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Study of Malondialdehyde Levels in sera, RBCs and Tissues homogenate of Iraqi Women With Breast Tumors

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Abstract:

Breast tumors patients generally have more oxidative stress than normal females. This was clear from a highly significant elevation (P<0.05) in malondialdehyde level in RBCs, serum and tissue of all patients groups with breast cancer as compared with control group. In this study we had found that free radicals in malignant breast tumors were higher than benign tumors, therefore the MDA might be used as a marker for prognosis of the disease.

Key words: Breast tumors, malondialdehyde, free radicals, lancer.

Introduction:

The breast is a large 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].

Breast tumors may be described, 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)[1].

The important risk factors are: sex, age, family history & genetic factors. Reactive Oxygen species “ROSs” are the main cause of breast cancer. Some of ROSs are defined as a free radicals, any atom or molecule having an unpaired electron in its outer orbit as (O₂⁻, OH⁻, COO⁻, CO⁻), others, are not radicals but active metabolites of oxygen, ex (H₂O₂, HOCI). ROSs formation sources are mitochondrical respiratory chain, phagocytes, redox reaction, radiation, cigarette, smoke environmental pollution. Main ROSs are: (superoxide radical O₂⁻, Hydrogen peroxide H₂O₂, hydroxyl radical OH⁻, nitric oxide NO, singlet oxygenO₂⁻)[1].

The major deleterious effect of intracellular free radical generation is lipid peroxidation [2,3]. Lipid peroxidation is the oxidative deterioration of poly unsaturated lipid under influence of free radicals, which has several biochemical effects, that are not limit to the involved lipid, but also membrane. Alteration in membrane structure and function enhance and ultimate consequences such as the loss of membrane lipid, the production of toxic lipid break down product, and the peroxidative damage to near by membrane protein[4,5]. Seven & al, and Turkmen & et al., found relationship between lipid peroxidation & breast tumor, one study shows alteration in lipid peroxidation with advanced breast cancer due to change in oxidant antioxidant status[6]. Another study shows decreased lipid peroxidation in breast cancer in comparison to benign breast disease [7].

MDA is the most marker that is used to investigate the presence of lipid peroxidation in biological system[8].

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The pathways that lead to marker from lipid peroxidation are illustrated in figure (1). MDA is a three carbon molecule with two aldehyde groups which renders it highly reactive with other bio-molecules[9]. MDA circulates in plasma either bound to protein or in free form[7,10]. Another portion of MDA is generated in vitro, from decomposition of lipid hydroperoxides during sample preparation[11]. MDA is a significant by-product during enzymatic synthesis of prostaglandin[12]. It can be removed by renal clearance[13]. It plays a significant role in DNA damage, sister-chromatid exchanges and carcinogenesis. MDA was higher in the breast cancer group & patients with stage II disease also showed the highest levels of MDA[14]. It can be stated that in advanced breast cancer the lipid metabolism is altered and these changes can be related to an alteration in oxidant-antioxidant status[15].

Figure (1) The Reactions of Lipid Peroxidation[16]
Materials & Methods:

Chemicals:
All the chemicals were from BDH Company & were analyte gradient
- Trichloro acetic acid (TCA)
- Sodium
- Sodium arsenite
- Thiobarbituric acid (TBA)
- Sodium hydroxide
- Normal salin
- Sodium azide
- Sodium dihydrogen phosphate
- Disodium hydrogen phosphate
- Bovine serum albumin
- Folin-Ciocalteau (phosphor molybdo tungstic)
- Copper sulfate (hydrate)
- Sodium, potassium tartarate
- Sodium carbonate
- Sodium hydroxide

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

Group I: Consisted of (33) premenopausal patients with benign breast tumors.

Group II: Consisted of (19) premenopausal 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 diseases that may interfere with our study were excluded.

All patients were admitted for diagnosis & surgery to (Al- Yarmok Teaching Hospital, Baghdad Teaching Hospital, Nursing Home Hospital). 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, Amyp glucos, Paracetol), non of patients were exposed 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).

Tumor tissues were surgically removed from breast tumor patients by either mastectomy (cancer patients) or lumpectomy (benign tumor patients).
The specimens were cut off and immediately rinsed with ice-cold normal saline solution, & stored at (-20°C) until homogenization. The frozen tissue was thawed & worked as follow:
1) The blood & adipose tissue were removed with enough cold normal salin (0.9% NaCl).
2) Tissue was weighted.
3) Sliced finely with scalpels in Petri dish standing on ice bath.
4) The slices were further minced with scissors then homogenized by using a manual homogenizer in buffer solution for malondialdehyde measurement: weighted tissue was homogenized in (20mM) phosphate buffer pH 7.4
5) For Glutathione reductase measurement [50 mM tris (hydroxyl methyl) methyl amine – (0.1 mM) EDTA] pH 7.6 was used.

The buffer was added in a ratio (1:3) (weight: volume) gradually to tissue. The homogenate was filtered through (4) layers of x-ray gauze size (90 cm x 5 mm), then centrifuged at (3000 r.p.m) at (4°C) for 10 min (for MDA) and at (10000 r.p.m) at (4°C) for 10 min (for GSSG- Red).
4) An aliquot was removed (supernate of the sample for determining protein concentration).
5) Sample immediately was frozen at (-20°C) until assay, the supernatant was used through the study[17,18].

Solutions:
Phosphate buffer (20 mM, pH= 7.4) for (MDA) assay was prepared by dissolving (0.284 gm) of NaH2PO4 in (100ml) distilled water (reagent A) and dissolving (0.24 gm) of Na2HPO4 in (100ml) distilled water (Reagent B). Then (25ml) of reagent A were mixed with (10ml) of reagent B and adjusted pH. Volume was completed to (100ml) distilled water & adjusted pH again.

Total protein determination of breast tumor tissue homogenate:
The total protein of breast tumor tissue homogenate was determined by Lowry et al. Method[19] using bovine serum albumin (BSA) as the standard protein.
1) Reagent A: Alkaline sodium carbonates solution (2% Na2CO3 in 0.1N NaOH).
2) Reagent B: Copper sulfate- sodium potassium tartarate solution (0.5% CuSO4.5H2O in 1% Na,K tartarate).
This solution was prepared freshly by dissolving (0.1g) of (Na,K- tartarate) in 10ml of CuSO4.5H2O.
3) Reagent C: Alkaline copper solution: Fifty milliliters of reagent A were mixed with (1 ml) of reagent B prepared immediately before assay.
4) Reagent D: Folin Ciocalteu, reagent was prepared freshly by the dilution of commercial reagent with an equal volume of distilled water.
5) Standard bovine serum albumin (BSA): (0.2 mg/ml) was prepared as stock solution, working BSA solutions were prepared by serial dilutions of stock solution.

Procedure:
1) From each of standard (BSA) (25, 50, 75, 100, 125, 150, 175, 200, 225) µg/ml, (1000) microliter of was pipetted in a set of test tubes.
2) In a test tube (20) microliter of breast tumor homogenate were pipetted and the volume was made up to (1000µl) with distilled water.
3) To all assay tubes (5000) microliters of reagent C was added, then the contents were mixed by vortexing and allowed to stand for 10 min at room temperature (25°C).
4) From reagent D (500) microliter was added drop by drop with vigorous mixing to all assay tubes. The mixture was left to stand for 30 min at room temperature 25°C.
5) The absorbance of developing colour was read at (600nm) against the appropriate blank.
6) Blank contained all reagents except (BSA).

Calculations:
The standard curve was obtained by plotting the absorbance against the corresponding concentrations of standard protein and used to determine the unknown protein concentration of the sample (breast tumor homogenate) as shown in fig (2).

![Figure (2) Standard curve of protein cons.](image)

Measurement of erythrocyte malondialdehyde (MDA) levels:

Reagents:
1) Reagent A: (28% TCA) was prepared by dissolving (28gm) TCA in (100ml) of (0.1M) NaAsO₂.
2) Reagent B: (1% TBA) one gram of TBA was dissolving in (100ml) of (0.05M) NaOH.
3) Reagent C: (0.1M salin azid) was prepared by dissolving (0.65gm) of NaN₃ in (100ml) normal salin (0.9% NaCl).

Procedure (1):
1) One milliliter of reagent A was added to one ml of (10%) aliquote suspension of RBCs in reagent C, the mixture was centrifuged.
2) Two milliliter of the supernatant were mixed with (0.5ml) of H₂O and (0.5ml) of reagent B.
3) The mixture was incubated in water bath at (100°C) with cover for 15 minute until color development.
4) The tubes were cold under tap water, and the extent of MDA production was estimated by reading the absorbance at λ = 532nm.
5) Blank was included all assay mixture except TBA.

Calculations:
MDA concentration was calculated using a molar absorbivity coefficient of (1.56×10⁵M⁻¹cm⁻¹) and the results were expressed as (nmol MDA/gm Hb)[20].

\[
\text{MDA}_{\text{con. in Rbc/nmol/gm Hb}} = \frac{A \times 10^6 \times \text{d.f.}}{\varepsilon \times \text{Hb} \times b}
\]

A: Absorbency
\(\varepsilon\): Extinction coefficient = 1.56 × 10⁵ M⁻¹cm⁻¹
Hb: hemoglobin concentration g/L
b: light path (1cm).
d.f: dilution factor = 30.

Measurement of serum MDA levels:

Procedure (2):
1) One & three quarter milliliter of reagent C was added to (0.25ml) of serum.
2) One milliliter of reagent A was added to one ml of mixture (serum in reagent C) then centrifuged.
3) The procedure was followed as (the steps 2-5) in procedure (1).

Calculations:
MDA con. In serum was expressed as \(\mu\text{mol/L}

\[
\text{MDA}_{\text{con. in serum/μmol/L}} = \frac{A \times 10^6 \times \text{d.f.}}{\varepsilon \times b}
\]

d.f = 18.
Measurement of tissue MDA levels:

**Procedure (3):**
1) One & three quarter milliliter of reagent C was added to (0.25ml) of tissue supernatant.
2) One milliliter of reagent A was added to one ml of mixture (tissue supernatant in reagent C) then centrifuged.
3) The procedure was followed as (the steps 2-5) in procedure (1).

**Calculations:**

MDA con. in tissue supernatant was expressed as μmol/g protein:

\[ MDA \text{ con. in tissue homogenate } \mu \text{mol/g protein} = \frac{A \times 10^6 \times d.f}{e \times b \times \text{protein con. g/l}.} \]

\[ \text{d.f} = 18 \]

**Discussion:**

The human breast tumor tissue was taken through our study & was classified according to the patient’s age (pre or post menopausal) and each type was divided to malignant & benign undergo histopathological examination.

The homogenization condition are optimized by systematic of parameter such as the extraction medium, time, temperature and type equipment used to reduce major problems.

Homogenization was carried out in a cold medium (i.e., 4°C) to avoid protein denaturation.

The filtration of the tissue homogenate through four (4) layers of x-ray gauze were used to remove any suspended pieces of unhomogenized fragments & blood vessels and eliminate fibers of connective tissue, while the centrifugation of the homogenate at 3000 r.p.m (for MDA) helped for further elimination.

MDA level is the most convenient marker used to detect oxidative stress & lipid peroxidation, although there are some factors that contribute to limit its utility[21,22]. Elevated level of MDA has been reported in several disorders associated with oxidative stress such as cataract[23], cardiovascular disease[24], cancer[25], renal failure[26], inflammatory diseases, and several others. MDA forms a (1,2) adduct with thiobarbituric acid (TBA) & produces the following compound:

![Chemical structure of TBA and MDA](image)

Reddish chromogen absorbs light at (532 nm)[27,28].
As shown in Table (1), patients with breast tumors had a significant elevation in erythrocytes MDA level as compared with that in normal controls.

Table (1): Erythrocytes MDA level (µmol/g Hb) in patients with breast tumor (pre & post M.P.) & in controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Malignant</th>
<th>benign</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post M.P.</td>
<td>Pre M.P.</td>
<td>Post M.P.</td>
</tr>
<tr>
<td>No. of samples</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>0.1402</td>
<td>0.1324</td>
<td>0.1237</td>
</tr>
<tr>
<td>SD</td>
<td>0.0111</td>
<td>0.0091</td>
<td>0.0074</td>
</tr>
<tr>
<td>T-test</td>
<td>5.396</td>
<td>7.363</td>
<td>2.395</td>
</tr>
<tr>
<td>Probability</td>
<td>0.005</td>
<td>0.002</td>
<td>0.05</td>
</tr>
</tbody>
</table>

MDA levels in malignant breast tumors for both post & pre menopausal were found significantly higher (P<0.05) than their corresponding normal controls & also a significant increase was noticed in level of MDA in benign tumor (P<0.05) for both pre & post menopausal compared to that observed in normal controls. In malignant tumors’ patients MDA levels for pre M.P. were more than post M.P. in reverse for the benign tumors patients, post M.P. were more than pre M.P., we demonstrated that MDA levels in malignant tumors were more than benign.

The increases in MDA levels reflects to an increase in lipid peroxidation initiated by ROSs. So that the imbalance between antioxidants production & removal of ROSs lead to oxidative stress[29].

A study in Iraq has shown an increase of MDA levels in erythrocytes & plasma of patients with breast cancer before & after radio therapy[30]. In blood malignancies, increased MDA level is reported in RBCs of patients with leukemia[31,32,33,34], malignant lymphoma[35], and oral cancer[36].

The level of MDA serum shows an increase in patients with breast tumors when compared with normal control, as shown in Table (2).

MDA levels in malignant & benign breast tumors for both pre & post M.P. were significantly higher (P<0.05) than normal control.

Table (2): Serum MDA level (µmol/L) in patients with breast tumor (pre & post M.P.) & in controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Malignant</th>
<th>benign</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post M.P.</td>
<td>Pre M.P.</td>
<td>Post M.P.</td>
</tr>
<tr>
<td>No. of samples</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>3.584</td>
<td>21.25</td>
<td>10.44</td>
</tr>
<tr>
<td>SD</td>
<td>2.196</td>
<td>5.018</td>
<td>4.0948</td>
</tr>
<tr>
<td>T-test</td>
<td>4.924</td>
<td>8.877</td>
<td>7.02</td>
</tr>
<tr>
<td>Probability</td>
<td>0.005</td>
<td>0.001</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Post M.P patients with benign & malignant tumors had a serum MDA level more than in pre M.P. Generally malignant tumor’s patients showed an increase in MDA level compared to that found in benign tumor’s patients.

Turkmen[37], stated that in advanced breast malignancy, the lipid metabolism is altered in serum & plasma[37].

Look[38], concluded that plasma MDA level in patients with breast cancer significantly more than those in controls (P<0.001)[38,39], but Ray [14], Seven [7], found that MDA production in plasma decreased in patients with breast cancer in comparison to benign breast tumor groups[7,14].

Our results also are in a good agreement with that of several authors who have reported an increase in MDA level in other forms of cancers including brain tumor[40], cancer of esophagus[41], lung cancer[41] and breast cancer[42]. They attributed that the increase in the level of serum MDA
is due to the increase in the rate of lipid peroxidation of “PUFAs”.

Iraqi studies show also an increase in MDA for patient’s with leukemia & oral cancer compared to the control group[43].

Table (3) shows MDA levels in the tissue of breast tumors. We found that the level of MDA in Pre M.P. for both malignant & benign was more than post M.P. patients.

Table (3): Tissues MDA level (µmol/g protein) in patients with breast tumor (pre & post M.P.)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Malignant</th>
<th>Benign</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre M.P.</td>
<td>Post M.P.</td>
</tr>
<tr>
<td>No. of samples</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>694.386</td>
<td>1312.141</td>
</tr>
<tr>
<td>SD</td>
<td>540.527</td>
<td>608.506</td>
</tr>
<tr>
<td>T- test</td>
<td>2.923</td>
<td>2.161</td>
</tr>
<tr>
<td>Probability</td>
<td>0.015</td>
<td>0.054</td>
</tr>
</tbody>
</table>

A high significant increase (P<0.05) was noticed in the level of MDA in malignant tumors patients as compared with benign tumors for both pre & post M.P.

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