

Study the effect of isolated human epidermal growth factor receptor (HER2) from plasma on histopathological aspects in some organs of adult female albino rats

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Abstract

This study included an attempt to isolate and purify Human Epidermal Growth Factor Receptor (EGFR) from healthy human plasma using different biochemical technique. Two peaks had been isolated by gel filtration chromatography from the precipitate produced by using ammonium sulfate. The first peak (peak A) had a high level of EGFR. Furthermore, the purity of isolated EGFR peak A had been identified by the high-performance liquid chromatography and by gel electrophoresis technique. The results obtained from high performance liquid chromatography showed that there was a good identity in retention time between the standard and the isolated EGFR peak A. The approximate molecular weight of partially purified EGFR peak A was 75318 ± 100 and 73029 ± 100 dalton using gel filtration chromatography and gel electrophoresis technique respectively. The effect of the isolated EGFR peak A on some histopathological aspects in adult female albino rats had been studied. The result showed that the treatment with three different concentrations of peak A causes alteration in the normal architecture of the liver and kidney characterized by infiltration of lymphocytes, coagulative necrosis, hemorrhage, increase in collagen fibers deposition, hyperplasia, pre-neoplastic lesion with bizarre nucleus, and adenoma. These irreversible pathological changes which occurred in these organs may be converted to malignance if take longer time (more than one month). According to the findings of this study, high levels of HER2 cause irreversible pathological changes in the liver and kidney, which can progress to cancer over time.

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Introduction

Human epidermal growth factor receptor 2 (HER2) is a transmembrane glycoprotein receptor that has tyrosine kinase activity (1). It is a member of the human epidermal growth factor (EGF) receptor family, which consists EGFR (HER1), HER2 (also known as ErbB2), HER3, and HER4 (2). It is encoded by the proto-oncogene HER2, which is found on the long arm of chromosome 17q 21 (3). The abbreviation ErbB comes from the name of a viral oncogene

to which these receptors are symmetric: erythroblastic leukemia viral oncogene B (4). EGF receptor family is structurally featured by an N-terminal extracellular ligand binding portion (ECD), a single alpha-helix transmembrane segment (TM), and an intracellular protein tyrosine kinase (5). Because HER2 is an orphan receptor (no specific ligand for HER2 has been identified) (6), it relies on heterodimerization with other HER receptors or homodimerization with itself when expressed at very high levels on the cell surface (7), which results in activation of

the HER2 signaling pathways (8,9). HER2 dimerization leads to autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiates a variety of downstream signaling pathways specifically phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (3,10), which than regulated cellular function included cell migration, differentiation, proliferation, apoptosis, cell cycling, angiogenesis and tumorigenesis (11-13). In normal tissues HER2 gene expressed at low level (14), and the amplification of HER2 gene result in overexpression of HER2 receptor which correlated with certain types of cancer (15,16). Moreover, it has been found that among all ErbB receptor dimers, the heterodimers containing HER2 receptors have the highest mitogenic potential (17).

The aim of this articles is to study the histopathological role of isolated HER2 in some organs of adult female albino rats.

Materials and methods

Ethical approve

This article is approved by University of Mosul, Collage of Science issued in 2020/9/17 with approval number 25044.

Samples

Fresh plasma 30 ml was obtained from a 29-year-old healthy male with the help of Mosul's blood bank (the plasma was taken ready and frozen).

Precipitation and separation of protein using ammonium sulfate

Proteinous material has been precipitated with ammonium sulfate at 4°C (18). Ammonium sulfate has been gradually added to plasma at the saturation 0-75% with slow stirring at 4°C for 1 hour. The mixture was kept in the fridge at 4°C for 24 hours. The precipitated protein was isolated by centrifuging it for 30 minutes at 12000xg, then dissolved it in the smallest amount of distilled water. The proteinous solution was then sealed in a tight test tube for the next step.

Dialysis

Proteinous solution put in semi permeability tube (cellophane tube) which tied from one end of it, and immersion in 0.1M ammonium bicarbonate solution at 4°C for 24h, ammonium bicarbonate solution was changed every 6 h (18).

Gel filtration chromatography

This technique utilizes a 100*1.6 cm column filled with gel (Sephadex G-100). The previously prepared protein solution was added to this column, and the fractions were retrieved at a flow rate of 58 ml/h. Protein and HER2 concentrations were determined at each step of the isolation process.

Lyophilization

Peaks A had been acquired from the column and dried using freeze-drying at Tikrit University's department of biology.

HER2 assay

The level of HER2 was determined using a competitive-enzyme linked immunosorbent assay (ELISA) technique with a Bioassay Technology Laboratory kit (China). This test was done out in an immunity laboratory at Al-Salam hospital in Mosul.

Determination of protein concentration

The modified Lowry method was used to determine protein concentration using standard bovine serum albumin (19), and using bovine serum albumin as a standard (it is extinction coefficient equal to 0.67).

High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography technology was used to ascertain the purity of the HER2 peak A obtained from lyophilization by applied it on C18 RP-HPLC (20).

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS -PAGE)

We used Sodium dodecyl sulphate poly acrylamide gel electrophoresis technique (SDS-PAGE) for separating compound depending on their molecular weight (18). This technique was used to ascertain the purity of the HER2 peak A obtained from lyophilization, as well as to estimate the molecular weight for it. It is done by using Vertical Slab Electrophoresis Apparatus.

Experimental animals

Twenty adult female albino rats weighing 200-240 g were obtained from the house of laboratory animal at the University of Mosul's veterinary medicine collage. The animals were housed in plastic cages and under laboratory conditions. The rats had unlimited access to both water and food (21).

Experiment groups

Four groups of rats were formed, each group included 5 rats. All treatment by intraperitoneal injection. first group (T1) control group inject with normal saline, T2, T3, T4 inject with purified HER2 protein peak A by dissolving it in normal saline at concentration 5, 2.5, 1.0 mg/Kg respectively. Following one month of treatment with peak A (HER2) which isolated and purified from plasma by gel filtration (Sephadex G-100), Diethyl ether was used to euthanize all the animals. The liver and kidney were immediately removed and fixed in 10% neutral buffer formalin for 72 hours before beginning the histological slide preparation process. Following fixation, the specimens were

dehydrated in a series of increasing alcohol concentrations and embedded in paraffin wax (22). A 5-micron-thick section was stained with hematoxylin and eosin and examined under a light microscope (23).

Results

HER2 isolation and purification from healthy human plasma

The proteinous precipitate resulted from plasma by ammonium sulfate has a specific activity of HER2 is 0.19 compared to plasma 0.12, whereas the filtrate had no specific activity of HER2 and thus was ignored. After the dialysis the specific activity of HER2 is (0.23).

Gel filtration chromatography

Gel filtration chromatography was performed to separate the proteinous precipitate solution gained by ammonium sulphate precipitation from human plasma. As shown in figure 1 two peaks were obtained A and B the elution volume of them was 93, 161.8 ml respectively. peak A has a high specific activity of HER2. Table1 illustrate purification steps of HER2. HER2 specific activity rises from 0.12 in plasma to 5.42 in peak A, while the protein concentration was lowering from 1974.0 mg in plasma to 31.2mg in peak A, and the times number of purification increased to 45.19 for peak A (Table 1). The result of all purification steps for HER2 were listed in (Table 1).

HPLC

To determine the purity of HER2 peak A, HER2 standard solution was introduced into the HPLC system to know its retention time under the following conditions; Flow rate: 0.5

ml/min, Temp. at ambient, Wave length: 205, Mobile phase (60:40 v/v) acetonitrile in 0.1% trifluoroacetic acid. The results in figure 2 and table 2 indicated that there were two peaks for HER2 standard, the first and the second peaks were appeared at 0.970 min and 1.127 min respectively.

While, the results in figure 3 and table 2 illustrate there is three peaks were appeared after introducing a sample of HER2 peak A into HPLC system under the same condition of standard. The first, second and the third peak were appeared at 0.983, 1.103 and 1.217 min respectively. After comparing the chromatogram of standard with that of the sample, it was found that there was a good identity between the retention time of the standard with the sample.

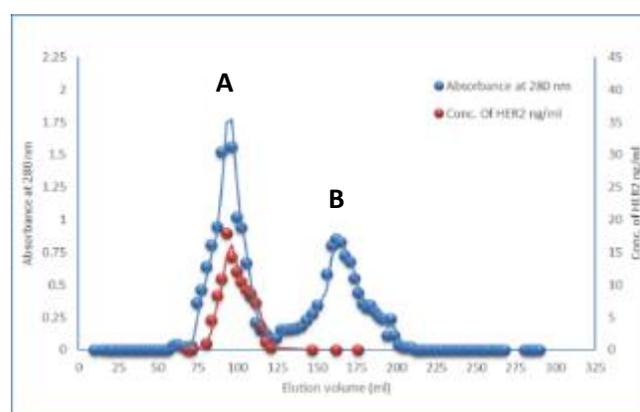


Figure 1: Elution of protein precipitate solution resulted from plasma by ammonium sulfate on Sephadex G-100. Column dimension is (100*1.5cm) at the flow rate is (36 ml/h).

Table 1: Result of partial purification of HER2 from the plasma

Purification Steps	Total Volume (ml)	Total protein (mg)	HER2 (ng)	Specific activity	Recovery %	No purification
Plasma	30	1974.0	237	0.12	100	1
Proteinous precipitate solution	25	1032.5	202	0.19	85.2	1.58
Dialysis	23	770.5	179.4	0.23	75.6	1.91
Gel filtration	6	31.2	169.2	5.42	71.4	45.19

To check the purity of isolated HER2 peak A from plasma, SDS-PAGE and HPLC were used as shown below.

Table 2: obtained data from HPLC of HER2 standard solution and isolated HER2(sample)

Name of the solution	Peak	Ret. Time (min)	Height	Area
Standard	1	0.970	719	3648
	2	1.127	731	9419
Sample	1	0.983	736	5954
	2	1.103	1258	10319
	3	1.217	856	7156

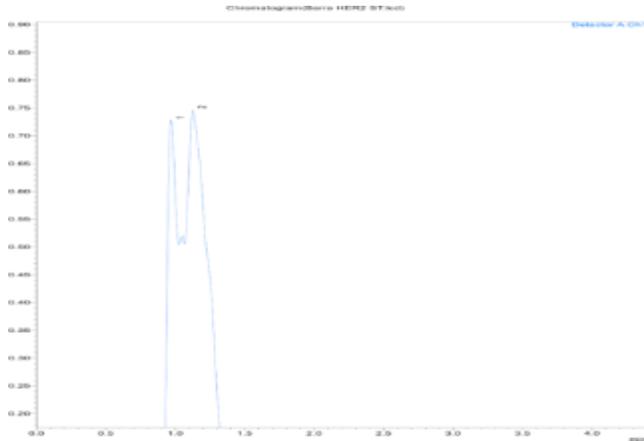


Figure 2: chromatogram of HER2 standard solution

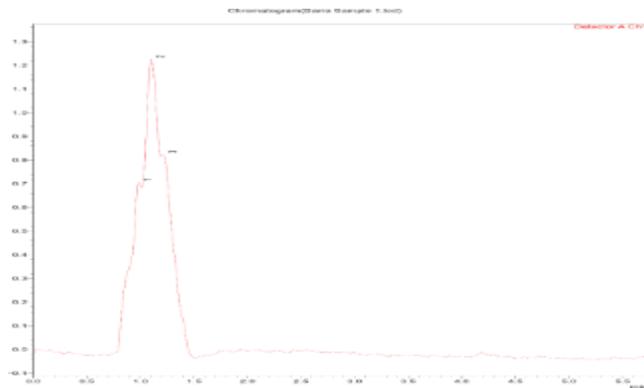


Figure 3: chromatogram of sample solution peak A from gel filtration (Sephadex G-100)

SDS-PAGE electrophoresis

Figure 4 explain the SDS-PAGE electrophoresis of HER2 peak A and the marker substances.

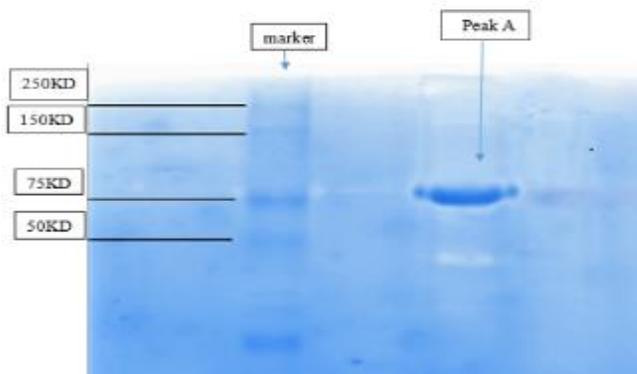


Figure 4: SDS-PAGE electrophoresis of partially purified HER2 peak A from plasma, HER2 standard solution and marker substances.

Determination of the molecular weight of HER2 by gel filtration chromatography using Sephadex G-100

The approximate molecular weight of peak A as a source of HER2 was determined from the elution volume on a Sephadex G-100 column. The calibration curve obtained by using known molecular weight protein is shown in figure 5. The molecular weight of peak A is approximately equal to 75318 ±100 Dalton as shown in figure 5.

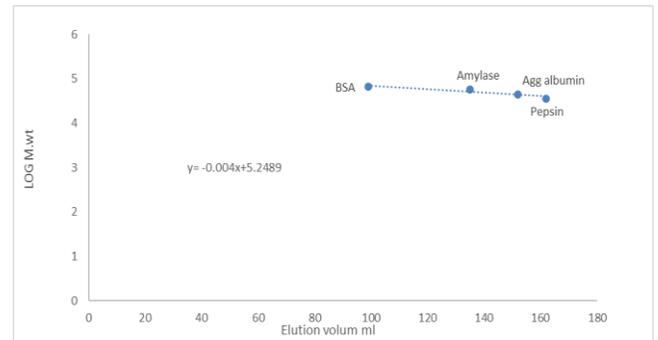


Figure 5: Standard curve for molecular weight determination by gel filtration (Sephadex G-100)

Determination of molecular weight by SDS-PAGE electrophoresis

The approximate molecular weight of peak A as a source of HER2 can be determined from the relationship between the molecular weight of markers and the traveled distance of them on SDS-PAGE than compared it with that for peak A. The calibration curve obtained by using markers is shown in figure 6. The molecular weight of peak A is approximately equal to 73029 ±100 Dalton as shown in figure 6.

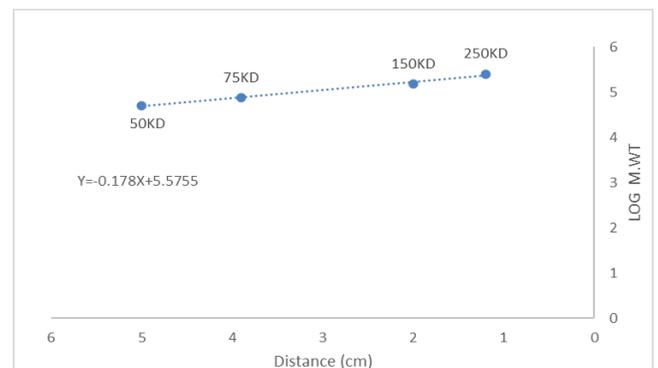


Figure 6: Standard curve for molecular weight determination by SDS-PAGE.

Histopathological study

The results of the histological examination showed moderate to severe pathological changes in the liver of female albino rats after treatment with purified HER2 peak

A at concentration 1.0, 2.5, 5.0 mg/kg respectively for one month compared with control (Figure 7), this changes characterized by hyperplasia of bile caniculi, necrotic changes in hepatocytes and infiltration of macrophages around portal area (Figure 8) and hyperplasia of bile caniculi, necrotic changes in hepatocytes ,increase in collagen fibers deposition, and congestion of blood vessels (Figure 9) at 1.0 mg/kg concentration of HER2peak A. The histological changes of the liver at 2.5mg/kg characterized by hyperplasia of bile caniculi, necrotic hepatocytes, hyperplasia of Kupffer cells (Figure 10) and fibrosis around portal area, necrotic hepatocytes, hyperplasia of fibrocytes (Figure 11).

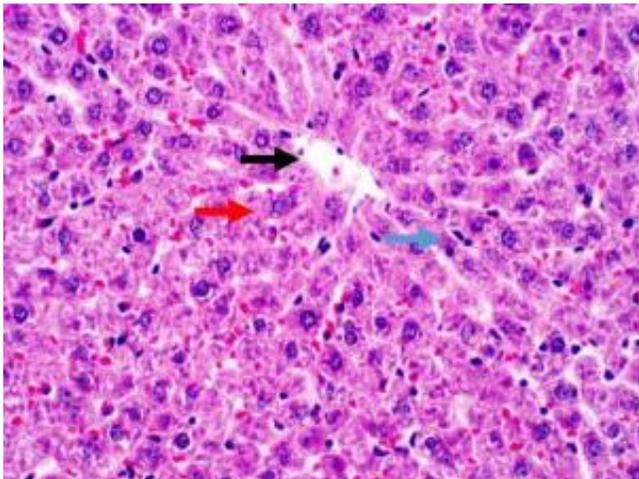


Figure 7: Showed normal architecture of central vein (arrow), Kupffer cells (arrow), hepatic cords (arrow). H&E. 400x.

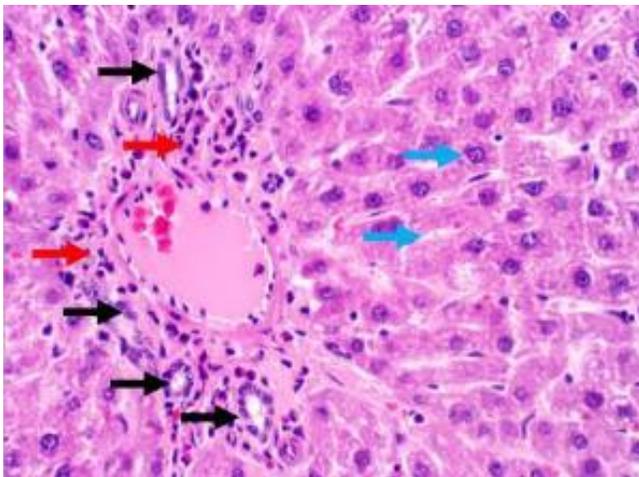


Figure 8: Showed hyperplasia of bile caniculi (arrow), necrotic changes in hepatocytes (arrow), infiltration of macrophages around portal area (arrow). H&E. 400x.

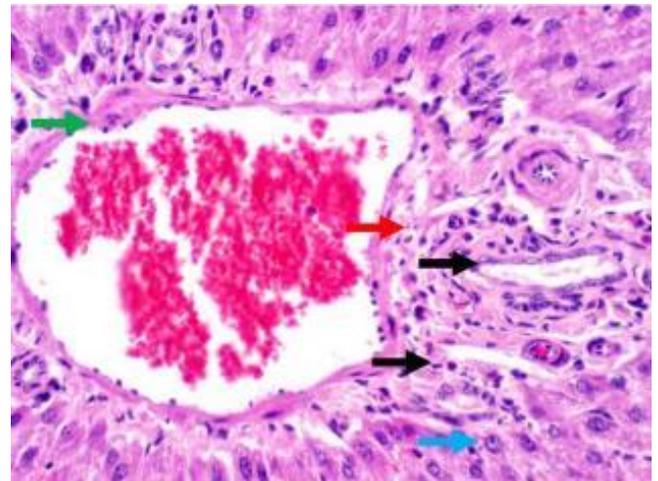


Figure 9: Showed hyperplasia of bile caniculi (arrow), necrotic changes in hepatocytes (arrow), increase in collagen fibers deposition (arrow), and congestion of blood vessels (arrow). H&E. 400x.

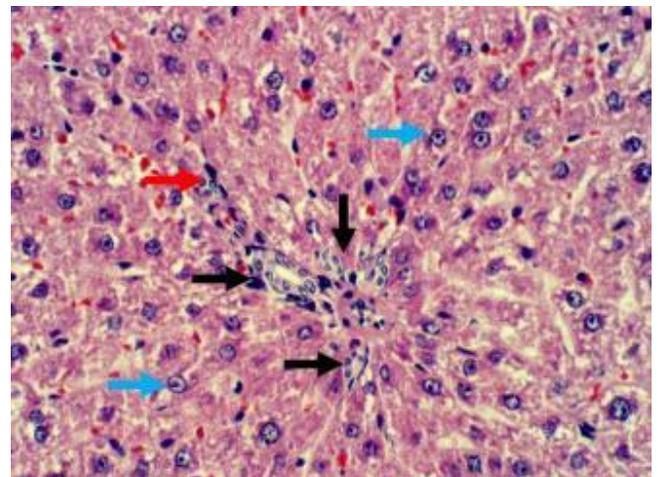


Figure 10: Showed hyperplasia of bile caniculi (arrow), necrotic hepatocytes (arrow), hyperplasia of Kupffer cells (arrow). H&E. 400x.

The histological changes of the liver at 5.0mg/kg characterized by the presence of hepatocellular adenoma scattered between normal tissue as a glial distribution with polymorphic nucleus, other hepatocytes showed some pre-neoplastic lesions with bizarre nucleus, with hemorrhages (Figure 12) and presence of hepatocellular adenoma as nodule, with deposition eosinophilic material in this lesion, infiltration of lymphocytes, with hemorrhages (Figure 13). Figure 14 showed the normal glomerular tuft and normal renal tubules. The histological changes of the kidney after one month of treatment with purified HER2 peak A at

concentration 1.0mg/Kg can be characterized by normal glomerular tuft, with coagulative necrosis in the epithelia cells that lining renal tubules, infiltration of inflammatory cells, with interstitial hemorrhages (Figures 15 and 16), compared with control group. While at 2.5 mg/Kg the histopathological changes of the kidney characterized by necrotic epithelial cell, infiltration of lymphocytes, interstitial hemorrhages (Figure 17) and hyper cellularity of glomerular tuft, necrotic renal tubules, edema (Figure 18), compared with control group.

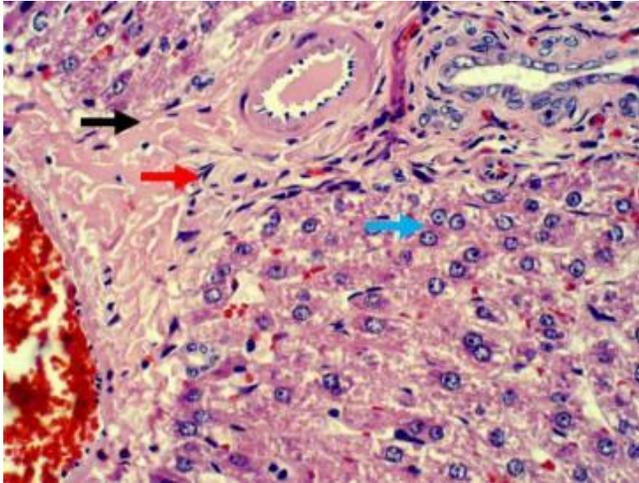


Figure 11: Showed fibrosis around portal area (black arrow), necrotic hepatocytes (blue arrow), hyperplasia of fibrocytes (red arrow). H&E. 400x.

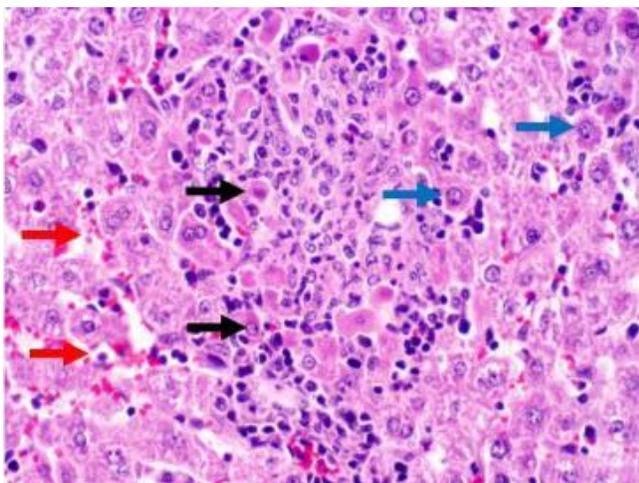


Figure 12: Showed presence of hepatocellular adenoma scattered between normal tissue as a glial distribution with polymorphic nucleus (black arrow), other hepatocytes showed some pre-neoplastic lesions with bizarre nucleus (blue arrow), with hemorrhages (red arrow). H&E. 400x.

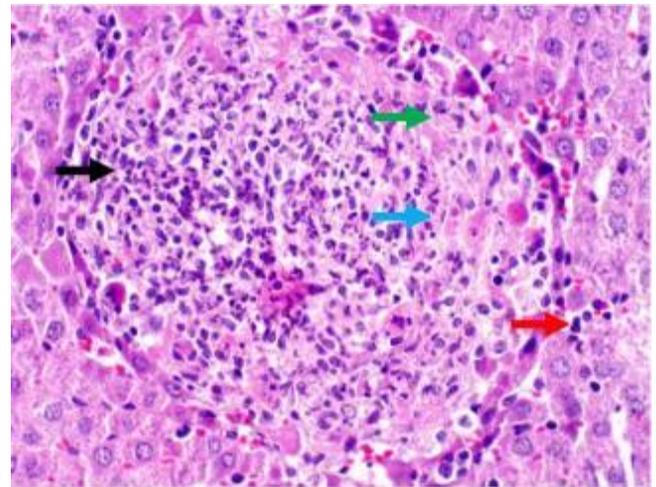


Figure 13: Showed presence of hepatocellular adenoma as nodule (black arrow), with deposition of eosinophilic material in this lesion (green arrow), infiltration of lymphocytes (red arrow), with hemorrhages (blue arrow). H&E. 400x.

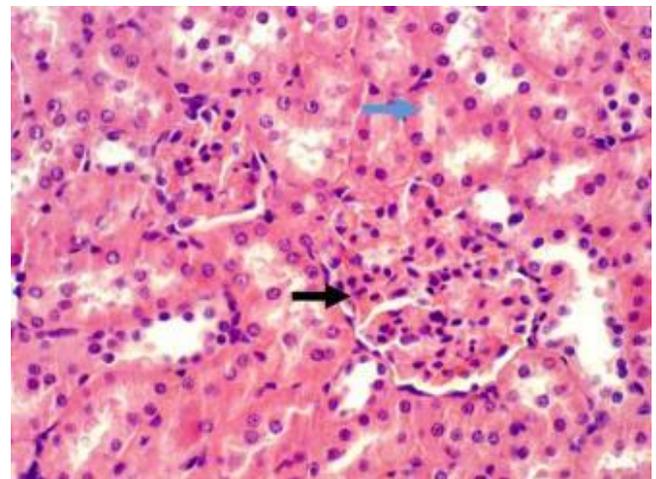


Figure 14: Showed normal glomerular tuft (black arrow), and normal renal tubules (blue arrow). H&E. 400x.

Finally, at 5 mg /kg the histopathological changes of the kidney characterized by increase in Bowman's space, coagulative necrotic changes in renal tubules, infiltration of inflammatory cells, with edema (Figure 19) and vacuolar degeneration in epithelia cells lining renal tubules, and deposition of eosinophilic material in this tubules, infiltration of inflammatory cells, with hemorrhages, (Figure 20), compared with control group.

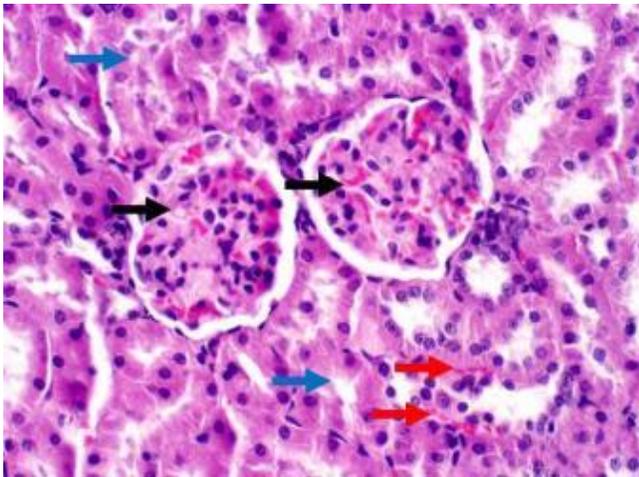


Figure 15: Showed normal glomerular tuft (arrow), with coagulative necrosis in the epithelia cells that lining renal tubules (arrow), with hemorrhages (arrow). H&E. 400x.

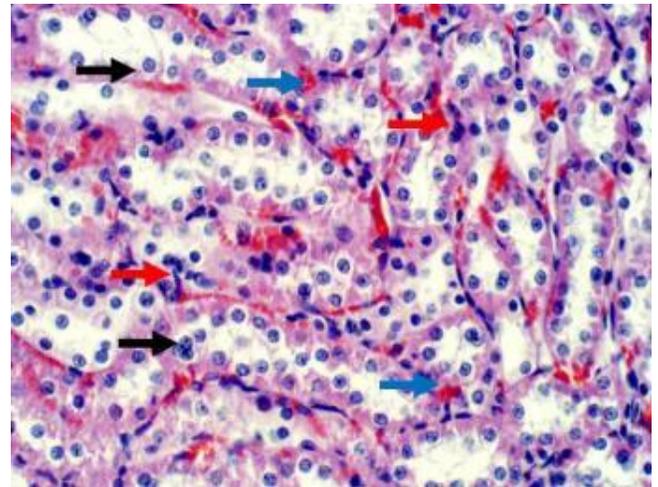


Figure 17: Showed necrotic epithelial cell (arrow), infiltration of lymphocytes (arrow), interstitial hemorrhages (arrow). H&E. 400x.

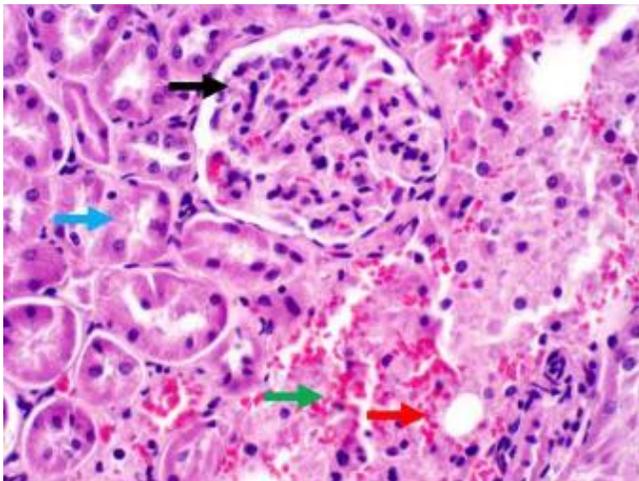


Figure 16: Showed normal glomerular tuft (arrow), with coagulative necrosis in the epithelia cells that lining renal tubules (arrow), infiltration of inflammatory cells (arrow), with interstitial hemorrhages (arrow). H&E. 400x.

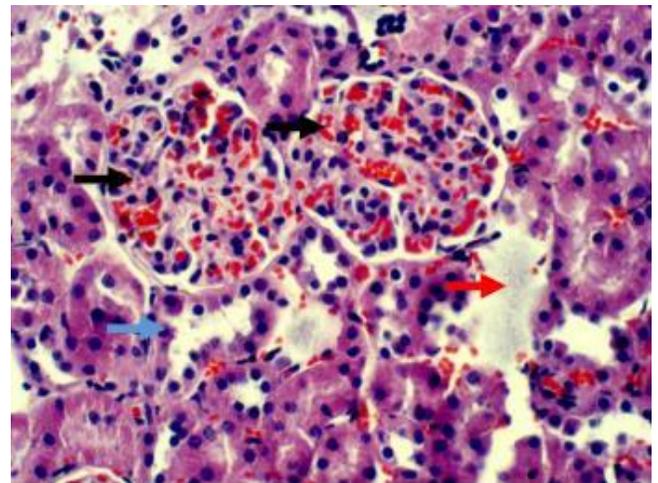


Figure 18: Showed hyper cellularity of glomerular tuft (arrow), necrotic renal tubules (arrow), edema (arrow). H&E. 400x.

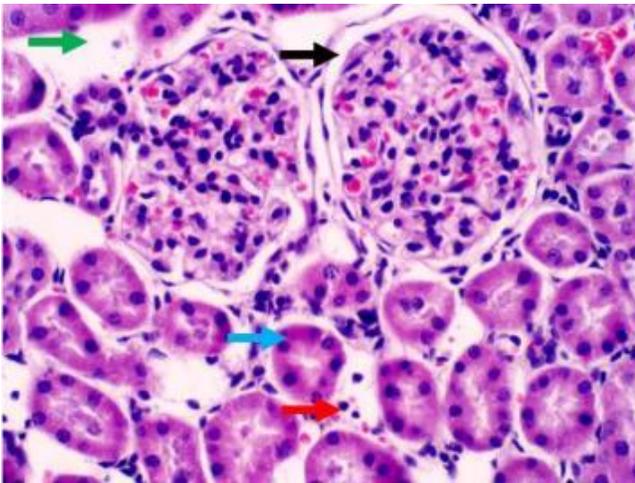


Figure 19: Showed increase in Bowman's space (arrow), coagulative necrotic changes in renal tubules (arrow), infiltration of inflammatory cells (arrow), with edema (arrow). H&E. 400x.

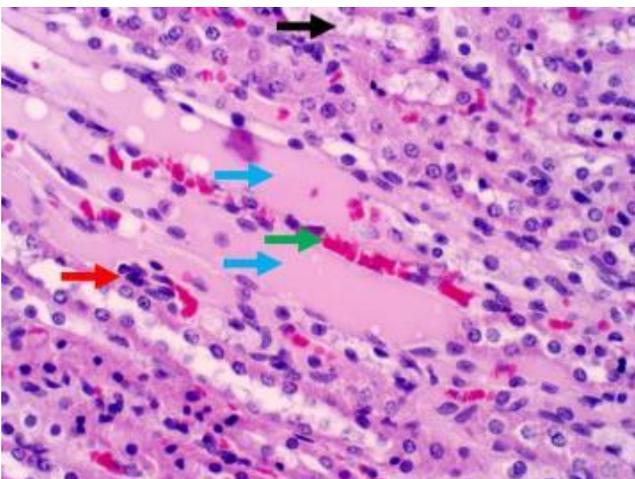


Figure 20: Showed vacuolar degeneration in epithelia cells lining renal tubules (arrow), and deposition of eosinophilic material in this tubules (arrow), infiltration of inflammatory cells (arrow), with hemorrhages (arrow). H&E. 400x.

Discussion

HER2 is a transmembrane glycoprotein belonging to the receptor tyrosine kinase family (24). In this study we attempted to isolate and partially purify HER2 from the healthy human plasma and also determined the approximate molecular weight of peak A which contained high concentration of HER2. The result was agreed with a previous study of Spanov *et al.* (25) and Vega *et al.* (26) which calculate the molecular weight based on the amino

acid sequence of HER2 (70200 Dalton), and it is higher than those approved by other studies Kanthala *et al.* (27) and Hart *et al.* (28).

In addition to transmembrane signaling, HER2 may also exist in the nucleus and mediate diverse nuclear signaling effects including DNA repair and cell cycle arrest (29). HER2 overexpression was found in approximately 20-30% of breast cancer patients (30), and it is the most aggressive of all the breast cancer types, is unresponsive to treatment, highly angiogenic, proliferative and has the lowest survival rate (31,32), in addition to high ability to develop liver metastatic than the other subtypes (33). HER2 overexpression is linked to the proliferation and progression of certain aggressive cells, which is caused by signal transduction mediated by the activation of the PI3K/AKT and Ras/Raf/MEK/MAPK pathways, resulting in negative biological and clinical outcomes (34). Atypical HER2 activation was able to stimulate the PI3K/AKT and MAPK/ERK pathways in several types of cancer, like lung, prostate, and breast cancer (35,36). Both of these signaling pathways are involved in cell survival, cancer cell apoptosis inhibition, and metastasis (37,38). HER2 overexpression in breast cancer cells increases CXCR4 expression (39). The ability of HER2 to increase CXCR4 translation via activation of a phosphatidylinositol 3-kinase/Akt/mTOR signaling pathway appears to be a major mechanism for the HER2-promoted increase in CXCR4 expression (40). The high level of HER2 increases lipoperoxidation and that causes alteration of the physical properties of the cell membrane which can lead to devastation of the cell membrane permeability and release of a large number of phospholipids and the event of hypoxia and lymphocyte infiltration in response to tissue injury (41). Another study observed significantly more tumor-infiltrating lymphocytes (TILs) in HER2-positive Ductal Carcinoma in Situ (DCIS) (42). HER2 overexpression is an important inducer of clonal proliferation in cancer cells. Increased growth caused necrosis because clonal expansion exceeded the available oxygen/blood supply. Furthermore, necrotic debris may elicit inflammatory responses (43,44). The degeneration of the liver tissue may be resulted from increased oxidative stress due to HER2 overexpression, this leads to induce Kupffer cells to over production of oxygen radical, cytokines and TNF-alpha, which leads to development of liver injury (45). HER2 signaling pathways are implicated in renal physiology through nephrogenesis, tissue repair, and electrolyte balance, and the high level of blood HER2 are linked with an increased risk of incident chronic kidney disease (46).

Conclusion

The results from this study concluded that the high levels of HER2 causes irreversible pathological alteration in the

liver and kidney, that may be converted to malignant at the passage of time.

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Conflict of interest

The authors declare that no conflict of interest exists.

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دراسة تأثير عامل النمو البشرية البشري المعزول من البلازما على النواحي المرضية النسيجية في بعض أعضاء إناث الجرذان البيضاء البالغة

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الخلاصة

تضمنت هذه الدراسة محاولة لعزل وتنقية مستقبل عامل نمو البشرية البشري من البلازما لشخص سليم باستخدام تقنيات كيميائية حيوية مختلفة. حيث تم عزل قمتين بروتينيتين بواسطة كروماتوغرافيا الترشيح الهلامي من الراسب البروتيني الناتج من استخدام كبريتات الأمونيوم. أظهرت القمة البروتينية الأولى (القمة أ) مستوى عالٍ من مستقبل عامل نمو البشرية البشري. علاوة على ذلك، تم التعرف على نقاء مستقبل عامل نمو البشرية البشري المعزول في القمة أ بواسطة كروماتوغرافيا السائل عالية الأداء وتقنية الهجرة الكهربائية للهلام. أظهرت النتائج التي تم الحصول عليها من كروماتوغرافيا السائل عالي الأداء وجود تطابق جيد في زمن الاستعادة بين مستقبل عامل نمو البشرية البشري القياسي والمعزول من القمة أ. كما تم احتساب الوزن الجزيئي التقريبي لمستقبل عامل نمو البشرية البشري المنقى جزئياً من القمة أ وكان 100 ± 75318 و 100 ± 73029 دالتون باستخدام كروماتوغرافيا الترشيح الهلامي وتقنية الهجرة الكهربائية للهلام على التوالي. تمت دراسة تأثير مستقبل عامل نمو البشرية البشري المعزول من القمة أ على بعض النواحي المرضية النسيجية في إناث الجرذان البيضاء. أظهرت النتائج أن الحقن بثلاث تراكيز مختلفة من القمة أ يسبب تغيراً في البنية الطبيعية للكبد والكلية يتميز بارتشاح الخلايا الليمفاوية والنخر التجلطي والنزيف وزيادة ترسب ألياف الكولاجين وتضخم وافة ما قبل الورم مع نواة غريبة وأورام غدية. ويمكن أن تتحول هذه التغيرات المرضية الغير عكسية إلى ورم خبيث إذا استغرقت وقتاً أطول (أكثر من شهر واحد). وفقاً لنتائج هذه الدراسة، فإن المستويات العالية من HER2 تسبب تغيرات مرضية لا رجعة فيها في الكبد والكلية، والتي يمكن أن تتطور إلى السرطان بمرور الوقت.