Abbreviations

AdoMet- Dc	S- adenosyl methionine decarboxylase
BRCA	Breast cancer
BSA	Bovine serum albumin
CEA	Carcino- embryonic antigen
Cu	Copper
DNA	Deoxyribonucleic acid
E- coli	Escherichia coli
EMA	Epithelial membrane antigen
Fe	Iron
HCG	Human chronic gonadrotropin
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
µg	Microgram
Mn	Manganese
Mg	Milligram
NBT	Nitoblue tetrazolium salt
Ng	Nanogram
ODC	Ornithine decarboxylase
O_2^-	Superoxide radical
RNA	Ribonucleic acid
Ros	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
SPD	Spermidine
SPM	Spermine
SSAT	Spermidine/spermine N^1 acetyltransferase
Uv-Vis	Ultraviolet visible spectrophotometer
Zn	Zinc

Acknowledgments

I would like to express my sincere thanks and grate appreciation to my supervisor **Dr.Shahbaz A.Maki** and **Dr.Abdul wahab R.Hamad** for their supervision, suggestion throughout my study.

Deep gratitude should expressed to staff of chemistry Department for the kind help throughout this work.

Thanks go to the Hadel and Rawa'a in Medical Research Center, College of Medicine for their continued support.

Special and dearest thanks to my family **(my father, my mother, brothers, sisters)** for their patience, encouragement, and continues support during my study.

Enas

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Summary

Polyamines, spermidine and spermine constitute a group of cell components that are important in the regulation of cell proliferation and cell differentiation.

The enzyme superoxide dismutase catalyzed the breakdown of superoxide anion (O_2^-) and provides the first line of defense against oxygen toxicity. This work is an attempt to look for possible biochemical marker and causes of breast cancer based on the above polyamines.

The levels of spermine and spermidine and superoxide dismutase levels were measured in serum of two women groups. Group one contains (66) breast cancer patients while the second group contains (20) healthy women used as controls.

Blood samples from the two groups were prepared for quantitative determination of polyamines using high performance liquid chromatography (HPLC), while SOD was determined using uv-vis spectrophotometer.

It has been found that spermidine is significantly increased ($P<0.05$) in patients with breast cancer (0.184 ± 0.037) compare with normal subjects (0.14 ± 0.01), also spermine level was significantly higher ($P<0.05$) in breast cancer patients (1.105 ± 0.379) compared with control (0.22 ± 0.08) and with spermidine.

SOD level was significantly lower in breast cancer patients (330.141±54.527) when compared to the control group (535.875±39.855) with ($P<0.05$).

From the result of the present study, it can be concluded that detection of polyamines and superoxide dismutase in serum were useful and valuable as diagnostic marker for breast cancer

جامعة النهرين
كلية العلوم
قسم الكيمياء

التغيير في البولي أمين و الأنزيمات في مصل دم سرطان الثدي لدى الإنسان

رسالة مقدمة إلى

كلية العلوم- جامعة النهرين

و هي جزء من متطلبات نيل درجة الماجستير في علوم الكيمياء

من قبل

إيناس جبار حسن
بكالوريوس علوم كيمياء (جامعة النهرين ٢٠٠٢)

٢٠٠٥م ١٤٢٦هـ

الخلاصة

يشكل متعدد الأمين(سبيرميدين، سبيرمين) مجموعة من مكونات الخلية المهمة في عمليات تنظيم، تكاثر و تخليق الخلايا.

أنزيم السوبر اوكسايد دزميوتيز(SOD) يعمل على تحفيز انحلال أنيون السوبر اوكسايد(O_2^-) و يوفر الحماية الأولية في الدفاع ضد سمية الأوكسجين.

تتضمن الرسالة قياس مستوى متعدد الأمين(سبيرميدين، سبيرمين) و إنزيم السوبر اوكسايد دزميوتيز (SOD) في أمصال مجموعتين من النساء، تتكون المجموعة الأولى من (٦٦) مصابة بأورام الثدي الخبيثة، بينما تتكون المجموعة الثانية من (٢٠) نساء طبيعيات تم اعتمادها كمجموعة سيطرة.

تم حساب مستوى متعدد الأمين في جهاز السائل عالي الكفاءة الكروموتوغرافي بينما تم حساب مستوى SOD بواسطة جهاز السبيكتروفوتوميتر فوق البنفسجي.

أظهرت النتائج إن متوسط تركيز السبيرميدين في مصل دم المرضى (٠,١٨ ± ٠,٠٣٧) أعلى من تركيزه في مصل دم مجموعة النساء الطبيعيات(٠,١٤±٠,٠١) وان القيم كانت معتمدة ($P<0.05$) وكذلك وجد إن متوسط تركيز السبيرمين في مصل دم المرضى (١,١٠٥±٠,٣٧٩) أعلى من تركيزه

في مصل دم مجموعة الأصحاء(٠,٢٢±٠,٠٨) وكذلك أعلى من تركيزالسبيرميدين ،

وقد وجد إن تركيز إنزيم SOD في مصل دم المرضى(٣٣٠,١٤١±٥٤,٥٢٧) اقل من تركيزه في مصل دم مجموعة النساء الطبيعيات(٥٣٥,٨٧٥±٣٩,٨٥٥) .

من خلال نتائج هذه الدراسة، يمكن إثبات إن الكشف عن متعدد الأمين و إنزيم السوبر اوكسايد دزميوتيز يكون ذات قيمة و فائدة كعلامة تشخيصية لسرطان الثدي.

بسم الله الرحمن الرحيم

((وان يمسسك الله بضر فلا كاشف له ألا هو وان يمسسك بخير فهو على كل شيء قدير))

صدق الله العظيم

(الأنعام ١٧)

2.1. SAMPLES

2.1.1. Grouping

All blood samples were collected from the Institute and Hospital of Radiology and Nuclear Medicine in Baghdad. The first group consisted of 66 blood samples of breast cancer female patients, their ages ranged from 24-70 years. Twenty healthy volunteers were used as a second group from which blood samples were collected from Al-Kadumia Hospital and their ages were matched with patients group.

2.1.2. Samples Collection

Blood samples are drawn using 10 ml syringes with steel needles. A 3ml blood sample was drawn from each patient. The whole blood was immediately transferred to a plain tube. These samples were allowed to stand at room temperature for 10 minutes for clotting; the clots were separated from the wall of the tube using a wooden applicator stick. The tube was centrifuged for 10 minutes at 3000(r.p.m). The serum was then transferred to a second tube using micropipette and stored at -20 C° until the day of analysis. These processes were performed at the Chemistry Dept, College of Science, Al-Nahrain University. The serum samples were used for the determination of polyamines (spermidine and spermine) using HPLC as well as for determination of Superoxide Dismutase. All analysis was done at Medical Research Center, College of Medicine, at Al-Nahrain university.

2.2. INSTRUMENTS

2.2.1. HPLC Analysis [81]

Analysis of benzolyated polyamines (spermidine and spermine) was performed on a Shimadzu High Performance Liquid Chromatography (HPLC) model (SCL-10AVP) with 10µl sample loop, UV absorption detector (254nm), and a Shim-PackC_{18}, 5 µm particles, ODS column (250 X 4.6 mm i.d).

2.2.2. Reagents and Solvents

Benzoyl chloride, spermidine and spermine standards were obtained from Sigma (St.Louis, Mo, USA); Methanol (HPLC grade) was obtained from Fisher Scientific (Ottawa, Canada).

2.2.3. Derivatization [81]

Stock solution (1ml) of 2% benzoyl chloride in methanol was added to 500µL of serum in a 10ml screw-capped vial. A 1ml of 2M sodium hydroxide was added and the mixture vortexed for 30s and incubated at 37°c for 18-20 minute. The reaction was terminated by addition 2.0ml of saturated aqueous sodium hydroxide solution followed by 3.0ml of diethyl ether. This solution was vortexed for about 1-2 minutes, then centrifuged at 3000 r.p.m for 10minute to separate the aqueous and organic solvent phases. The upper ether phase containing benzolyated polyamines was transferred to another set of screw-capped tubes and evaporated to dryness.

300μL of methanol were added to the residual, this methanol solution was filtered through Millipore Hv filters (0.45μm) to remove particulates.

2.2.4. Chromatographic Separation of Polyamine Derivatives

The mobile phase methanol: water (60:40 %) was used for the separation of spermidine (spd) and spermine (spm). Chromatograms of standards spermidine and spermine are shown in figures (2.1) and (2.2) respectively. Benzolyated polyamines were separated within 15min

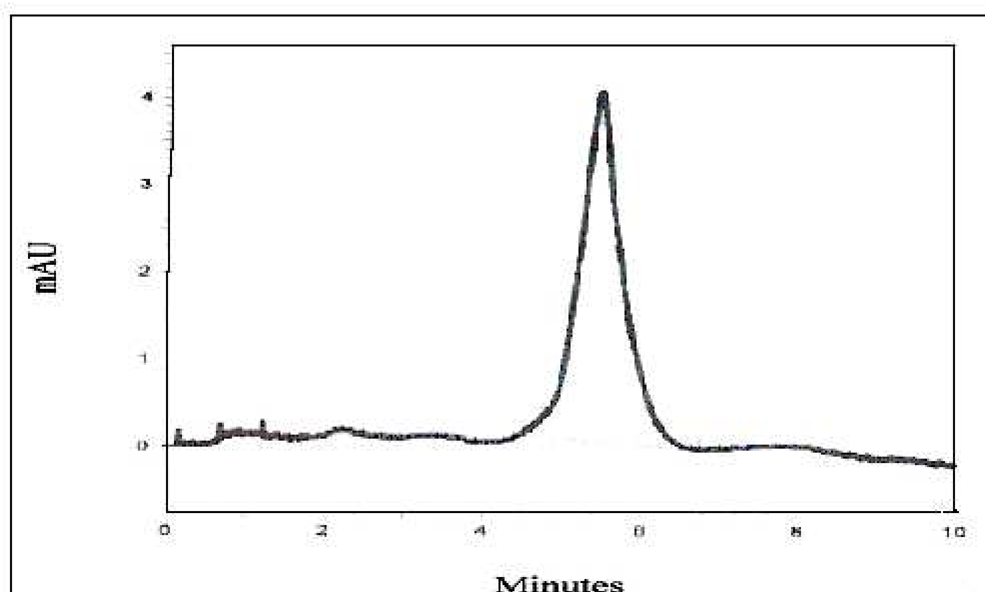

Figure (2.1): Chromatogram of spermidine standard on ODS column (25×0.46 cm i.d), mobile phase (60:40%) methanol: water, flow rate 1ml/min and uv detection wavelength at 254nm.

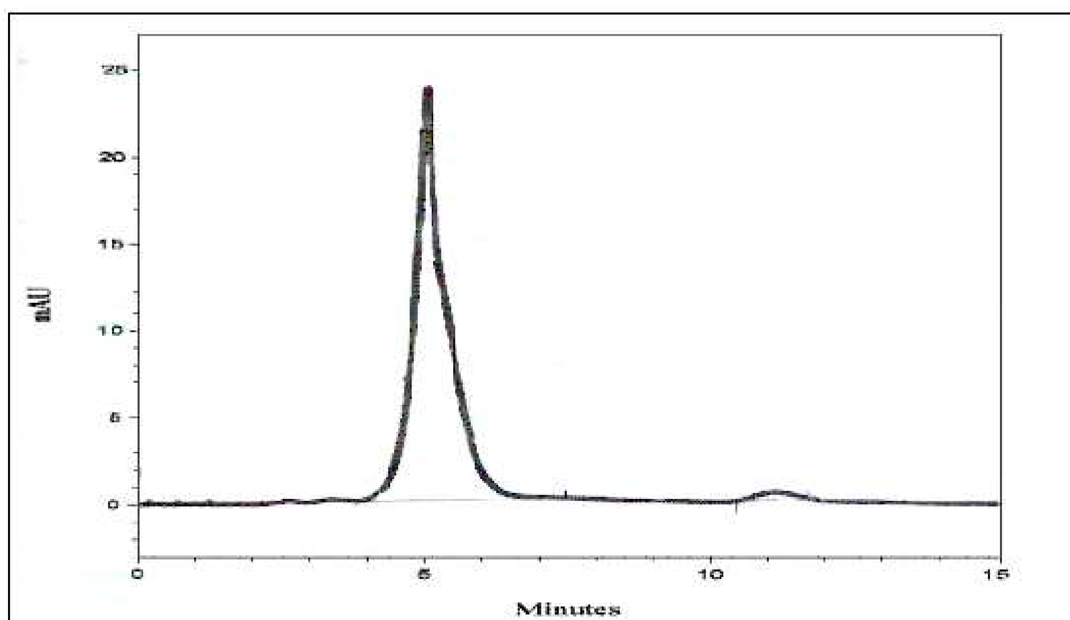

Figure (2.2): Chromatogram of spermine standard, the condition is the same in figure (2.1).

The chromatograms for the separation of normal and patient samples are shown in figure (2.3) and (2.4) respectively.

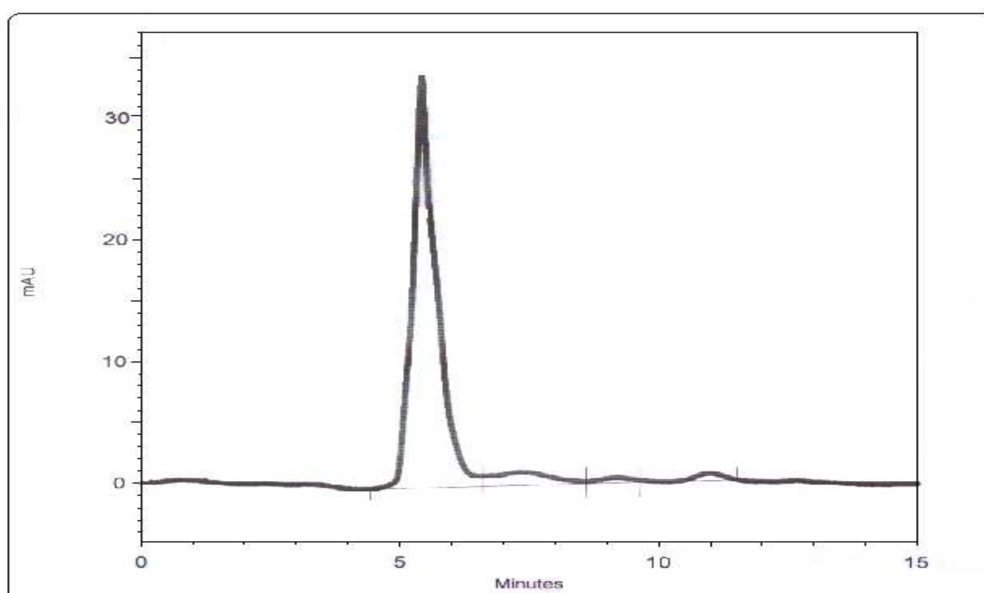

Figure (2.3): Chromatogram of polyamine (spd& spm) in serum of normal, the condition is the same in figure (2.1).

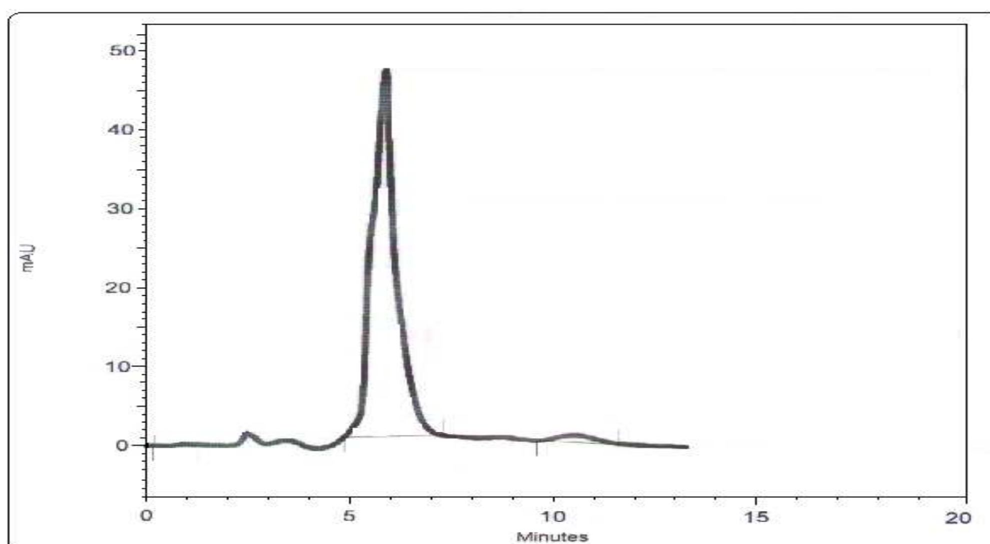

Figure (2.4): Chromatogram of polyamine (spd& spm) in serum of breast cancer patient, the condition is the same in figure (2.1).

2.2.5. Quantitative Analyses

Standard solutions of spermidine and spermine were prepared by subsequence dilution with distilled water. The prepared concentration ranges for polyamine (spermidine and spermine) were from (0.002-1.0) μmol/ml. Above derivatization method was also used for standard spermidine and spermine. Calibration curves give a linear response for spermidine and spermine as shown in figures (2.3) and (2.4) with their linear equations (2.1 and 2.2) respectively.

$Y = 1E+7X - 2E+6$ $R^2 = 0.9796$ .... (2.1)

$Y = 47193X + 2522$ $R^2 = 0.9929$ .... (2.2)

The samples were run on HPLC using the same method. A calibration curves were constructed using the peak area for each concentration of the standard.

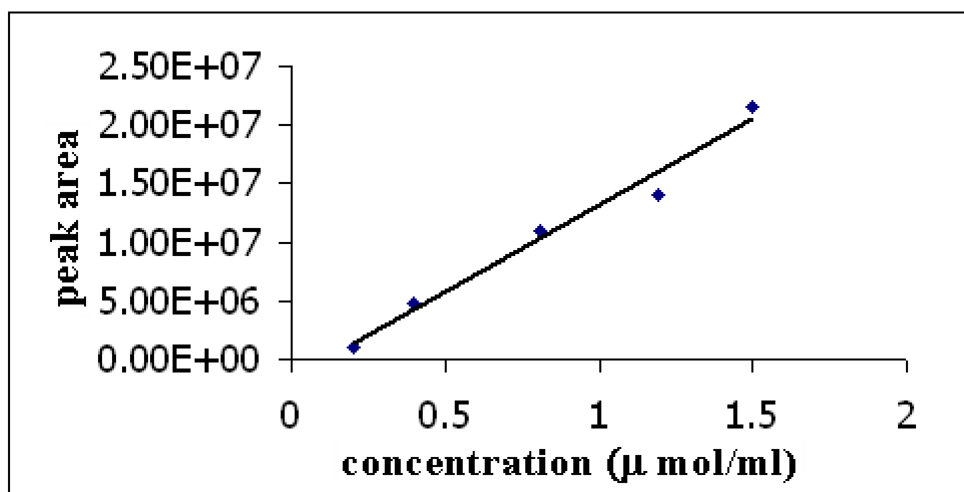

Figure (2.5): calibration curve for spermidine.

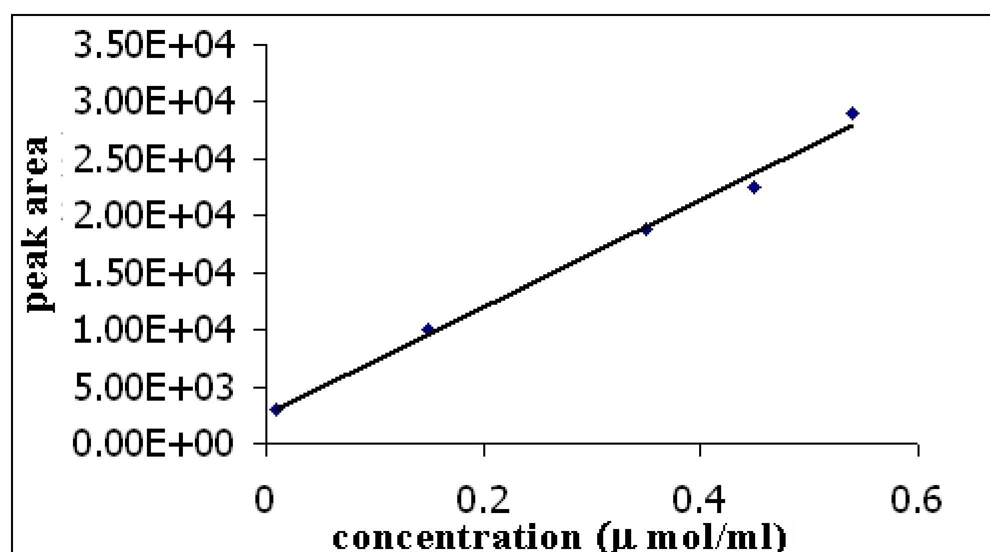

Figure (2.6): calibration curve for spermine.

2.3. SUPERROXIDE DISMUTASE

2.3.1. Reagents

- Standard SOD solution.

The stock solution consisted of 2mg of Cu, ZN SOD (Sigma) dissolved in 25ml distilled water; this solution was refrigerated until

used. Before use in the assay, the stored solution was diluted to 600μL with distilled water.

-Xanthine Oxidase solution (XO)

This solution was prepared freshly by diluting 50μL of XO (stock solution 1.8μg/ml) (Sigma) to 2.0ml with ice-cold 2mol/L ammonium sulfate.

-Xanthine (0.3mM)

1.3mg solid Xanthine (Fluka) was dissolved in 50ml distilling water.

-EDTA (0.06mM)

0.011gm solid EDTA (Fluka) was dissolved in 50ml distilled water.

-NBT (150μmol/L)

3.06mg NBT (Sigma) was dissolved in 25ml distilled water.

-Sodium Carbonate (4M) (PH 10.2)

1.06gm Na_2CO_3 (Fluka) was dissolved in 25ml distilled water.

-Bovine serum albumin (BSA)

1gm BSA (Sigma) for each litter.

-$CuCl_2$ (0.8mM)

2.7gm $Cucl_2$ (Sigma) was dissolved in 25ml distilled water.

2.3.2. Superoxide Dismutase Assay Reagent

A 40ml Xanthine solution, 20ml EDTA, 20ml NBT, 12ml Na_2CO_3 and 6ml BSA were combined

2.3.3. The Superoxide Dismutase Assay[82]

Each tube contained:

2.45ml of SOD assay reagent and 0.5ml of diluted serum or Cu,Zn SOD standard (0-270) ng/ml were incubated in a water bath at 25°c (blank without serum). Then 50μL of Xanthine Oxidase solution was added. The sample was incubated for 20min. To terminate the reaction, a 1ml of (0.8mM) $CuCl_2$ solution was added.

2.3.4. Spectrophotomtric determination

Cintra 5 (Australia) Ultraviolet \ Visible Spectrophotometer was used for the determination of Superoxide Dismutase. The absorbance for each sample was measured at 560nm and the percent of inhibition is calculated using equation (2.3) [82]:

$$\%\text{Inhibition} = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100\ \% \qquad .....(2.3)$$

Using the standard inhibition curve, the SOD concentration was determined as shown in figure (2.5).

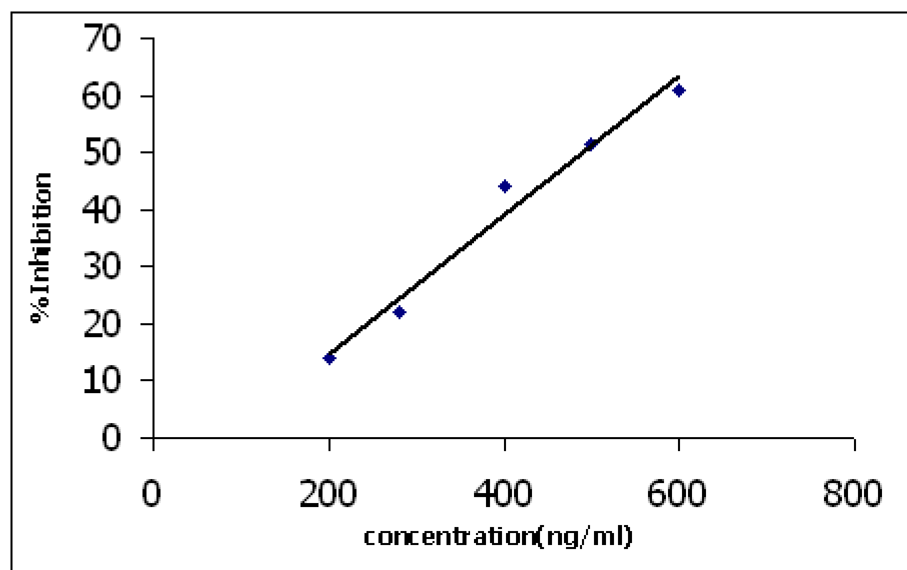

Figure (2.7): (SOD) standard curve of the inhibition by Cu, Znsod of NBT reduction in the xanthine-xanthine oxidase system.

3.1. Polyamines

3.1.1. Spermidine (SPD) & Spermine (SPM) Levels

Benzolyated polyamines were resulted from mixing serum with benzoyl chloride, sodium hydroxide and saturated aqueous sodium chloride solution with diethyl ether. HPLC was used to separate spermidine and spermine .The area under the peak was calculated to determine the concentration of spd & spm in the sera for patients and normal human. Statistical analysis was employed and the result was found that the mean ±SD of spermidine and spermine were (0.184±0.037) and (1.105±0.379), respectively .

Derivatization of polyamines at 37^0C gave the best peak slope and maximum peak area. Additional peak usually appeared when the reaction was carried out above 37^0C possibly due to degradation of polyamines at higher temperatures. Decrease peak areas resulted when derivatization was carried out below 37^0C suggesting incomplete reaction. Polyamine standards were derizatized for periods ranging from 5-20 min. The best peak slopes and maximum peak areas were obtained when the reaction mixture was incubated for 18-20 min., while an increase in duration of

incubation there was a corresponding decrease in peak area and extra peaks appearance.

According to the method of Redmond and Teseng[82],which suggested that the derivatization of polyamines involved the addition of benzoyl chloride to the sample suspended in an alkaline solvent. Benzoyl chloride which is only sparingly soluble in aqueous solutions, was dissolved in water-miscible solvents, such as methanol, prior to adding to the polyamine solution in water. Furniss et al.[81] recommended that following the reaction, the mixture should be washed with alcohol to remove excess benzoyl chloride. Reacting benzoyl chloride in methanol probably aids in removing any excess reagent [81].

The results were shown that there was a significant difference in the level of polyamines between each age and the control group, at the p values of (< 0.05). at all ranges of age. Spermine was found to be higher than spermidine. Spermine at age 41-60 was higher(1.141±0.405) than the other age groups, while spermidine at age over 60 was higher (0.2045±0.043) than the other age groups. However spermine at the same age was lower (0.845±0.359) than the other age as shown in table (3.1) and figure (3.1)

Spermidine levels was significantly higher ($p<0.05$) in breast cancer patients (0.184±0.037) compared with the healthy human

(0.14±0.01). Spermine levels was appeared to be significantly higher (1.105±0.379) in breast cancer patients compared with control (0.22±0.08) as shown in table (3.2) and figure (3.2).

The polyamines (spermidine and spermine) are essential for the growth and proliferation of all cells. Further more, quickly dividing cells especially cancer cells, synthesize and absorb large amounts of polyamines [83]. In general, high concentrations of polyamines are formed in rapidly growing cells, and low concentrations are present in quiescent cells [84]. Many types of cancer cells, including breast [85], colon [86], and hair of cancer patient[87], have also been reported to have high intracellular polyamine content [88]. Intracellular concentrations of the naturally occurring polyamines have been closely linked with the rate of cell growth and differentiation [89].

Table (3.1): The mean values for the SPD and SPM in each age compared to the control group with their (P) value.

Age range(year)	NO. of subjects	mean±SD(μmol/ml) of SPD	mean±SD(μmol/ml) of SPM	P value
20-40	12	0.1665±0.035	1.0917±0.289	<0.05
41-60	48	0.186±0.037	1.141±0.405	<0.05
Over60	6	0.2045±0.043	0.845±0.359	<0.05
control	20	0.14±0.01	0.22±0.080	-

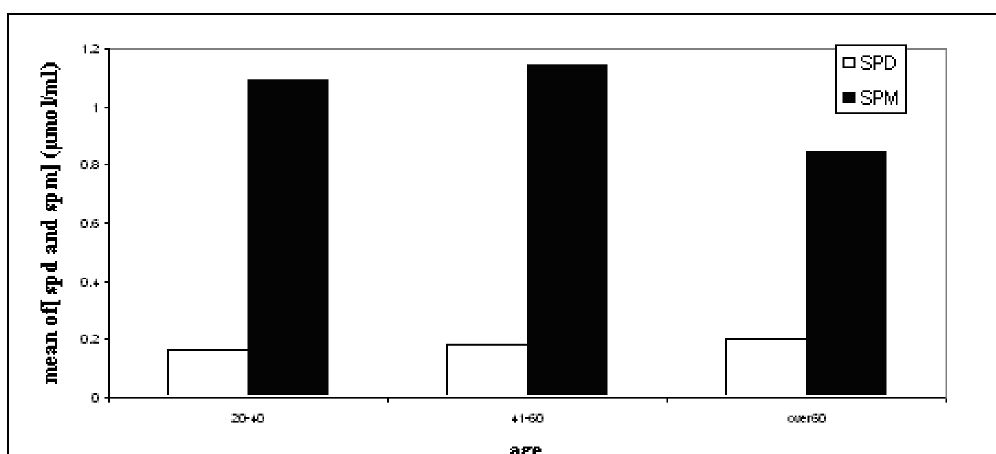

Figure (3.1): relation between mean of spermidine (SPD) and spermine (SPM) with age

Table (3.2): The mean values of SPD and SPM compared to control group with their p values.

polyamine	Mean±SD(μmol/ml) Of patient	mean±SD(μmol/ml) Of control	P value
SPD	0.184±0.037	0.14±0.01	<0.05
SPM	1.105±0.379	0.22±0.08	<0.05

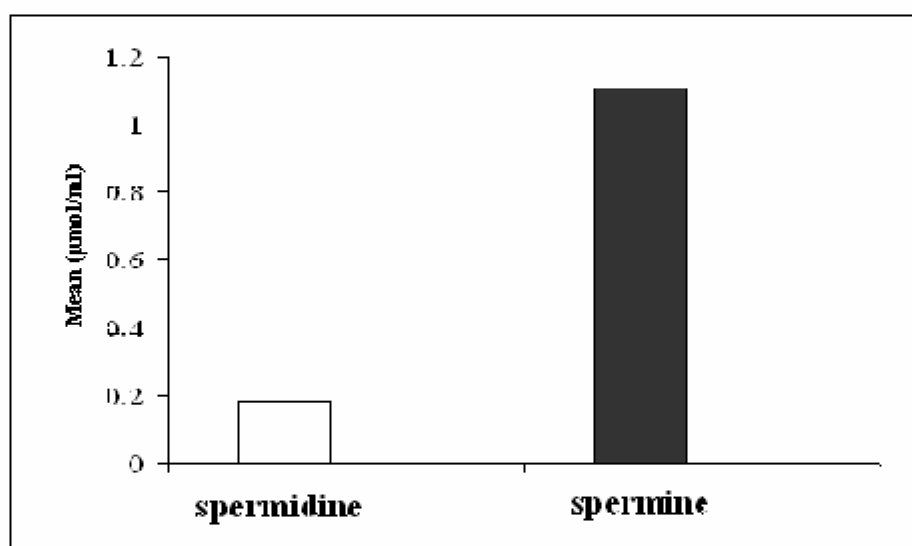

Figure (3.2): Levels of SPD and SPM in breast cancer patients.

The importance of the polyamines in cells function is reflected in a strict regulatory control of their intracellular levels. Adequate cellular polyamine levels are achieved by a careful balance between biosynthesis, degradation, and uptake of the amines. Some of the regulatory mechanisms involved in maintaining a balance of the cellular polyamine pools are truly unique [90]. The biological half-lifes

of the two regulatory enzymes ornithine decarboxylase and s-adenosyl methionine decarboxylase which are (5-60 min), are among the shortest known mammalian enzymes, allowing the cell to rapidly change the cellular polyamine levels [91]. The polyamine degradation pathway consisted of the highly regulated enzyme SSAT and the constitutively expressed polyamine oxidase. In addition to regulation of polyamine levels by biosynthesis and degradation, cells are equipped with an efficient transport system for utilization of exogenously derived polyamines [92].

The biosynthesis of polyamines is increased by a great variety of physiological growth stimuli. Polyamine deficiency results in an arrest of cell proliferation, which can be reversed by supplementation with external polyamines. Polyamine deficiency can also, under certain circumstances, resulted in programmed cell death or apoptosis [93]**.** Polyamine deficiency may be achieved by treating cells with specific inhibitors of the polyamines biosynthetic enzymes. In view of the fact that constitutive overproduction of ornithine decarboxylase has been observed in many types of cancer cells. The ornithine decarboxylase gene appeared to be of central importance in the regulation of cell growth. When the ornithine decarboxylase gene is transfected into cells and over expressed, the cells go through malignant transformation [94]**.**

3.2. Superoxide Dismutase Level

Using the standard inhibition curve, the SOD concentration was determined and listed in table (3.3). Superoxide Dismutase is thought to play a very important role in protecting living cells against toxic oxygen derivatives.

Table (3.3): Mean values of the SOD for patients compared to the control groups with their P value.

enzyme	Mean±SD(μmol/ml) Of patient	Mean±SD(μmol/ml) Of control	P value
SOD	330.141±54.527	535.875±39.855	<0.05

The enzyme catalyzes the dismutation of superoxide radicals O_2^- into O_2 and H_2O_2 [95]. Many methods have been devised to measure superoxide dismutase activity. We use the method of Yamanaka et al. [96] as follows (a) increased the concentration of xanthine oxidase to keep the absorbance at 560 nm equal to 0.25 in the blank tube. The accuracy of an absorbance measurement is higher at the higher absorbance. (b) increase the $CuCl_2$ concentration to ensure full termination of the reaction . (c) added

xanthine oxidase at 30-s intervals, then measure 40 sample for SOD content within 55min.

The Levels of SOD were Found to be significantly lower in breast cancer serum (330.141±54.527) compared to the control subjects (535.875±39.855)at ($p<0.05$) as shown in figure (3.5). Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxidase. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Additionally ROS have been implicated in cancer. Although SOD was lower in tumors and correlated well with the degree of tumor differentiation[97].

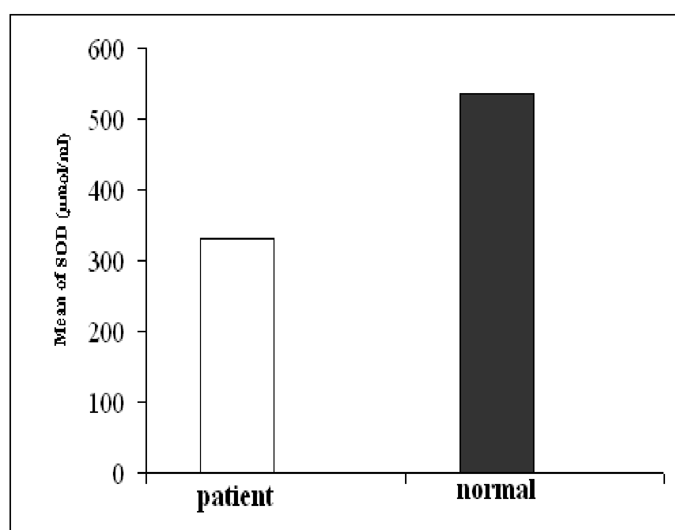

Figure (3.3): Level of SOD of normal and breast cancer patients.

It is speculated that the level of O_2^- scavenging activity by SOD is related to the appearance of cancer[98], so far, there are several examples that clearly indicate the correlation between SOD activity and human diseases such as breast cancer (decrease in SOD activity). Furthermore, since SOD activity is dramatically decreased in breast cancer, the SOD activity level of breast cancer patient end to be low. This decrease in SOD activity also decreases the breast cancer patients defense capability against oxidative stress [98].

3.3. The Relationship between SOD and Polyamines

Poduslo and Curran[99] have found in their study that modification of superoxide dismutase (SOD) with the naturally occurring polyamines; spermidine (SPD), spermine (SPM), and putrescine (PUT) dramatically increases the permeability-coefficient surface area product at the blood-brain barrier and blood-nerve barrier after parenteral administration. Because of this increased permeability, the efficient delivery of polyamine-modified SOD (polyamines-SOD) across these barriers may enhance its therapeutic usefulness in treating ischemic neuronal degeneration, neurodegenerative disease, or even aging as an important antioxidant therapeutic strategy [99]. The singlet oxygen-mediated damage to SOD and catalase may result in the perturbation of

cellular antioxidant defense mechanisms and subsequently lead to a pro-oxidant condition [100].

As well as polyamines-SOD was modified in their present experiments by activating carboxylic acid groups to the reactive ester with water-soluble carbodiimide and then reacted with polyamines as the nucleophilic reagent. Preservation of SOD enzyme activity while maximizing the permeability was accomplished by adjusting the ionization of the protein carboxylic acid with pH. These studies will allow an evaluation of the therapeutic usefulness of polyamines-SOD in animal models of neuronal degeneration [99].

Conclusions

In this work, the following conclusions have been reached out:

1. The level of polyamines (spermidine and spermine) were significantly higher in breast cancer patients compared with normal peoples.
2. The level of SOD was significantly lower in breast cancer patients compared with control subjects.

Future works

1. Study the level of polyamines and superoxide dismutase in blood, tissues and other fluids in human body and it's related other types of cancer.
2. Study the relationship between polyamines and antioxidants in cancer disease.
3. Study the polyamines and other parameters in different disease such as, diabetic disease, heart disease…etc.

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الإهداء

إلى من برؤيته يمس شغاف قلبي

والدي الغالي

إلى من راني قلبها قبل أن تراني عينها

والدتي الحنونة

إلى نبضات قلبي و أقمار مستقبلي

أخوتي و أخواتي

إيناس

Future works

1. Study the level of polyamines and superoxide dismutase in other body fluids and tissues in human and it's relationship to other types of cancer.
2. Study the polyamines and other parameters in different disease such as, diabetic disease, heart disease…etc.

1.1. Cancer Disease

1.1.1. The Nature of Cancer

Cancer is a group of more than 100 different diseases. cancer occurs when cells become abnormal and begin to divide and forming more cells without any control or order. Malignancy signs include a loss in the cell's distinctive shape and boundaries. [1, 2]

A cancerous cell develops when it ceases to respond to growth-inhibiting signals and gains the ability to multiply uncontrollably. The cells divide when new ones are not needed and form mass of excess tissue called tumor. This tumor, in turn, can damage healthy tissue in its area. The cancerous cells from this tumor can also break away and travel through the blood or the lymphatic system to form new tumor in other parts of the body. The spreading of the cancer in this fashion is called metastasia. [3]

1.1.2. Causes of Cancer

Cancer has many different causes and sources. One of these sources is a group of chemicals called carcinogens [4]. These carcinogens have been found in industrial pollutants, pesticides, and food additive. People who are exposed to these carcinogens through their occupation or other activities have been known to be more susceptible to cancer. [3, 5] There is clear evidence that the risk of certain types of cancer can be reduced by avoidance the smoking, and the choice of proper diet [1, 5] and the development of cancer have also been closely linked to heredity.

A genetic mutation (a change in the genetic code) could be passed to a child through the sperm or egg. A family history of cancer could be due to a dominant that makes cancer more likely to be passed down from generation to generation.

1.1.3. Breast Cancer

Breast cancer is a malignant growth that begins in the tissues of the breast.Breast cancer is the most common type of cancer in women, with approximately one in nine women developing the disease in their lifetime. Although breast cancer is known to affect women, it also can be equally devastating to men. Male breast cancer accounts for 1% of all diagnosed breast cancer (6). These cancerous cells continue to grow and eventually form into a tumor. Although more than 80% of breast lumps are not cancerous, a process known as a biopsy is the only way to diagnose it for sure.(7,8)

In Iraq, breast cancer remained the common tumor for females accounting for more than 30% of the registered female caners [as listed in table (1.1)], with a sharp increase in the incidence of this tumor in a younger age groups (9).

Table (1.1): The commonest cancers in Iraq (1995-1997) inclusive [(9)].

Primary site	ICD*	No. of cases	%of total
Breast	174	3486	30.1
Bronchus & Lung	162	483	4.2
Urinary Bladder	188	466	4.1
Ovary	183	462	4.0
Skin	173	461	4.0
Stomach	151	345	3.0

ICD: International Classification of Diseases.

1.1.4. Breast Cancer Factors

A risk factor is something that increases the chance of getting a disease or its condition. As with most cancers, age is a significant factor. The breast cancer deaths occur in women aged 50 and older. Some families appear to have a genetic tendency for breast cancer. Two variant genes have been found to account for this: BRCA1 and BRCA2[(10)]. These facts suggested that breast cancer is caused by the growth of genetically damaged cells. Such genetic damage is known to gradually accumulate in the cells of the body over time. Women carrying mutated BRCA1 and/or BRCA2 genes have a head start in this process.[(11)] Hormonal influences are important because they encourage cell growth. High levels of hormones during a woman's reproductive years, especially when they are not interrupted by the hormonal changes of pregnancy, appear to

increase the chances that genetically damaged cells will grow and cause cancer.[12]

Women who started menstrual periods early (before age 12) or went through menopause late (after age 55) are at higher risk. Also, women who have never had children or who had them only after the age of 30 have an increased risk. Use of hormone replacement therapy has been shown to increase the risk of breast cancer. Obesity is controversial as a risk factor. Excessive alcohol use (more than 1-2 drinks a day) has been associated with an increased risk of breast cancer, chemicals that are found in pesticides and other industrial products are possible increased risk of breast cancer. People exposed to radiation, particularly during childhood, may face an increased risk for breast cancer in adulthood.[6, 13]

1.1.5. Tumor Marker

These substances are normally present in small amounts in the blood or other tissues. Cancer cells can sometimes make these substances. When the amount of these substances rises above normal, cancer might be present in the body.[14]Tumor are graded according to their degree of differentiation as (1)well differentiation (2)poorly differentiation, or (3)ana- plastic [without form]. Tumor markers are the biochemical or immunological counterparts of the differentiation state. In general, tumor markers represent re-expression of substances produced normally by embryogenically closely related tissues. [15]

Few marker are specific for a single individual tumor (tumor-specific marker), most are found with different tumor of the same tissue type (tumor-associated markers). They are present in higher quantities in cancer tissue or in blood from cancer patients than in benign tumors or in the blood of normal subjects. Few tumor markers are specific for the organ where the tumor resides. (16)

In general, tumor markers may be used for diagnosis and prognosis of carcinomas and for monitoring effects of therapy as well as targets for localization and therapy. Ideally, a tumor marker should be produced by the tumor cells and be detectable in body fluids. (17)

Markers produced by cancers include enzymes and isozymes, hormones, oncofetal antigens, carbohydrate epitopes recognized by monoclonal antibodies, receptors, oncogene product, and genetic changes. Enzymes were one of the first groups of tumor markers identified (18). Their elevated activities were used to indicate the presence of cancer. Hormones as tumor markers were used for detection and monitoring of cancer. (19)

Finally, genetic markers emerging with great potential for diagnostic application, two types of markers antigen and suppressor gene mutations and oncogene products could prove to be clinically useful beyond the current application of tumor markers. Their areas of application could include establishing the risk factor of developing cancer in each individual by analyzing the genetic predisposition of his or her chromosomes. Gene markers could provide an insight into

the progression from normal to benign, from benign to primary disease, and from primary disease to metastatic stages. [20, 21]

1.1.6. Tumor Markers and Breast Cancer [22]

The microscopic distribution of large number of tumor and other cellular and sub cellular markers has been studied in normal and abnormal breast tissues. These markers included, among other, carcino-embryonic antigen [CEA], human chronic gonadotropin [HCG], placental lactogen, alphalactalbumin, pregnancy-specific beta-1 glycoprotein, lactoferrin, secretory component, blood group iso-antigens, neuron-specific enolase, S-100, and epithelial membrane antigen [EMA].

CEA was the most frequently expressed tumor marker, while HCG was the least frequent. None of the purified tumor marker used seem to have a consistent significant relationship to prognosis. Recently, there has been suggestion that there may be a relationship between recurrence and survical in breast cancer patients and lectin binding.

1.2. History of Polyamines

The relationship of polyamines to cell proliferation has been a subject of intense interest in recent years [23]. In 1965 Dykstra and Herbst were the first who's suggested that polyamines might be involved in the proliferative response of cells and tissues [24]. These workers demonstrated increases in tissue levels of spermidine early in the regenerative process. In recent years [25], it has been suggested the mechanisms that regulate the activity of

ODC are an integral part of growth-control programs. The enzyme also attracted considerable attention in 1969 when Russell and Snyder reported that ODC has an extremely short half-life. This result suggested that ODC also play a role in regulating growth [26]. In 1971, Hershko and coworkers found that accelerated rates of polyamine biosynthesis, with increase in cellular polyamine levels, simply could have been non-essential by-products of the pleiotypic growth response of cell [27].

Although these correlations were highly suggestive, they clearly did not prove that increased cellular polyamine levels were essential for cell multiplication. In1974, Morris and Fillingame were observed the correlation between stimulation of cell growth and large increase in the rate of polyamine biosynthesis [28].also they reviewed some of the properties of ODC, its regulation and its relationship to all of polyamine biosynthesis [29]. This correlative approach was extended to its ultimate in the statistical study in 1975 by Heby and coworkers [30].

It was only with the isolation of well-defined, putrescine auxotroph of E-coli that the growth requirement for polyamines was proved [31]. Studies with these mutants have provided considerable insight into the physiology of the polyamine-deficient state [32]. In 1978 Tabor and Tabor studied that mutants have also recently revealed a polyamine requirement for growth in yeast[33].Interest in the regulation of polyamine biosynthesis in animal cells was triggered by the finding that growth processes are closely related to cellular polyamine synthesis [34].

This brief review is not intended to be a comprehensive synopsis of the subject of ODC, but rather an overview of the

various mechanisms for the regulation of mammalian ODC. These are in some cases quite complex. It may be that attempting to describe an overall unified mechanism for ODC regulation is like trying to explain the specific role of polyamines in cell. In fact, the reason why there are so many mechanism of ODC regulation is related to the ubiquity of polyamines themselves.

1.2.1. Polyamines

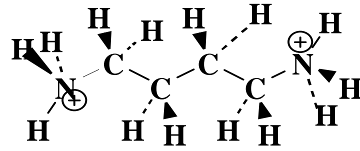
Polyamines are small, low molecular weight, ubiquitous organic polycations.(35,36) That have been implicated in a wide variety of physiological function including protein translation , membrane stabilization, cell growth, and cell proliferation .(37)

The most common polyamine are putrescine, spermidine, and spermine (shown in figure 1.1), which are present in eukaryotic organisms at abundant levels (high micro molar to millimolar). (35)

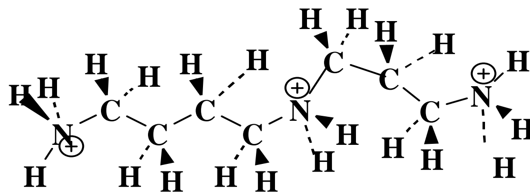
An important property of these specific polyamines is that they are positively charged at physiological pH, and consequently, they have a high affinity toward negatively charged cellular molecules. Thus polyamines are very soluble in water, and they exert strong cation –anion interactions with macromolecules, mainly with DNA and RNA (38), and may stimulate DNA and RNA biosynthesis, DNA stabilization and packaging of DNA in bacteriophage. (36)

The most marked synthesis and accumulation of polyamines occur in rapidly growing tissues. (39) However detailed knowledge of what they do and how they do it is far less clear. They are intimately

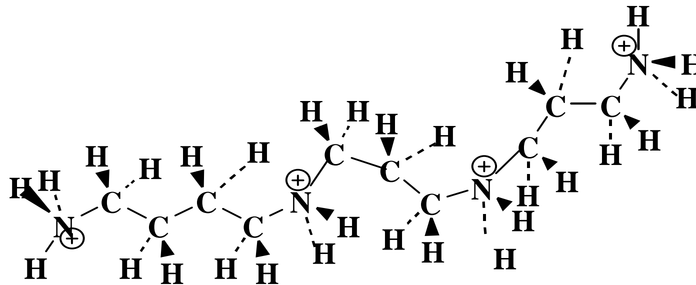
involved in cell replication and they have been thought of as markers for the onset of various cancers. (40)



PUTRESCINE



SPERMIDINE



SPERMINE

Figure (1.1): structure of polyamines

1.2.2. Biosynthesis of Polyamines

In humans, polyamines originally come from the amino acid called ornithine. Each step of the pathway produces one of three different polyamines. In the first step, a specific enzyme [ornithine decarboxylase] converts ornithine into polyamine known as putrescine. In the second step, two enzymes, [AdoMet- DC and

SSAT] work together to change the putrescine into asparate polyamine called spermidine. Finally, with help of the same two enzymes, the spermidine transformed into the polyamine called spermine [41] as shown in figure [1.2]

Humans synthesize about 0.5 mmol of spermine per day. Spermidine and spermine are growth factors for cultured mammalian and bacterial cell and function in the stabilization of intact cells, subcellular organelles and membranes. Pharmacologic doses of polyamines are hypothermic and hypotensive. Because of their multiple positive charges. [42]

Polyamines also exert diverse effects on protein synthesis and inhibit certain enzymes, including protein kinases. [37]

1.2.3. Polyamine in Cell and Tissue Growth

The putrescine portion of spermidine and spermine derives from L- ornithine and the diamino propane portion from L-methionine by S- adenosylmethionine. Ornithine decarboxylase and S-adenosylmethionine decarboxylase are inducible enzymes with short half-lives. Spermine and spermidine syntheses are being contrast, neither inducible nor unusually labile enzyme [42].

The enzymes of mammalian polyamine biosynthesis two, ornithine decarboxylase and S- adenosylmethionine decarboxylase[43]. These are of interest with respect to their regulation and their potential for enzyme directed chemotherapy. The half- life of ornithine decarboxylase, about 10 minutes, is among shortest of any mammalian enzyme, and its activity responds rapidly

and dramatically to many stimuli. Ten to 200- fold increases in ornithine decarboxylase activity follow administration to cultured mammalian cells of growth hormone, corticosteroids, testosterone, or epidermal growth factor [44].

Polyamine added to cultured cells induces synthesis of a protein that inhibits ornithine decarboxylase activity. S-Adenosylmethionine decarboxylase contains pyruvate rather than pyridoxal phosphate as its cofactor, has a half- life of 1-2 hours and responds to promoters of cell growth in a manner qualitatively similar to ornithine decarboxylase. S- Adenosylmethionine decarboxylase [shown in figure 1.2] is inhibited by decarboxylated S-adenosylmethionine and activated by putrescine [42].

1.2.4. Polyamine Catabolites

The key regulatory enzyme for polyamine catabolism is the spermidine/spermine N^1acetyltransferase [45, 46]. That oxidizes spermine to spermidine and subsequently oxidizes spermidine to putrescine [37, 47]. Both amino propane moieties are converted to β-amino propanaldehde. Subsequently, putrescine is partially oxidized to $NH4^+$ and CO2. A major fraction of putrescine and spermidine are, excreted in urine as conjugates, principally as acetyl derivatives[48] as shown in the figure [1.3].

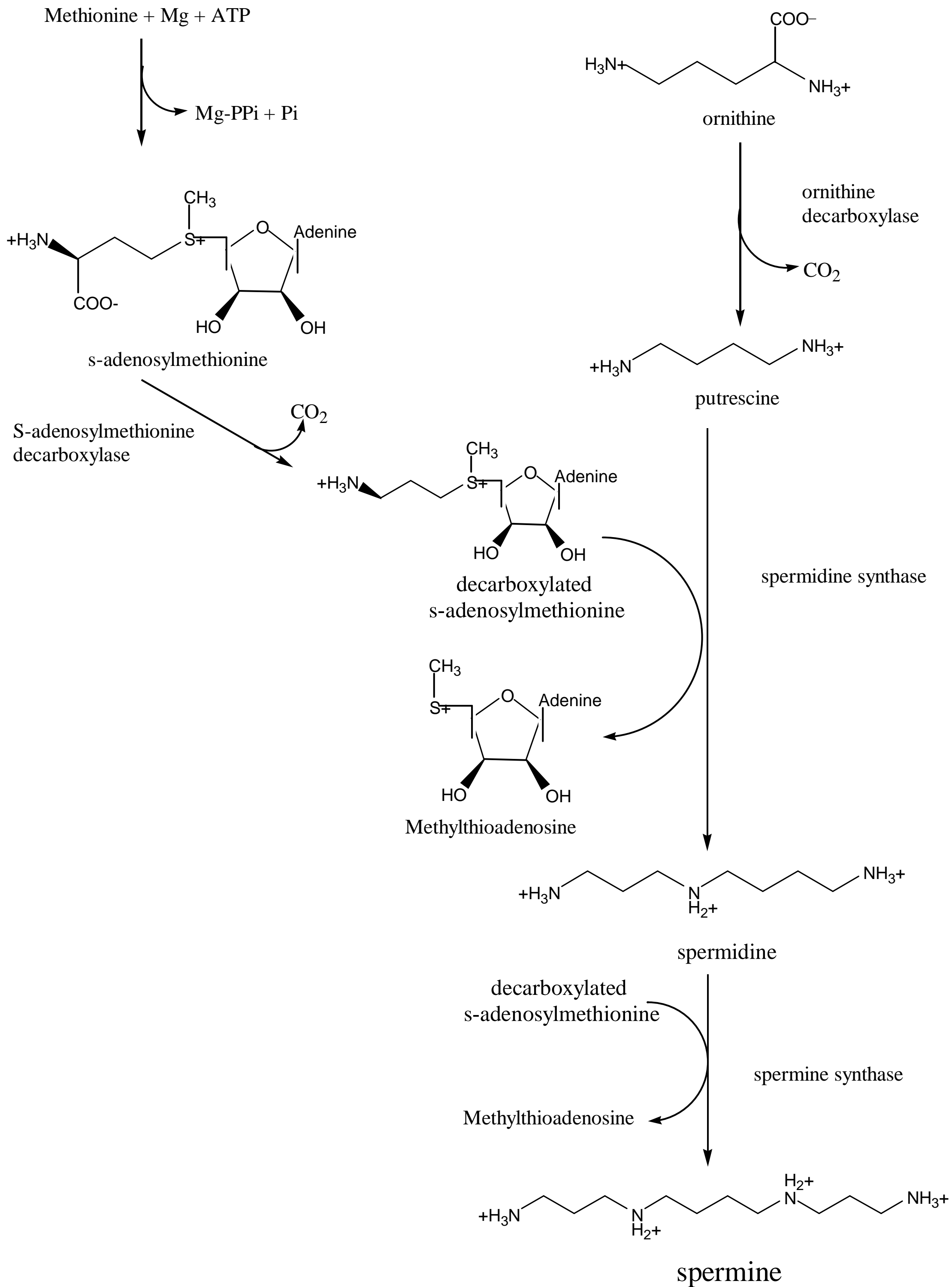
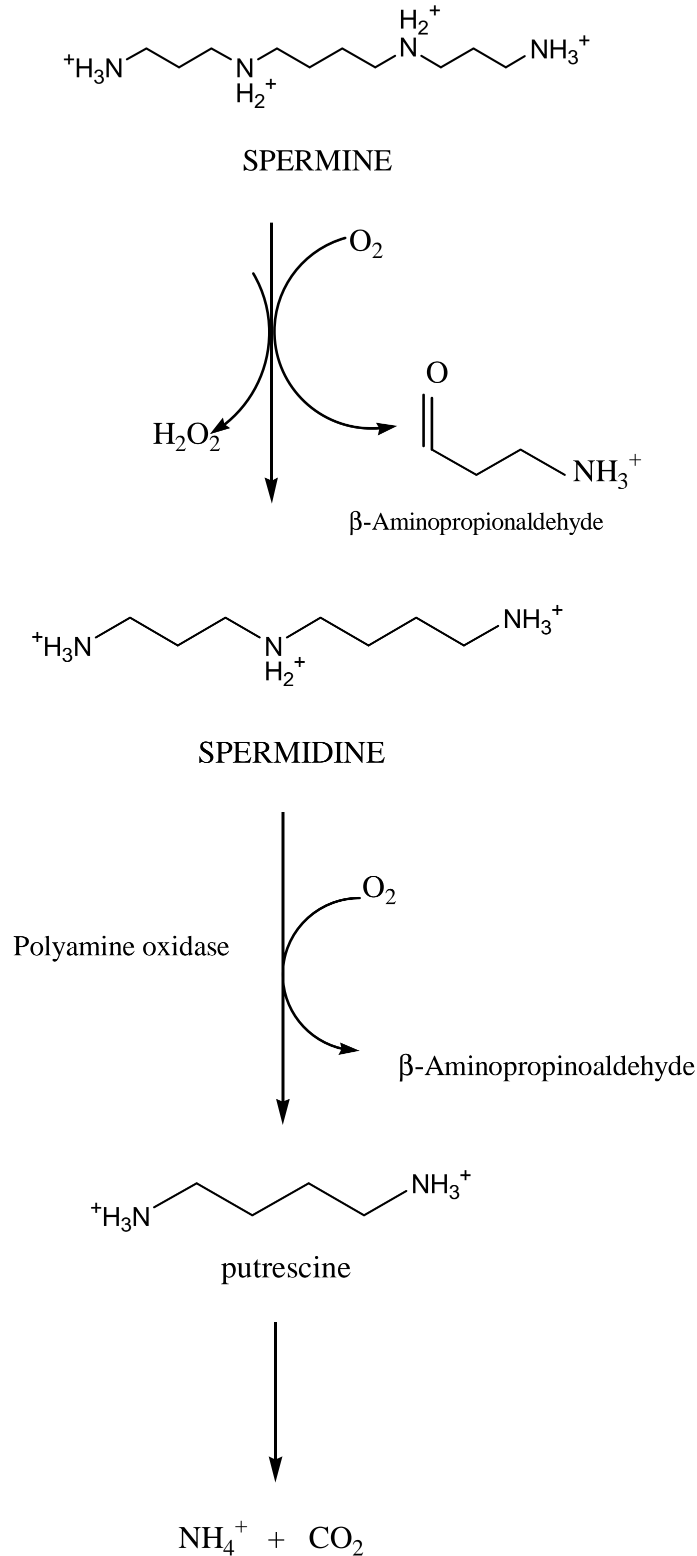


Figure (1.2):The utilization of putrescine and S-adenosylmethionine for the formation of spermidine and spermine[41]



Figure(1.3): catabolism of polyamines,(structures is abbreviated to facilitate presentation)[47].

1.3. Enzymes

Enzymes are bio-polymers that catalyze the multiple dynamic processes which make life possible [49, 50].

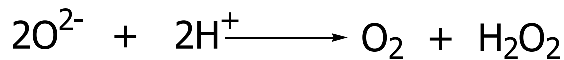
As the determination of the rates at which physiologic events take place, enzymes play central roles in health and disease. The breakdown of foods to supply energy and chemical building blocks, proteins, membranes, and the DNA that encodes genetic information and the harnessing of energy to produce cell movement are all made possible by the carefully coordinated action of enzymes[51].

All physiologic processes occur in an ordered, regulated manner and homeostasis is maintained. Homeostasis can be profoundly disturbed in pathologic states [52]. The severe tissue injury that characterizes liver- cirrhosis can profoundly impair the ability of cells to form the enzymes which catalyze a key metabolic process such as urea synthesis. The resultant inability to convert toxic ammonia to nontoxic urea is then followed by ammonia intoxication and ultimately hepatic coma. A spectrum of rare but frequently debilitating and often fatal genetic diseases provides additional dramatic examples of the drastic physiologic consequences that can follow impairment of the activity of a single enzyme [50].

1.3.1. Superoxide Dismutase

1.3.1.1History of Superoxide Dismutase

Superoxide dismutase (SOD) is one of the primary antioxidant enzymes in eukaryotic cells; it catalyzes the destruction of the O^{2-} free radical [53].



$$2O^{2-} + 2H^{+} \longrightarrow O_2 + H_2O_2$$

SOD was found to protect oxygen- metabolizing cells against harmful effects of superoxide free- radicals by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen [54] SOD was also found to protect hyaluronate against depolymerization by free-radical and indicated that exogenous SOD might have an anti-inflammatory effect [53]. The SOD deficiency might lead to Heinz body hemolytic anemia [51]. Superoxide dismutase is widespread in nature, and present in all oxygen- metabolizing cells [56].

There are two main form of SOD in eukaryotic cells: A blue-green Cu (+2)-Zn (+2) enzyme comes from human and bovine erythrocytes, a wine-red Mn (+3) protein which was found in E-coli, and chicken, and rat liver mitochondria, and a yellow Fe (+3) enzyme from E-coli [53, 57, and 58].

1.3.1.2. Copper- Zinc Dismutases (Cu-Zn-SOD)

The copper-zinc dismutase is a remarkably conserved family, with respect to their gross structural properties [53, 59]. Without

exception , the purified enzymes have been shown to be a dimmer (molecular weight usually 31,000 to 33,000) containing nearly 2.0g-atoms of copper and 2.0g-atoms of zinc per mole (58,60). The two subunits are apparently identical and are associated solely by non covalent interaction. subunit molecular weights estimated by physical methods (15,000 to 17,000) are in good agreement with the results of the complete amino acid sequences, now known for the enzymes from bovine and human erythrocytes , from yeast, and from horse liver (subunit molecular weight 15,600 to 16,000) (61).

These sequence data and the crystal structure of the bovine erythrocyte enzyme demonstrated that each subunit contains one intrachain disulfide bond and one active site , containing an atom of copper and atom of zinc bridged by the imidazole ring of histidine (His 61), a feature unique to enzymes of this class. The Cu is coordinated by a further three histidine ligands and a water molecule, while Zn is linked by two additional histidines and an aspartic acid (54, 62) as shown in figure [1.4]

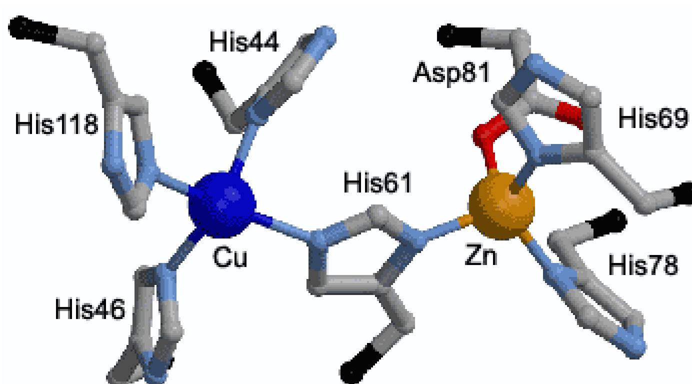


Figure (1.4): structure of Cu-Zn dismutase (54)

1.3.1.3. Manganese / Iron Dismutases

Enzymes of the manganese /iron family are not as uniform in such basic properties as molecular weight, subunit structure, and metal content. Some are dimers; others are tetramers [63]. The contents of the metal ranged from about 0.5 to 1g-atom / mol of polypeptide subunit chain [60]. Only one type of metal is present, manganese or iron. Regardless of subunit structure, the constituent polypeptide chain of this dismutase is larger than that of copper-zinc dismutase. The subunit molecular weight ranged from 19,000 to 22,000 g/mol which in agreement with values of 22,000 and 23,000 g/mol for the polypeptide of the two enzymes of this family for which complete sequence data were available from, the dimeric Mn-containing enzymes from E-coli. [64] Neither of these two enzymes contain half-cystine, the two subunits are identical in each dimer [65]. Although Mn/Fe dismutases are generally dimeric, other multimeric states exist. Tetramers have been identified in (1) all manganese-containing enzymes from eukaryotes, (2) manganese enzymes from three bacteria, Mycobacterium phlei, thermus aquaticus, and thermus thermophilus, and (3) one iron-containing dismutase, which form Mycobacterium tuberclosis. One trimmer has been identified, in the manganese enzyme from Mycobacterium. The molecular weight of the trimmer ranged 62,000 to 65,000[60].

1.3.2.Reactive Oxygen Species and Superoxide Dismutase

A given amount of the oxygen taken in by the body is always converted to $O_2^{\cdot}$, H_2O_2, hydroxy radical ($^{\cdot}OH$) and other molecules by various enzymatic metabolism system [66,67] among these

molecular species. The life spans of $^{\cdot}OH$ and $O_2^{\cdot-}$, which has unpaired electron, are the shortest . $^{\cdot}OH$ has the highest reactivity and it reacts with various molecules with diffusion controlled rate [68]. Although it is believed that O_2^- may not be directly react with lipids, proteins, sugars or nucleotides, it is transformed into $^{\cdot}OH$ when it interacts with metal ions and it react with nitric oxide (NO) to quench the physiological activities such as vascular relaxation [64].

At the same time, O_2^- generates peroxinitrite ($ONOO^{\cdot}$), which causes oxidative damage. In order to protect the body from highly toxic ROS, the body has acquired anti oxidative stress mechanisms including SOD. These anti oxidative stress mechanisms are localized in tissues and inside the cell where reactive oxygen species (ROS) are generated [69,70].

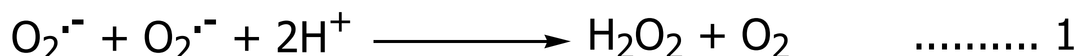
1.3.3. Superoxide Dismutase and Cancer

Reactive Oxygen Species (ROS) are generated endogenously by all aerobic cells because of the metabolism of oxygen. ROS at high concentrations exert harmful effect on living organisms including damage to DNA and cell membranes. ROS at low concentration perform essential metabolic functions in regulating signal transduction pathways and transcription factors. Intensive investigations have revealed possible effects of ROS on tumor inition and transformation [70, 71]. If the amount of ROS exceeds the limit of the defense mechanism of the body for any reason, serious disease may be induced, the typical example is cancer [53, 68,and 72]. Lifetime exposure to exo-endogenous estrogens appears to be closely related to development and progression of breast cancer [73].

A variety of cancer cells are known to express reduced levels of antioxidant enzymes, especially MnSOD, when compared with their normal counterpart [59, 74]. Furthermore, the increased expression of MnSOD has been found to suppress the malignant phenotype of human breast cancer cells suggestion that MnSOD is a tumor suppressor gene in human breast cancer [75].

1.3.4. Catalytic Mechanism of Superoxide Dismutase

The very nature of the dismutation of superoxide to dioxygen and hydrogen peroxide creates a complication for all physiological catalysts. At neutral pH superoxide radical is predominantly in the anionic form, $O_2^{\cdot -}$, being the conjugate base of the hydroperoxyl radical ($OH_2^{\cdot}$), who's pka is (4.8). Any catalyst must circumvent the electrostatic repulsion and consequent slow reaction between two superoxide anion indicated in the chemical equation as shown in equation 1 [64].

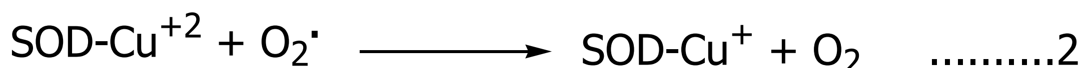


$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{.......... 1}$$

The active site of the cytosolic enzyme in eukaryotes contains a copper ion and a zinc ion coordinated to the side chain of a histidine residue. The negatively charged superoxide is guided electrostatically to a positively charged catalytic site at the bottom of a channel [76]. $O_2^{\cdot -}$ binds to Cu^{2+} and the guanido group of an arginine residue.

An electron is transferred from superoxide to cupric ion (Cu^{2+}) to form cuprous (Cu^{1+}) and O_2, which is released [77]. A second

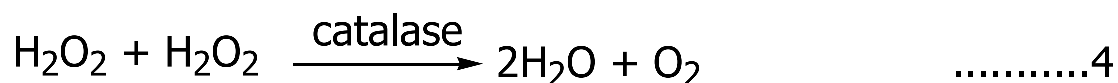
superoxide enters the active site and binds to Cu^{+}, arginine, and H_3O^{+}. The bound $O_2^{\cdot -}$ acquires an electron from Cu^{+} and two protons from its binding partners to form H_2O_2 and regenerate the Cu^{2+} state of enzyme[(78)].as shown in equations 2 and 3 and all that explain in figure (1.5)



$$SOD\text{-}Cu^{+2} + O_2^{\cdot} \longrightarrow SOD\text{-}Cu^{+} + O_2 \quad ....2$$

$$SOD\text{-}Cu^{+} + O_2^{\cdot -} \longrightarrow SOD\text{-}Cu^{+2} + H_2O_2 \quad ....3$$

The hydrogen peroxide formed by SOD and by the uncatalyzed reaction of hydroperoxy radicals is scavenged by catalase, ubiquitous hem protein that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen as shown in equation 4[(79)].



$$H_2O_2 + H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad ....4$$

X-ray absorption spectroscopy has definitively established that the valence of Zn^{2+} does not change during catalysis of that reaction [(64)].

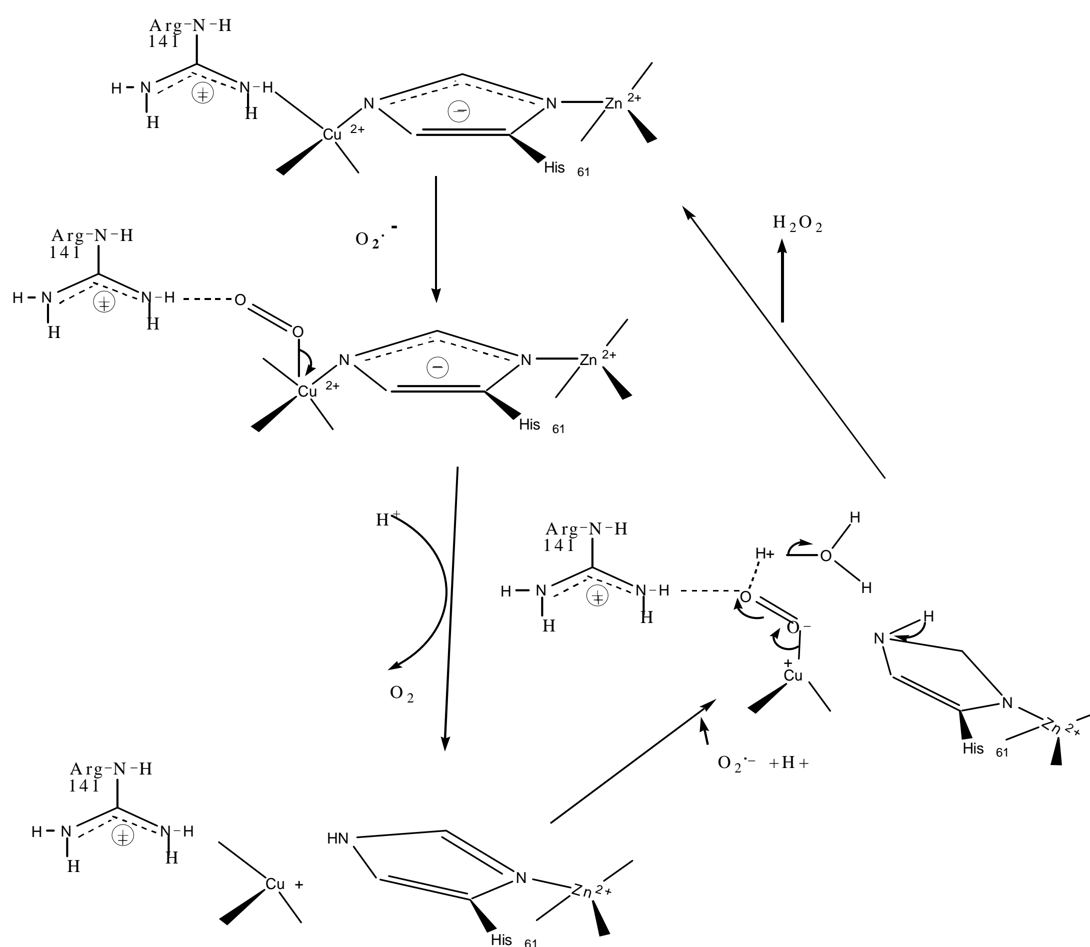


Figure (1.5) proposed catalytic mechanism of SOD

1.4. Aim of the work

This study was to measure the concentration of new tumor marker based on the levels of the polyamine (spermidine and spermine), and SOD in breast cancer patient and compared them with that of control subject. Moreover, study the biochemical change in breast cancer patient will be studied.

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Supervisors Certification

We certify this thesis was prepared under our supervision at Al-Nahrain University as partial requirements for the **Degree of master of science in chemistry**.

Signature :
Name: Dr.Shahbaz A.Maki
Date:

Signature :
Name: Dr.Abdul wahab R.Hamad
Date:

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature :
Name: Dr. Shahbaz A. Maki
Head of Chemistry Department
College of Science
Al-Nahrain University
Date:

Examiners certifications

We certify that we have read this thesis, titled **"Alteration in polyamines and enzymes in human breast cancer serum "** and examined the student **(Enas Jabbar Hasan)** in its contents and that, in our opinion, it is accepted as a thesis for the **degree of Master of Science in Chemistry**.

Signature:
Name:prof. Dr. Lu'ay Qaseem
Date:
(**Chairman)**

Signature:
Name:Dr. Abeer Hazem
Date:
(Member)

Signature:
Name: Ass. Prof. Dr.Tariq Hfthy
Date:
(Member)

Signature:.
Name: Ass. Prof. Dr. Shahbaz A. Maki
Date:
(Supervisor)

Signature:
Name:Prof. Dr. abdul Wahab R.Hamad
Date:
(Supervisor)

Approved by the University Committee on Graduate Studies.

Signature:
Name: Dr.Laith Abd Al-Aziz
Adress:Dean of College of Science at Al-Nahrain University
Data:

Al-Nahrain University
College of Science
Department of Chemistry

Alteration in polyamines and enzymes in human breast cancer serum

A thesis
Submitted to the college of science at Al-Nahrain University as a partial Fulfillment of the Requirements for the Degree of Master of Science in chemistry.

By
Enas Jabbar Hasan
B.Sc.(Al-Nahrain University 2002)

2005 1426