Natural honey pre-treatment protect against immune suppression in cyclophospamide exposed wistar rats

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Abstract

Background :Honey is a natural compound with numerous therapeutic functions ranging from anti-inflammatory, anti-oxidant, anti-microbial, anti-hypertensive and hypoglycemic activities. The aim of this study was to investigate the immunomodulatory activity of natural honey on cyclophosphamide induced suppression of humoral immunity in Wistar rats.

Methods :Wistar rats with mean body weight of 125±25g were divided into 5 groups (1-5 ;n-5) Group 1 (control)received only saline ,while groups 2-5 were treated with 30mg /kg bw of Cyclophosphamide (Cyp)intraperitoneally on days 19, 20 and 21. Groups 3-5 received 1.0g, 2.0g and 4.0g per kg bw natural honey orally for 21days in addition to the Cyp injections. Rats were weighed pre-treatment and post-treatment respectively. Blood samples were collected for measurements of hematological parameters and serum immunoglobulin G and M concentrations. Spleen were harvested, weighed and measured respectively.

Results : Compared to the Control group , group 2 had significant reduction in haemoglobin concentration (10.6g /dl) , lymphocytes ($0.4x10^9$/L) and total white blood cells ($0.4x10^9$/L) counts. IgG and IgM concentrations were equally reduced at 960.4 ± 37.3mg/dL and 173.6±1.2mg/dL respectively. Body and spleenic weights, heamatological parameters and IgG concentrations were increased in groups 3-5 on a dose dependent manner ;the highest increase been observed in group 5. IgM concentration was significantly increased in groups 3-5 relative to group 2, but in reverse dose dependent style .

Conclusion :Natural honey pre -treatment with Cyp treatment improves haematological and leucocytic parameters as well as serum IgG and IgM concentrations, thereby potentially protecting tissues from the deleterious effects of short term Cyp treatment.

Key words :Cyclophophamide, Haematological parameters, Humoral immunity, Honey

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Introduction

Cancer is among the leading cause of death around the world, accounting for 13 percent of all global registered deaths; 70 percent of the death occuring in middle and low income countries ,with Nigeria recording about 10,000 cancer deaths and 250,000 new cases annually.[1] According to Global Cancer Incidence, Mortality and Prevalence,[1] [a database of the International agency for Research on Cancer (IARC) the global cancer burden stood at 18.1 million new cases with 9.6 million deaths in 2018 ;with 5-year prevalence estimated to be 43.8million . 57% of new cancer cases as well as 65% of cancer deaths in 2012 occurred in less developed regions of the world that included Central America and parts of Africa and Asia.[1] It is estimated that new cancer cases per year in 2030 shall rise to 23.6 million.[2] The number of patients across the world who will require first-line chemotherapy will rise to 15 million in 2040 (from 9.8 million in 2018) ; an increase of 53% [3].

Cyclophosphamide (Cyp) is one of the first line chemotherapeutic agents. It is a cytotoxic drug that suppresses both humoral and cellular immunity. Its immunosuppressive and cytotoxic effects may bring an impairment of host defence mechanism leading to significant morbidity and mortality, which is a major limiting factor in cancer treatment.[4] Since cytotoxic drugs affect dividing cells ,many of the side effects are concentrated on renewable tissue such as hair ,bone marrow ,and mucous membranes .The side effects vary from person to person, with the type and severity of the side effects depending on the drugs used, dosages and how the body responds to the drugs. The commonest side effects of cytotoxics include hair loss, nausea and damage to the mucosae of gastrointestinal tract, the mouth (resulting in diarrhea and mouth sores), damage to bone marrow with increased risk of infection and communicable diseases. Other systemic manifestations include fever, paraesthesia, encephalopathy, vomiting, constipation, interstitial pneumonitis, congestive cardiomyopathy, angina, heart failure, sterility, haemorrhagic cystitis. The negative side effects of chemotherapeutic treatments can severely impact the quality of life for patients and may result in discontinuation of therapy.[5] Therefore ,therapies which can prevent progression to malignancy, reduce the required dosage of conventional drugs, or lessen the severity of adverse effects are of considerable benefit.

Honey has been used for more than 2000 years as traditional medicine in different cultures due its nutritional and medicinal properties. Reports have

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highlighted multiple roles for honey in enhancing immune responses, including the induction of inflammatory cytokine production by macrophages,[6] stimulation of neutrophil migration and enhanced antibody production.[7] There are wide varieties of honey in use including manuka honey ,pasture honey ,jelly bush honey and jungle honey, etc., largely based on the flower sources.[8] Therefore ,this study was carried out to determine if the use of natural honey along with cyclosphosphamide can alleviate the side effects from the drug and improve quality of life of cancer patients.

Methods

Injectable Cyp, manufactured by Cadila[7] healthcare limited ,marketed by Zydus Celexa, India was procured and constituted as 500mg Cyp dissolved in 25mls of injection water for intraperitoneal injection. Immunoglobulin ELISA kits were equally procured for the study. All other reagents used were of analytical grade.

Freshly harvested honey without additives was purchased from a bee farm in Jato Aka, Benue State, Nigeria. The honey was dissolved in physiological saline solution and prepared freshly each time for treatment and administered orally.

A preliminary study was carried out to determine the effective immunosuppressant, but non-lethal dose of Cyp before commencement of treatment.[9]

Twenty five Wistar rats, aged 6-8 weeks (mean weight=125$\pm$25g) were procured from the disease free stock of the Animal house unit, College of Health Sciences, Benue State University (BSU), Makurdi and randomized into experimental groups 1-5 (n=5). The animals were housed in wooden cages and acclimatized for 10days before commencement of treatments. The rats were maintained in standard condition at room temperature and relative humidity. All the rats had free access to rat chow and portable water *ad libitum* and cared for in accordance with international guidelines for animal care.[10]

The treatment proctocol were as follows:

- Group 1 (control) Rats: Had intraperitoneum injection of 1ml of physiologic saline from days 1-21 each.
- Group 2 Rats: Had intraperitoneum injections of 1ml of physiologic saline from days 1-21, with 30mg/kg BW Cyp on days 19, 20, 21 each.
- Group 3 Rats: Had oral 1.0g/kg BW of honey from day 1-21 with injectable 30mg/kg BW Cyp on days 19, 20, 21 each.
- Group 4 Rats: Had oral 2.0g/kg BW of honey from day 1-21 with injectable 30mg/kg BW Cyp on days 19, 20, 21 each.
- Group 5 Rats: Had oral 4.0g/kg BW of honey from day 1-21 with injectable 3mg /kg BW Cyp on days 19,20, 21 each .

Each animal was weighed daily before treatment administration throughout the study period.[11] At the end of treatment period ,the animals were fasted overnight . Under chlorofoam anaesthesia[12], 5ml of blood samples were collected from each animal with 3ml separated into a plain vacutainer while the remaining 2ml was received into an EDTA bottle .Blood sample in the vacutainer was allowed to clot at room temperature and thereafter centrifuged at 3500 rpm at 4^{O}C (degrees Celsius) for 10min. The consequent supernatants (sera) was collected and stored at -20^{O}C for estimation of immunoglobulins G and M using ELISA techniques.[13] Estimation of haematological parameters was performed on the blood sample collected in the EDTA using the Diatron-Abacus 5 hematology analyzer[14].

Date obtained were compiled using Micosoft Excel 2016 and reported as mean $\pm$ SD .Differences between groups were estimated using one way ANOVA with Turkey *post hoc* test using SPSS software for windows version 21 (IBM. Corp, Armonk, N.Y(USA). Difference was considered significant when P=0.05.

RESULTS

Table 1 showed that rats treated with Cyp alone had a significant (P<0.05)weight loss compared to the Control while rats in groups 3-5 had significant (P<0.05)total body weight gain relative to those of group 2. However , this relative weight gain was still significantly lower (P<0.05)when compared with the Control group .

Similarly, the mean spleenic weight showed a significant (P<0.05)decrease in groups 2-5 compared to that of group 1. Honey pre treatment in groups 3-5 induced a significant (P<0.05)spleenic weight increase relative to group 2.

As shown in Table 2, there was significant (P<0.05) decrease in all the haematological parameters studied in group 2 (Cyp alone) compared to the Control group . Pre treatment with honey in groups 3-5 on the other hand caused a significant (P<0.05)improvement in the studied haematological parameters relative to group 2 findings in a dose dependent manner .These improved haematological parameters however ,were significantly lower (P<0.05) compared to those of Control group except for MCV in group 5 which showed insignificant relationship .

It was observed that there was significant (P<0.05) reduction in the mean concentrations of IgG and IgM in group 2 relative to the Control group (Table 3). On the

other hand ,pre -treatments with honey in groups 3-5 showed significant (P<0.05)elevation and depression of IgG and IgM concentrations respectively relative to group 2 in a dose dependent manner. The respective immunoglobulin concentrations were all significantly lower (P<0.05)compared to those of the Control group .

These findings suggest that short term Cyp treatment profoundly suppresses total body and spleenic weights, heamatological and immunological parameters in the Wistar rats. However, pre-treatment with honey tended to protect against Cyp-induced the haematological and immune suppression.

Table 1: Effect of Honey on Weight parameters in Cyp-induced immunosuppression in Wistar rats

Variable	Study group (Mean±SD				
	Control	30mg/kg Cyp	1g/kg honey + 30mg/kg Cyp	2g/kg honey+ 30mg/kg Cyp	4g/kg honey + 30mg/kg Cyp
Pre-treatment weight(g)	132.0±1.1	133.2±0.8	131.9±0.7	131.6±0.8	131.3±0.7
Post-treatment weight(g)	142.9±1.2	121.4±0.8*	139.3±0.9*#	140.3±0.7*#	140.6±0.6*#
Change in weight(g)	10.9±0.5	-11.8±0.8*	7.4±0.2*#	8.6±0.2*#	9.3±0.3*#
%Change in weight(g)	8.2±0.4	-8.8±0.6*	5.6±0.1*#	6.6±0.2*#	7.1±0.3*#
Spleen weight(g)	0.8±0.1	0.4±0.1*	0.5±0.1*	0.6±0.1*#	0.6±0.1*#

*Significantly different from control group with P<0.05, #Significantly different from 30mg/kg Cyp group with P<0.05, SD= Standard Deviation

Table 2: Effect of honey on Haematological parameters in Cyp-induced Wistar rats

Parameter	Treatment group (mean±SD)				
	Control	30mg/kg Cyp	1g/kg honey+ 30mg/kg Cyp	2g/kg honey+ 30mg/kg Cyp	4g/kg honey + 30mg/kg Cyp
WBC (10^9/L)	24.1±1.4	0.4±0.1*	1.1±0.03*	2.0±0.1*#	2.7±0.2*#
NEU (10^9/L)	2.0±0.1#	0.08±0.02*	1.1±0.01*#	1.4±0.03*#	1.6±0.01*#
LYMPH (10^9/L)	18.1±1.5	0.4±0.0*	1.0±0.02*	1.5±0.1*	2.5±0.1*#
MON (10^9/L)	1.7±0.4	0.06±0.02*	0.1±0.0*	0.1±0.01*	0.2±0.01*
RBC (10^{12}/L)	7.5±0.2	5.1±0.05*	5.6±0.2*#	6.0±0.1*#	6.6±0.2*#
PCV(%)	44.3±0.7	32.2±1.2*	35.8±0.7*#	36.9±0.5*#	38.3±0.5*#
HGB (g/dl)	15.8±0.6	10.6±0.5*	12.1±0.1*#	13.4±0.1*#	13.9±0.1*#
PLT (10^9/L)	553.5±7.5	371.7±9.2*	438.0±11.9*#	479.2±1.6*#	486.8±5.0*#
MCH (pg/cell)	20.5±0.6	16.4±0.25*	17.6±0.6*#	18.3±0.5*#	22.0±0.04*#
MCHC (g/dl)	40.0±0.5	31.7±0.5*	33.5±0.4*#	34.4±0.3*#	35.1±0.2*#
MCV (fl)	58.3±0.6	54.1±0.2*	55.0±0.2*	56.0±0.8*#	57.5±0.4#

*significantly different from control with p<0.05; # significantly different from 30mg/kg group SD= Standard Deviation
Values are expressed as Mean ± SD. Treated groups are compared with Control group. PCV = Packed Cell Volume; Hb = Haemoglobin; WBC = White Blood Cells; NEU = Neutrophils; LYMPH = Lymphocytes; MON = Monocytes; PLT = Platelets; RBC = Red Blood Cells; MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration; MCV= Mean Corpuscular Volume

Table 3: Effect of Honey on Immunoglobulin concentration in Cyp-induced Wistar rats

Variable	Control	Variable Treatment group (Mean±SD)			
		30mg/kg CYP	1g/kg honey + 30mg/kg Cyp	2g/kg honey + 30mg/kg Cyp	4g/kg honey + 30mg/kg Cyp
IgG (mg/dL)	1496.7±54.7	960.4±37.3*	1099.2±50.3*#	1237.4±22.4*#	1342.7±15.4*#
IgM (mg/dL)	355.1±15.8	173.6±1.2*	247.6±7.3*#	201.6±4.4*#	197.3±1.3 *#

* significantly different from control with p<0.05; # significantly different from 30mg/kg Cyp group SD= Standard Deviation
IgG= immunoglobulin G, IgM= immunoglobulin M

DISCUSSION

The immunomodulatory activity of honey was explored by evaluating its effects on Cyp-induced immunosuppression .Cytotoxic agents are non specific in their actions ,targeting almost all cells in the body killing healthy cells as well as cancer cells .In the present study , short term treatment with Cyp induced total body weight loss by about 9% in the experimental animals. This finding is supported by other studies which showed significant body weight loss in mice with exposure to Cyp at 80mg/kg/d for 3 consecutive days,[15,16] while a 5-10mg/kg Cyp treatment for 28– 30 days (long duration treatment) by oral gavages induced an initial significant body weight loss followed by weight gain.[17] This is an indication of the cytotoxic properties of Cyp on cellular multiplication in growing tissues particularly. The mechanism of weight loss may be related to the effect of the metabolic product of Cyp. Cyp is metabolised by the liver cytochrome P450 mixed function oxidase system, converting it to hydroxycyclophosphamide, which is subsequently metabolized to aldophosphamide. Aldophosphamide is cleaved to the active alkylating agent phosphoramide mustard and acrolein. The phosphoramide mustard forms a highly reactive cyclic aziridinium cation, which can react with the N(7) of the guanine and cytidine DNA molecule. The two reactive moieties in the molecule can form intrastrand and interstrand cross-links DNA, thereby inhibiting DNA multiplication (hence mitosis)[18] and equally promoting cellular apoptosis.[19] The outcome of this cellular action is prevention of tissue growth as well as the promoting of tissue loss. Another possible mechanism underscoring Cyp-induced weight loss lies in the fact that Cyp's active metabolite acrolein can interfere with antioxidant defense system, leading to the production of highly reactive oxygen species (ROS), which are a group of free radicals.[20,21] that can oxidatively damage hepatic cells with the consequence of causing digestive abnormalities , thereby making the body prone to weight loss.

Similarly, significant reduction in the mean spleen weight induced by Cyp treatment was observed in this study. This finding is supported by findings of immune organ atrophy and weight in animals treated with Cyp[22] due to the fast depletion of lymphocytic population in the lymphoid tissues of the organ .[23] The physiological basis for this phenomenon equally lies in the ability of Cyp metabolite to induce cellular DNA injuries , especially in the fast replicating cells of the spleen (the major lymphoid organ) and bone marrow leading to apoptosis and atrophy in these organs.

These actions of Cyp, in addition to the induction of ROS by Cyp metabolite acrolein in the liver and bone marrow,[24] has a severe adverse outcome on the general population of myeloid cell lines, as evidenced by the global suppression (by about 30%) of haematological parameters with resultant anaemia, erythrocytopenia, pan leucocytopenia and thrombocytopenia .In a study,[25] a single intraperitoneal injection of 200mg/kg bw Cyp induced in significant decreases in total red blood cells (RBCs), white blood cells (WBCs), thrombocyte counts as well as the haematocrit values in CP-treated fish, lending support to the findings of the present study.

IgG and IgM are the major immunoglobulins that provide humoral immunity for body defense against pathogens. It was observed in that immunoglobulins G and M were significantly reduced in all the Cyp exposed groups compared to the concentrations in the Control group ,a finding that is consistent with observation made in a study that a high single dose Cyp injection depressed B lymphocytes and abolished their function on the 7th day in the spleen and bone marrow, subsequently causing reduction in immunoglobulin populations.[26] The observed pan leucocytopenia and hence neutropenia, monocytopenia and especially lymphocytopenia impact adversely on humoral immunity. This may pave way for overwhelming body infections. Elsewhere similar observations have been made by other researchers who reported an imbalance of various leukocytic subpopulations in the peripheral blood of mice exposed to Cyp.[27,28]

In addition to the afore-mentioned mechanisms of action of Cyp in affecting mitosis and promoting apoptosis, Cyp suppressively target B lymphocytes (hence depressed IgG and IgM) as well as disruption of cell adhesion molecules (CAM) and cytokines.[19] T cell absolute population are equally suppressed by Cyp,[4] while the fine balance between T helper cell 1 and 2 (Th1/Th2) subpopulations is completely lost.[29]

The observed suppressive actions of Cyp in this study were significantly ameliorated by honey pre-treatment of Cyp exposed animals with improvements in spleenic and body weights, immune and haematological parameters earlier depressed by Cyp treatments. Similar findings supporting the outcome of our present study have been documented in other studies which showed the positive effect of honey treatments on body weight, immune and heamatological indices.[25,30] This protective action of honey may likely be attributed to the intrinsic properties of honey, including its high osmolarity and acidity, as well as the presence of flavonoids (such as quercetin, catechin, kaempferol, luteolin, and apigenin) and phenolic acids (such as caffeic acid, ellagic acid, gallic acid, syringic acid, chlorogenic acid, p-coumaric acid, ferulic acid, and the fiavonoids chrysin, kaempferol, catechin, quercetin, galangin, luteolin, pinocembrin, pinobankskin, and myricetin)[31] which are responsible for its antibacterial and antioxidant

activities.[32]

Additionally, honey is known to contain over 200 compounds, consisting mainly of sugars (75% monosaccharides: glucose and fructose ; 10%-15% disaccharides: sucrose, maltose, etc.) and water, as well as enzymes, vitamins (Vitamin B6, ribofiavin, niacin, thiamine, etc.), minerals, volatile compounds, and pigments.[33] In addition to its antimicrobial, antioxidant and tissue-protective activities, Manuka, Pasture, Nigerian Jungle, and Royal jelly honeys are found to increase IL-1β ,IL-6, and TNF-α production.[7] There are over 300 varieties of honey recognized.[34] These varieties are defined by their components of the flower sources and the degree of protection offered by honey against Cyp effects was more dependent on honey type than its duration and dose.

The production of short-chain fatty acid (SCFA) by honey may also play a key role in the protective actions of honey on Cyp-induced side effects as fermentation agents of SCFA, especially nigero-oligosaccharides as well as other non-sugar ingredients, possess strong immunomodulatory properties.[35 35] Manuka and pasture honeys have strong demonstrable ability to stimulate the production of the cytokine tumour necrosis factor -alpha (TNF-α) cytokines ,interleukin-1 and 6 (IL-1β ,and IL-6) by cells.[6] TNF-α produced by bone marrow -derived macrophages (BMDM) and human monocytes is essential for macrophage activation, stimulation of angiogenesis and re-epithelialisation during early wound healing, a key activity in tissue recovery from Cyp effects. While cytokines equally play important role in wound healing, IL-6 enhances proliferation of keratinocytes and attract neutrophils while interleukin-1 (IL-1β), stimulates the release of important wound - healing growth factors. Production of TNF-α are potently stimulated by Type II arabinogalactan proteins (AGPs) also. AGPs, found in Kanuka honey, are derived from essential polysaccharide polymers found in the cell walls and tissues of plants and can be transported to honey from plants by bees during the honey -making process.[36] Thus, honey ameliorate Cyp induced haematological and immune suppressive effects on cells by inducing phenolic antioxidation and suppressing the production of ROS, which is a key marker of tissue inflammation.

Even though the study was well designed to satisfy the overall aim ,the study was by no means totally exhaustive and limited in scope owing to paucity of funds.

It is hereby recommended that Cyp use as cytotoxic therapy be preceeded by honey treatments as well as close monitoring of the individual to curb any debilitating side effects that may arise.

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