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Potency of the Natural Honey in Homeostasis of Four Liver enzymes in Rats Induced by Doxorubicin

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ABSTRACT

Previous histological studies have confirmed the curing potency of ingested natural honey on both liver and kidney's cellular structures in mice and other experimental animals. Forty four male Wister rats were divided into four groups, control (G1); honey only (G2); Doxorubicin (DOX) (G3) and DOX with natural honey (G4), respectively. The experiments lasted 7 weeks following subcutaneous administrating 5mg/Kg b.wt./week of DOX and treatment with 1 mL/L honeyed water. Four most common liver enzymes e.g. Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST) and Gamma-Glutamyl Transferase (GGT) in the blood as well as differential counting of WBC were tested to assess the impact of curing potency of the ingested natural honey via stabilizing the levels of the enzymes. The biochemical assessments have shown curing potency of natural honey against the toxicity impact of DOX on the above liver enzymes. Differential counting of WBC have shown insignificant changes in most WBCs but significant elevation of eosinophil in DOX rats (G3) in comparison with other groups indicating development of allergy. It has been concluded that lower or mild doses of DOX have less toxic effects on the body with a rather better impact of honey. Perhaps regular ingestion of the natural honey could provide a natural remedy on general health e.g. functions of the liver and positive impacts on other chronicle diseases. Further researches are recommended using higher doses of DOX via using other administration methods e.g. intravenous.

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Introduction

Liver is the largest solid organ in the body an essential for human and animals with over 500 different functions most of them are the removal of toxins, waste products, foreign substances from the blood stream, breaks down poisonous substances like alcohol and drugs [1]. Liver, also secretes certain amount of a few enzymes directly to the blood stream, but, when injured, for any reason, it releases extra amount of them, mainly, Alanine transaminase (ALT), Alkaline Phosphatase (ALP), Aspartate transaminase (AST) and Gamma-Glutamyl Transferase (GGT). Hence, elevated liver enzymes often indicate a consequence of either liver trauma, inflammation or damage to liver cells (hepatocytes) leaked into the bloodstream measureable via blood tests [2]. Moreover, any harm to liver might also be attributable to various causes i.e. viruses, over-the-counter drug prescription or toxicities. Changes in the level of enzymes of liver or kidney can be measured by biochemical tests at laboratories they deem useful in checking body's health following treatment of patients undergoing hepatic dysfunction and/or abnormality occur may be considered as biochemical marker of liver dysfunction [3]. While decline in levels of liver or kidney enzymes detected by biochemical measurement would therefore indicate an improvement of the function of these organs [4]. Recently, structural improvement has been seen in liver cells treated by short-term ingestion of natural honey in mice [5]. Other evidences indicate that honey can exert several health-beneficial effects including antioxidant [6], anti- inflammatory [7], antibacterial [8], antidiabetic [9], respiratory, gastrointestinal [10], cardiovascular, and nervous system protective effects [11]. Healthy experimental animals could be subject model to measure the levels of hepatic enzymes before and after creating a model of liver and kidney failure via ingesting them with Doxorubicin (DOX) which has a toxic effects on body organs [12]. The theory postulates that the impact of ingestion of natural honey might reasonably drop down the leaked liver enzymes by these organs following subcutaneous admission of Doxorubicin, measured by biochemical kits e.g. ELISA and hematology techniques. The ingested natural honey, following structural improvement in hepatocytes, might indicate improvement in the homeostasis of liver enzymes. The blood specimens of the experimental animals can provide a solid information of homeostasis e.g. plasma and formed blood cells. Differential counting for White Blood Cells (WBC) has been considered in this project to confirm the biochemical results obtained in this thesis. This idea has been inspired by citation of role of honey in the holy book, the Qura'n as a remedy for many or general health disorders.

All types of natural honey, a sweet viscose liquid are extracted from nectar of flowers by honeybees (Apis mellifera; Family: Apidae) flavorful with very great variations in composition and characteristics due to its geographical and botanical origin [13]. Most ancient population, including the Greeks, Chinese, Egyptians, Romans, Mayans, and Babylonians, consumed honey both for nutritional aims and for its medicinal properties [14], while other application include cosmetic, therapeutic, and industrial values [15]. Honey, a balanced diet has also been reported to contain over 180 substances and is considered as an important part of traditional medicine and equally popular for male and female in all ages [16]. It also has numerous uses and functional applications worldwide i.e. in food systems, religious and magical ceremonies as well as in human and veterinary medicine [17,18]. The present research was designed to assess the impact of ingestion of natural honey, following intravenous injection of DOX, via biochemical measurements of levels of some enzymes secreted by the liver as well as differential counting of WBCs.

Materials and Methods

Forty four healthy male adult sibling rats weighed 250±25gm (4+ months old), obtained from National Research Center, Tikrit University, were quarantined for 2 weeks for settlement and maintained under constant conditions i.e. 20±2 oC and 12- hour light/dark cycles with normal diet and water ad libitum. Rats were then allocated into four groups i.e. 10 rats control group (G1) feed with natural diet and filtered tap water without honey; group-2 (G2), feed only natural honey in their drinking water (4mL/L) in tap water, subject to change every 3rd day; group-3 (G3), subcutaneously given 5mg/Kg b.wt. Only Doxorubicin diluted in saline on weekly basis and left in the cages further two weeks, while group-4 (G4) received DOX with honey.

At the beginning of week 10, rats were anesthetized by a piece of cotton socked with chloroform placed in a big glass jar to eliminate pain suffering according to legal ethical guidelines approved by the Ethical Committee of the Federal Legislation and National Institute of Health Guidelines in USA were dissected. The body of the anesthetized rats was opened with a sharp scissors, the chest was opened and the rib cage was separated apart to expose the heart. The heart was then cut off to let the blood bleed and the fresh blood samples were collected directly from the heart in EDTA vials and sent to Azadi hospital for biochemical analysis. Enzyme levels were Assessed using ELISA kits according to and the unit used was international unit per liter blood (iu/L) [19]. A drop of blood was added on a clean slide for blood smear, dried, labeled with a pencil, and kept in slide tray for differential counting using Lieshmanie stain for 10 minutes. Slides were washed using distilled water, dried out at room temperature, and the formed WBCs were counted using a drop of Mustered oil. Two separate blind counting were performed, the data were analyzed expressed as percentage. The enzyme levels were analyzed bio-statistically using Minitab software to verify the significant improvement in the level of the enzymes. Histograms and tables were prepared accordingly and included in the result section.

Results

Experimental animals, G3 and G4 stayed quite all the way along the experiments but were alerted to occasional fights with development of red skin rash had led to sporadic injuries all over their bodies due to scratch in comparison with their counter parts the control G1 and G2. Two rats died from G3 and G4, perhaps due to deadly fights (1.6% mortality). The mean enzyme levels of both ALT in untreated control group rats [G1] (134±20.5 iu/L) and AST (125.0±45 iu/L) were so close to honey treated group (G2) (120.4±17 iu/L) and (124±21 iu/L), respectively. The mean levels of these two enzymes showed significant elevation ($p \leq 0.04$) in comparison with G3 (DOX) group ALT (176±26 iu/L) and AST (249±41 iu/L), respectively; meanwhile, had significantly ($p \leq 0.05$) dropped following treatment with natural honey (G4) (129±28.5 iu/L) and AST (139±24 iu/L) in comparison with G3 (Table.1).

Likewise, the arithmetic means of Alkaline phosphatase (ALP) in an only honey treated control group (G2) showed an insignificant drop ($p \leq 0.06$) (289±66 iu/L) in comparison with control group (G1) (453±49iu/L) but a significant increase ($p \leq 0.05$) in ALP level (558±iu/L) was detectable, in comparison with G3 (DOX) group. However, the level of ALP was significantly dropped ($p \leq 0.05$) in natural honey (G4) (451± iu/L) (Fig.8). In general arithmetic means of serum Gamma-Glutamyl Transferase levels (GGT) were much lower than other three enzymes (ALT, ALP and AST). It appeared 7.8 iu/L in control group (G1) while its level significantly dropped down in G4 (8.43 iu/L). The GGT enzyme level of the only honey treated control [G2] (10.72+) was insignificantly higher than the sham control but was significantly elevated ($p \leq 0.01$) in G3 [DOX] group (13.5±3.1 iu/L). It's was significantly dropped when treated with natural honey (G4) (8.4±1.6 iu/L) (Fig.9).

Table 1: Mean Levels and ±SD of 4 Different Liver Enzyme (ALT, AST, ALP and GGT) of Rats Following Treatment with DOX and Honey for 7 weeks (*): Significant Differences ($p \leq 0.05$- $p \leq 0.01$); iu/L International Unit per Liter

Groups/ (n=10) Means±SD	ALT+SD (iu/L)	AST+SD (iu/L)	ALP+SD (iu/L)	GGT±SD (iu/L)
Control (G1)	134±20	124±17	453±49	7.83±2.2
Honey only (G2)	119±17	124±21	289±66	10.72±1.7
DOX (G3)	*176±26	*249±25	*557±57	*13.5±3.1
DOX + Honey (G4)	*129±28	*139±24	*451±52	*8.43±1.6

Differential WBC Counting

The differential blood counting (Table-2) revealed few alterations represented in a slight but significant elevation of Neutrophil percentages ($p \leq 0.05$) in G2 group in Comparison with other three groups. Another significant elevation ($p \leq 0.05$) in eosinophil percentage was developed in DOX treated rats (G3). Difference in the percentages of other WBC counting were insignificant.

Table 2: Differential Counting of White Blood Cells (WBC) for Experimental Rats. (*) Represents Significant Differences (p≤0.05)

Enzymes	Neutrophils (%)	Basophils (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
G1	12.50±1.82	79.70±7.11	5.2±1.10	2.2±0.41	0.3±0.10
G2	*16.60±2.2	77.0±10.14	4.0±1.04	2.2±0.24	0.2±0.10
G3	13.67±1.33	76.83±12.7	5.0±2.31	*4.17±1.0	0.33±0.11
G4	14.50±2.01	79.50±9.94	3.25±1.05	2.75±0.62	0.0±0

Discussion

Stability in health generally, is linkable, to the body homeostasis or regular function of body organs including all body enzymes i.e. liver kidney and others. These enzymes show up and down to refer to status of homeostasis although not always [20]. Biochemical methods to measure body enzymes e.g. blood plasma assessment represent the most useful ways, as an indicator, in assessing the impact of various diseases in human.

Dealing with rats is not an easy task in comparison with mice due to their over activity, size and strength in comparison with mice. Injection of DOX has been an easy either to achieve. Two rats of each groups G3 and G4 died during the duration of the experiments with some external body wounds which might be attributed to either skin rash or/and fight triggered between them due to stress. Both, red skin and rashes appeared in the G3 experimental rats could be attributed to the allergy developed due to increase in eosinophil in their skin which provoked the rats to scratch their skins causing injuries and perhaps occasional fights. Such a skin rash in rats appears unique in comparison to human being. This is an expected phenomenon in between male animals kept together due to unreleased compromised sexual male hormone. These animals were immediately disregarded from the experiments. Accordingly, the number of each group, eventually, ended up on 10 rats only.

These experiments lasted only 7 weeks which could be considered as acute term (short-term) experiments, where, in such short time, neither a clear impact of DOX nor the natural honey could have expressed enough their impact on the target enzymes. Such an experiment needs to be prolonged on for extra months (3-4 months) as chronic experiments to confirm the clear impact of the honey on. Similarly, the dose of DOX was mild or relatively low enough (5mg/Kg b.wt.) to produce rather Sharp impact on enzymes [5]. Perhaps higher doses may have more representative and reliable effects on the enzymes. It is reported that healthy liver cells store most of the ALT, but small amounts are also found in the muscles and blood. In human, the ALT is most commonly increased in response to liver disease or liver damage, caused by alcohol, drugs, supplements, or toxins.

ALT is commonly used as a way of screening for liver disorders but other expected interpretation has also been valid. Other causes of high ALT include obesity, anorexia, biliary disease, muscle damage and disease, heart attack, hypothyroidism, and infections and diseases that can impair liver function [21]. Alcohol breakdown could cause free radical which damage and increase levels of inflammatory markers [22]. Excessive alcohol consumption leads to death of liver cell indicated by elevated levels of ALT ranges of ALT values showed reference specificity in patients (0-40 iu/L) [23,24]. In human, the test results should always be interpreted using the reference range from the laboratory that produced the result as well as gender difference [25].

The normal range is around 7-35 iu/L in women and 7-40 iu/L in men with some lab-to-lab variability in ranges due to differences in equipment, techniques, and chemicals used [21]. Therefore, the difference in levels of ALT detected in this project, in rats, varies than that of human confirms species variation. The means of ALT in control rats (G1) was (134±20.5iu/L) but an [] dropped to be similar to those of group (G2) (120±17 iu/L). The decline in ALT levels between G1 and G2, although insignificant, may refer to the improving impact of honey on ALT's level. This reading was in concomitant with another study done on rats [26]. The values of ALT in both G1 and G2 showed significance differences to G3 (DOX) group. This result is likely to indicate the toxic effects of DOX on liver function and more likely structural damage to hepatocytes [5]. The significantly dropped in level of ALT treated with natural honey (G4) is a positive impact of the natural honey to cure the toxicity of DOX. It may extend to cover any other disorder might have cause the administration in experimental animals. In other words, the curing potency of the natural honey had reversed and/or halted the toxic effect of the DOX within a short treatment time. In order to confirm the results of ALT other parallel tests have also been involved in this project e.g. ALP, AST and GGT.

The liver is one of the main sources of ALP, but some may also be made in the bones, intestines, pancreas, and kidneys [27]. The level of ALP could either elevate or dropdown depending on various reasons, causes or factors. Blood levels of ALP also found to increase by 2-4 folds during pregnancy, which refers to extra ALP produced by the placenta [28]. It has also been reported that high levels ALP may double indicate damage to both liver cells as well as to any type of bone disorder. Only tests on liver enzymes are involved in the present study while similar projects deems important to include search other structures e.g. bone marrow cells. In human normal age's level ranged between 44-147 iu/L, or 41-133 iu/L [29]. But may elevates two folds in common healthy athletes and fitness enthusiasts [30,31]. Low levels of ALP occur in hypothyroidism, pernicious anemia, zinc deficiency and congenital hypophosphatemia [32].

It therefore is not an easy task to precisely interpret the results unless specific reasons are narrowed down to produce an unbiased interpretation. In this research, although, the rats were healthy animals but other factors of enzymatic alteration cannot be neglected. While ingestion of natural honey (G2) had dropped the ALP level in comparison with those of control the administration of DOX was significantly elevated (p≤0.01) to 557±63. By ignoring interference of any other causes, such a result clearly indicates the toxic impact of DOX on liver enzyme ALP. Meanwhile, the ingestion of the natural honey caused significant improvement in ALP level.

Natural honey, generally, has many varieties distributed worldwide dependent on a few environmental factors but it is so popular in most countries as a sweet food used as an additive to various other foods. In Turkey, other natural honeys e.g. "mad honey", comprised of the nectar of grayanotoxin-containing flowers is generally consumed as food, but is also used as an alternative medicine

element for stomachache, digestive disorders, hypertension and sexual stimulation among the general public [33]. The outcome of this Research clearly proves the consumption potency of the natural honey in curing side effects of DOX used as medication against cancer and other disorders. It also confirms the fact that reduction in enzyme leakage from liver would indicate improvement in function and therefore, a reflection of improvement in cellular structure of hepatocyte [5].

The AST enzyme is similar to ALT in that both enzymes are associated with liver parenchymal cells. The difference is that ALT is found predominantly in the liver, with clinically negligible quantities found in the kidneys, heart, and skeletal muscle, while AST is found in the liver, heart (cardiac muscle), skeletal muscle, kidneys, brain, and red blood cells [34]. The ALT is a more specific indicator of liver inflammation than AST, which may be elevated also in diseases affecting other organs i.e. myocardial infarction, acute pancreatitis, acute hemolytic anemia, severe burns, acute renal disease, musculoskeletal diseases, and trauma [35].

Laboratory tests should always be interpreted using the reference range from the laboratory that performed the test. In this research the AST level had significantly elevated in DOX treated group indicating its existence and action in liver while other organs i.e. kidney or heart have not been assessed for confirmation. Its proportion (125±17 iu/L) were seen much higher than in human male samples (8-40 iu/L). Such a difference might refer to species specificity or other reasons which unknown at this stage but needs further investigation.

The Serum GGT is an enzyme found throughout the body, but especially in the liver which is considered as a marker of hepatic injury used extensively in humans, but has rarely been used in rats because of relevant scant research [36]. In contrast to humans of serum activities up to 30 iu/L basal levels of serum GGT in rats are much low or hardly detectable [37]. The serum GGT activity in the rat was confirmed as a diagnostic of bile duct cell necrosis hortly after the insult and as an indicator of possible bile duct hyperplasia 11.9±1.8 iu/L [36]. A high level of GGT in the blood, may therefore, refer to any sign of liver disease or damage to the bile ducts, accordingly to its function. The difference in its amount in both human and rats is unknown at this stage but it is most likely to be due to size difference of the livers in between human and rats or species differences.

Most recently, GGT levels in human were partially purified and studied biochemically, had shown elevation in its level at various liver diseases e.g. hepatitis-B, heart failure, stroke [38,39]. In the present research the GGT was studied in rats following subcutaneous administration of DOX which causes hepatocyte necrosis [5]. The level of GGT in the rats were as low as 8-13 iu/L in the plasma in comparison with human. The present observations support the use of serum GGT activity in the rat as diagnostic hepatocyte necrosis indicator following administration of DOX in acute or short term experiments. This result is in concomitant with other early studies which confirmed low normal serum values and a lack of reported correlations between serum GGT and specific liver lesions, where serum GGT has not been viewed as an effective indicator of liver dysfunction or injury in rats [40].

Information about GGT in experimental animals i.e. rats are still scanty. The arithmetic means of serum GGT in the two control group (G1 and G2) of rats were as low as (7.83±1.16 iu/L). Nevertheless, the ingestion of natural honey yet caused a significant drop GGT in DOX treated rat group (G3) back to normal. An elevated GGT level suggests that a condition or disease is damaging the liver cells hepatocytes but does not indicate specifically the mechanism of the damage. In general, the higher the level, the greater the damage to the liver. Elevated serum levels of GGT are markers of oxidative stress, resulting from factors including alcohol, heavy metals, cardiovascular disease and diabetes (Ref.). Currently, the histology of the liver in these rats are under extensive research to correlate the impact of DOX and DOX plus the potency of ingestion of natural honey on hepatocytes. Such a result would further confirm the impact of ingestion of natural honey on liver after admission of DOX in experimental rats. Further researches are underway to explore the impact of higher doses of DOX.

Differential Counting of WBC

The level of ALP, an important marker in the blood is checked through routine blood tests which depends on factors i.e. age, sex, or blood type [41]. Abnormal levels of ALP in the blood could also indicate issues relating to the liver, gall bladder or bones, kidney tumors, infections as well as malnutrition [42]. A blood smear is usually taken and stained to categorize each leukocyte into specific "leukocyte alkaline phosphatase indices". This marker is designed to distinguish leukocyte types and determine different enzyme activity from each sample's extent of staining [43].

Results of WBCs proportion (percentages) being blindly and independently counted by two different researchers showed have been insignificant different in these four groups. The WBCs seem unaffected, except in Eosinophils ($p \leq 0.04$) was revealed as a significantly elevation in only DOX experimental animals. The unaffected WBC might be attributed to various reasons relevant to DOX used in this project i.e. low dose of DOX, high resistance of rats, gender difference as male are more resistant than female, short-term or acute impact of the experiments rather chronic, or the subcutaneous administration of DOX produced unaffected results, or the blood has not been the target organ to be affected by DOX. This not wrong by assuming the differences recorded in liver enzymes.

This elevation in eosinophil, ironically, implies an allergy kick off in the body as a response of immune system to the admission of DOX. Consequently, it might had led in developing red skin and skin rash. These two consequences are likely to force the animal to stress and scratch their skins. Moreover, they could lead to provoke the animals to occasional fight between themselves which cause wounds in the body. It might end up in mortality of these animals at certain stage. Accordingly, both could be attributed to the impact of DOX into body as an antigen antibody reaction initiated by the immune system against DOX which led to increase in the histamine in the blood. Eventually, ended up in the rash spots and consequent wounds in the skin. The level of histamine has not been assessed to confirm the cause of allergy and skin rash in this research which deems necessary to explore this issue for future studies.

While the elevated liver enzymes don't signal a chronic, serious liver problem, however, in most cases, their levels are only mildly and temporarily elevated. Following administration of certain medication might need to monitor the blood chemistries to make sure the medication is not causing further harm to the liver; meanwhile, regular impact of the natural honey improved the cellular metabolism of hepatocyte leading to improvement of the levels of enzymes.

Conclusion

It is likely that regular ingestion of natural honey would confirm the divine statement in the holy book, the Quran (Quran, Nahl,

V.68-69) of honey as a cure to various disorders May human being can undergo.

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