Determination of glutathione peroxidase (GSH-Pex) activity in sera & RBCs of Iraqi Women With Breast Tumors

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Abstract:

Breast tumor patients generally have more oxidative stress than normal females. This was clear from non significant decrease in RBCs and serum of GSH-Pex activity in all groups of patients. The study had found that free radicals in malignant breast tumors were higher than benign tumors; therefore the GSH- Pex might be used as markers for prognosis of the disease.

Key words: glutathione peroxidase, Breast Tumors, free radical ,Antioxidants.

Introduction:

The breast is a large compound racemose gland, consists of a few ducts, which are connected to the nipple and open to the surface. The main function of the breast is the production and expression of milk. Development of the breast requires the co-ordinate action of many hormones[1].

The breast tumors may be described, as either benign or malignant (cancerous). The national center of cancer in Iraq predicts that there is an increasing of incidence breast cancer in Iraqi women for years (1975-2000).

The important risk factors are: female sex and age, age of menarche and menopause, family history & genetic factors. ROSs are the main cause of breast cancer which encompasses all highly reactive oxygen containing molecules, including free radicals. Some of ROSs are defined as a free radicals, any atom or molecule having an unpaired electron in its outer orbit as ($O_2^{\bullet-}$, OH•, COO•, CO•). others, are not radicals but active metabolites of oxygen, ex (H2O2, HOCl). ROSs formation sources are mitochondrial respiratory chain, phagocytes, redox reaction, radiation, cigarette, smoke environmental pollution. Main ROSs are: (super oxide radical $O_2^{\bullet-}$, Hydrogen peroxide H2O2, hydroxyl radical OH•, nitric oxide NO, singlet oxygen $\mathbf{O_2^-}$)(1). A first line body defense against oxidative stress produced by generation of free radical and reactive oxygen species ROSs are antioxidants which can defined as (any substance which delays or inhibits oxidative damage to a target molecule[1].

Antioxidants are classified to enzymatic (GSH-Pex, GSSG-Red, SOD, CAT…) non enzymatic (vit.C, vit.E, bilirubin GSH)(2).

GSH-Pex (EC 1.11.1.9) is a tetramer protein with molecular weight (70 kDa) composed of four identical subunits each containing one atom of selenium, which decomposes H2O2, various hydro and lipid peroxides[2,3]. It is present in mammalian red cells & that it protected hemoglobin from oxidative break down [4,5].

There are at least five GSH-Pex isoenzymes found in mammals. The

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$$2GSH + H_2O_2 \xrightarrow{GSH-Pex} 2H_2O + GSSG$$

classical or cystolic glutathione peroxidase (cGSH- Pex) was the first mammalian selenoprotein to be characterized[6,7,8]. Lateron, phospholipid hydrolipid glutathione peroxidase (pH GSH-Pex)[9,10], plasma GSH-Pex (PGSH- Pex)[11], agastrointestinal form of GSH- pex (GI GSH- Pex)[12], and non selenium dependent GSH- Pex[13] were identified[14,15,16].

Diamond[17,18], also raise the possibility that reduced levels of certain selenoproteins, including GSH-Pex, may increase the risk of promote the development of breast cancer[17,18]. Turkmen[19], found relation between the level of GSH- Pex and breast cancer[19], wherease Ray et al., found that GSH- Pex activities significantly raised in all stages of breast cancer patients and this may be due to the response of increased ROS production in the blood [20].

Materials & Methods:

Chemicals:

Nicotinamid adenine dinucleotid phosphate (reduced) disodium salt.	BDH chemical Ltd, England
Disodium hydrogen phosphate.	BDH chemical Ltd, England
Ethylene diamin tetra acetate disodium salt.	BDH chemical Ltd, England
Sodium bicarbonate	BDH chemical Ltd, England
Potassium ferric cyanide	BDH chemical Ltd, England
Glutathione reductase type III	Sigma chemicals, USA
Sodium azide	Sigma chemicals, USA
Glutathione (reduced)	Sigma chemicals, USA
Hydrogen peroxide	Merck, chemicals Germany

Patients:

Four groups of breast tumor patients were included in this study:-

Group I:	Consisted of (33) pre-menopausal patients with benign breast tumors
Group II:	Consisted of (19) pre-menopausal patients with malignant breast tumors
Group III:	Consisted of (5) postmenopausal patients with benign breast tumors
Group IV:	Consisted of (16) postmenopausal patients with malignant breast tumors
Group V:	Consisted of (34) healthy subjects

The patients were recently diagnosed and were not undergone any type of therapy. Patients suffered from any other diseases that may interfere with our study were excluded.

All patients were admitted for diagnosis & surgery to (Al-Yarmok Teaching Hospital, Baghdad Teaching Hospital, and Nursing Home Hospital).

Table (1): The host information of breast tumor patients(the studied) and healthy subjects studied

Group	Patients	No.	Age	Type of tumor
I	pre-menopausal benign breast tumors	33	16-49	- Fibrocystic changed adenosis
				- Axillary lymph nodes
				- Fibro adenoma
				- Duct papilloma
II	pre-menopausal malignant breast tumors	19	16-49	- Infiltrattive ductal carcinoma
				- InSitu carcinoma
III	postmenopausal benign breast tumors	5	50-65	- Duct Papilloma
				- Axillary Lymph nodes
				- Fibrocystic Changed
IV	postmenopausal malignant breast tumors			- Infiltrattive ductal carcinoma. - Fat necrosis. Several patient's with metastasis & recurrence breast carcinoma after mastectomy & radio or chemotherapy.
V	Control	34	20-45	

Non of the patients were on a special diet, or taking any antioxidants (Vitamins E, Vitamins C,… etc) or treated with antioxidant drugs except (Voltarin, Ampy Glucose, Paracetol), non of patients were exposured directly to radiation and didn't drink alcohol or smoke, negative genetic factor to have cancer with very clearly irregular menstrual cycle in most patients.

Sample collection:

Blood samples (5 ml) were collected from the patients just before surgery by vein puncture & from the healthy controls. Tow & half milliliter of aspirated blood (2.5ml) was immediately transferred into plan tube with anticoagulant (Potassium-EDTA) (Ethylene diamine tetra acetate tripotassium) (1.5 mg/ ml blood) for erythrocyte isolation and their study, blood samples were centrifuged at 4000/ r.p.m for 10 minutes after allowing the blood to stand at room temperature (25°C), plasma & buffy coat were removed and erythrocytes were washed two times with cold normal saline (0.9% NaCl) pH= 7.0. The packed cell volume (PCV) stored at (-20°C) until assay. Another (2.5ml) of aspirated blood were transferred into plan tube without anticoagulant for serum separated by centrifugation at 4000 r.p.m for 10 minutes (serum kept at -20°C until assay) after allowing the blood to clot at room temperature (25°C).

Erythrocyte glutathione peroxidase GSH-pex. Assay[21,22,23,24]:

Reagents:

1) Reagent A: [Sodium phosphate buffer (0.15M) pH=7.0]: (0.81153g) Na_2HPO_4, (11.1420g) NaH_2PO_4, (1.68g) Na_2-EDTA was dissolved in one liter D.W.
2) Reagent B: (0.025g) of NADPH-Na_4 was dissolved in (10ml) D.W. (freshly prepared).
3) Reagent C: (0.0005g) of GSSG-Red (type III) was dissolved in (10ml) phosphate buffer.
4) Reagent D: (0.039g) of NaN_3 was dissolved in (10ml) D.W.
5) Reagent E: (0.000921g) of GSH was dissolved in (10ml) D.W. (freshly prepared).
6) Reagent F: (Double- strength Drabkin's reagent): One gram of $NaHCO_3$, (0.2gm) $K_3[Fe(CN)_6]$,

(0.052 gm) KCN was dissolved in (500ml) deionized water.

7) Reagent G: (0.008ml) of (30%) H_2O_2 solution was added to (100ml) D.W. (prepared immediately before assay).

Procedure:

Hemolysate preparation:

1) To a set of test tubes containing (0.1ml) of washed packed cell volume, (0.4ml) D.W. was added to it & frozen at (-20°C) then thawed. The freezing and thawing were repeated thrice. Then hemolysate was centrifuged to remove the depressed cells.
2) To a tube containing (1.9ml) D.W., (0.1ml) of supernatant was added and mixed.
3) One milliliter of mixture (supernatant +D.W.) was combined with (1ml) of reagent (F) and left for 20 min. Hemolysate was used as sample.
4) Enzyme assay:

Reagents	Blank (ml)	Test (ml)
Reagent A	1.0	1.0
H_2O	1.5	1.4
Reagent E	0.1	0.1
Reagent C	0.1	0.1
Reagent D	0.1	0.1
Reagent B	0.1	0.1
Sample	—	0.1
Reagent G	0.1	0.1
Final volume	3.0	3.0

The reaction was initiated by the addition of reagent G to the assay mixture and change in absorbance was monitored for (10min.) period of time (at λ= 340nm) and (25°C).

Calculations:

$$\text{GSH - Pex activity in RBC U/gm Hb} = \frac{\frac{\Delta A}{t} * V_T * 1000}{\varepsilon * V_S * l * \text{Hb(gm/L)}}$$

ΔA: Difference in absorbence
t: Time interval (10min.)
V_T: Total volume of assay = 3.0ml
V_S: Sample volume = 0.1ml
ε: Extinction coefficient = 6.22m $M^{-1}.cm^{-1}$
l: length of light path (1cm).
Hb: hemoglobin concentration

$$\text{So : GSH - Pex activity in RBC U/gm Hb} = \frac{48.23\,\Delta A}{\text{Hb(gm/dl)}}$$

Serum glutathione peroxidase (GSH-pex.) assay:

Procedure:

1) One milliliter of serum was combined with (1ml) of reagent F & left for 20 min. mixture was used as sample.
2) The procedure was followed as step [4] in procedure of the erythrocyte glutathione peroxidase assay

Calculations:

$$\text{GSH - Pex activity in Serum U/L} = \frac{\frac{\Delta A}{t} * V_T * 1000}{\varepsilon * V_S * l}$$

Discussion:

Mills (1957) was the first who demonstrated that glutathione peroxidase presents in mammalian red cells, and that it protects hemoglobin from oxidative break down by hydrogen peroxide[25].

GSH- Pex may catalyse the conversion of lipid peroxide in to lipid alcohol without free radical activity, hence terminate the branching chain reaction of lipid peroxidation & this prevents & ameliorates damage to the cell membrane, the recycling procedure for the determination of GSH- Pex activity depends on the oxidation of GSH to GSSG by the enzyme in presence of NADPH & exogenous glutathione reductase (GSSG-Red) which regenerate GSH from GSSG[21,26].

$$2\,GSH + H_2O_2 \xrightarrow[Se]{\text{GSH-Pex}} 2H_2O + GSSG \xrightarrow[NADPH \quad NADP]{\text{GSSG-Red}} 2\,GSH$$

The rate of enzyme activity was monitored by following the decrease in absorbance at (340 nm) as a function of NADPH exhaustion[22].

Table (2) shows GSH – Pex activities in Rbcs of patients with breast tumors were non-significantly differences (P>0.05) in comparis on to that observed in normal controls.

Table (2): Erythrocytes GSH – pex activities (U/g Hb) in patients with breast tumor (pre & post M.P.) and in controls

Groups	Control	Malignant		Benign	
		Post M.P	Pre M.P	Post M.P	Pre M.P
No. of samples	7	6	5	4	6
Mean	0.958857	0.854933	0.82760	0.8260	1.055750
SD	0.21184	0.173298	0.165968	0.132333	0.359721
T- test		- 1.469	- 1.768	- 2.008	0.660
Probability		0.202	0.152	0.138	0.539

GSH- Pex activity in RBCs of malignant breast tumors for both pre & post M.P. and also in benign tumors post M.P. had a non-significant decrease (P>0.05) compared to that in normal control, except in benign tumors for pre M.P, GSH- Pex activity was non-significant increased (P>0.05) when compared with control. We found that GSH - Pex activity in post M.P. was more than in pre M.P. for malignant disease, while in post M.P. was less than pre M.P. for benign disease. The results show that the activity in malignant was more than the benign for post M.P, in reverse for pre M.P.

Raising GSH- Pex activity may contribute to the need of body to counteract the toxicity of ROS, but the depressed activity may cause by the consumption of all enzyme activity during defense against ROS, or tumor cells[27].

Error in gene expression of enzyme can also cause delay or decrease enzyme activity & this error come from harm DNA due to oxidative stress damage[28].

The defect in GSH-Pex may lead to the accumulation of ROS, especially $O_2^{\bullet}$ in plasma. Super oxide Can easily be converted to H_2O_2 by metal ions present in plasma, and H_2O_2 can penetrate through Rbc, & neutralized by GSH- Pex[29].

Our results are in a good agreement with Hopkins [23]., who found that in patients with breast carcinoma a wide variation in red- cell GSH- Pex, but the mean value was significantly less than normal, and in some cases may be related to the general state of protein metabolism [23, 25].

Seven [30], demonstrated that GSH-Pex activity in Rbcs was significantly increased in benign and malignant breast tumors when compared to normal controls and showed that in relation to increased oxidative stress[30], Arruda,[31] reported significant decrease in GSH-Pex activity in Rbcs of patients with leukemia.

The results of another study in leukemia results show significant increase in Rbc GSH-Pex activity for newly diagnosed leukemia patients as compared with that of controls[32].

Serum GSH-Pex activities in post M.P for both malignant and benign tumors patients were non significantly decreased (P>0.05) in comparison to that observed in normal subjects, where is, in pre M.P for both malignant and benign tumors patients

had significantly decrease (P>0.05) compared to normal subjects. Table (3)

Table (3): Serum GSH- Pex activities (U/L) in patients with breast tumor (pre & post M.P.) and in controls

Groups	Control	Malignant		Benign	
		Post M.P	Pre M.P	Post M.P	Pre M.P
No. of samples	7	3	5	3	11
Mean	11.11300	11.012367	7.90940	10.65833	9.066273
SD	2.06191	1.158792	0.321699	2.478972	2.804064
T- test		- 0.150	- 22.268	- 0.318	- 2.421
Probability		0.894	0	0.781	0.036

We noticed that post M.P. patients had GSH- Pex activities more than pre M.P. patients in both malignant & benign disease. Serum enzyme activity in post M.P. for malignant tumor patients was more than benign, the contrast is true with pre M.P. patients.

This result is in agreement with Gavriliuk & Shraiman[29], who reported significantly decrease in GSH- Pex activities in plasma, Leukocytes, and lymphocytes of acute Leukemias. Ray et al., found significant raised in GSH- Pex activity in serum for all stages of breast cancer patients (P<0.001) except in stage IV was found a smaller alteration (P<0.02)[20,33], On other hand Gary[34]., found plasma & Rbcs GSH-Pex activities of patients with benign & malignant breast diseases were significantly different as compared to the control groups[34].

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تقدير فعالية الكلوتاثايون بيروكسيديز في امصال وكريات الدم الحمراء لنساء عراقيات مصابات باورام الثدي

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الخلاصة:

تكون النساء المصابات بسرطان الثدي عموما اكثر عرضة للاجهاد التاكسدي مقارنة بالنساء غير المصابات. ولكن لم يكن هناك انخفاض معنوي في فعالية ال (GSH-Pex) في الامصال وكريات الدم الحمراء في جميع مجاميع المرضى. وقد اظهرت الدراسة ارتفاع نسبة الجذور الحرة في الاورام الخبيثة مقارنة بالحميدة، ولهذا يمكن استخدام الـ (GSH-Pex) كدالة لتقدم المرض.