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Evaluation of Haemolytic Activity in Smokers by Using Non-Linear Regression

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Abstract

Background and Objective: Smokers are poorly protected against pathogens. Many studies have focused on the concentrations of complement components in comparison to smokers while ignoring the practical sequencing of complement components. There are numerous methods for estimating haemolytic activity (CH50%), all of which need a large number of samples and dilution, in addition to a typically expensive test kit. This novel study attempts to use statistical analysis and use the non-linear regression 'power equation' to extract the CH50% by using 5 serum dilutions only. **Materials and Methods:** The power equation can multiply the five practical dilutions into hundreds of mathematical loops within the sample range. The (CH50%) value is highly accurate for both the study and comparison sample and was evaluated in11 smokers. **Results:** The results were contrasted with a control composed of 11 individuals, matched by age and sex. The power equation showed a 6.48% significant reduction in (CH50%) in smokers compared with non-smokers, where a 17.54% reduction was observed. **Conclusion:** The current study suggests a decrease in the function of the classical complement pathway (CH50%) in smokers. On the other hand, the study provided a new statistical pattern, linking the practical values with default values within the range of dilution and formulating an equation that could be used to extract the value of CH50% with high accuracy.

Keywords: Complement, erythrocyte, haemolytic assay, power equation, smokers, serum pathway, liposome immunoassays, cell lysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For several years, the functional integrity of the complement system (classical pathway) has been measured in the clinical laboratory through standard haemolytic complement (CH50%) assays. Tittered complement components in patients' sera need to associate with antibody-sensitised sheep erythrocytes in the solution. The titre (CH50 unit) at which 50% haemolysis occurs is proportional to the functional activity of the classical serum pathway. Different methods for measuring total classical complement operation have been established. The CH50% procedure uses solution-sensitised sheep erythrocytes[1]. The rate of cell lysis is proportional to the overall activity of the classical complement present in the serum.

The CH50 units are described as follows: Low: <100, Normal: 100-300 and High: >300[1]. Liposome immunoassays (LIA) uses dinitrophenyl (DNP)-coated liposomes containing the enzyme glucose-6-phosphate dehydrogenase. Since the serum is combined with liposomes and a substrate containing anti-DNP antibodies, nicotinamide adenine dinucleotide and glucose-6-phosphate, enabled liposomes lyse and enzyme colourimetric reactions to arise in proportion to the overall classical complement activity. The LIA units are evaluated as follows: Low: <23, Normal 23-60 and High >60[2]. Enzyme Immunoassays (EIA) integrate the concepts of the haemolytic assay with the use of a neoantigen-specific monoclonal antibody (C5b-9 complex) developed because of complement activation. The level of the C5b-9 polymerized end product is equivalent to the functional behaviour of C1 through C9. The interpretation of the EIA unit is as follows: Low: <60, Normal: 60-140 and High: >140[3]. In this study's analysis, a new, practical and statistical method for the total haemolytic of complement titration (1-100%) is described. It is simple, economical and innovative.

Conversely, the Inhalation of tobacco smoke is meant to activate the complement pathway that initiates the production of inflammatory cytokines that recruit leukocytes to the respiratory fluid[4]. Other studies have shown the complement haemolytic activity consumption in serum after incubation with tobacco smoke condensate[5,6].

A very few of the researches have investigated the Complement Haemolytic function (CH50%) in smokers, likely due to the inherent challenges. Consequently, this study chose the serum from heavy smokers due to the ability of tobacco components to activate the classical pathway presence of a substance called tobacco glycoprotein (TGP), which is a polyphenol-rich glycoprotein extracted from tobacco leaves. In this regard, the classical complement pathway is activated via a process that tends to require direct interaction with the complement component 1q (C1q). Tobacco smoke condensate has also yielded a protein with TGP-like action (TGP-S). It has a comparable functional activity to TGP and shares a binding location on C1q[7]. The presence of a compound such as TGP-S stimulates the classic pathway activation in the serum of smokers, indicating a persistent inflammatory condition in heavy smokers compared with normal individuals.

So, the current study aimed to update Mayer's method to calculate the total haemolytic activity by deriving a mathematical equation extracting the value of decomposition from 1-100 by using only five serum dilutions for each sample of heavy smokers and estimating the value of haemolytic activity (CH50%) relative to the control group.

MATERIALS AND METHODS

The study was carried out at the Department of Biology, Advanced Immunology Laboratory, Iraq from (March to September, 2021).

Participants: In the current study, 22 participants were involved, split into two categories. Group one involved 11 smokers, about (25-45) years of age and the average number of cigarettes smoked was (40-60) per day. The participants had been heavy smokers for at least five years from the start date of the study. All smokers were healthy and had no disease manifestations. Group two consisted of non-smokers of age and sex with no signs of disease or autoimmune disorders.

Sample collection: Venepuncture was used to collect blood samples. Those taken from participants were allowed to coagulate for 30 min at room temperature. Subsequently, samples of the extracted Serum were isolated by centrifugation from the blood at 2500 rpm for a quarter-hr. Serum samples were obtained and maintained at -70°C in the deep refrigerator.

Evaluation of haemolytic activity CH50% in serum

Veronal buffered saline (VBS): VBS (5x) solution was prepared in compliance with procedure[8].

Sensitisation of sheep red blood cells with haemolysin: By diluting at 1:50 with VBS, haemolysin (anti-SRBCs antibody from MyBio Source the USA) was prepared. At 600 g for 5 min, the mixture was centrifuged (LABtech. China) and then the supernatant was eliminated. With a combination of VBS and SRBCs, the cell was washed twice again. The cells were re-suspended with VBS to prepare a 10% solution. The same

volume of haemolysin was then used to obtain cells from the previous step with continuous rolling. The combination was incubated in a water bath at 30°C for30 min while being mixed every 15 min. The SRBC's (sensitised) final solution was kept overnight in a refrigerator[9].

CH50% assay: Clean glass tubes have been duplicated in the geometric sequence 1/8, 1/16, 1/32, 1/64 and 1/128. The next two-fold serial manure dilutions were conducted in the test serum and in each duplication for each label, the control group in VBS was prepared, starting with the (300 µL VBS with 100 µL serum) tube. Next, 200 µL of the sample was added to the test tube. The second transfer of 200 µL was made to the next tube. These steps were replicated until the final dilution (1/128). A 200 µL suspension-sensitized SRBCs equal volume was added to all tubes. In addition, For two tubes, a 200 µL suspension-sensitised SRBCs aliquot was added as a blank and anther two tubes containing 200 µL of sensitised SRBCs with 200 µL of DW were labelled as 'total lysis'. At 37°C for 30 min in a water bath, the tubes were softly mixed every 15 min while incubating. The resulting mixture was centrifuged at 1,500 g for 5 min to sediment the RBC after the incubation period. After that, 100 µL of supernatant was added to each well from each tube. A 100 µL of DW had been added to each well. The good result was read using a 540 nm wavelength plate spectrophotometer reader (Rt-2100c microplate Reader. USA)[10].

Analysing results: For each specimen, the average absorbance was evaluated after the blank absorbance was subtracted from the absorbance reported for all specimens. For each dilution, the percentage lyse was calculated by using this equation[11]:

$$\text{Iysis (\%)} = \frac{OD_{540(test)}\text{-}OD_{540(blank)}}{OD_{540(total\ Iysis)}\text{-}OD_{540(blank)}} \times 100$$

Statistical analysis: A non-parametric test, dependant on optical density readings, was used to evaluate CH50. The statistical non-parameterised Mann-Whitney U test was used to compare two independent samples, estimating the correlation coefficient and the non-linear regression coefficient at a significant level $p \leq 0.05$. Statistical analyses were conducted using SPSS.24 statistical analysis program software.

RESULTS AND DISCUSSION

Comparison using the Mann-Whitney U test of two independent samples at each dilution: The Mann-Whitney U test was used to compare two independent samples depending on the mean rank in the comparison. The results presented a significant decrease in haemolytic complement activity in four dilutions, namely, 1/8, 1/16, 1/32 and 1/64 compared to control. The results also revealed that there was no significant difference in 1/132 dilutions in haemolytic complement activity compared to control because the significant value of this dilution was 0.064, which is greater than 0.05 in Fig. 1a-e: (a) Dilutions (1/8), (b) Dilutions (1/16), (c) Dilutions (1/32), (d) Dilutions (1/64) and (e) Dilutions (1/132) and Table 1.

Correlation between haemolytic activity and dilutions in smokers compared with control: The Pearson correlation scale was used to analyse the relationship of the correlation. The bootstrap technique was used to prove the hypothesis that the data should be distributed according to a normal distribution to calculate the value of the correlation with high accuracy. The results showed that there was a strong and significant inverse relationship between the percentage of dilution and haemolytic activity in smokers because the value of the correlation coefficient that appeared is equal to -0.702 in Table 2. Both the upper and lower limits of the confidence limits have similar and negative signs. The same result was observed between both dilution and haemolytic activity in control samples, the value of the correlation appeared equal to -0.773, which is significant because both the upper and lower limits of the confidence have negative and similar signs.

Impact relationship analysis between haemolytic activity and dilutions: The non-linear regression model represented by the power equation was used to describe the effect of the independent variable (dilution) and the dependent variable (haemolytic activity), as shown by the equation:

Table 1: Haemolytic activity for five dilutions in smokers compared to the control samples

Dilutions	Mean rank control	Mean rank smoker	Independent samples Mann-Whitney U test by Rank's	p-value
1/8	14.27	8.73	91.00	0.047*
1/16	15.27	7.73	102.00	0.006*
1/32	16.68	6.32	117.50	0.000*
1/64	15.86	7.14	108.50	0.001*
1/132	14.06	8.95	88.50	0.064

*$p \leq 0.05$

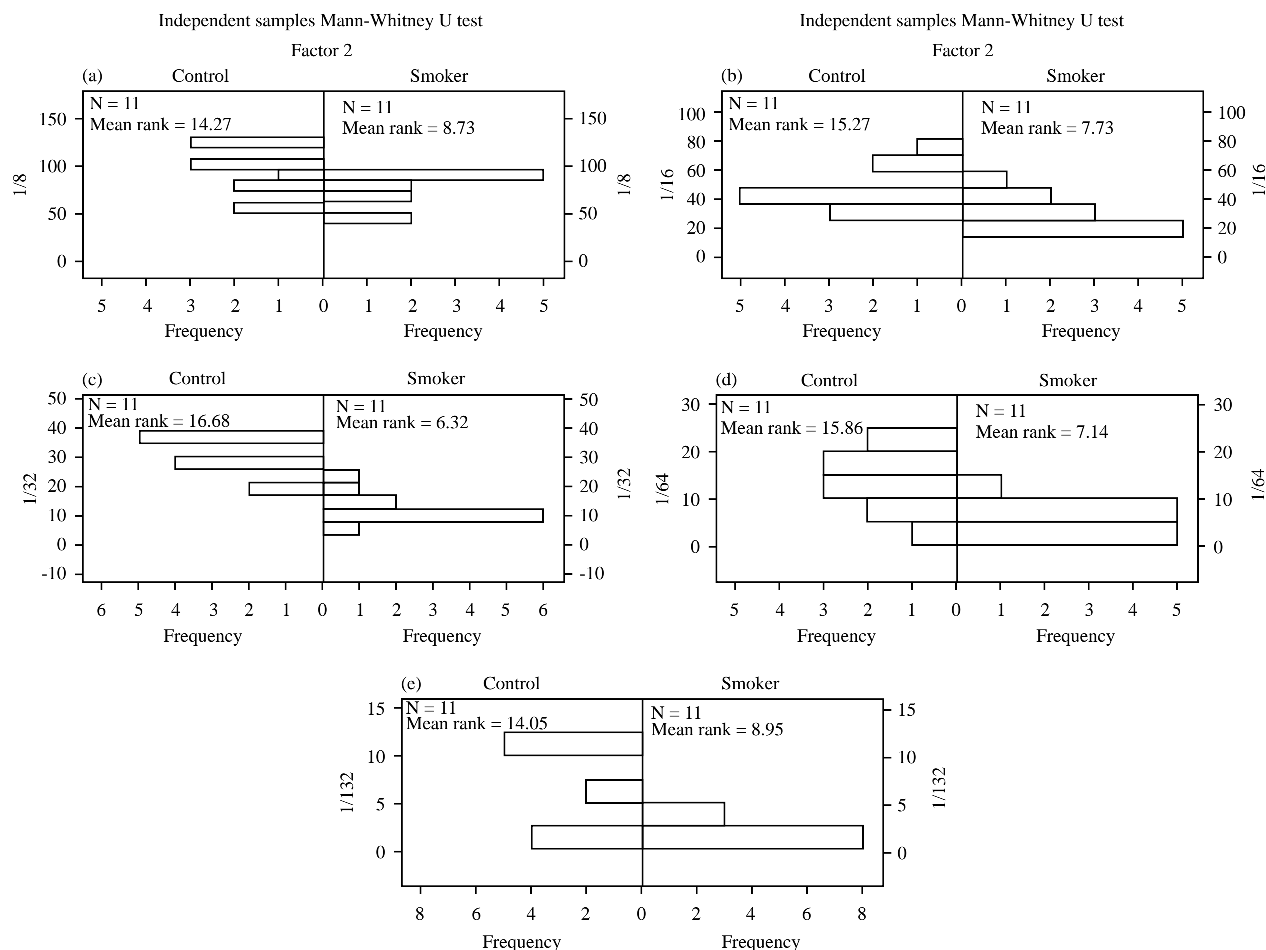

Fig. 1(a-e): Mann-Whitney U test of two independent samples at each dilution, (a) Dilutions 1/8, (b) Dilutions 1/16, (c) Dilutions 1/32, (d) Dilutions 1/64 and (e) Dilutions 1/132

Table 2: Correlation between haemolytic activity and smoker dilution relative to control

Correlations				Smoker	Control
Dilutions	Pearson correlation			-0.702	-0.773
	N			5	5
	Bootstrap[c]	95% confidence Interval	Lower	-1.000	-1.000
			Upper	-0.644	-0.739

[c]Unless otherwise noted, bootstrap results are based on 1,000 bootstrap samples

$$Y = (\beta_0)(X)^{\beta 1}$$

Y = Dependent variable (haemolytic activity)
X = Independent variable (dilution)
β_0,β_1 = Constant and slope coefficients, respectively

CH50% smokers = 6.84%
CH50% control = 17.54%

Smokers samples: The power model was used to analyse the relationship of the dilution effect on haemolytic activity. The results showed a significant and negative effect of dilution on haemolytic activity. In other words, the increase in the level of dilution reflects the decrease in the percentage of haemolytic activity. As the dilution percentage increases by one unit, the haemolytic activity will decrease by 1.342. Based on the value of the coefficient of determination (R^2), the results showed that dilation caused 99% of changes in the value of the haemolytic activity, as shown in Table 3.

Control samples: The power model was used to analyse the relationship of dilution effect on haemolytic activity. The results showed a significant and negative effect of dilutions on

Table 3: Coefficients between haemolytic activity and dilutions in smokers

Coefficients	Coefficients (B)	R^2	T	Significance	Equation
Dilutions	-1.342	0.998	-35.905	0.000	$Y = (1235.246)\ (X)^{-1.342}$
Constant	1235.246		7.410	0.005	$Y = (1235.246)\ (50)^{-1.342} = 6.48$

Table 4: Coefficients between haemolytic activity and dilutions in control

Coefficients	Coefficients (B)	R^2	T	Significance	Equation
Dilutions	-0.913	0.994	-23.287	0.000	$Y = (626.484)\ (X)^{-0.913}$
Constant	626.484		7.065	0.006	$Y = (626.484)\ (50)^{-0.913} = 17.54$

haemolytic activity. In other words, the increase in the level of dilution reflects the decrease in the percentage of haemolytic activity, since as the dilution percentage increases by one unit, the haemolytic activity will decrease by 0.913. Based on the value of the coefficient of determination (R^2), the results showed that dilation caused 99% of changes in the value of the haemolytic activity, as shown in Table 4.

The Mann-Whitney U assay showed the value of haemolytic activity CH50% of complement components for both the smokers and control samples as equations for two separate samples. In this regard, five dilutions were used, where X represented serum dilutions in both smokers and control samples and Y represented the value of haemolysis of SRBCs by the complement compounds, as seen in Fig. 2.

The haemolytic activity test provides an opportunity to assess the classical pathway of the complement system. The CH50% test reflects the dilution value of serum causing sheep blood cells to degrade. The increase in haemolytic value is directly related to higher classical pathway activity of the complement; this increase is not an indication of a pathological condition but rather the presence of active immune responses. Conversely, a decrease in the CH50% value indicates a defect in the classical complement pathway, the reduction could be due to impairment in the synthesis complement system proteins or due to a structural defect. The classical complement system dysfunction is due to the presence of genetic factors, especially in people with autoimmune diseases or frequent respiratory infections such as pneumonia, meningitis and abscesses[12]. Chronic bacterial infection and autoimmune deficiency can be associated with defects in complement components 1-4. The study by authors[13] showed that deficiency of complement component 3 is frequently associated with serious bacterial infections, the complement also plays an important part in resistance to repeated infections. As an immune response that stimulates the production of antibodies[14]. The haemolytic activity can be impaired when there is an imbalance in the concentration of complement components[15] and the presence of liver disease[16]. Most of the complement proteins are provided by the liver. Individuals who smoke heavily will

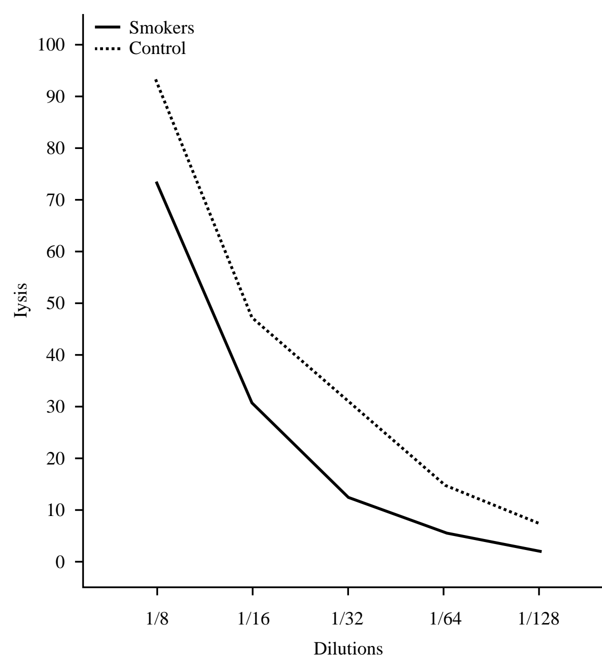

Fig. 2: Haemolytic activity CH50% in smokers compared to the control samples

release toxins into the bloodstream, contributing to the occurrence of inflammation and an increase in the severity of changes to the hepatic epithelium[17]. Many studies indicate that cigarette smoking causes tissue changes in the liver and these changes are at several levels-including immune, toxic and genetic changes that directly affect the vital activities of the liver[18-20].

Smoking affects both specific and non-specific immune responses and serves a diverse purpose in immune management by exacerbating harmful immune responses[21]. Several immunological or inflammatory mediators, including pro-inflammatory and complement proteins, have been linked to smoking[22,23]. Cigarette smoke causes the production of many harmful free radicals, resulting in damage to the function of neutrophils and macrophages that are responsible for displaying foreign antigens to T-cells which, in turn, stimulate the humoral immune response through the activation of B-cells and the formation of antibodies[24]. Another

study showed a decrease in the percentage of haemolysis with complement components in the serum after incubation with high and intense levels of cigarette smoke[25]. The presence of an oxidative environment inside the lung combined with cigarette smoke creates an imbalance in the immune response against infectious agents, not only on the complement pathway but on all immune responses within the lung tissue[26].

CONCLUSION

The current study suggests there is a defect in non-specific defences, which is represented by a decrease in the haemolytic activity of complement (CH50%) in smokers. The imbalanced immune responses resulting from smoking tobacco products makes the lung a suitable environment for infectious agents and their frequency-making smokers less resistant to infectious agents, compared to non-smokers. In addition, the study provides a new statistical pattern linking the practical values with default values within the range of dilution, determining the value of CH50 with high accuracy and organising an equation that could be used to extract the value of CH50, small sample size and a few dilutions. The step of introducing statistical equations into practical experiments introduces a new approach that could reduce the time, effort and cost involved. Furthermore, these equations could obtain highly accurate results when applied to other research within the same field of experimentation.

SIGNIFICANCE STATEMENT

This study uncovers a deficiency in the classical complement component pathway's mechanism of action in smokers, which is one of the most essential innate host defenses. On the other hand, this study will aid in the development of a new statistical pattern, linking realistic values with default values within the dilution range and constructing an equation that can be utilized to accurately extract the value of CH50% with less effort and cost.

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