v) enthanol (9). The blank contained distilled water instead of the source of enzyme. The enzyme activity was calculated by means of molar extinction coefficient $\varepsilon = 18\ cm^2/\mu mole$ for NADPH and was expressed as units/min/mg hemoglobin (1 unit = 1 nmole of NADPH oxidized). All assays were done in triplicate.

Extraction of lipid from serum

1 ml of serum was extracted with 50 ml chloroform-methanol (2:1) and the lipid extract was freed from non-lipid contaminants by phase partition (10).

Extraction of cholesterol from serum

The cholesterol fractions (free and esterified) were extracted from the serum by a modification of a double extraction procedure (11). 3 ml isopropanol-water-10N NaOH (250:125:10) mixture were pipetted into a 10 ml glass stoppered tube. 1 ml serum was added and mixed on a vortex type mixer for 3 min. After standing for 5 min, 3 ml of n-octane were added and mixed again for 3 min. Octane was separated from the lower isopropanol-water-sodium hydroxide mixture. After 10 min, 2 ml of the upper octane layer were removed and octane was evaporated under a stream of nitrogen.

Table 2. Glutathione Peroxidase (GSH-Px) in erythrocytes.
Peroxide: Cumene hydroperoxide Unit: Units/min/mg Hgb (540 nm)

Indian immigrants				Danes			
Code	Age	Sex*	GSH-Px	Code	Age	Sex*	GSH-Px
S. H.	35	M	7.5	J. J.	26	M	8.2
S.	33	M	10.9	E. A.	21	M	7.1
J. S. B.	36	M	8.8	T. N.	21	M	9.0
K. C. S.	41	M	7.1	K. A.	22	F	7.7
V. K. S.	29	M	10.9	O. L.	22	M	6.7
R. B.	34	F	9.5	U. W.	20	M	9.1
M. U.	27	F	11.9	H. W.	32	F	9.1
T. A.	25	M	15.0	L. F.	42	F	6.7
C. H.	25	M	8.8	M. N.	37	M	8.1
S. U. R.	30	M	9.0	J. R.	46	M	6.6
S. C. R.	32	M	5.6	M. A.	20	F	9.4
Mean ± SD		9.5 ± 2.6				7.9 ± 1.0	
Range		5.6 – 15.0				6.6 – 9.4	
Median		9.0				8.1	
Decile 10%		7.1				6.7	
Decile 90%		11.9				9.1	

* M: male, F: female

Preparation of fatty acid methyl esters

The methyl esters of total serum and cholesterol fraction were prepared by transesterification, the lipid (1–10 mg) was dissolved in 2 ml of 5% HCl in dry methanol and 0.5 ml of dry benzene. The mixture was refluxed for 2 hr at 80–100 °C and cooled. Two volumes of water were added and the methyl esters so formed were extracted three times with 3 ml of diethyl ether. The pooled extracts were dried over sodium sulphate-sodium bicarbonate and taken up in 100 µl of chloroform for analysis.

Analysis of fatty acid methyl esters

During the course of fatty acid analysis the following gas chromatographs equipped with hydrogen flame detectors were used: Beckmann GC 72–5 and Perkin Elmer F-11. The electrometers of these instruments were interfaced with a Hewlett Packard 3380A series reporting integrator (Hewlett Packard, Avondale, Pa, USA) for identification and quantitation of fatty acids.

The samples were analysed on columns packed with 20% (w/w) ethylene glycol succinate on a silica No 545 (mesh 60–100) and 10% EGSS-X on celite AAW (mesh 80–100), glass 2 m × 3 mm I.D. Nitrogen was used as a carrier gas (flow = 60 ml/min). The temperature of the injector block was 260 °C and of the detector 245 °C. The column oven temperature was maintained at 180 °C for the former and 185 °C for the latter type. The fatty acids were identified by means of methyl ester standards from Ihe Hormel Institute, Minnesota, USA.

Serum Cholesterol

Determination of serum cholesterol was done as described earlier (12).

Statistical assays

The mean, standard deviation, median and 10 and 90% deciles were computed. The level of significance was determined by means of Wilcoxon's non-parametric

Table 3. Glutathione Peroxidase (GSH-Px) in erythrocytes.
Peroxide: t-butyl hydroperoxide Unit: Units/min/mg Hgb (540 nm)

Indian immigrants				Danes			
Code	Age	Sex*	GSH-Px	Code	Age	Sex*	GSH-Px
S. H.	35	M	5.8	J. J.	26	M	9.6
S.	33	M	10.7	E. A.	21	M	8.3
J. S. B.	36	M	10.0	T. N.	21	M	9.5
K. C. S.	41	M	9.0	K. A.	22	F	7.8
V. K. S.	29	M	12.3	O. L.	22	M	6.3
R. B.	34	F	10.0	U. W.	20	M	9.2
M. U.	27	F	14.4	H. W.	32	F	10.4
T. A.	25	M	16.4	L. F.	42	F	6.8
C. H.	25	M	9.5	M. N.	37	M	9.3
S. U. R.	30	M	11.1	J. R.	46	M	8.4
S. C. R.	32	M	8.1	M. A.	20	F	7.0
Mean ± SD		10.7 ± 2.9				8.4 ± 1.3	
Range		5.8 – 16.4				6.3 – 10.4	
Median		10.0				8.4	
Decile 10%		8.1				6.8	
Decile 90%		14.4				9.6	

* M: male, F: female

method (Geigy, Documenta Geigy, 1965) (13). The level of significance was set at 5% level. Spearman Ranks correlation test was used for the correlation purpose. The level of significance was set at 5% level.

Results

The results of the assay of glutathione peroxidase (GSH-Px) activity using three different peroxides in Indian immigrants and Danes are presented in Tables 1–3. The glutathione peroxidase (GSH-Px) activities were significantly increased in Indian immigrants as compared to Danes when t-butyl hydroperoxide was used as peroxide donor ($p \leq 5\%$). By using hydrogen peroxide and cumene hydroperoxide, no significant difference was found between the two groups. Related to median values the glutathione peroxidase (GSH-Px) activity increased by 16% in Indians as compared to Danes when t-butyl hydroperoxide was used as peroxide. Range was found to be wider for the Indians as compared to Danes with all the three peroxides studied.

Using Spearman Ranks correlation test, a significant correlation was found in the Indian group ($p \leq 1\%$, $R \leq 0.9$) with all the three peroxides studied. This significant correlation could not be traced for the Danish group.

The fatty acid composition of total serum lipids and cholesterol esters expressed as relative percent are shown in Tables 4 and 5. Triplicate analyses were performed in all cases. In case of total serum fatty acids Wilcoxon's test showed a significant decrease in C14:0 ($p \leq 1\%$), C16:0 ($p \leq 1\%$), C18:0 ($p \leq 1\%$) and C20:3 ($p \leq 1\%$), and a significant increase in C16:1 ($p \leq 1\%$), C18:1 ($p \leq 1\%$) and in C18:2 ($p \leq 5\%$) in the Indian group compared to the Danish group. However, no significant difference could be seen for C18:3 and C20:4.

For cholesterol fatty acids no significant difference was seen between the two groups. Spearman Ranks correlation test showed no correlation between the different GSH-Px's and fatty acids of total serum and cholesterol fraction.

Serum cholesterol was significantly lower in the Indian group as compared to the Danish group ($p \leq 5\%$).

Discussion

The recent discovery that the GSH-Px is a seleno-enzyme has considerably widened the possibilities for the elucidation of biochemical role of this trace element as well as for the detection of selenium deficiency states. Thus a close relation between tissue and red blood cell GSH-Px activity and selenium in chicken (14), rats (15), cattle (16) and sheep (17), and man (18) has already been demonstrated. However, it deserves mention that while GSH-Px located in erythrocytes is selenium dependent, several other tissues also contain a selenium independent form of the enzyme (19). Assuming that the variations in red blood cell GSH-Px activity mainly are genetically based and that selenium as part of the enzyme molecule amounts to a considerable part of the erythrocytes' total selenium content, the GSH-Px activity of these cells may be used as a measure of selenium states in various selenium deficiency diseases (20).

Table 4. Total serum fatty acid composition in Indian immigrants and Danes: Comparative study.
(Values expressed as percentages of total fatty acid methyl esters.)

Fatty Acids	Indian immigrants (n = 11) Mean ± SD	Median	10% Decile	90% Decile	Danes (n = 11) Mean ± SD	Median	10% Decile	90% Decile
C14 : 0	2.9 ± 0.9	3.0	2.1	3.9	4.0 ± 1.3	3.8	2.6	5.3
C16 : 0	23.6 ± 3.0	22.7	21.4	28.1	28.5 ± 2.2	28.1	26.7	32.0
C16 : 1	3.3 ± 0.5	3.2	2.8	4.1	2.1 ± 0.9	1.8	1.4	3.1
C18 : 0	8.4 ± 0.7	8.4	7.5	8.9	14.0 ± 1.2	13.6	13.1	15.5
C18 : 1	23.0 ± 3.8	22.4	18.8	27.7	14.7 ± 1.6	14.9	12.7	16.3
C18 : 2	30.3 ± 6.5	33.5	20.5	36.4	25.4 ± 2.9	25.9	25.9	28.1
C18 : 3	0.7 ± 0.5	0.6	0.4	0.7	0.8 ± 0.3	0.7	0.7	1.2
C20 : 3	1.7 ± 0.4	1.7	1.3	2.2	2.9 ± 0.8	2.6	2.6	4.1
C20 : 4	6.2 ± 1.6	6.4	4.1	7.6	7.3 ± 1.4	6.7	6.1	8.6
Mean serum cholesterol ± SD (mg/100 ml)			183 ± 37			216 ± 29		

Table 5. Fatty acid composition of serum cholesterol esters in Indian immigrants and Danes: Comparative study.
(Values expressed as percentages of total fatty acid methyl esters.)

Fatty Acids	Indian immigrants (n = 11) Mean ± SD	Median	10% Decile	90% Decile	Danes (n = 11) Mean ± SD	Median	10% Decile	90% Decile
C14 : 0	3.1 ± 1.4	3.0	1.4	5.2	2.4 ± 0.7	2.4	1.5	3.2
C16 : 0	20.8 ± 4.6	19.6	17.2	27.4	21.0 ± 2.6	20.6	18.6	24.4
C16 : 1	3.8 ± 1.5	3.8	3.2	5.9	4.0 ± 0.7	4.0	3.5	4.9
C18 : 0	4.0 ± 1.5	3.6	2.6	6.0	5.4 ± 1.7	4.8	4.1	7.9
C18 : 1	27.1 ± 4.1	27.5	22.6	32.9	26.1 ± 2.7	25.0	23.1	29.3
C18 : 2	35.6 ± 9.2	39.4	20.9	43.7	34.7 ± 4.6	33.9	29.9	39.7
C18 : 3	0.6 ± 0.3	0.6	0.3	0.8	0.6 ± 0.2	0.6	0.3	0.8
C20 : 3	0.4 ± 0.1	0.4	0.3	0.6	0.6 ± 0.3	0.5	0.4	1.1
C20 : 4	4.4 ± 1.5	4.1	2.5	5.9	4.9 ± 1.6	4.5	3.9	6.8

The results of the present study demonstrate a significant increase in GSH-Px activities in Indian group as compared to Danish group when t-butylhydroperoxide was used as a substrate ($p \leq 5\%$) and also a significant correlation was found in the Indian group with all the three peroxides studied whereas this significant correlation was missing in the Danish group.

This difference may be explained on the basis that the peroxidase activity may be due to a selenium containing enzyme and an enzyme not containing selenium (19). If this is correct it is tempting to suggest that the deficiency in the Danish group concerns an enzyme containing selenium.

It is generally accepted that the fatty acid pattern, which is ester-bound in the serum lipids, reflects to some degree the fatty acid pattern of the dietary fats. This relation was confirmed in the present study as the total serum fatty acid patterns between the two groups differed considerably reflecting the differences of the food habits of the two groups, especially in case of Indians whose food besides containing animal protein and fat, consists substantially of cereals, vegetables and vegetable oils.

Summary

A comparative data on erythrocyte glutathione peroxidase (GSH-Px) activity, serum cholesterol and serum fatty acid pattern between Indian immigrants and Danes have been presented. The erythrocyte GSH-Px activity in the Indian immigrants has been found to be significantly increased as compared to that in the Danish group when t-buthylhydroperoxide was used as a substrate. Also a significant correlation ($R \leq 0.9$) was found in the GSH-Px activities in the Indian group with the three peroxides, viz., hydrogen peroxide, cumene hydroperoxide and t-butylhydroperoxide, used. This correlation was, however, missing in the Danish group. Serum cholesterol was found to be significantly lower in the Indian group as compared to the Danes. Also total serum fatty acid patterns between the two groups differed considerably.

Zusammenfassung

Vergleichende Daten über Glutathionperoxidase (GSH-Px)-Aktivität der Erythrozyten, Serum-Cholesterin und Serum-Fettsäuremuster von indischen Emigranten und Dänen werden dargestellt. Die GSH-Px-Aktivität in Erythrozyten indischer Emigranten wurde im Vergleich zu der von Dänen, bei Verwendung von t-Butylhydroperoxid als Substrat, signifikant erhöht. Auch bei Verwendung dreier Peroxide, nämlich Wasserstoffperoxid, Cumenhydroperoxid sowie t-Butylhydroperoxid, wurde eine signifikante Korrelation (R 0,9) in der indischen Gruppe gefunden. Eine solche Korrelation konnte jedoch bei der dänischen Gruppe nicht festgestellt werden. Der Serum-Cholesteringehalt lag bei der indischen Gruppe signifikant niedriger als bei der dänischen Gruppe. Auch die Gesamtfettsäuren-Muster des Serums beider Gruppen unterschieden sich beträchtlich.

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