CHAPTER 2

AIM OF THE WORK

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The aim of this study is to estimate Metallothionein concentrations in *Andara dulotii* mussel samples as a biomarker of heavy metals exposure to monitoring pollution of Abu qir bay (El-Maadiya region), and study the risk assessment of this pollution on human health through the determination of oxidative stress biomarkers (malondialdehyde, glutathione content, glutathione peroxidase activity, catalase activity and superoxide dismutase activity), and the gene expression of insulin-like growth factor II (IGF-2) in blood of fishermen.

CHAPTER 3

MATERIAL & METHODS

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A. Material

1. Chemicals

Absolute ethanol, chloroform and hydrochloric acid, nitric acid, β-mercaptoethanol, hydrogen peroxide, milliQ water were of HPLC grade purchased from Riedel deHaën, (Germany). potassium dihydrogen phosphate, di potassium hydrogen phosphate, di sodium hydrogen phosphate, sodium dihydrogen phosphate, ethylene diaminetetraacetic acid, sodium citrate, 5, 5`-dithiobis-2-nitrobenzoic acid , Sucrose, phenylmethylsulphonylfluoride, leupeptine, thiobarbituric acid, Tris-HCl, Pyrogallol, trichloroacetic , agarose gel, ethidium bromide, reduced glutathione, sodium azide, nicotinamide adenine dinucleotide phosphate, glutathione reductase, and freeze dried mussel tissues (*Mytilus edulis*) were from Sigma-Aldrich Chemical Co., (St. Louis, MO, U.S.A.). Potassium hydroxide, sodium hydroxide and sodium chloride from ADWC (Egypt). IQeasy plus blood RNA extraction kit and Maxime RT-PCR Pre Mix were from iNTRON biotechnology, (Korea). DNA primer sequences were from Jena, Bioscience Germeny. Master Mix, magnesium chloride and PCR markers were from Promega, Madison, WI, USA.

2. Study area

Boughaz El-Maadiya is a shallow narrow channel of about 2 m depth, 20 m width and 100 m length. The hydrographic and biological characteristics of the water in El-Maadiya connection depend on the water exchange between Abu-Qir Bay and Lake Edku [(241)]. Abu Qir bay is a semicircular shallow basin about 35 k east of Alexandria city between Abu Qir peninsula (west) and the Rosetta branch of the Nile (East), with a shoreline extending about 50 km (figure 11) [(242)]. It lies between 30° 4'- 30 21' East and 31 16'-31 30' North. It is relatively shallow with a depth ranging from less than 1 m along the coast, increasing gradually away from the shore to reach a maximum depth of about 15 m [(241)]. In Abu-Qir Bay, great amounts of industrial wastes are discharged into the bay through Tabia pumping station (figure 12). These wastes are coming from about 36 factories extending from Kafr El-Dawar to Alexandria in a cultivated area. Tabia pumping station is pumping highly polluted water of El-Amia Drain, which receives the drainage from El-Behira district and

the industrial wastes of the factories. These waste discharges originated from five main activities; fertilizer industry, pesticides industry, textile manufacturing, paper industry and food processing and canning. The main industrial plants are Rakta pulp and paper companies, National paper company, Beida dyes company, Misr rayon company, Misr textile company, Kaha company, Ammonia urea plant, Abu Qir Fertilizer Co, Power-plant, AgroChem for pesticides industries and in the last years backing plant of cement [(241)]. Tabia pumping Station is located in the south-western part of Abu-Qir Bay, nearly in midway between the city of Abu-Qir and Boughaz El-Maadiya. It pumps out an average amount of 1.5 to 2 million m^3 of polluted water per day [(241)].

Figure 11: Map of Mediterranean Sea, Abu-Qir bay show study area (El Maadiya region) [(242)].

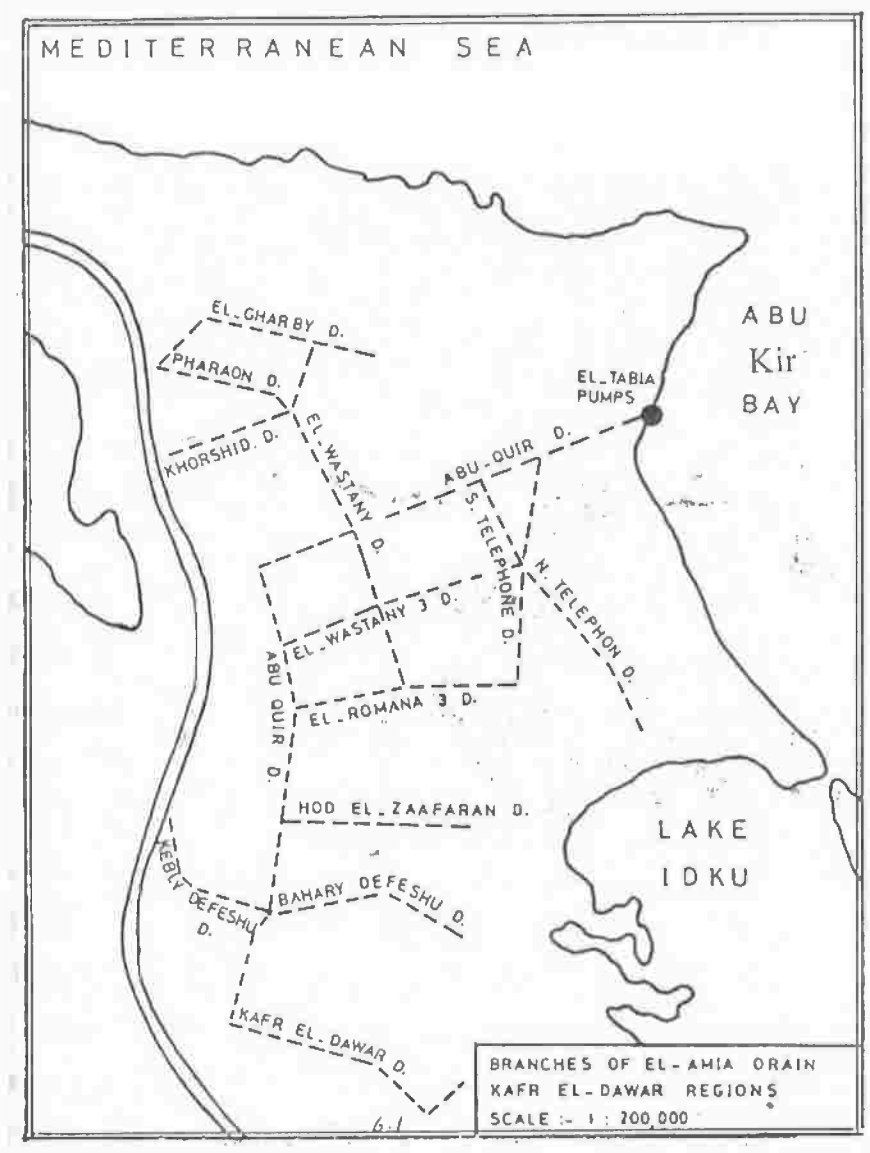

Figure 12: Map of El-Tabia Pumping Station (EL-Amia Drain) [242]

3. Sampling

3.1. Biological Matrix

Andara dulotii mussel samples (Figure 13) were collected from Mediterranean Sea, Abu-qir bay "El-Maadiya region" and acclimated to laboratory conditions at 15°C for three days in EDTA free synthetic seawater, pH 7.9-8.0, at 35 osmolarity & salinity (Viarengo *et al.*, 1997) [243], for determination of metallothionein and five studied metals (cadmium, lead, chromium, copper and zinc) [244].

Figure 13: *Andara dulotii* mussel samples collected from Mediterranean Sea, Abu qir bay "El-Maadiya region".

3.2. Human Subjects

A total of fifty (56) male subjects with an age range of (20-55 years old); and weighed from 62-85 Kg, and divided into two groups;

A).Control subjects (Group I): Includes (12) healthy control male person who are living in El-Maadiya region but works in other jobs rather than fishing.

B).Volunteer Fishermen (Group II): includes (44) professional Fishermen volunteers living in El-Maadiya region.

Based on questionnaire (Appendix 1), the information on age, weight, area of residence, smoking history, consumption of caffeine, medication used, history of acute or chronic illness were recorded for each subject. Also, their food habits were taken in concern, thyroid dysfunction, diabetes, and liver disease were excluded.

Blood Samples: were collected from all subjects enrolled in this study for the assay of: Metallothionein [245, 246], Heavy Metals (Cd, Pb, Cr, Cu and Zn) [247], Malondialdehyde (MDA) [248], Glutathione (GSH) content [249], Glutathione Peroxidase (GPx) activity [250, 251], Catalase (CAT) activity [252], and Superoxide Dismutase (SOD) activity [253]. RNA was extracted from peripheral blood mononuclear cells by commercially available kit for study of Insulin-Like Growth Factor II (IGF-2) expression level [254].

B. Methods

I. Oceanographic study

1. Determination of metallothionein in mussel samples [243]

1.1. Principle of the method

Metallothionein concentration was evaluated utilizing a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the tissue homogenate.

1.2. Reagent preparation

1. *Extraction buffer (pH 8.6)*

20 mM TRIS-HCl (MW: 157.6).

300 mM Sucrose (MW: 342.30)

For 100mL of solution; amount of 0.315 grams of TRIS-HCl were dissolved in milliQ water, adjust the pH = 8.6 with HCl and bring the solution to volume with milliQ water, then dissolve 10.2 grams of sucrose in Tris-HCl freshly prepared and finally adjust the volume to 100 ml with milliQ water. Note (Store at 4°C up two month).

2. *Antiproteolitic cocktail*

0.006 mM leupeptine hemisulfate (MW: 475.6),

0.5 mM PMSF (phenylmethylsulphonylfluoride) as antiproteolytic agents (MW: 174.19),

0.01% β-mercaptoethanol as a reducing agent,

Freshly prepared homogenizing buffer contains for 15 ml extraction buffer: 45 µl leupeptine hemisulfate (1.0 mg/ml), 22.5 µl PMSF (58 mg/ml ethanol) and 15 µl of β-mercaptoethanol (10 %).

3. *Re-suspension solution (C_l)*

250 mM NaCl (MW: 58.44)

For 100ml of solution; amount of 1.416 grams of NaCl were dissolved in milliQ water. Note (Store at 4°C)

4. *Destabilizing solution (C_2)*

1.0 N HCl

4.0 mM EDTA (MW: 372.2)

For 100mL of solution; amount of 0.149 grams of EDTA (0.5M) were dissolved in milliQ water and 8.84 ml of HCl (35%) was added and complete the volume to 100 ml by milliQ water, store at 4°C up to two weeks.

5. *Solution C*: ($C_1 + C_2$; 1:1 V/V)

6. *Reaction buffer*

Phosphate Buffer Stock solution (0.2 M, pH 7.6)

For 250 ml; amount of 6.0 grams NaH_2PO_4 were dissolved in 50 ml milliQ water and 7.1 g Na_2HPO_4 were dissolved in 50 ml of milliQ water then mixed and the pH was adjusted (pH 7.6). Finally, 29.22 grams NaCl were added to obtain a final concentration of 2.0M NaCl and completed to the volume equal 250 ml by milliQ water. Stored at; 4°C up to two weeks.

Working Solution

Amount of 0.170 grams DTNB were dissolved in 100 ml phosphate buffer stock solution (0.2 M, pH 7.6). (Preparation of this solution is immediately before use. Protect from light with foil and stir well to dissolve the DTNB. Stored at room temperature and discarded after each analysis).

7. *Standard solution "GSH reference"*

4 mM sulfhydryl reference standard solution reduced solution, GSH, MW: 307.33

(0.012 grams reduced GSH dissolved in10 ml re-suspension solution Store at 4°C and discard after each analysis).

1.3. Procedures

1. *Sample preparation*

Mollusks gills and digestive glands were rapidly dissected out, stored 24 hours under liquid nitrogen and then stored at – 80 °C.

2. *Metallothionein extraction*

1. An amount of 0.5 gram of tissue was homogenized at (0-4°C) adding 1.5ml of homogenizing buffer. The homogenate transferred into 2.0 ml micro-tube and centrifuge at 16,000 x g at 4°C for 40min.

2. Taken a 0.3 ml of supernatant (determined its total protein concentration) and transfer into 1.5 ml micro-tube, add 0.315 ml of cold absolute ethanol (stored - 20°C) and 20 µl of chloroform (stored RT). Mix and centrifuge at 16,000 x g at 4°C for 5min.

3. All the supernatant was collected and transferred into 2ml tube; add 12 µl of 37% HCl (stored RT) and 1.5 ml of cold absolute ethanol (stored -20°C).

4. Incubated at -20°C for 1h, and centrifuged at 16,000 x g at 4°C for 5min.

5. Discard the supernatant, dry the pellet with a gently stream of nitrogen to remove the ethanol residues.

6. Store the pellet at -80°C after drying with liquid nitrogen (if not continue the assay).

3. *Spectrophotometric assay (Ellman's reaction)*

♦ **Sample:** the pellet was re-suspended with 50 µl of solution C ($C_1 + C_2$; 1:1 v/v)**,** 1.950 ml of reaction buffer was added at room temperature, mix briefly, incubated 2.0 min at RT and centrifuged at 16,000g at room temperature for 2 minutes.

♦ **Blank**: a volume of 50 µl of solution C ($C_1 + C_2$; 1:1 v/v)**,** 1.950 ml of reaction buffer was added at room temperature, mix briefly, incubated 2.0 minutes at RT and centrifuge at 16,000g at room temperature for 2.0 minutes.

♦ **Standards**

Preparation of standards

Standard	GSH reference (µl)	Sol. C (µl)	reaction buffer (ml)	nmol [SH]
A	2.5	47.5	1.950	10
B	5	45		20
C	10	40		40
D	15	35		60
E	20	30		80

Mixed briefly, incubated 2 minutes at room temperature and centrifuge at 16,000g at room temperature for 2 minutes.

♦ Read samples, blank and standards with spectrophotometer at 412 nm.

1.4. Calculation

The absorbance values (412 nm) were interpolated over the standard curve (Figure 14) to obtain the concentration (nmol) of sulfhydryl groups, i.e. cysteine residues, due to metallothionein present in the sample: (nmol Cys ^{MT}). To obtain the concentration of MT (µg/g of tissue) the next equation was applied:

$$(\mu g\ MT) * g^{-1} = \frac{ABS \times 142.85 \times 121.61}{0.1 \times 23 \times 0.055 \times 100}$$

Where:

* ABS = Absorbance of sample relative to [SH].

* g = amount of tissue equivalent to 0.3 ml of supernatant subject to precipitation.

* 142.85 = Slope of standard curve.

* 121.61 = molecular weight of cysteine.

* 23 = number of cysteine residues present in the mussel metallothionein.

* 0.1 = weight of tissue used in extraction.

* 1.0 µM –SH = 0.055 µM metallothionein.

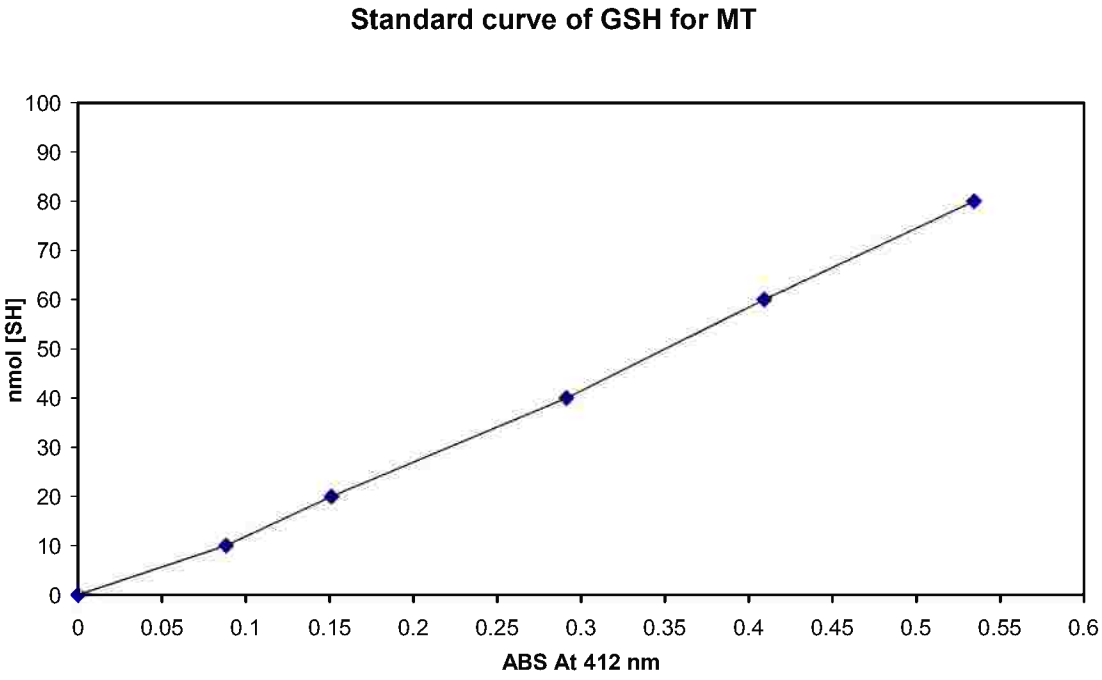

Figure 14: Standard curve of GSH (nmol [SH]) for metallothionein.

2. Determination of some heavy metals in mussel samples [244]

1. Mussel gills and digestive glands were rapidly dissected out and then stored at (-80°C) for determination of cadmium, lead, chromium, copper and zinc.

2. An amount of 1.0 gram of mussel sample was digested with 4.0 ml of concentrated nitric acid in a Teflon vessel, covered tightly and allowed predigested at room temperature over night.

3. The digestion block was placed on a preheated hot plate at 80 °C for three hours.

4. The samples were cool to room temperature and then transferred to a 25 ml volumetric flask.

5. All digested solution were analyzed and measured using atomic absorption spectrophotometer (SPECTR AA- plus version) working with air/acetylene flame and D_2 –background correction. The water used was de-ionized.

6. All data are presented as concentration/weight of samples as $\mu g\ g^{-1}$.

7. The accuracy of the method was verified using standard reference material (freeze dried mussel tissues, *Mytilus edulis*).

II. Biochemical Studies:

1. Determination of metallothionein in human erythrocytes [245]

1.1. Principle of the method

Metallothionein concentration was evaluated utilizing a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the erythrocytes hemolysate.

1.2. Reagent preparation

1. *Extraction buffer (pH 8.6):*

20 mM tris-HCl (MW: 157.6).

300 mM Sucrose (MW: 342.30)

For 100mL of solution; amount of 0.315 grams of tris-HCl were dissolved in milli Q water, the pH was adjusted (pH 8.6) with HCl and bring the solution to volume with milliQ water, then an amount of 10.2 grams of sucrose were dissolved sucrose in Tris-HCl freshly prepared and finally adjust the volume to 100 ml with milliQ water. (This solution is stable for two months at 4°C).

2. *Antiproteolitic cocktail*

0.006 mM leupeptine hemisulfate (MW: 475.6),

0.5 mM PMSF (phenylmethylsulphonylfluoride) as antiproteolytic agents (MW: 174.19),

0.01% β-mercaptoethanol as a reducing agent,

Freshly prepared homogenizing buffer contains for 15 ml extraction buffer 45 µl leupeptine hemisulfate (1.0 mg/ml), 22.5 µl PMSF (58 mg/ml ethanol) and 15 µl of β-mercaptoethanol (10 %).

3. *Re-suspension solution (C_1)*

250 mM NaCl (MW: 58.44)

For 100ml of solution; 1.416 grams of NaCl were dissolved in milliQ water (Store at 4°C).

4. *Destabilizing solution (C_2)*

1.0 N HCl

4.0 mM EDTA (MW: 372.2)

For 100mL of solution; amount of 0.149 grams of EDTA (0.5M) were dissolved in milliQ water and 8.84 ml of HCl (35%) was added and complete the volume to 100 ml by milliQ water, (store at 4°C up to two weeks).

5. *Solution C*: ($C_1 + C_2$; 1:1 V/V)

6. *Reaction buffer*

Phosphate Buffer Stock solution (0.2 M, pH 7.6):

Amount of 6.0 grams NaH_2PO_4 were dissolved in 50 ml milliQ water and 7.1 g Na_2HPO_4 were dissolved in 50 ml in milliQ water then mixed and adjust (pH 7.6). Finally, 29.22 grams NaCl added to obtain a final concentration of 2.0M NaCl and completed the volume to 250 ml by in milliQ water. (Stored at; 4°C up to two weeks).

Working Solution:

For 100 mL; amount of 0.170 grams DTNB were dissolved in phosphate buffer stock solution (0.2 M, pH 7.6). Prepare this solution immediately before use. Protect from light with foil and stir well to dissolve the DTNB. (Stored at room temperature and discarded after each analysis).

7. *Standard solution "GSH reference"*

4.0 sulfhydryl reference standard solution reduced solution, GSH, MW: 307.33

Amount of 0.012 grams reduced GSH were dissolved in10 ml re-suspension solution Stored at 4°C and discard after each analysis.

1.3. Procedures

1. *Sample preparation*

Venous blood samples were drawn in trace element-free-evacuated tubes containing EDTA as anticoagulant, whole blood was centrifuged at 600 xg for 5 minutes to separate the cells from plasma. The buffy coat was removed from the erythrocyte pellet, and an

equal volume of ice-cold 0.9 % NaCl was added. After being inverted several times, the tubes were centrifuged again; this process was repeated twice. The washed cells were stored at – 80 °C.

2. *Metallothionein extraction*

• A volume of 0.5 ml of packed erythrocytes was homogenized at (0-4°C) adding 1.5 ml of homogenizing buffer "extraction buffer"+ antiproteolytic cocktail". The homogenate transferred into 2.0 ml micro-tube and centrifuge at 16.000 xg at 4°C for 40 minutes.

• A 0.3 ml of supernatant was taken (determined its hemoglobin concentration by the method of Drabkin) [246] and transferred into 1.5 ml micro-tube, then 0.315 ml of cold absolute ethanol (stored at - 20°C) and 20 µl of chloroform (stored at RT) was added. then mixing and centrifuge at 16,000 xg at 4°C for 5 minutes.

• All the supernatant was collected and transferred into 2ml tube; add 12 µl of 37%HCl (stored RT) and 1.5 ml of cold absolute ethanol (stored -20°C).

• The reaction tubes were incubated at -20°C for 1h, and centrifuge at 16,000 xg at 4°C for 5 minutes.

• The supernatant was discarded, then the pellet was dry the pellet with a gently stream of nitrogen to remove the ethanol residues.

• Store the pellet at -80°C after drying with liquid nitrogen (if not continue the assay).

3. *Spectrophotometric assay (Ellman's reaction)*

Sample: the pellet was re-suspended with 50 µl of solution C ($C_1 + C_2$; 1:1 v/v)**,** 1.950 ml of reaction buffer was added at room temperature, mix briefly, incubated 2 min at RT and centrifuge at 16,000g at room temperature for 2 minutes.

Blank: aliquot of 50 µl of solution C ($C_1 + C_2$; 1:1 v/v)**,** 1.950 ml of reaction buffer was added at room temperature, mixed briefly, incubated 2 minutes at RT and centrifuged at 16,000g at room temperature for 2 minutes.

1.4. **Calculation**

The absorbance values (412 nm) were interpolated over the standard curve (figure 14) to obtain the concentration (nmol) of sulfhydryl groups, i.e. cysteine residues, due to metallothionein present in the sample: (nmol Cys^{MT}). To obtain the concentration of MT (nmol MT) per gram of hemoglobin in erythrocytes apply:

$$(\text{nmol MT}) * g^{-1}\text{Hb} = \frac{(\text{nmol } Cys^{MT})}{g\,\text{Hb} * n^{\circ}cys}$$

Where:

* g Hb = amount of hemoglobin in grams equivalent to 0.3 ml of supernatant subject to precipitation.

* $n^{\circ}cys$ is the number of cysteine residues present in the human metallothionein = 20.

2. **Determination of some heavy metals in human blood** [247]

Atomic absorption spectroscopy (AAS) assay used to determined the concentration of cadmium, lead, chromium, copper and zinc in the whole blood samples of human subject.

• In this procedure, all calibrated flasks, pipette tips, containers and auto-sampler tubes used in the assay were made of poly propylene or high density poly ethylene or Teflon.

• All calibrated flasks, containers and auto-sampler tubes were pre-cleaned, typically soaked with HNO_3/HCl/de-ionized water (1+2+9)(V/V/V) for a minimum 24 hours and then rinsed with de-ionized water.

2.1. **Procedures**

An aliquot of 0.5 ml whole blood was diluted 5 fold with 0.14N nitric acid, and then digested at 80 ^{o}C until the solution become colorless and clear.

2.2. **Calculation**

The content of the blood metals will be obtained from the standard curve ranged from 0.25 mg/l to 5 mg/l.

3. Determination of Serum lipid peroxide (Malondialdehyde) [248]

3.1. Principle

Principle of this procedure is based on the reaction of lipid peroxides (MDA) with thiobarbituric acid in acidic medium forming a red pigment which extracted using n-butanol and measured at 530nm.

3.2. Reagent preparation

1. 2.0 g/dl trichloroacetic acid

Amount of 2.0 grams of trichloroacetic acid were dissolved in a volume of distilled water and complete the volume to 100 ml with distilled water.

2. 0.05 M Sulfuric acid

3. 0.05 M sodium hydroxide

Amount of 0.2 grams of NaOH were dissolved in 50 ml distilled water and complete the volume to 100 ml by distilled water.

4. 0.67 % thiobarbituric acid (TBA)

Amount of 0.67 grams of TBA were dissolved in 50 ml sodium hydroxide (0.05 M) and complete to 100 ml.

5. n-butyl alcohol (absolute).

6. Standard solution (Malonaldehyde-bis-diethyl-acetal 'MDA')

a). stock standard solution was prepared by diluting a 25 ul of 4.04 M MDA standard to 50 ml with 0.05 M sulfuric acid to give 2.02 mM MDA solution.

b). Working standard solution 15, 60, 100, 150 and 200µl aliquot stock solution (a) pipette to 50ml volumetric flasks, then completed to the mark with 0.05M H_2 SO_4. These give a series of working standard solution containing 0.6, 2.4, 6.05 and 8.0 Nm MDA respectively.

3.3. **Procedures**

1. To 0.5 ml serum, 2.5 ml trichloroacetic acid (2%) was added and the tube was left to stand 10 minutes at room temperature then centrifuged at 3500 rpm for 10 minutes.

2. After centrifugation the supernatant was decanted and the precipitate was washed once with 0.05 M sulfuric acid.

3. A volume of 2.5 ml of 0.05 M sulfuric acid were added, and then shaked till the precipitate completely dissolved.

4. An aliquot of 1.0 ml of TBA was added, the coupling of lipid peroxides with TBA was carried out by heating in a boiling water bath for 30 minutes.

5. After cooling, the resulting chromogen was extracted with 4.0 ml n-butanol by vigorous shaking.

6. Separation of the organic phase was facilitated by centrifugation at 3000 rpm for 10 minutes, and its absorbance was determined against water at 530 nm.

3.4. **Calculation**

A standard curve (Figure 15) was constructed relating n mole of MDA to optical density Figure (15). The MDA of each sample was determined from the standard curve and expressed in nmole/ml.

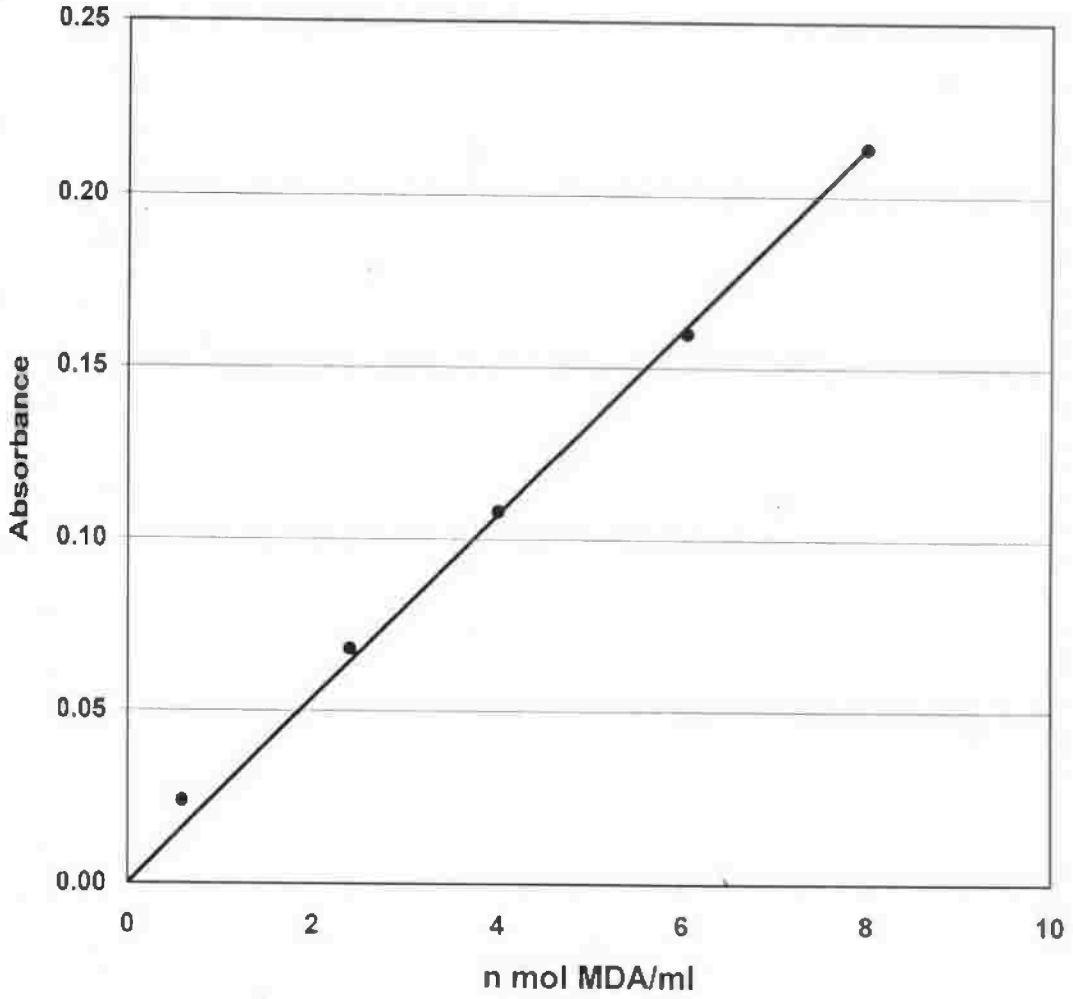

Figure 15: Standard curve of malonaldehyde-bis-diethyl-acetal (MDA) (nmole/ml).

4. Determination of blood glutathione content [249]

4.1. Principle

The method based on the reduction of 5,5' dithiobis- (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produces a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance measured at 405nm.

4.2. Reagent preparation

1. *Precipitating solution*

Amount of 0.372 grams of ethylenediamine tetraacetic acid (EDTA) and 82 grams of trichloroacetic acid (TCA) were dissolved in 500 ml of de-ionized water and complete the total volume to 1000 ml using distilled water. (This solution is stable for approximately 3 weeks at 4 ^{o}C).

2. *Phosphate buffer solution 01 M, pH 7.0*

Amount of 1.361 grams of potassium dihydrogen phosphate, 1.742 grams of dipotassium hydrogen phosphate and 0.0372 grams EDTA were dissolved in 50 ml de-ionized water and the pH was adjusted to 7.0, and then completes the total volume to 100ml with de-ionized water.

3. *DTNB reagent*

Amount of 40 mg of 5, 5' dithiobis-(2-nitrobenzoic acid) per 100 ml of 1 % sodium citrate. Sodium citrate has been selected for convenience, since its pH is appropriate both for the solubility and stability of the reagent. A phosphate buffer pH 7 to 8 was also used. (The DTNB reagent is stable for at least 13 weeks in the refrigerator).

4.3. Procedure

- an aliquot of 0.2 ml of venous whole blood added to 1.8 ml of distilled water, 3.0 ml of precipitating solution mixed with the hemolysate, the rate of addition was not critical. The mixture allowed to stand for approximately 5 minutes and then filtered.

♦ **Sample:** aliquot of 2.0 ml of filtrate were added to 8 ml of the phosphate buffer solution, then 1.0 ml of the DTNB reagent added.

♦ **Blank:** was prepared with 8 ml of phosphate buffer, 2 ml of diluted precipitating solution (3 parts to 2 parts of distilled water) and 1 ml of the DTNB reagent.

The optical density of sample and blank were measured at 412 nm using PRIME automatic photometer from (BPC BioSED srl - Italy).

4.4. Calculation

$$\text{Glutathione (mg/dl)} = \frac{O.D.}{13.6} * \frac{5}{0.2} * \frac{11}{2} * 307 * \frac{100}{1000}$$

Where:

13.6 = Molar extinction coefficient (ml $\mu mol^{-1}Cm^{-1}$).

5 = volume of precipitating solution in ml.

0.2 = Initial volume of the blood in ml.

11 = Total volume of reaction mixture in ml.

2 = Volume of filtrate in ml.

307 = Molecular weight of glutathione.

1000 = to convert μg to mg.

100 = to convert mg/ml to mg/dl.

5. Determination of erythrocytes glutathione peroxidase activity [250]

5.1. Principle of the method

Glutathione peroxidase (GPx) activity measures indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced up on reduction of hydro peroxide by GPx is recycled to its reduced state by GR and NADPH:

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{GPx} R\text{-}O\text{-}H + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the absorbance at 340 nm is directly proportional to the GPx activity in the sample.

5.2. Reagent Preparations

1. *Tris HCl buffer (50 mM, pH 7.6)*

Amount of 7.88 grams of Tris HCl were dissolved in 500 ml de-ionized water, then adjusted the pH to 7.6 and the volume completed to 1000 ml with de-ionized water.

2. *Reaction mixture*

To 100 ml of Tris HCl buffer (50 mM, pH 7.6) the following were dissolved (freshly each run): (0.0372 grams of Na_2EDTA (1.0 mM), 0.0615 grams of reduced glutathione (2.0 mM), 0.0260 grams of sodium azide (4.0 mM), 667 µl of NADPH 30 mM stock solution (0.2 mM) [25 mg of NADPH dissolved in 1.0 ml de-ionized water to give 30 mM stock solution) and 100 µl of glutathione reductase (1000 U).

3. *Hydrogen peroxide (8.8 mM)*

91 µl of H_2O_2 (30 %) diluted to 100 ml using de-ionized water freshly prepared each run.

5.3. Procedures

1. *Sample preparation*

♦ Heparinized venous blood was centrifuged and takes off plasma and leukocyte layer. The erythrocyte sediment was washed 3 times with isotonic NaCl.

♦ Erythrocytes were hemolysed by diluting fourfold with de-ionized water and freezing overnight.

♦ after centrifugation to remove cell debris, dilute the hemolysate fivefold with Drabkin's reagent.

2. *Spectrophotometric assay "modified Paglia and Velentine method"* [251]

1). An aliquot of 20 µl of fivefold hemolysate was transferred to a 1.0 ml quarts cuvette containing 980 µl of the reaction mixture (Tris buffer, 50 mmol/l, pH 7.6, containing per liter, 1.0 mmol of Na_2EDTA, 2.0 mmol of reduced glutathione, 0.2 mmol of NADPH, 4.0 mmol of sodium azide, and 1000 U of glutathione reductase), then incubate the mixture for 5 minutes at 37 ^{o}C.

2). Then the reaction was initiated by adding 10 µl of H_2O_2 8.8 mmol/l and follow the decrease in NADPH absorbance at 340 nm for 3 minutes, after 30 second (lag period), the decrease will be linear with time.

3). The non-enzymatic reaction rate (blank) was determined by substituting water for the hemolysate and recording the decrease in NADPH absorbance.

4). Determine the hemoglobin concentrations in the fourfold diluted hemolysate by the method of Drabkin [246].

5.4. Calculations

Determine the rate of ΔA_{340} / min. for the non-enzymatic reaction and subtract this rate from that of the sample reaction. Using the following formula to calculated the GPx activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00622 μM^{-1} cm^{-1}.

One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 37 ^oC.

$$\textbf{GPx Activity (U/g Hb)} = \frac{\Delta A340/min}{0.00622} * \frac{\text{Total reaction volume}}{\text{Sample volume}} * \frac{\text{Dilution factor}}{\text{Hb (g/l)}}$$

6. Determination of erythrocytes catalase activity [252]

6.1. Reagent preparation

Purity of reagents glassware, buffer and substrate should be free from heavy metals (catalytic decomposition of H_2O_2), and all reagents should be dissolved in de-ionized water.

1. *Phosphate buffer (50 mmol/l; pH 7.0)*

Amount of 6.81 grams of KH_2PO_4 were dissolved in water and make up to 1000 ml (solution A), and 8.90 grams of $Na_2HPO_4.2H_2O$ dissolved in water and make up to 1000 ml (solution B), then mix the solutions (A) and (B) in proportion 1 :1.5 (v/v).

2. *Hydrogen peroxide (30 mmol/l)*

A volume of 0.34 ml of 30% hydrogen peroxide was diluted with phosphate buffer (50 mmol/l; pH 7.0) to 100 ml.

6.2. Procedure

1. *Sample preparation*

• heparinized venous blood was centrifuged, and taken off plasma and leukocyte layer. The erythrocyte sediment was washed 3 times with isotonic NaCl.

• A stock haemolysate prepared by addition of 4 parts of water.

• The concentrated haemolysate is diluted to 500 times (v/v) by phosphate buffer immediately before the assay and determine in duplicate its hemoglobin content (by the method of Drabkin) [246].

2. *Spectrophotometric assay*

Assay conditions

Wave length 240 nm; light path: 10 mm; final volume 3 ml. Read at room temperature against blank containing enzyme solution or haemolysate without substrate.

Pipette successively into the cuvette:	**blank**	**sample**
phosphate buffer sample	1.0 ml	--
dilute Haemolysate	2.0 ml	2.0 ml
buffered H_2O_2 solution	--	1.0 ml
The apparatus adjusted at zero using blank cuvette, start the reaction by addition of H_2O_2 and follow the decrease in absorbance at 15 *s* intervals.		

The values for $\Delta A_{240} / \Delta t = 15\ s^{-1}$ should not be greater than 0.100 and not smaller than 0.020.

6.3. **Calculation**

A definition of catalase unit (U) is not feasible because of the abnormal kinetics. Therefore, it is acceptable for this enzyme to use a number of differently defined units. It is recommended that the rate constant of the first order reaction (*k*) should be used. The rate constant related to haemoglobin content (*k* / g Hb) can serve as the measure of the specific activity of erythrocyte catalase.

For a time interval of 15 second the following relationship is obtained:

$$\boldsymbol{k} = \frac{2.3}{15} \text{ x } \textbf{log } \frac{A_1}{A_2} = \textbf{0.153 x log } \frac{A_1}{A_2} \ s^{-1}.$$

To calculate the catalytic concentration *b* of the sample (unit *k*/l) or catalytic content z_c / m_s of the sample (unit *k*/g Hb) proceed as follows:

$$b = \frac{V}{v} \textbf{ x } \frac{2.3}{15} \textbf{ x log } \frac{A_1}{A_2} \qquad s^{-1}/l \quad (k/l)$$

$$b = \frac{3}{2} \text{ x } \frac{2.3}{15} \text{ x } \log \frac{A_1}{A_2} = 0.23 \text{ x } \log \frac{A_1}{A_2} \quad (k\text{l})$$

$$z_c / m_s = \frac{\alpha \times 2.3}{\rho Hb \times 15} \text{ x } \log \frac{A_1}{A_2} = 0.153 \text{ x } \log \frac{A_1}{A_2} \text{ s}^{-1} \text{ x g Hb} \quad (k\text{g Hb})$$

Erythrocytes catalase activity (U/g Hb) is given by:

$$\text{CAT activity (U/g Hb)} = 0.153 \text{ x } \log \frac{A_1}{A_2} s^{-1} \text{ x Hb } (g/l) \text{ x } 240{,}000$$

Where:

- $\mathbf{A_1}$ is the absorbance of the sample (A_{240}) at zero second.

- $\mathbf{A_2}$ is the absorbance of the sample (A_{240}) after first 15 second.

- V is total assay volume

- v is sample volume in assay mixture.

- α is the quotient of mass concentration of haemoglobin ρ_{Hb} (unit g/l) in blood or erythrocyte sediment, and in the cuvette.

- 240,000 is the molecular mass of calalase in grams.

7. Determination of erythrocytes Superoxide dismutase activity [253]

7.1. Principle of the method

The superoxide dismutase activity assayed based on the ability of the enzyme to inhibit the auto-oxidation of 10 mM pyrogallol-HCl solution [253].

7.2. Reagent preparation

1. *Pyrogallol (10 mM)*

Amount of 0.126 gram pyrogallol was dissolved in 10 mM HCl and the volume completed to 100 ml.

2. *Reaction Buffer*

Tris-HCl buffer (75mM, pH 8.2) 1.773 grams Tris-HCl dissolved in 50 ml de-ionized water and adjust pH 8.2 (Solution **A**),

EDTA (30mM); 1.675 grams EDTA dissolved in 25 ml de-ionized water (Solution **B**), solution **A** and solution **B** was mixed then completed to 150 ml with de-ionized water.

7.3. Procedures

1. ***Sample preparation***

Heparinized venous blood was centrifuged and takes off plasma and leukocyte layer. The erythrocyte sediment was washed 3 times with isotonic NaCl. 1 % erythrocytes lysate was prepared using de-ionized water (10 µl erythrocytes + 990 µl de-ionized water), by method of Drabkin's determined hemoglobin concentration of this lysate [246].

2. *Spectrophotometric assay*

Pipette successively into a cuvette	**Blank**	**Sample**
Working reaction buffer	1.45 ml	1.40 ml
Lysate (1 % erythrocytes)	-	0.05 ml
Pyrogallol stock (10 mM)	0.05 ml	0.05 ml
An increase of absorbance was recorded at 420 nm for 3.0 min		

7.4. Calculation

One unit of SOD activity is described as the amount of enzyme required to cause 50% inhibition of the rate of auto-oxidation of pyrogallol per ml reaction mixture at 420 nm.

$$\text{SOD Activity (U/g Hb)} = \frac{\Delta B - \Delta A}{\Delta B} \times \frac{100}{50} \times \frac{1}{Hb(g/ml)}$$

Where:

Δ A: the change of the absorbance of sample per minute.

Δ B: the change of the absorbance of blank per minute.

Hb(g/ml):hemoglobin concentration in g/ml of the 1% lysate.

III. Insulin-like growth factor 2 gene expression by nested RT-PCR [254]

1. Isolation of total RNA

Venous blood samples were withdrawn in sterile evacuated tubes containing EDTA as anticoagulant and total RNAs were isolated from peripheral blood mononuclear cells by blood RNA extraction kit.

1.1. *Kit Contents*

Label	Description
- Buffer RBL	Pre-lysis buffer
- Buffer RLB	Lysis buffer
- Buffer RWA	Washing buffer A
- Buffer RWB	Washing buffer B
- Buffer RE	Elution buffer
- gDNA Removal Spin Columns	gDNA Eliminator Column
- Spin Columns (red color O-ring)	
- Collection Tubes	

1.2. *Description*

The IQeasy^{TM} plus blood RNA mini kit used for (i) the preparation of total cellular RNA from up to 1.0 ml of whole blood, (ii) used to purify total RNA from whole blood (iii) and to separate RNA from proteins, salts and other reaction components. Contaminants and enzyme inhibitors such as hemoglobin and heparin are completely removed, leaving purified RNA ready to use during the procedures for purification of RNA from blood, erythrocytes are selectively lyses and leucocytes are recovered by centrifugation. The leucocytes were then lyses using highly denaturing conditions that immediately inactivate RNases, allowing the isolation of intact RNA. RNA is bound to silica membrane during a brief centrifugation step. Contaminants are washed away and total RNA is eluted in 30 µl or more RNase-free water for direct used in many gene expression profiling techniques. The total RNA yield from kit are 3 ~ 10 µg from 1.0 ml whole blood from normal healthy donor.

1.3. *Total RNA purification protocol*

According to kit manual

1. An aliquot of 1.0 ml whole blood was added to 15 ml tube.
2. Then, aliquot of 3.0 ml of Buffer RBL were added, and vortex.
3. Then, incubated for 10 minutes on ice. Centrifuge at 4000 rpm for 10 minutes at 4 ^{o}C to obtain white blood cells pellet. Carefully removed supernatant.
4. White blood cells pellet were re-suspend in 400 µl of buffer RLB and 4.5 µl of β-mercaptoethanol (14.2 M) to each sample. And then mix by vortex.
5. The lysate was applied to gDNA remover spin columns, and centrifuge for1.0 minute at 13000 rpm (R.T.). After centrifugation, transfer the flow-through into a new 1.5 ml tube.
6. Aliquot of 200 µl of absolute ethanol were added to collected lysate, and mixed well by pipetting or gently inverted 5-6 times (don't vortex).
7. Aliquot of 600 µl of the mixture from step 6 were Transferred into the spin column (red color O-ring) inserted in a 2.0 ml collection tube. Centrifuge at 13000 rpm at R.T. for 1.0 minute, and discard flow-through and collection tube altogether.
8. Aliquot of 700 µl of buffer RWA were added to the spin column, and centrifuged at 13000 rpm for 1.0 minutes, the flow-through was discarded and reuse the collection tube.
9. Aliquot of 700 µl buffer RWB were added to the spin column, and centrifuged at 13000 rpm for 1.0 minute to dry membrane. The flow through and collection tube were discarded together.
10. The spin column was placed in a new 2.0 ml collection tube, and centrifuged at 13000 rpm for 1.0 minute to dry the column membrane.
11. The spin column was placed into a new 1.5 ml tube, and 50 µl buffer RE directly onto the membrane. Incubated for 1.0 minute at room temperature and then centrifuged for 1.0 minute at 13000 rpm to elute.

2. *Synthesis of cDNA and amplification of nested PCR*

For synthesis of cDNA, one step RT-PCR kit beads were used which, contains (the four nucleotides dA, dG, dC, dT, Taq polymerase, magnesium chloride, reverse transcriptase and universal primer. The resulting cDNA was amplified by a nested PCR with two pairs of primers. The oligonucleotides were designed according to IGF-II sequence and synthesized by Jena Bioscience GmbH, Löbstedter, street 80, Germany. The sequences of the 2 external primer pairs used for the initial PCR amplification were IGF-II-1(sense), 5'-ATGGGAATGCCAATGGGGAAG-3' (nt251-271) and IGF-II-2(antisense), 5'-CTTGCCCACGGGGTATCTGGG-3' (nt 566-586),

The cDNA synthesis

To each one step RT-PCR bead add the following,

A volume of 2.0 µl of external primer (sense),

A volume of 2.0 µl of external primer (antisense),

A volume of 1.0 µl Purified RNA sample,

Then a volume of 15 µl nuclease free water.

The cDNA synthesis was preformed by incubation of reaction bead at 42 °C for 60 minutes (cDNA synthesis) then at 94 °C for 10 minutes (reverse transcriptase inactivation).

Initial PCR amplification steps

30 cycles:

♦ Denaturation step: 94 °C for 25 second

♦ Primer annealing: 55 °C for 30 second

♦ Extention step: 72 °C for 90 second.

Final extension step: 72 °C for 10 minutes.

The size of amplified gene fragment was 336 bp.

The sequences of the two internal primer pairs used for the second PCR amplification were IGF-II-3 (sense), 5'-TGCTGCATTGCTGCTTACCG-3' (nt 311-330) and IGF-II-4 (anti-sense), 5'-AGGTCACAGCTGCGGAAACA-3' (nt 461-480). After the end of the program,

To each one new PCR tubes add the following

A volume of 5.0 µl initial PCR product (336 bP),

A volume of 10 µl master mix,

A volume of 2.0 µl internal primer (sense),

A volume of 2.0 µl internal primer (antisense),

And then a volume of 1.0 µl $MgCl_2$

Second PCR amplification steps

Initial denaturation step: at 94 °C for 5.0 minutes.

30 cycles:

♦ Denaturation step: 94 °C for 25 second

♦ Primer annealing: 55 °C for 30 second

♦ Extension step: 72 °C for 90 second.

Finally extension step: 72 °C for 10.0 minutes.

The final product of nested PCR was 170 bp.

Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genome was used as a control. Primer sequence for GAPDH was GAPDH-1 (sense), 5'-ACCACAGTCCATGCCATCAC- 3' (nt 601-620) and GAPDH-2 (antisense), 5'-TCCACCACCCTGTTGCTGT A-3' (nt 1 033-1 052), the product of PCR was 452 bp (GAPDH gene transcript, 40 pmol/l).

The PCR products were electrophoreses on 2% agarose gel with ethidium bromide staining. The fragment sizes were evaluated using PCR markers (Promega) as molecular weight standards.

The bands were scanned and the data were analyzed using UVP-DOC-ITLS™ Image & acquisition and analysis software (Ultra-Violet product, Ltd, Cambridge UK) that analyze the relative band densities of IGF2 to the glyceraldehyde3-phosphate dehydrogenase band (as internal control).

VI. Statistical analysis

♦ Statistical analysis was performed using SPSS version 20 Chicago, IL, USA for windows.

♦ Results were expressed as (mean ± SD), and the mean values for different variables of fishermen and control subjects were compared using the t-test for independent variance.

♦ Correlation analysis between different parameters was performed by the Pearson's linear regression analysis.

♦ In statistical tests alpha was set to 0.05. Results with $p \leq 0.05$ were considered to be significant.

CHAPTER 4

RESULTS

RESULTS

I. Oceanographic results

Table (1) and Figure (16) depicted metallothionein concentrations in mussels collected from Mediterranean Sea El-Maadiya region.

The concentrations of the five studied metals (cadmium, lead, chromium, copper, and zinc) in mussels illustrated in table (2) and Figure (17).

Table (1): Metallothionein concentrations (µg/g ww) in mussels collected from Mediterranean Sea "El-Maadiya region".

Sample Number	Metallothionein Concentration (µg/g ww)
1	2.53
2	1.37
3	32.6
4	1.77
5	1.37
6	1.37
7	19.15
8	12.11
9	11.1
10	23.81
11	8.69
12	7.73
13	1.71
14	3.97
15	4.86
16	7.32
17	8.21
18	9.58
19	8.21
Mean	8.81
S. D.	8.38
S. E.	1.92

* ww = Wet weight

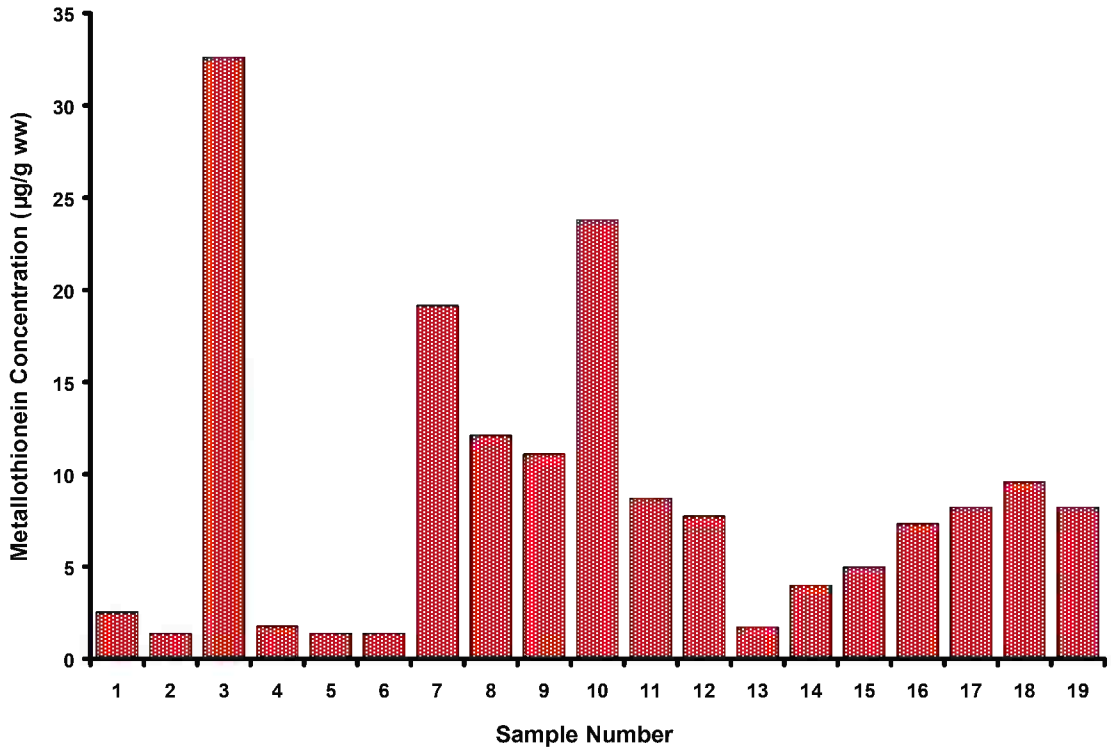

Figure 16: Metallothionein concentrations (µg/g ww) in mussels collected from Mediterranean Sea "El-Maadiya region".

Table (2): Concentrations (µg/g) of cadmium, lead, chromium, copper and zinc (µg/g) in mussels collected from Mediterranean Sea "El-Maadiya region".

Sample Number	Cadmium (µg/g)	Lead (µg/g)	Chromium (µg/g)	Copper (µg/g)	Zinc (µg/g)
1	1.11	50.32	10.58	1.77	9.97
2	0.72	47.48	8.78	1.23	2.48
3	0.57	45.86	9.07	0.75	6.28
4	0.81	15.42	11.14	0.75	3.06
5	0.86	16.23	11.05	1.07	1.53
6	0.78	15.42	9.16	0.80	5.04
7	0.72	13.80	10.95	1.71	3.74
8	0.72	13.80	9.54	1.39	6.02
9	0.75	18.67	10.10	1.39	3.62
10	1.00	12.99	9.54	1.28	3.08
11	0.95	19.48	8.69	0.64	5.20
12	1.13	20.29	13.31	1.28	3.20
13	0.80	118.49	8.88	1.28	2.31
14	0.59	15.83	9.07	1.39	3.99
Mean	0.822	30.29	9.99	1.195	4.25
S. D.	0.172	28.77	1.299	0.352	2.156
S. E.	0.046	7.69	0.347	0.094	0.576

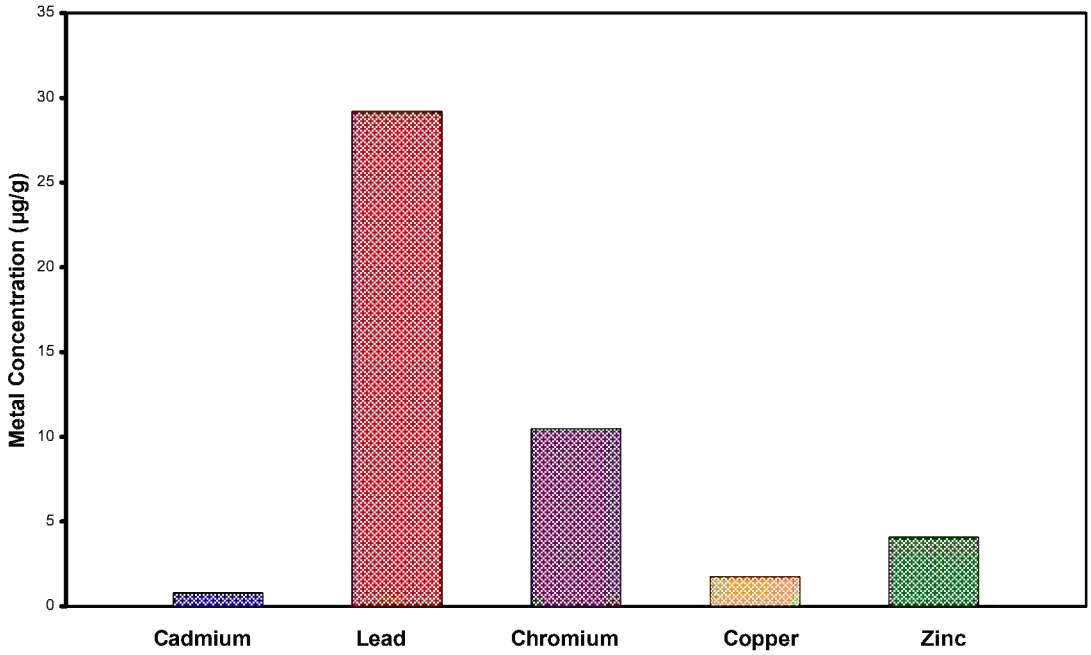

Figure 17: Mean concentrations (µg/g) of cadmium, lead, chromium, copper and zinc in mussels collected from Mediterranean Sea "El-Maadiya region".

II. Biochemical Results

Table (3) and figure (18) demonstrated metallothionein concentrations in erythrocytes of the control as well as the fishermen groups. The difference between variables was measured by independent t-test tabulated in table (4), revealed that there was a high striking significant increase in the levels of metallothionein of fishermen group than that of control group.

Table (5) and figure (19) illustrated the results corresponding to the five studied metals (Cd, Pd, Cr, Cu and Zn) concentrations in the blood of both control and fishermen groups.

The results corresponding to the serum malondialdehyde of healthy control and fishermen groups were tabulated in table (6) and figure (20). The Statistical analysis using independent t-test table (7) demonstrated that there was a striking high significant increase in the levels of malondialdehyde in serum of fishermen group when compared with that of control group.

Table (8) and Figure (21) described the levels of glutathione content in the whole blood of control and fishermen groups. The difference between variables measured by independent t- test (table 9) represented a sharp significant decrease in the levels of blood glutathione of fishermen group than that of control.

Table (10) and figure (22) represented the results corresponding to erythrocytes glutathione peroxidase enzymatic activity of healthy control and fishermen groups. The statistical analysis (independent t-test), table (11) revealed that there was a panic significant decrease in erythrocytes glutathione peroxidase activity of fishermen group than that of control group.

The data of erythrocytes catalase activity represented in table (12) and figure (23). Statistical analysis (independent t-test), table (13) revealed that there was a very high significant decrease in the enzymatic activity of erythrocytes catalase in fishermen group when compared with that of control group.

The data of erythrocytes superoxide dismutase activity illustrated in table (14) and figure (24). Statistical analysis (independent t-test), table (15) exhibited that there was no statistically significant difference observed between the two studied groups.

Table (3): Erythrocytes metallothioneins (MT) concentrations (nM/g Hb) of the healthy control group and the fishermen group.

	Group I (n = 12)	**Group II (n = 44)**	
	13	85	47
	8	22	19
	27	20	32
	22	77	23
	19	23	43
	6	37	40
	20	86	27
	18	37	23
	20	119	28
	30	25	11
	13	52	27
	9	151	16
		29	18
		143	42
		21	78
		20	20
		30	20
		37	24
		112	16
		50	16
		52	46
		73	23
Mean	17.1	44.1	
S.D.	7.5	34.2	
S.E.	2.2	5.2	

Where: Group I = Control group, Group II = Mediterranean Sea fishermen group.

Table (4): Statistical analysis (independent t-test) of erythrocytes metallothionein (MT) Concentrations (nM/g Hb) of the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group II	
$\bar{X}$	17.1	44.1	P = 0.009 *
n	12	44	
Range	(6 – 30)	(11 – 151)	
S.D.	7.5	34.2	
S.E.	2.2	5.2	

Where: * $p < 0.05$ is significant.

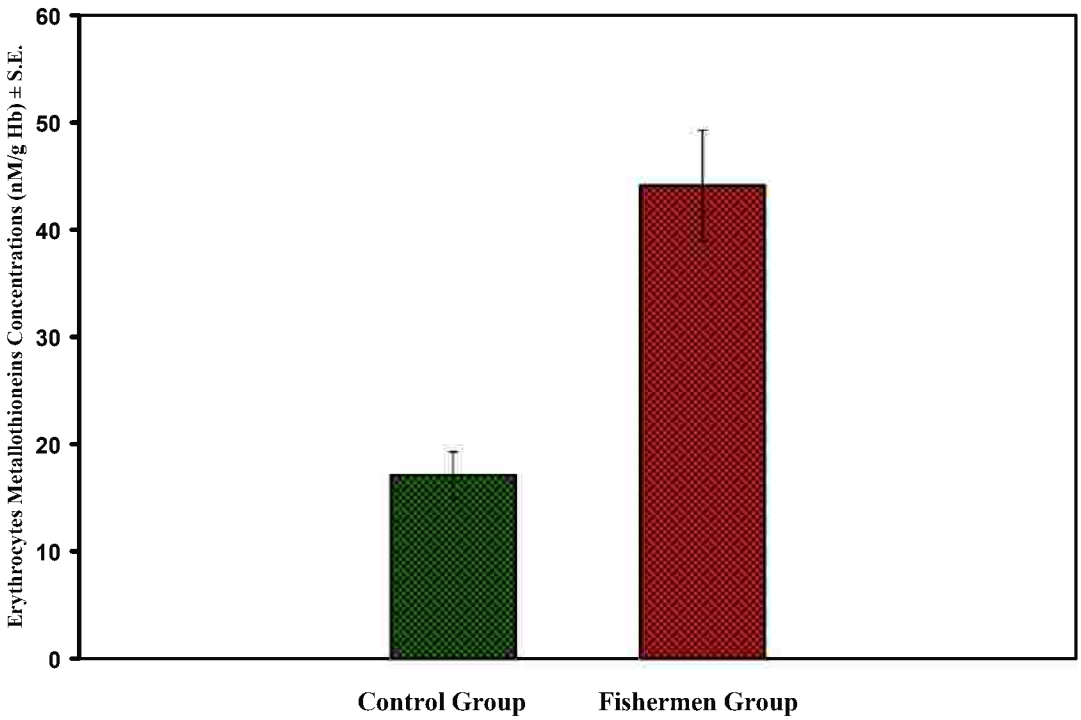

Figure 18: Mean erythrocytes metallothionein (MT) concentrations (nM/g Hb) of the healthy control group and the fishermen group.

Table (5): Whole blood concentrations (µg/ml) of cadmium (Cd), lead (Pb), chromium (Cr), copper (Cu) and zinc (Zn) of the healthy control group (Group I) and the fishermen group (Group II).

	Group I					Group II				
	Cd	**Pb**	**Cr**	**Cu**	**Zn**	**Cd**	**Pb**	**Cr**	**Cu**	**Zn**
	ND	0.108	0.206	1.262	0.043	0.208	0.235	0.283	1.172	0.077
	0.088	0.127	0.254	0.958	0.087	0.256	0.372	0.303	1.733	0.102
	0.012	ND	0.110	1.291	0.014	0.316	0.500	0.146	--	0.022
	ND	0.186	0.109	--	0.019	0.022	0.225	0.116	--	0.019
	0.149	0.196	0.261	1.171	0.093	0.073	0.304	0.134	0.925	0.023
	0.009	0.284	0.255	1.059	0.131	0.184	0.314	0.201	--	0.024
	ND	0.225	0.263	1.265	0.097	0.184	0.147	0.246	1.326	0.091
	0.001	0.167	0.134	0.128	0.023	0.004	0.235	0.198	--	0.024
						0.018	0.314	0.301	1.056	0.035
						0.083	0.108	0.288	--	0.030
						0.020	0.304	0.243	1.385	0.101
						0.113	0.186	0.221	--	0.037
						ND	0.225	0.224	1.385	0.100
						ND	0.069	0.186	1.297	0.038
						ND	0.186	0.191	1.384	0.041
						0.019	0.392	0.225	1.064	0.101
						ND	0.049	0.194	0.879	0.052
						ND	0.196	0.233	1.567	0.052
						ND	0.176	0.203	0.841	0.053
						ND	0.147	0.221	0.868	0.053
						ND	0.206	0.264	1.064	0.052
						0.161	0.235	0.277	1.036	0.056
						0.247	0.265	0.270	--	0.064
						0.162	0.147	0.277	1.081	0.058
						0.291	0.147	0.297	--	0.063
						0.190	0.235	0.333	0.778	0.067
						0.305	0.157	--	1.413	0.087
						0.129	0.196	0.274	0.805	0.075
						0.228	0.167	0.310	--	0.078
						0.229	0.225	0.264	--	0.077
						0.132	0.294	0.282	1.557	0.080
						0.159	0.255	0.246	1.146	0.076
						0.238	0.225	0.288	1.794	0.083
						0.115	0.167	0.300	1.009	0.088
Mean	0.052	0.162	0.199	1.019	0.063	0.157	0.224	0.244	1.19	0.061
S.D.	0.065	0.085	0.07	0.411	0.044	0.094	0.091	0.053	0.292	0.026
S.E.	0.029	0.030	0.025	0.156	0.016	0.018	0.016	0.009	0.06	0.004

ND: Not detected

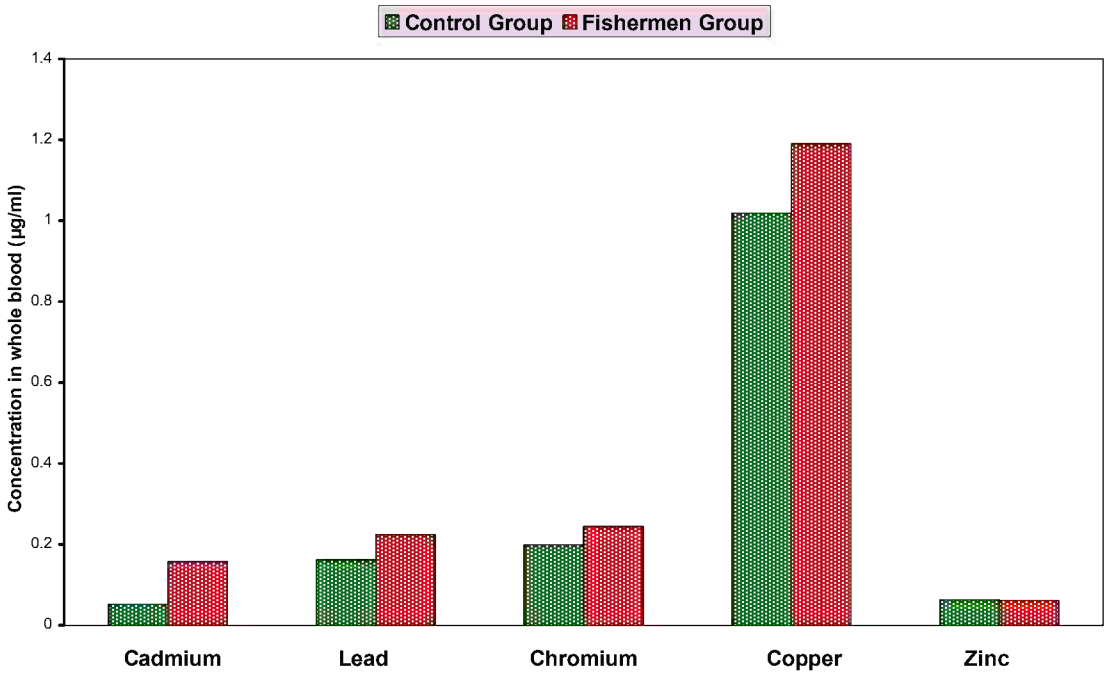

Figure 19: Mean blood concentrations (µg/ml) of cadmium, lead, chromium, copper and zinc of the healthy control group and the fishermen group.

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Table (6): Serum malondialdehyde (MDA) concentrations (nM/ml) of the healthy control group (Group I) and the fishermen group (Group II).

	Group I (n = 10)	**Group II (n = 40)**	
	2.0	3.8	4.0
	2.3	3.3	3.4
	1.5	4.0	3.1
	2.4	3.0	3.0
	2.2	2.6	3.0
	2.1	2.8	3.6
	2.6	5.0	3.4
	1.5	2.3	2.2
	0.8	3.1	3.1
	1.3	2.7	3.3
		2.3	3.7
		8.9	3.4
		2.4	4.0
		3.6	4.0
		3.7	3.8
		4.9	3.8
		3.2	3.0
		6.1	2.5
		5.4	2.4
		4.5	2.2
Mean	1.87	3.56	
S.D.	0.57	1.23	
S.E.	0.18	0.19	

Table (7): Statistical analysis (independent t-test) of Serum Malondialdehyde (MDA) Concentrations (nM/ml) of the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group II	
$\bar{X}$	1.87	3.56	P = 0.000 *
n	10	40	
Range	(0.8 – 2.6)	(2.2 – 8.9)	
S.D.	0.57	1.23	
S.E.	0.18	0.18	

Where: * $p < 0.05$ is significant.

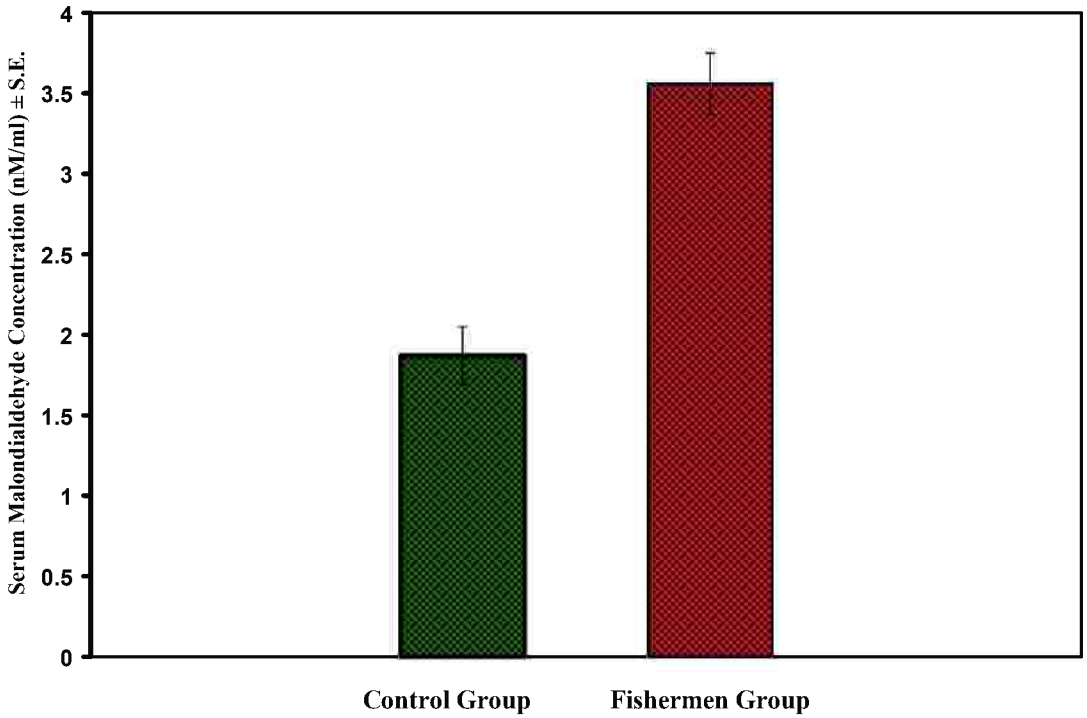

Figure 20: Mean serum malondialdehyde (MDA) concentrations (nM/ml) of the healthy control group and the fishermen group.

Table (8): Whole blood glutathione content (mg/dl) of the healthy control group (Group I) and the fishermen group (Group II).

	Group I (n = 10)	**Group II (n = 40)**	
	35	29	21
	37	22	25
	35	26	27
	42	22	24
	40	29	22
	43	23	23
	41	32	28
	38	20	28
	41	23	28
	44	27	20
		25	22
		28	18
		25	19
		20	27
		25	30
		26	26
		20	32
		29	18
		24	18
		24	20
Mean	39.6	24.4	
S.D.	3.2	3.9	
S.E.	1.0	0.6	

Table (9): Statistical analysis (independent t-test) of glutathione content in whole blood (mg/dl) of the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group I	
$\bar{X}$	39.6	24.4	P = 0.000 *
n	10	40	
Range	(35 – 44)	(18 – 32)	
S.D.	3.2	3.9	
S.E.	1.0	0.6	

Where: * $p < 0.05$ is significant.

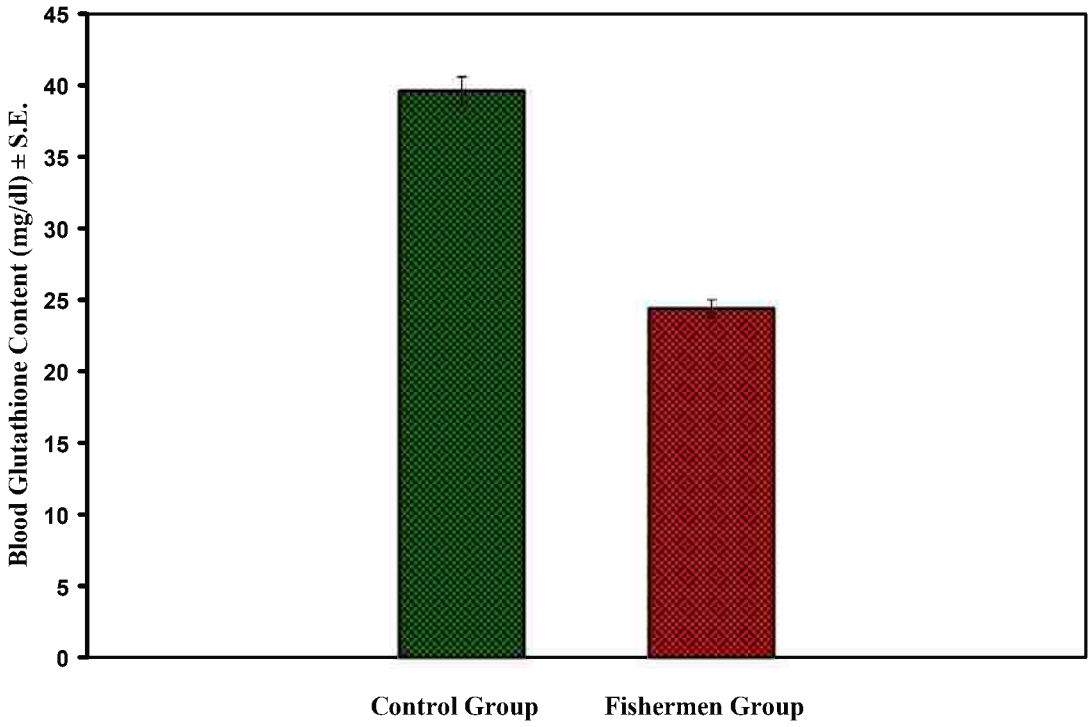

Figure 21: Mean whole blood glutathione content (mg/dl) of the healthy control group and the fishermen group.

Table (10): Erythrocytes glutathione peroxidase enzymatic activity levels (U/g Hb) of the healthy control group "Group I" and the fishermen group "Group II".

	Group I (n = 10)	**Group II (n = 40)**	
	146	118	113
	146	115	105
	260	76	115
	311	92	119
	167	90	145
	206	89	93
	244	110	139
	241	136	140
	149	126	89
	116	101	178
		95	150
		80	168
		131	164
		143	141
		104	152
		121	109
		153	145
		172	179
		152	140
		126	170
Mean	198.6	127.1	
S.D.	63.4	28.6	
S.E.	20.0	4.5	

Table (11): Statistical analysis (independent t-test) of erythrocytes glutathione peroxidase enzymatic activity levels (U/g Hb) of the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group I	
$\bar{X}$	198.6	127.1	P = 0.000 *
n	10	40	
Range	(116 – 311)	(76 – 179)	
S.D.	63.4	28.6	
S.E.	20.0	4.5	

Where: * $p < 0.05$ is significant.

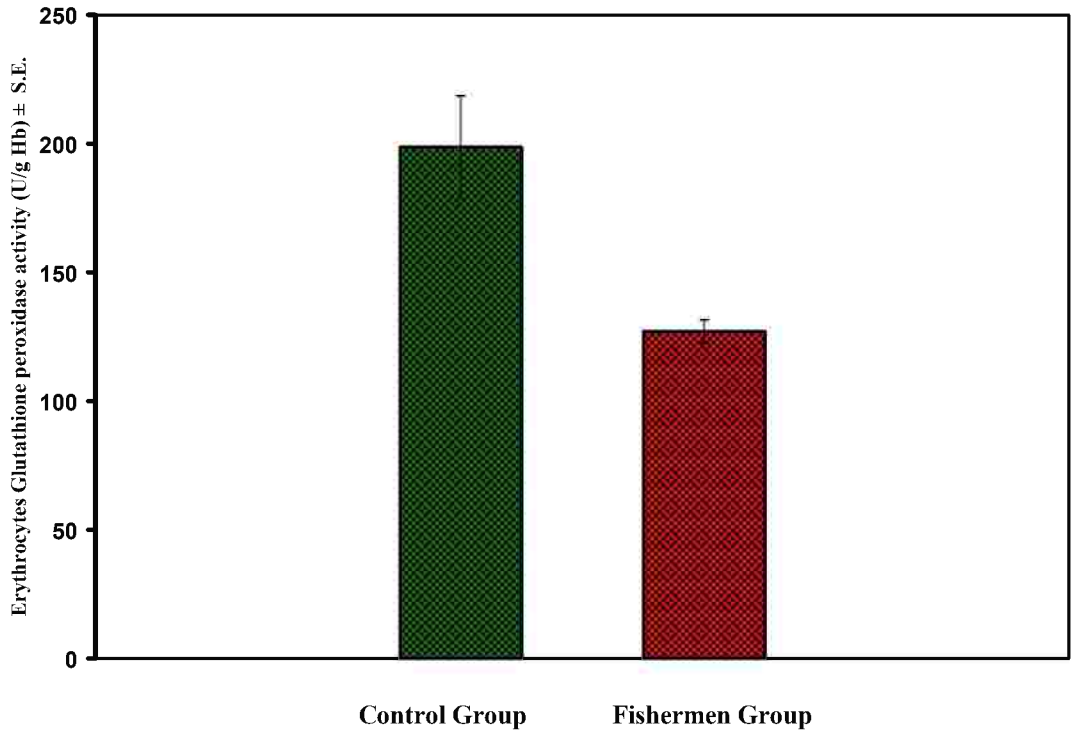

Figure 22: Mean erythrocytes glutathione peroxidase enzymatic activity levels (U/g Hb) of the healthy control group and the fishermen group.

Table (12): Erythrocytes catalase enzymatic activity levels (U/g Hb) of the healthy control group "group I" and the fishermen group "group II".

	Group I (n = 10)	**Group II (n = 40)**	
	926	635	539
	991	550	633
	977	649	662
	985	690	510
	928	654	699
	843	456	662
	805	574	670
	874	670	517
	874	501	670
	877	443	556
		612	529
		648	526
		639	386
		563	509
		503	702
		618	646
		533	308
		569	499
		574	569
		528	554
Mean	908	573.9	
S.D.	63.6	87.9	
S.E.	20.1	13.9	

Table (13): Statistical analysis (independent t-test) of erythrocytes catalase enzymatic activity levels (U/g Hb) of the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group I	
$\bar{X}$	908	573.9	P = 0.000 *
n	10	40	
Range	(805 – 991)	(308 – 702)	
S.D.	63.6	87.9	
S.E.	20.1	13.9	

Where: * $p < 0.05$ is significant.

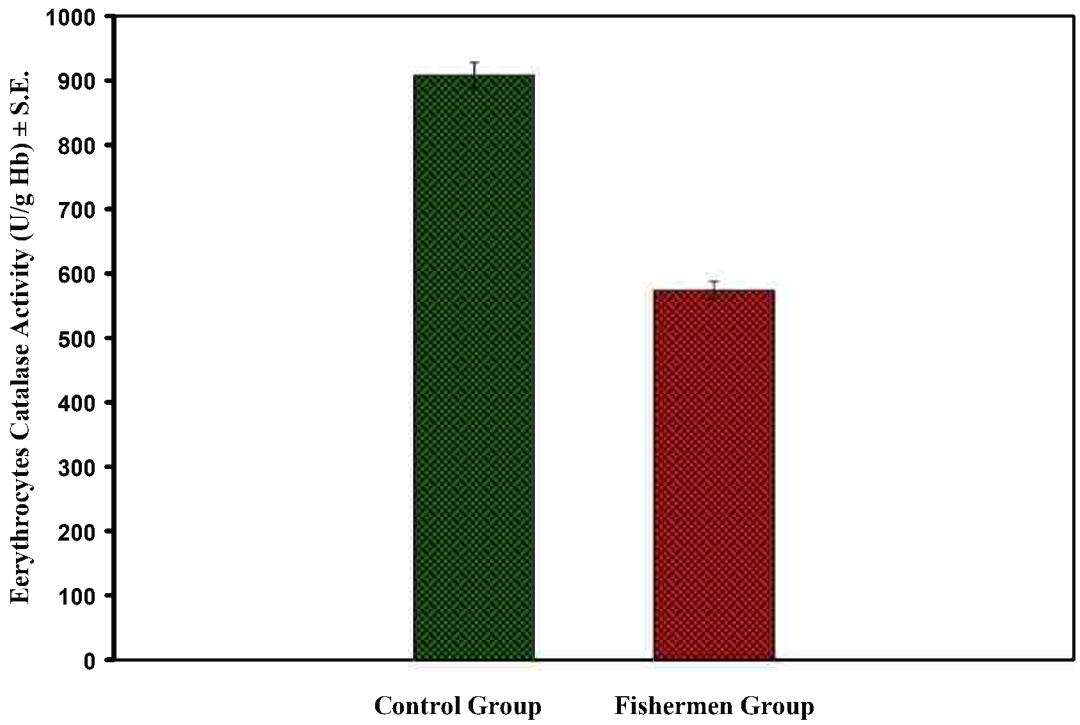

Figure 23: Mean erythrocytes catalase enzymatic activity levels (U/g Hb) of the healthy control group and the fishermen group.

Table (14): Erythrocytes superoxide dismutase enzymatic activity levels (U/g Hb) of the healthy control group "group I" and the fishermen group "group II".

	Group I (n = 10)	**Group II** (n = 40)	
	88	121	90
	131	94	121
	113	76	133
	68	104	118
	93	75	89
	88	115	116
	69	108	125
	97	110	88
	72	127	92
	126	71	81
		76	70
		77	80
		35	72
		83	118
		109	150
		77	138
		138	120
		89	82
		96	137
		106	131
Mean	94.5	101	
S.D.	22.7	25.0	
S.E.	7.2	4.0	

Table (15): Statistical analysis (independent t-test) of erythrocytes Superoxide dismutase enzymatic activity levels (U/g Hb) of the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group II	
$\bar{X}$	94.5	101	P = 0.462 *
n	10	40	
Range	(68 – 131)	(35 – 150)	
S.D.	22.7	25.0	
S.E.	7.2	4.0	

Where: * p < 0.05 is significant.

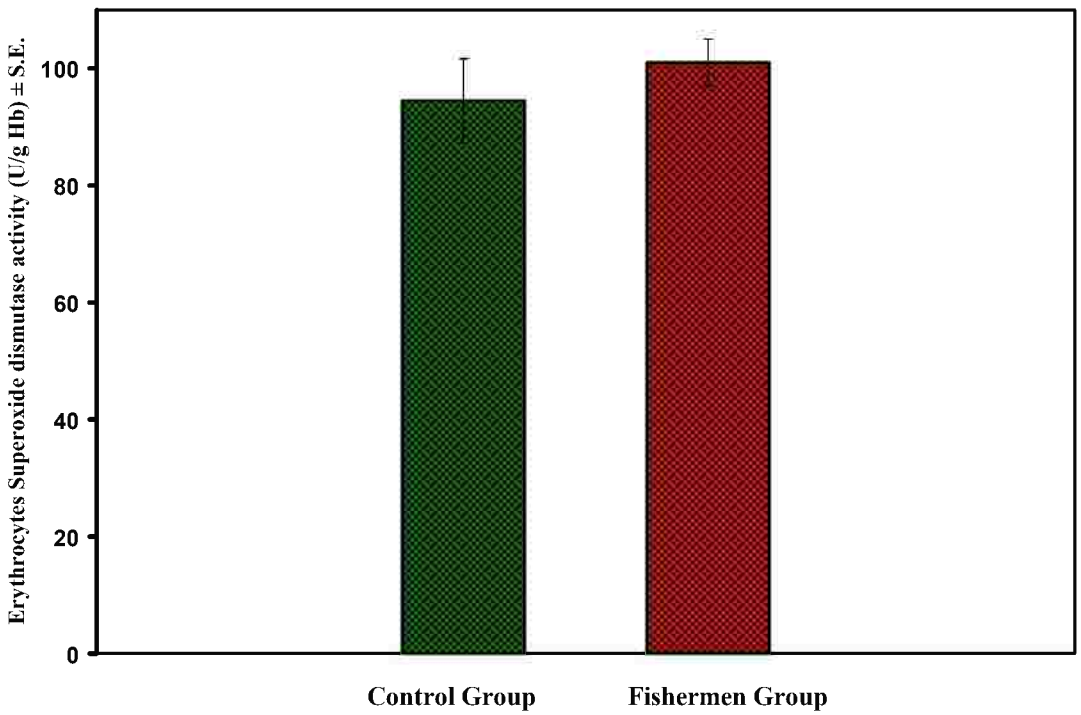

Figure 24: Mean erythrocytes superoxide dismutase enzymatic activity levels (U/g Hb) of the healthy control group and the fishermen group.

III. Amplification of IGF-2 mRNA results

Figure (25) illustrated the amplification fragments (170 bp) of insulin-like growth factor-2 (IGF-2) genome in circulating blood samples of control group, detected with different primer pairs by nested PCR.

Figure (26) demonstrated the amplification fragments (452 bp) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genome (as control genome) in circulating blood samples of control group, detected with different primer pairs by nested PCR.

Figure (27) depicted the amplification fragments (170 bp) of insulin-like growth factor-2 (IGF-2) genome in circulating blood samples of fishermen group, detected with different primer pairs by nested PCR.

Figure (28) showed the amplification fragments (452bp) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genome (as control genome) in circulating blood samples of fishermen group, detected with different primer pairs by nested PCR.

Table (16) and figure (29) represented the relative gene expression levels of insulin-like growth factor-2(IGF-2) the healthy control group and the fishermen group.

Table (17) showed the statistical analysis (independent t-test) of relative gene expression levels of insulin-like growth factor-2 (IGF-2). It manifested that there was a striking significant increase in the gene expression of fishermen group than that of control group.

Figure 25: The amplification fragments (170 bp) of IGF-2 genome in circulating blood samples of control group lane (1–7). Where M: (100 bp) DNA molecular weight marker.

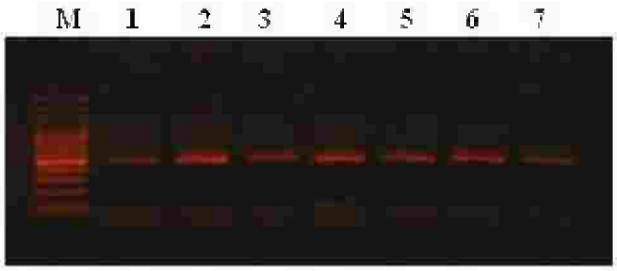

Figure 26: The amplification fragments (452 bp) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genome (as control genome) in circulating blood samples of Control group lane (1 – 7). Where M: (100 bp) DNA molecular weight marker.

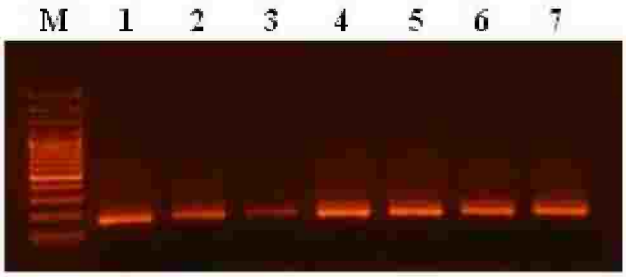

Figure 27: The amplification fragments (170 bp) of IGF-2 genome in circulating blood samples of fishermen group lane (1–7).Where M: (100 bp) DNA molecular weight marker.

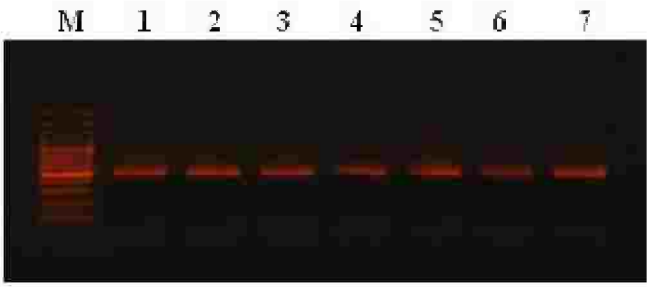

Figure 28: The amplification fragments (452 bp) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genome (as control genome) in circulating blood samples of fishermen group lane (1 – 7). Where M: (100 bp) DNA molecular weight marker.

Table (16): Relative gene expression levels of insulin-like growth factor-2(IGF-2) for the healthy control group "group I" and the fishermen group "group II".

	Group I (n = 12)	**Group II** (n = 36)	
	0.38	0.95	1.57
	0.69	1.68	1.7
	0.30	1.82	0.8
	0.33	0.96	2.43
	0.37	0.48	0.89
	0.15	0.44	0.49
	0.23	1.0	1.22
	0.82	2.14	0.37
	0.48	0.91	0.71
	0.82	0.53	0.65
	0.26	2.1	0.95
	0.64	0.78	0.57
		0.58	0.89
		1.44	0.71
		1.68	1.03
		0.77	1.66
		1.89	0.4
		1.21	1.78
Mean	0.46	1.12	
S.D.	0.23	0.57	
S.E.	0.07	0.1	

Table (17): Statistical analysis (independent t-test) of relative gene expression levels of insulin-like growth factor-2 (IGF-2) for the healthy control group "group I" and the fishermen group "group II".

	Group Statistics		Test of significance
	Group I	Group II	
$\bar{X}$	0.46	1.12	p = 0.000 *
n	12	36	
Range	(0.15 – 0.82)	(0.37 – 2.43)	
S.D.	0.23	0.57	
S.E.	0.07	0.1	

Where: * $p < 0.05$ is significant.

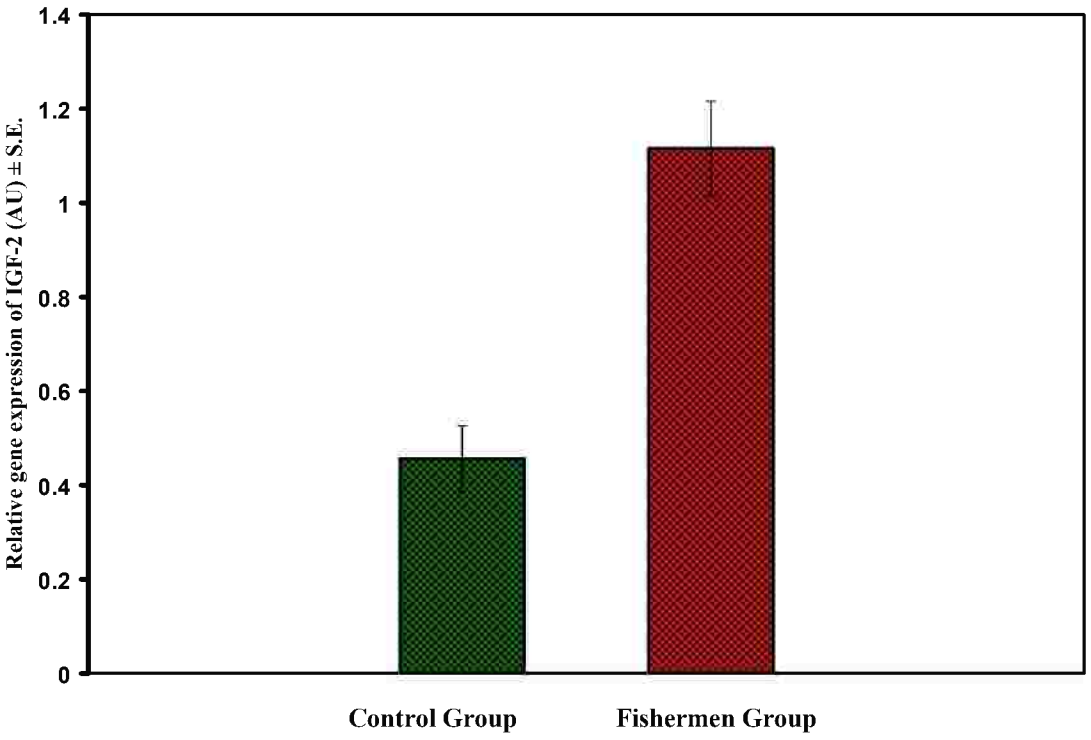

Figure 29: Mean relative gene expression levels of insulin-like growth factor 2 (IGF-2) for the healthy control group and the fishermen group.

The correlation coefficient between parameters:

Figure (30) clarify the data of correlation coefficient between blood glutathione content and metallothionein level of the two studied groups. The results indicated that there was a high negative significant correlation between them ($r = -0.321$, $p < 0.030$).

Figure (31) display the results of correlation coefficient between blood glutathione and the expression of IGF-2 gene of two studied groups. The results elucidated that there was a high negative significant correlation between them ($r = -0.422$, $p < 0.007$).

Figure (32) exhibited the data of correlation coefficient between erythrocytes metallothionein and erythrocytes glutathione peroxidase enzymatic activity. The results verified that there was a negative significant correlation between them ($r = -0.295$, $p < 0.042$).

Figure (33) display the data of correlation coefficient between the expression of IGF-2 gene and erythrocytes glutathione peroxidase enzymatic activity of the two studied groups. The results verified that there was a negative significant correlation between them ($r = -0.366$, $p < 0.017$).

Figure (34) elucidate the data of correlation coefficient between metallothionein and catalase enzyme activity in the blood of the two studied groups. The results indicated that there was a high negative significant correlation between them ($r = -0.360$, $p < 0.013$)

Figure (35) depicted the results of correlation coefficient between the expression of IGF-2 gene and catalase enzyme activity in blood of the two studied groups. The results verified that there was a negative significant correlation between them ($r = -0.385$, $p < 0.013$).

Figure (36) illustrated the data of correlation coefficient between metallothionein and the expression of IGF-2 gene in the blood of the two studied groups. The results proved that there was a well remarkable positive significant correlation between them ($r = 0.442$, $p < 0.002$).

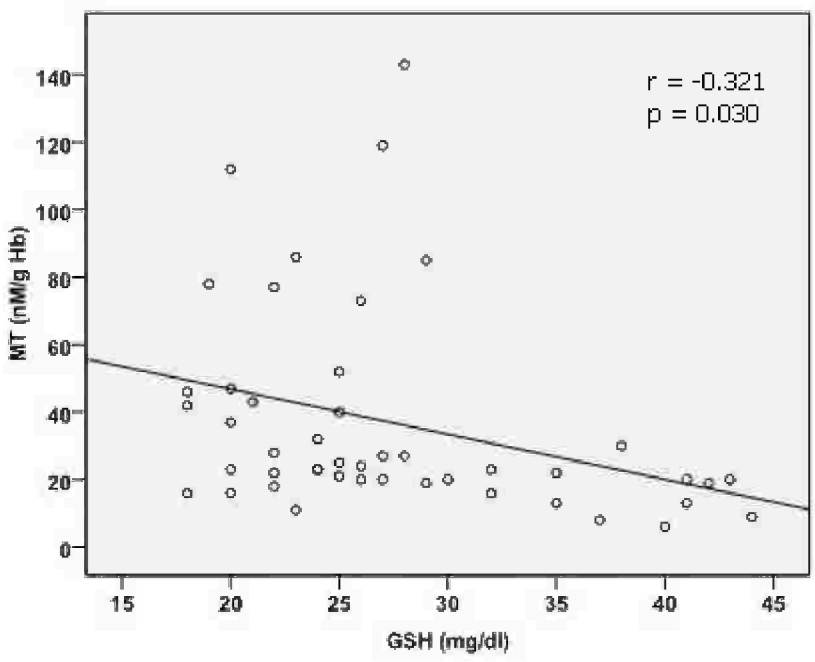

Figure 30: Pearson correlation between erythrocytes metallothionein concentration (MT) and blood glutathione content (GSH).

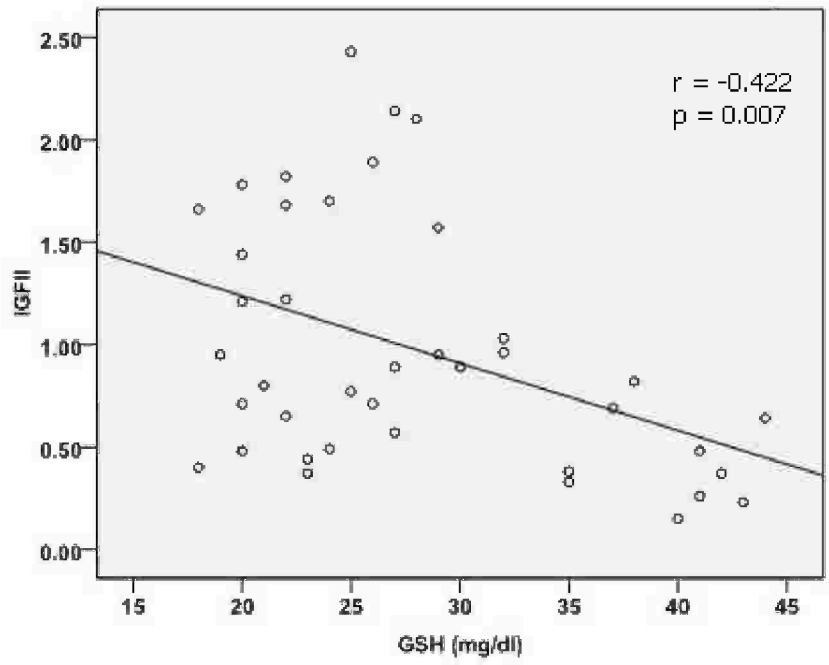

Figure 31: Pearson correlation between expression of insulin-like growth factor-2 gene (IGF II) and whole blood glutathione content (GSH).

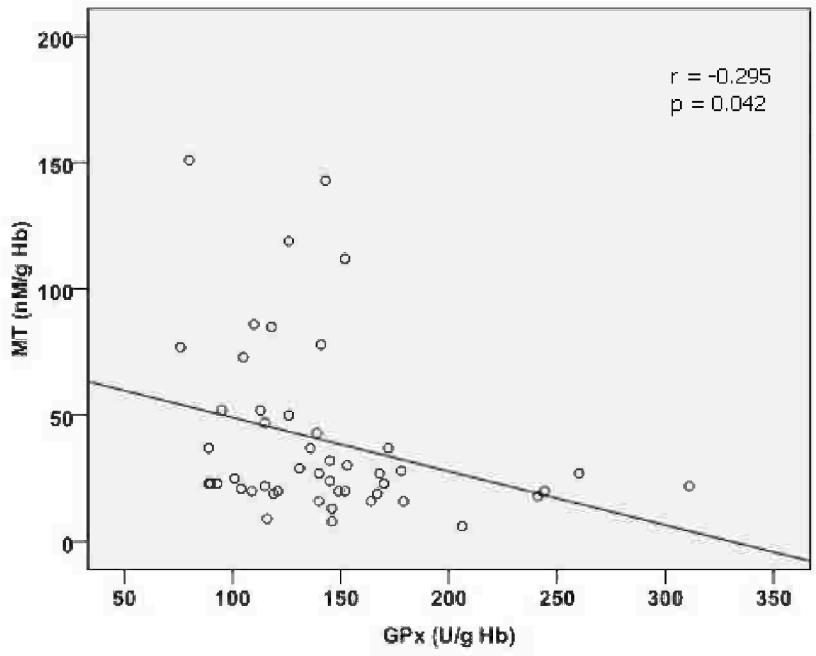

Figure 32: Pearson correlation between erythrocytes metallothionein concentration (MT) and erythrocytes glutathione peroxidase enzymatic activity (GPx).

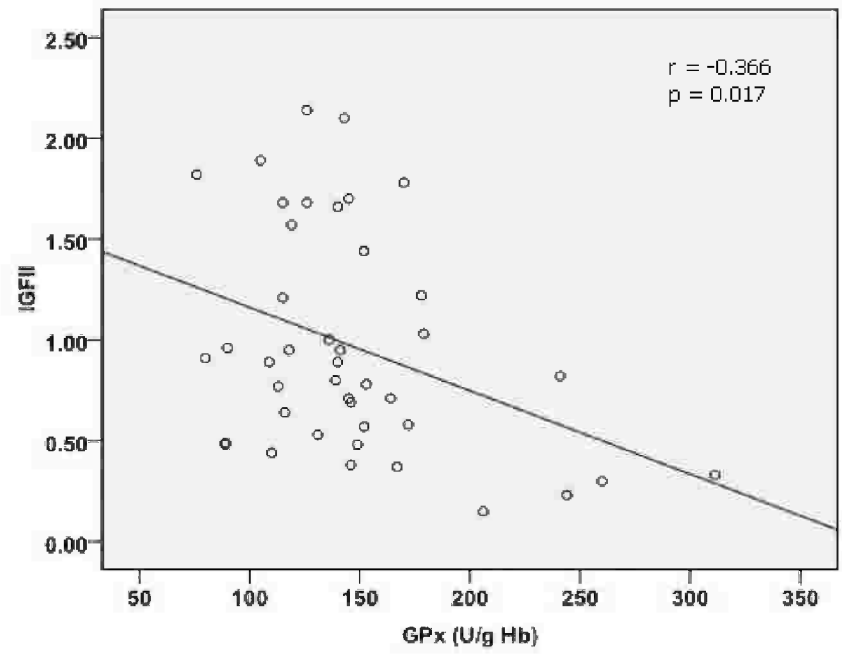

Figure 33: Pearson correlation between expression of insulin-like growth factor 2 gene (IGF II) and erythrocytes glutathione peroxidase enzymatic activity (GPx).

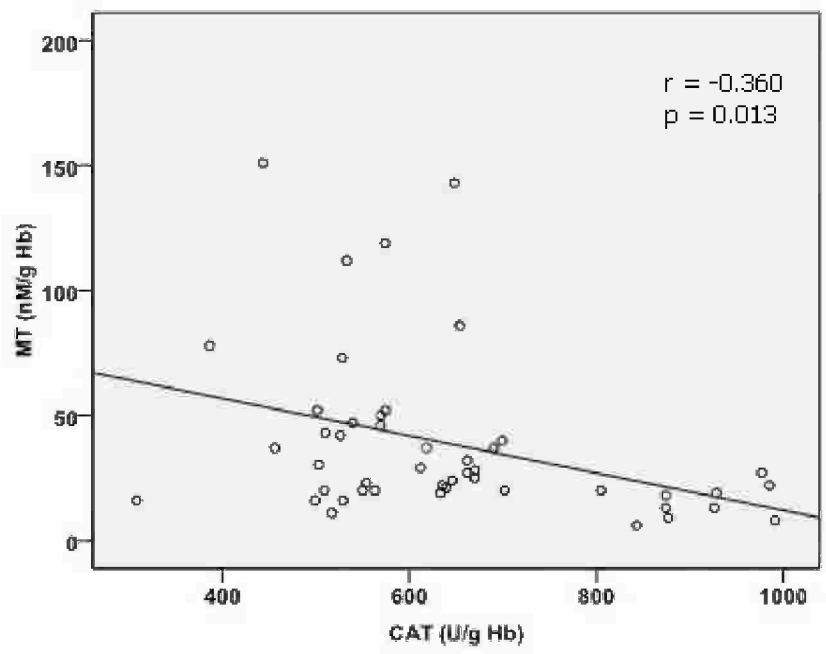

Figure 34: Pearson correlation between erythrocytes metallothionein concentration (MT) and erythrocytes catalase enzymatic activity (CAT).

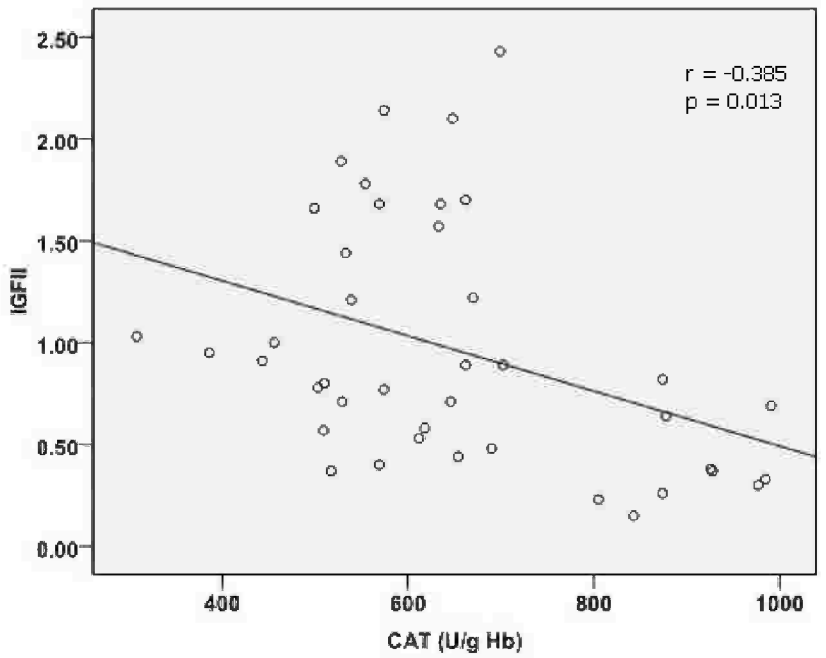

Figure 35: Pearson correlation between expression of insulin-like growth factor-2 gene (IGFII) and erythrocytes catalase enzymatic activity (CAT).

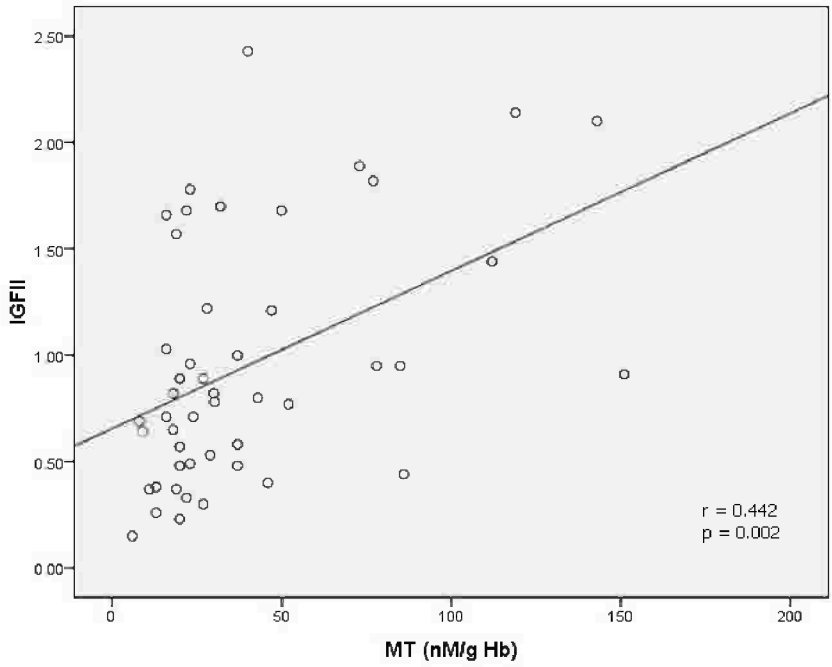

Figure 36: Pearson correlation between erythrocytes metallothionein concentration (MT) and expression of insulin-like growth factor-2 gene (IGFII).