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A Study of Lymphocyte Transformation, Phagocytosis Activity, Immunoglobulin's level of Hepatitis B Viruses Infected Patients

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Abstract:

Background: HB is in the list of leading causes of illness and death globally, and in Asia alone, the number of affected people is unnaturally high. Earlier studies have hypothesized that, the virulence factor of HB is modulated by the immune response. The aims of this study were to determine the level of phagocytic cells and the response of T-lymphocyte proliferation in adult patients in Iraq who contracted HBV.

Materials and Methods: These parameters include hepatitis B surface antigen (HBsAg) and other HBV indicators in sixty patients with hepatitis B virus (HBV) serum determined with the use of enzyme-linked immunosorbent assay (EIISA) kits. The basic procedure of obtaining PBMs was by the Ficoll-Hypaque density gradient centrifugation technique. Using flat-bottomed microtiter plates, three sets of cells were cultured: One of them is on a lymphocyte suspension and the other on diluted blood.

Results: The healthy group, than indicated a very significant increase ($P<0.05$) in the liver function enzymes. Compared with members in the control group, all the patients had a significant decrease ($P<0.05$) in both the lymphocyte count and the neutrophil count. The present study revealed that the lymphocyte transformation was significantly low in healthy carriers when compared with the controls ($P<0.05$).

Keywords: Hepatitis B Viruses, Lymphocyte Transformation, Phagocytosis Activity, Patients.

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Introduction

It perform many metabolic and immunologic roles hence is a vital core multi-specialty organ that, if infected, maybe deadly. Liver transplant may be regarded one of the best ways to treat terminal liver diseases, while there are several therapeutic approaches. This is further limited by shortage of available organs, problems in matching the HLA, immunologic side effects and issues to do with the costs. Additionally, this choice does not guarantee its survival in the long term [1]. There are several redundant pathways that regulate the inflammatory movement of the liver and can only occur in chronic infection, autoimmune diseases and cancer [2]. In liver illness, immunological dysregulation could also be used as a therapeutic objective. Autoimmunity is often characterised by a pathological immune response, for example, overactive or prolonged immune reaction that fails to contain an assault on the liver's tissues in several liver diseases. The outcome is a collateral damage type situation in which the body over stimulates the immune system and it then turns on the body's own cells. Fibrosis could be produced and hepatocyte death might occur in a situation where an inflammatory response becomes chronically sustained and ongoing, which is not necessarily symptomatic of an increased response [3, 4]. The chronicity of hepatocyte toxicity may be overwhelming to the body reparative systems.

Picking up from the point made in the previous text, it could also be illustrated thus: Hepatitis of the whole liver [each] of any of these diseases can be complicated by [any of] the types of Hepatitis Such diseases include; Through immunologic stimulus is one of the probable causes of liver diseases They can also be caused by immunologic stimulus where the common stimulus is viruses. It means that a viral component or life activities, for example replication evokes an immunological reaction that could be acute or chronic. This results in different outcomes in various hosts and is in turn dependent upon the invader pathogen [5-8]. Chronic sequelae of this illness consist of liver degradation and, in many parts of the world, HBV is the leading cause of liver failure. Hepatitis B virus (HBV) cases continue to occur anew, although there is a vaccine for this disease; poor immunization and the fact that immunizations are hard to come by and costly in areas where the disease is most prevalent play a part in this. This means that despite of the babies receiving the anti-HBV birth-dose vaccine in the recommended time of up to 5%, viral breakout will still result in new infection. In addition, persons who have the disease can take the shot and that will not prevent their getting ill [9, 10]. It has therefore been argued that immunization must be complemented by appropriate treatment of infected persons for the infection to be completely eliminated. The substantial players in humorally mediated immunity are the B-lymphocytes and T-lymphocytes, respectively [11]. To date, limited research attention has been directed to peripheral blood lymphocyte (PBL) in HBV patients. PBL is followed by the decrease in T-lymphocyte count in both acute and chronic hepatitis and reduction in different described mitogens responses in chronic liver disease. These studies have considered PBL in structural as well as functional context.

MATERIALS and METHODS

Patients Groups:

Serum hepatitis B surface antigen (HBsAg) and other hepatitis B virus (HBV) indicators were discovered in 60 patients with hepatitis B virus (HBV) using enzyme-linked immunosorbent assay (EIISA) kits.

Each group of patients was then separated into:

A-There were eight individuals diagnosed with acute hepatitis B (AHB) whose HBsAg levels were less than six months.

B-Liver function tests demonstrated normalization in seven patients with chronic hepatitis B healthy carriers (CHB-HC), although HBsAg levels remained elevated for over six months.

C-Liver function tests (T.S.B, SGOT, SGPT, and S.ALP) indicated increasing levels in fifteen patients with chronic hepatitis B, whose HBsAg remained for more than six months.

Based on the results of the clinical examination and the laboratory tests that were conducted earlier, these patients were clinically diagnosed.

Human Study Groups

In this study, the groups divided into:

A- Forty patients with HBV infections: 1- Twenty patients with **AHB**. 2- Twenty patients with **CHB**. Blood samples had been collected from (20) males and (20) females.

B- Twenty healthy adult volunteers (**NC**) Blood samples had been collected from twenty normal adult volunteer, used as normal control (NC).

Patients with AHB had Anti-HBe but Anti-HBc-IgM had not been observed.

Patients with CHB had HBeAg, Anti-HBeAg and Anti-HBc-IgM.

Blood Sample Collecting

- Each participant had 10 milliliters of their venous blood collected in an aseptic manner into a sterile test tube with a screw lid. This tube already contained 200 units of preservative-free heparin (20 units/ml). Then split into the following sections:
- To separate serum for testing immunoglobulins and complement component levels, 2.5 ml of blood was used.
- Using the stain approach, 0.5 ml of blood was morphologically assessed for the lymphocyte transformation and phagocytosis assays.
- Use either "separated lymphocyte" or "whole blood" in a 7-milliliter blood sample for the lymphocyte transformation assay, which measures the presence of 3H* -thymidine.

Viability and Lymphocyte Separation Assay

The fundamental method for isolating peripheral blood mononuclear cells (PBMs) was the Ficoll-Hypaque density gradient technique.

Before being put into a centrifuge tube, the heparinized blood was mixed with the same volume of Hanks Balance Salt Solution (HBSS) (1:1). Using a sterile Pasteur pipette, the mixture was layered with lymphoprep in a 2:1 ratio. After 30 minutes at ambient temperature, the tube was centrifuged at 2000 rpm. A sterile test tube was rinsed twice with HBSS after collecting the buffy interface layer, which consists mostly of lymphocytes and a small number of monocytes. Using a neubar hemocytometer, the final pellete was resuspended in 1 ml of full RPMI-1640 media and adjusted to 106 cell/ml.

Follow these steps to find out if the trypano blue dye exclusion test is viable:

After mixing 5 microliters of cell suspension with 200 microliters of 0.2% trypan blue dye, waiting 1-3 minutes, and counting cells using a hemocytometer until at least 100 cells were obtained, the reaction was stopped. Trypano blue dye did not stain the live cells.

Viability = Viable cells counted / Total cells count (viable + dead cells) × 100

The viability is always judged to be greater than 95%.

Separated Lymphocytes for Lymphocyte Transformation Assay

Following the protocols outlined in [12, 13], the T-lymphocyte proliferative response was executed. A different concentration of PHA (250□g/ml) was used to initially identify the optimal concentration for lymphocyte proliferation, and serial dilution was prepared in the following way: range of concentrations in milligrams per milliliter The RPMI-1640

Transformation of Lymphocytes with the Use of Whole Blood and Culture Method

In a lymphocyte transformation assay, whole blood was mixed with RPMI-1640 medium (which does not contain FCS) at a ratio of 1:15 (v:v).

The following was the procedure for the culture technique:

Using flat-bottomed microtiter plates, three sets of cells were cultured: one with a lymphocyte suspension and another with diluted blood. In this way:

"Separated lymphocyte"

Blank = 200µl of RPMI – 1640 medium.

Control = 100µl of RPMI-1640 medium +100µl of 106 cell/ml in suspension medium.

Test = 100µl of cell/ml in suspension medium + 100µl PHA (Pharmicia, Sweden).

"Whole blood"

Control = 100µl RPMI-1640 medium +100µl of diluted blood

Test = 100µl diluted blood + 100µl of PHA, (Pharmicia, Sweden)

- The plate covered and sealed with adhesive tape, then the culture incubated at 37C° for 72 hours with humidified condition containing 5% CO2.
- At the end of the incubation period, 50µl of (20µci/ml) of 3H*- thymidine added and the plate covered and re-incubated again in the same condition for 20 hours before harvesting.

Procedure for Harvesting

1. Before being dried by air, the fiberglass filter paper was cleaned in water for two seconds.
2. The pulp from the initial twelve wells of the plate was transferred to fiberglass filter paper by the section head of the apparatus.
3. Three steps were taken with the harvested material on fiberglass filter paper: first, a 20-second water wash to remove any non-incorporated surplus 3H*-thymidine; second, a 5-second wash with 5% TCA; third, a 10-second wash with methanol; and finally, a 10-second air drying.
4. After being transported from the second plate's 12 wells into the fiberglass filter paper by the section head, the culture was transferred to the second position on the filter paper.
5. After harvesting was complete, the culture obtained from all eight raw materials (96 wells) on the plate was transferred onto fiberglass filter paper in the form of discs. These were then left to dry on silver foil at room temperature.
6. Filberglass filter discs were filled with blood that had been bleached with 150l of 3% w/v hydrogen peroxide (H2O2) and then dissolved in 0.4 ml of scintillation fluid by heating it at 80C□ for 30 minutes.
7. After transferring the grown cells from the discs to the vials, which contained 10 ml of scintillation fluid, the discs were let to cool at room temperature.
8. After letting the vials sit overnight, they were counted.
9. The 3H*-thymidine in the vials was measured using a □-counter, and the results were recorded as a count per minute (CPM). The average count per minute of the three sets was added together, and the final result was reported as the stimulation index (SI).

S= mCPM of culture with stimulation / mCPM of culture without stimulation

Using the Stain Method for Lymphocyte Transformation Assay

The following procedure had been followed:

- Using two separate tubes for each test, mix 250 □l of heparinized blood with 2.5 ml of full RPMI-1640 medium in sterile silicon coated tubes.
- One tube was treated with 250 □l of mitogen (PHA) from the Saddam center for cancer and molecular genetics research in Iraq (the test tube), while the other tube was left untreated (the control tube).
- The tubes were incubated at 37°C with 5% CO2 in a humidified environment for 72 hours; daily shaking was required.
- Centrifugation at 2000 rpm for 10 minutes was used to separate the cells at the end of the incubation time.
- The sediment cells were treated with 5 milliliters of KCl, a hypotonic solution, and left to incubate at 37 degrees Celsius for half an hour. - ACentrifugation was then performed for 10 minutes at 2000 rpm.
- After adding 5 milliliters of fixation solution to the sediment cells, they were refrigerated at 4 degrees Celsius for 15 minutes.
- A colorless suspension of sediment cells was achieved by washing the sample three to four times with fixing solution and centrifugation.
- After transferring a single drop of sediment cells onto two separate slides using a Pasteur pipette, allowing them to dry at room temperature, staining them with giemsa for 10 minutes, washing them with D.W., and finally, counting 200 cells under a microscope using an oil immersion lens, the process was inspected under a microscope.
- This equation had been used to determine the transformed cell ratio:

Transformed cells % = No. of transformed cells / Total no. of cells counted.

Phagocytosis assay

- combine 250 □l of heparinized blood with 106 /ml of s. aureus bacterial solution in a 1:1 ratio in a sterile test tube.For 30 minutes while being constantly shaken, the mixture was incubated in a water bath set at 37°C.
- To make the smear, just drop a little of the liquid onto the slide.
- Two sets of slides were prepared for every tube.
- The slides were prepared as follows: they were allowed to dry naturally, fixed with 100% methanol, stained for 10 minutes with Giemsa stain, and then washed with D.W.
- In order to determine how many neutrophils and monocytes were consumed by the microbe, the slides were viewed using an oil immersion lens.
- This is the percentage of cells that are phagocytic:

No. of phagocytic cells % = No. of phagocytic cells No. of 100 cells phagocytic & non phagocytic cells.

Statistical Analysis

We utilized analysis of variance (ANOVA) for our statistical analysis, one-way analysis of variance for our group comparisons, and the least significant difference (LSD) for our source identification.

RESULTS and DISCUSSION

Currently, it is estimated that at least 296 million persons worldwide have chronic hepatitis B virus (HBV) infection with approximately 820, 000 deaths in 2019. HBsAg should be tested for diagnosing the HBV infection; and in addition, IgM hepatitis B core antibody (IgM anti-HBc) test is needed to diagnose the acute infection during the window period where both HBsAg and anti-HBs are negative [14–16]. Whereas, HBV DNA examination is useful for evaluating the replication status and treatment strategy, aminotransferases, platelet count, and elastography are the fundamental assets for determining histological activity and stage in liver disease. The best approach to the elimination of chronic HBV infection is universal immunization of newborns that comes with the birth dose vaccine.

Two new fully grown up vaccines, one of which is a boosted antigenicity and the other a staggered release in the pipeline, have in the two most developed markets of the United States and the European Union. Pegylated interferon and analogues are beneficial in the management of cirrhosis and hepatocellular carcinoma from formation [17–19]; however, they cannot eradicate the virus, and HBsAg, clearance is sparse in frequency.

Treatments should be considered if the patient has severe liver disease, has high viral load indicated by high levels of HBV DNA, or they have cirrhosis. There is active research on new antiviral and immunomodal therapy to target a functional cure, which is the loss of HBsAg. To effect hepatocyte lysis in the infected cells, the cellular immune response is comprised of the viruses' component antigens. Although cirrhotics and those with chronic or acute hepatitis do not exhibit HBV antigens on the surface of hepatocytes, healthy carriers during the prodromal phase of acute hepatitis and persons with normal liver function show the presence of antigens. In both acute and chronic infection, studies employing lymphocyte transformation and lymphokines production assays, the cellular immune response to HBV Antigens has been demonstrated [20, 21]. For this reason, these outcomes suggest that the cellular immuno- response to HBV is far more prevalent in acute hepatitis than it is in chronic hepatitis. T-cell mechanisms participate in hepatocyte overdose and entail activated macrophages and natural killer (NK) cells the level of which increases during the early phase of a viral infection. Macrophages are emphasized in their phagocytosis activities. When the resting small lymphocyte is transformed into lymphoblast, a conspicuous change in shape is said to be the lymphocyte transformation.

The biochemical processes, which transpire during blastogenesis in lymphocyte cultures, include mitogen or antigen mediated early change in outer cell membrane, synthesis of protein, RNA and DNA, cell division and morphological transition to more 'blast like' cell [22, & 23]. Lymphocyte stimulation remains an in vitro method that is applied to Immunodeficiency, autoimmune, infectious illness, and cancer patients to examine their cellular immune response. The enzymes pokeweed mitogen (PWM) enhance both B and T-lymphocytes whereas , mitogens such as phytohemagglutinin (PHA), concanavalin A (conA) enhance T-lymphocytes alone. Concentrations of Glutamic Oxaloacetic Transaminase in the Blood: S.GOT levels (Mean ± S.D) in Acute Hepatitis B [AHB], Chronic Hepatitis B [CHB] and Control group were [24.5 ±6.07, 33.02±3.95 and 12.03 ± 2.03] respectively Figure 1. Compared to the healthy control group, the AHB and CHB groups had significantly higher levels. Protein Carboxypeptidase Levels in the Blood: Figure 2 displays the results, which demonstrate that compared to the control group, Acute Hepatitis B [AHB] and Chronic Hepatitis B [CHB] were significantly higher [26.87 ± 3.06, 34.41 ± 5.97 and 9.90 ± 1.60] respectively. Serum Alkaline Phosphatase (S.ALP) Levels were more significant elevated in Acute Hepatitis B [AHB], Chronic Hepatitis B [CHB] than those of Control group and recorded [26.51 ± 3.72, 33.23 ± 5.00 and 12.87 ± 1.09] respectively.

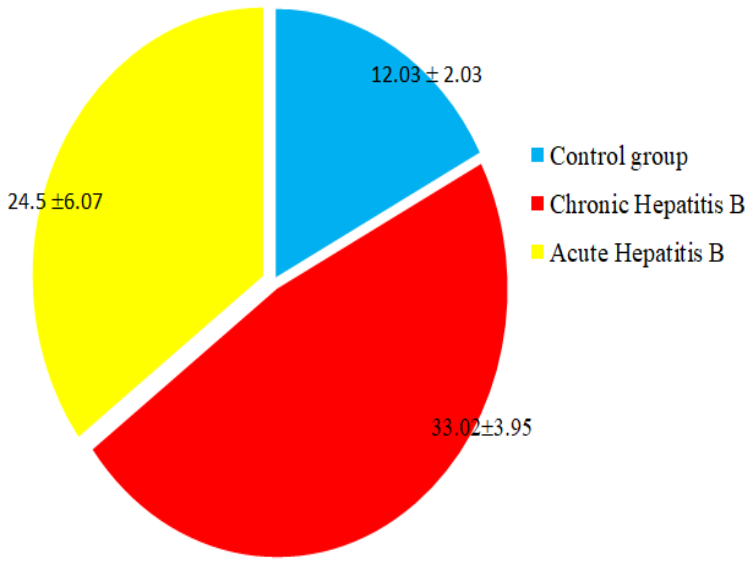

Figure 1. Serum Glutamic Oxaloacetic Transaminase (S.GOT) Levels in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

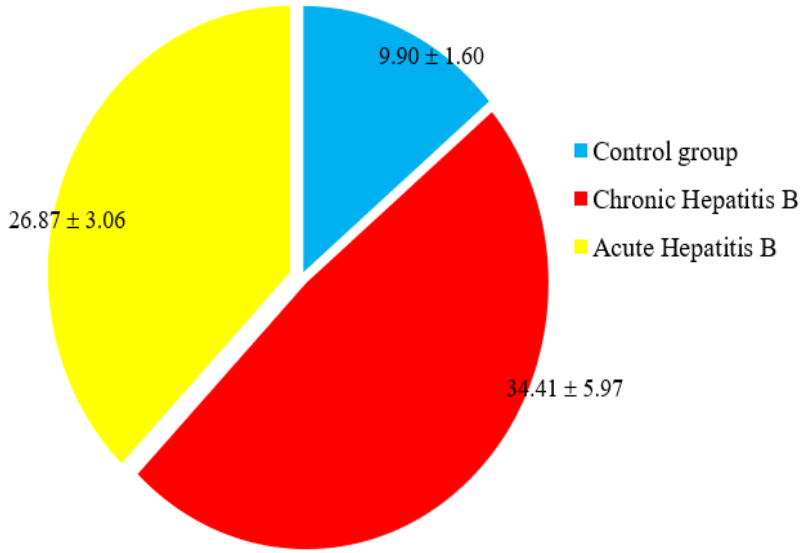

Figure 2. Serum Glutamic Pyruvic Transaminase (S.GPT) Levels in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

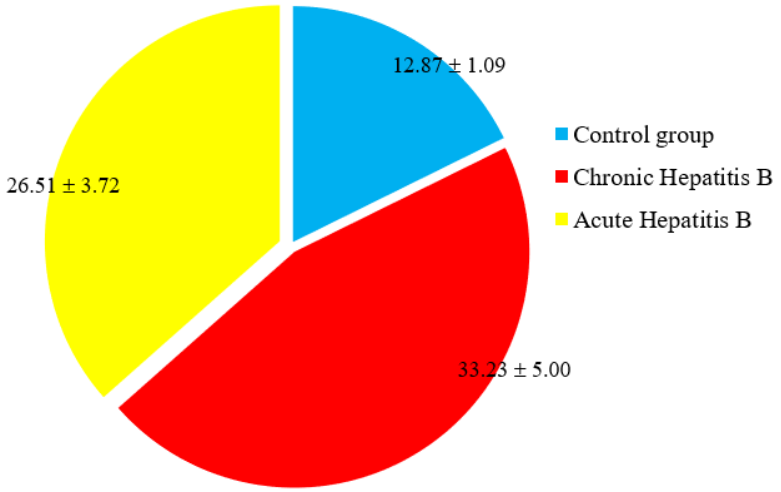

Figure 3. Serum Alkaline Phosphatase (S.ALP) Levels in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

The authors did find a low correlation between the degree of increase of liver enzymes and the severity of liver injuries in viral hepatitis, but it does not mean that increases in the level of liver enzymes are not associated with the presence of liver disease. High T.S.B. level could be an example of improper bile discharges; hepatitis B virus infection is one of the possible causes of the biliary tract blockages which can be connected with paranchymal dysfunction. Since S.GPT is present in the cytosol of hepatocytes while S.GOT is present in the mitochondria as well as in the cytosolic fraction, increased enzymatic values can suggest that the disease process is advancing. The ALP enzyme is found mostly in placenta, intestines, liver, and bones or bone. Stimulation of the manufacture of an enzyme by the hepatocytes as well as the biliary tract epithelium due to the blockages is often suggestive of a dys functionality of the biliary system except when in association with bone disease or pregnancy – ALP enzyme levels are abnormally high.

W.B.Cs findings which are shown in Figure 4 assessed that they (Mean ± S.D) were significantly less $P<0.05$ in AHB (7.30 ± 1.89) and CHB (9.05 ± 1.99) than those of Control group (11.07 ± 2.05). Lymphocytes count findings which

are displayed in Figure 4, showed that the count of lymphocytes were significantly less P<0.05 in AHB (19.35±3.83) and CHB (18.71 ± 3.71) than those of Control group (26.16 ± 4.08). Neutrophils counts illustrated in Figure 6 showed a significant less P<0.05 in AHB (47.76 ± 4.61) and CHB (42.19 ± 3.99) than those of Control group (44.21 ± 4.00)

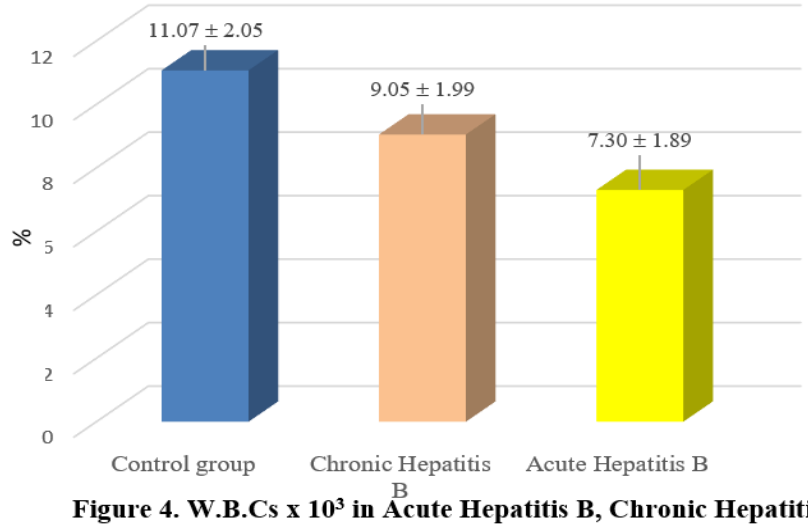

Figure 4. W.B.Cs x 10^3 in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

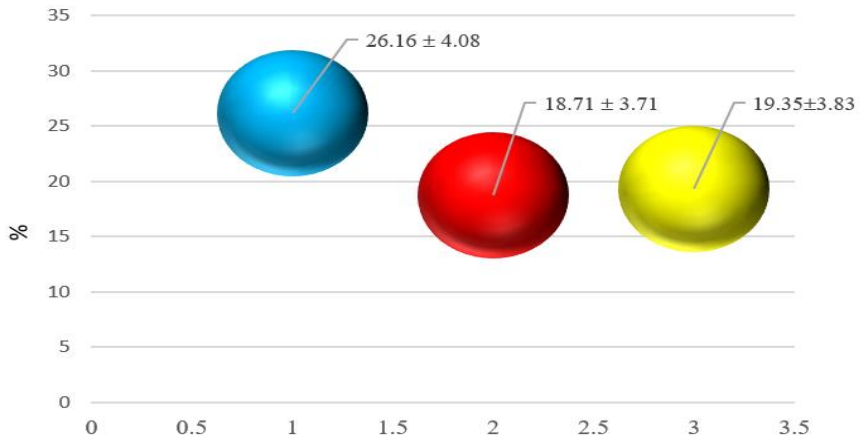

Figure 5. Lymphocyte counts in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

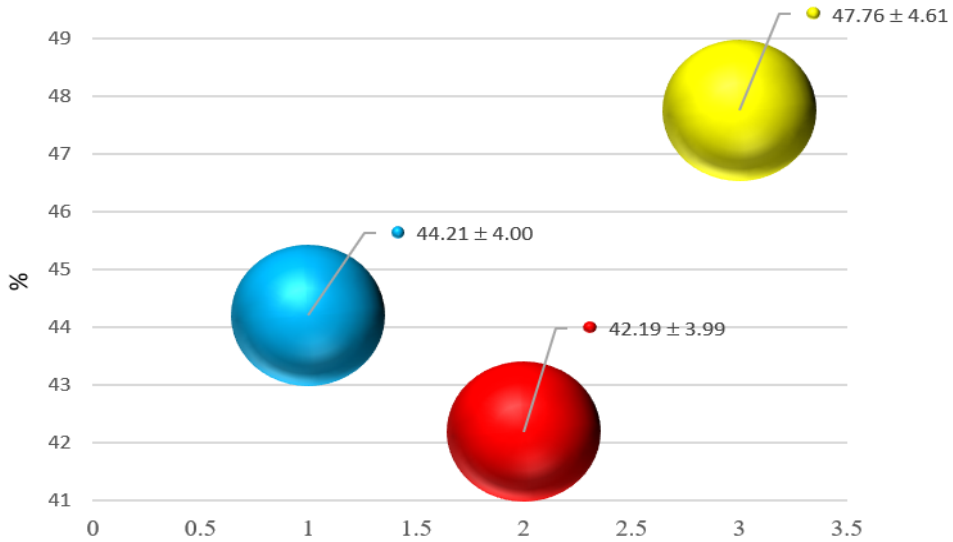

Figure 6. Neutrophils counts in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

Some of the various causes of decline in the white blood cell count include viral infections. From time to time, portal hypertension and hypersplenism are said to precipitate or exacerbate a mild leukopenia, one of such haematological derangement noticed in liver diseases [24]. The detection of viral nucleic acids in PMNs or potential viral synthesis in or neutrophil phagocytic activity results in the lowest neutrophil concentrations. The direct consequences of HBV damage the reduction of the quantity of monocytes within the body. The counts of monocytes in normal controls and CHB patients were nearly equal, as mentioned in the literature [25, 26]. In contrast to healthy carriers, percentages of lymphocytes are found to be low in acute and chronic Hepatitis B, other studies indicated.

Stain Method for Lymphocyte Transformation: The lymphoblast percentage from the total lymphocytes was determined by measuring the morphological change of lymphocytes under the oil immersion lens of the light microscope. The results were expressed as an assay. The transformed cells (Mean ± S.D) were significant less $P<0.05$ in AHB (29.47 ± 5.68) and CHB (27.11 ± 3.99) than those of control group (50.46 ± 9.80). These findings agreed with the previous studies reporting that the capacity of lymphocyte to be transformed as response to Acute, healthy carriers, and those with chronic hepatitis B all have lower PHA levels than the control group. Changes in plasma lipoproteins are a prevalent cause of the poor T-cell response to mitogens as PHA in chronic liver disease, according to some writers. Because there are fewer lymphocytes in the peripheral blood of patients with AHB and CHB, the Tlymphocyte response to PHA is diminished. Immunosuppressive factors, linked to high viral replication levels, were responsible for the decreased lymphocyte PHA capacity in AHB and CHB; these findings demonstrated the function of these factors in the immunological pathogenic processes. Studies have shown that healthy carriers have a normal lymphocyte response to PHA, which means that there is no major liver damage and no overall impairment of the immune system's cellular response, with the exception of the persistence of HBsAge. Using PHA as a mitogenic stimulation did not reveal any functional difference between PBL from healthy people and patients with CHB. Utilizing flow cytometry for a more precise assessment of the expression on the surface of several activation antigens, he conducted the proliferative response with more targeted T-cell activation techniques. This includes IL-2 receptors as well as the proliferation response following T-cell activation in vitro via CD3 and CD2 molecules. Their theory postulated that CHB patients' immunological responses were due to either an insufficient number of activated T-lymphocytes in the body or a selective deficiency in lymphokine synthesis.

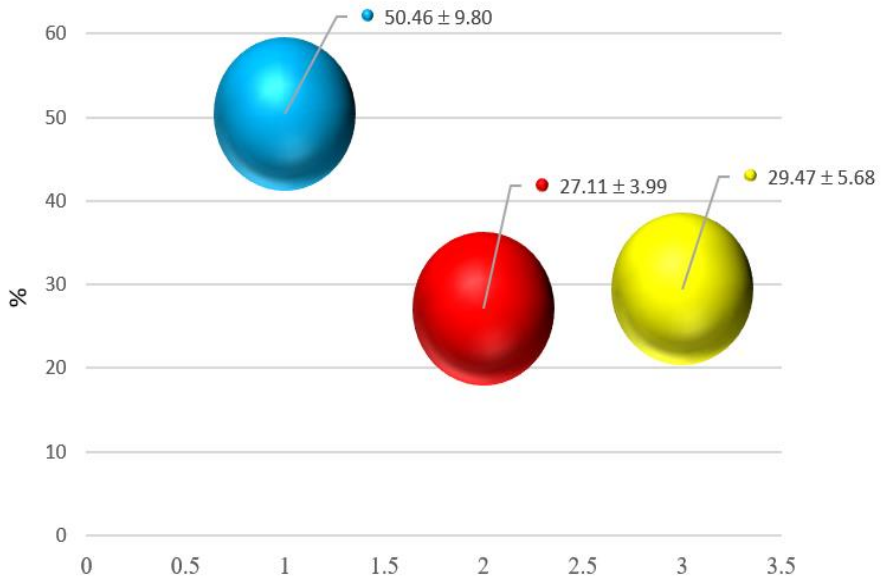

Figure 7. Transformed Lymphocyte cells in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

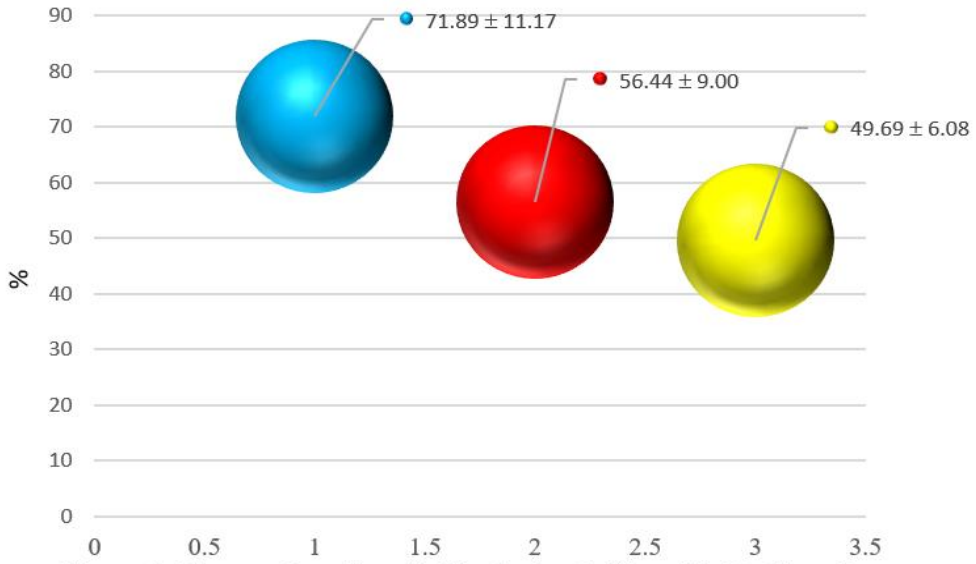

Figure 8. Phagocytic cells activities in Acute Hepatitis B, Chronic Hepatitis B patients and healthy Control groups.

Phagocytic cells activities in patients and control groups: The findings of phagocytic cells activities (Phagocytosis of *Staph. aureus* by phagocytic cell) illustrated in (Figure 8). showed that phagocytic cells activities (Mean ± S.D) were a highly significant decreased P<0.05 in AHB (49.69 ± 6.08) and CHB (56.44 ± 9.00) than those of control group (71.89 ± 11.17). Consistent with earlier research, this study discovered that zymosan stimulation decreased O2-production and that CHB patients exhibited significantly less bactericidal action. It is postulated that the inhibitory effects on neutrophil phagocytosis activity in AHB patients may be due in large part to serum inhibitory substances and circulating immune complexes. Because the virus and complexes containing the virus harm circulating monocytes, the percentages and functions of these cells decline in AHB patients. On the flip side, a reduction in INF-□ activity during the replication phase is responsible for the diminished monocyte phagocytosic activity observed in CHB patients.

CONCLUSION

In the healthy group, there was a significantly significant rise (P<0.05) in the levels of liver function enzymes. In comparison to the control group, all patients exhibited a notable decline (P<0.05) in the counts of lymphocytes and neutrophils. The phagocytic cell activities were significantly reduced (P<0.05) in AHB and CHB compared to the control group, and the transformed cells (Mean , S.D.) were significantly lower in AHB and CHB.

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