Evaluation the Genotoxicity of PHB Nanoparticle by Micronucleus Assay

Jenan A.Ghafil[1], May. T. Flieh[2]

[1]Lecture, [2]Professor, College of Science, Department of Biology, University of Baghdad, Baghdad, Iraq

Abstract

Determining the toxicity of substances in vivo is one of the most important tests that judge whether or not they are used in the pharmaceutical field. In the present study, the genetic toxicity of treatment doses of Polyhydroxybutyrate (PHB), PHB nanoparticles, Cefotaxime and complex of PHB nanoparticlesand cefotaxime was evaluated. The effect of these substances on the number and percentage of white blood cells (WBCs) in mice was also tested (in vivo). Micronucleus assay was used to assess genotoxicity of above materials in vivo, as well as the technique of WBCs chamber was used to estimate the total number of WBCs in mice administrated with above substances. The differential count of WBCs was measured by staining the smears with leishman stain. The present study demonstrated that there were no significant differences (P>0.05) in the number of micronucleus cells in the mice injected with treatment doses of PHB nanoparticles, Cefotaxime and complex of PHB nanoparticlesand cefotaximewhen compared with mice injected with normal saline. Similar finding was obtained in terms of counting of total WBCs and differential count in mice injected with treatment doses of PHB, PHB nanoparticles, Cefotaxime and complex of PHB nanoparticlesand cefotaxime when compared with WBCs total count and differential count in mice injected with normal saline (P>0.05). It can be concluded that there is no toxic effect of treatment doses of PHB, PHB nanoparticles, Cefotaxime and complex of PHB nanoparticlesand cefotaxime on mice.

Keyword: *Polyhydroxybutyrate, micronucleus assay, Genotoxicity.*

Introduction

Polyhydroxilconoate (PHB), a polymer belonging to the polyester classes, is a biodegradable and biodegradable plastic of interest. The poly-3-hydroxybiotrate (P3HP) form of PHB is probably a polyhydroxyalconoate type, but other polymers of this class are produced by a variety of organisms: including poly-4-hydroxybiotrate (P4HPHP).Polyhydroxyhexanonate (PHH), polyhydroxyocyanate (PHO) and their copolymers [1].

In the medical field, many drugs are used to treat bacterial infections [2], and this requires determining their toxicity. The same applies to the PHB, as it has many applications in vivo, including anti-bacterial [3], as well as to contribute to limiting the activity of some diseases such as cancer in the human body [4]. To determine the possibility of its use, it is required to determine cytotoxicity of PHB.

There are a number of methods by which it is possible to determine the extent of the body's response to foreign substances, including by measuring the number and percentage of white blood cells[5]. These are useful for determining the state of the immune and cellular system in case the body is exposed to foreign substances[5].

There are many methods that can be used to determine the genetic toxicity of medicinal materials, the most important and the most common of which is the Micronucleus assay [6].A micronucleus (MNs) test is a test used in toxicological screening for

Corresponding author:
Jenan Atiyah Ghafil
jenenghafil@gmail.com
College of Science, Department of Biology, University of Baghdad, Baghdad, Iraq

potential genotoxic compounds. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens. This test is based on the formation of number of MN in treated cells [7].MNs are formed during anaphase from chromosomal fragments or whole chromosomes that are left behind when the nucleus divides. Over time, the assay has evolved to include a pretreatment with cytochalasin-B (Cyt-B), a cytokinesis blocking agent that inhibits cell division, thereby giving the cells a binucleated appearance. This enables more accurate scoring and the ability to sieve out the dividing cells from the nondividing ones, thereby reducing the incidence of false positives[7].

In the absence of a previous study dealing with the effect of PHB nanoparticles on the state of white blood cells in the body as well as the genotoxic effect of this substance, so the genotoxicityof PHB nanoparticles in vivo was evaluated by using the micronucleus assay and the effect of PHB nanoparticles on the status of white blood cells in terms of number of cells and percentage in vivo was evaluated by using animal models (mice).

Material and method

Polyhydroxybutyrate nanoparticles

Polyhydroxybutyrate (PHB) as a powder was purchase from Sigma-Aldrich, USA the PHB was derived from microbial fermentation. The preparation of PHB nanoparticles was prepared by adding 1 gmof PHB to 50 ml of distilled water and pH was adjusted to 4 by HCl (1 N). The mixture was put in ultrasonic path at 4500 kh for 25 second.The pH was readjusted to 10 by NaOH (1N). The mixture was mixed by magnaticstirrer for 2 h at 21 ^{o}C. The mixture was incubated at 21 ^{o}C for 18 h and then the pH was readjusted to 7 by HCl (1 N). The synthesis of PHB nanoparticles was evaluated by Atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), Ultraviolet (UV) spectrophotometer, X-ray powder diffraction (XRD) and Scanning electron microscopy (SEM).

Mice

BALB/c mice 6-8 weeks old, weighing 20-25 gm was procured from central animal house, AL-NahrainUniversity, Baghdad, Iraq. Animals were kept in clean polypropylene cages and fed on standard antibiotic free diet. The mice that used in current study were male.

Micronucleus assay

The standard method of Sousa et al. (2016)[8] with little medication was followed to evaluate Percentages of micronucleated polychromatic erythrocytes (PCEMNs) in bone marrow of mice after 48 hours of injection subcutaneous with Polyhydroxybutyrate (PHB), PHB nanoparticles and PHB nanoparticles plus cefotaxime (complex). Briefly, four groups of mice were used in this experiment. Each group consisted of three mice. Group A, mice injected under skin with treatment dose of PHB (1 mg).Group B, mice injected under skin with treatment dose of PHB nanoparticles (1 mg).Group C, mice injected under skin with treatment dose of PHB nanoparticles + cefotaxime (500 µg).Group D (Control group), mice injected under skin with normal saline. The mice were sacrificed 48 hours post administration of treatment doses of above materials under skin.All mice were dissected.Bone marrow cells were collected immediately after the sacrifice of animals.Leg bones of mice were collected and homogenized by mortar aseptically.The bone marrows were collected from homogenised bones.In a Falcon tube previously marked with the animal group. Bone marrow material was re-suspended with fetal bovine serum until homogeneous. The suspension was centrifuged for 5 minutes at 1,000 rpm and the supernatant was discarded and the pellet was rewashed three times with fetal bovine serum. At the end of procedure the pellet was re-suspended with 500 µl of fetal bovine serum then the tube was homogenization genteelly and smears were prepared dripping off 2 drops of suspension on the tip of a slide (previously labelled with the animal's group) and with the aid of another slide bent at a 45 degree angle to make the smear and the slides were air dried, two slides per animal were made and stained by leishman stain[9].

The analysis was performed in blind field in an increase of 100x (immersion objective) in a short time by the same observer.Micronuclei were measured in 2,000 polychromatic erythrocytes (PCEs) / animal in bone marrow of adult mice.

White blood cells (WBCs) total count and differential count

The standard method of Liu et al. (2020) with little modification was followed to count the number of WBCs in peripheral blood that collected by anticoagulant capillary tube (heparin) inserted in the lateral canthus from the retroorbital sinus of mice.The specific WBC pipette and WBCs solution were used. The blood specimen was diluted 1:20 in a WBC pipette with the diluting fluid (WBCs solution) and the cells were counted under low power of the microscope by using a counting chamber. The number of cells in undiluted blood is reported per microliter of whole blood[10].**The** standard method of Nurhayati*et al*. (2019) was followed with little modification to be in line with approach of the present study[11] to measure the percentages of WBCs in peripheral blood of mice post smear preparation and stained witleishman stain. The WBCs total count and the percentages of different types of WBCs were measured in blood that collected from different mice groups. Group A, mice injected under skin with treatment dose of PHB. Group B, mice injected under skin with treatment dose of PHB nanoparticles.Group C, mice injected under skin with treatment dose of PHB nanoparticles + cefotaxime. Group D (Control group), mice injected under skin with normal saline.

Statistical Analysis

All values have been taken as mean value and standard deviation (SD) calculated. The differences were analyzedusing Student's t-test employing Origin version 8.0 software.A value of $P < 0.05$ was considered to be statisticallysignificant.

Result

Micronucleus assay

To ensure the presence or absence of the toxic effect of PHB, PHB nanoparticles, PHB nanoparticles plus cefotaxime, the percentage of micronucleated polychromatic erythrocytes (PCEMNs) in the bone marrow of mice was estimated after they were injected with the therapeutic dose of the following substances, PHB (1 mg) and PHB nanoparticles (1 mg) and the complex consisting of a mixture of PHB nanoparticles plus cefotaxime (0.5 mg). The results of number of PCEMNs in bone marrow of upper groups were compared with a number of PCEMNs in bone marrow of control group (mice injected with normal saline).

The results showed that there were no significant differences in number of PCEMNs in bone marrow of teat groups of mice and number of PCEMNs in bone marrow of control group of mice ($P<0.05$) (Fig. 1). This finding proves that no toxic effect of treatment dose of PHB or PHB nanoparticles or PHB nanoparticles plus cefotaxime (complex). The micronucleus test suggested that the PHB, PHB nanoparticles and PHB nanoparticles plus cefotaxime have no biological toxicity in order to not change the incidence of polychromatic erythrocytes. That confirmed the safety of using of these compounds in vivo.

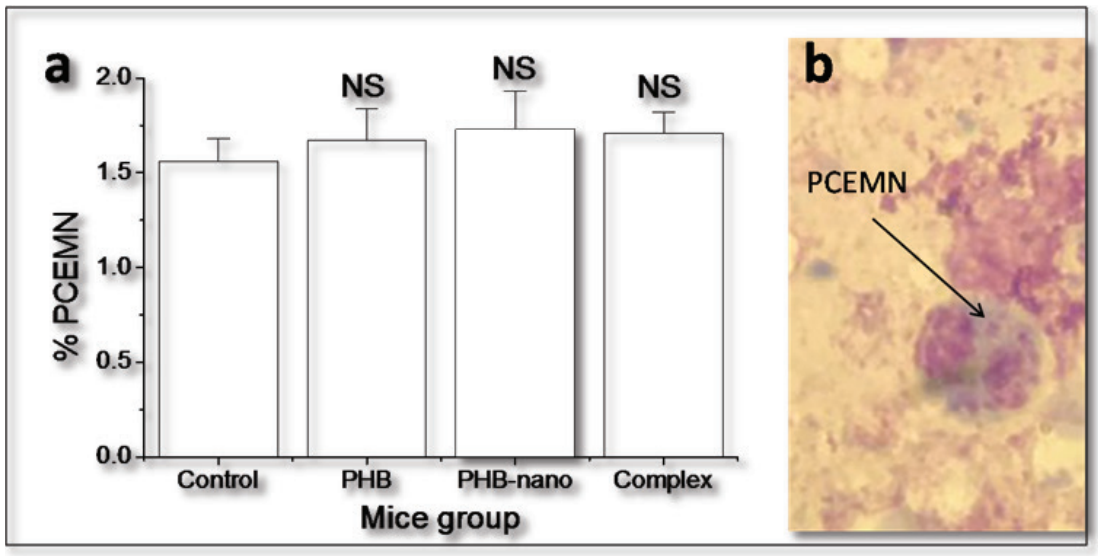

Figure 1 a, Percentages of micronucleated polychromatic erythrocytes (PCEMNs) in bone marrow of mice after 48 hours post injected subcutaneous with normal saline (control), Polyhydroxybutyrate (PHB), PHB nanoparticles and PHB nanoparticles plus cefotaxime (complex). NS, non significant difference from control group. b, micronucleus in PCEMN.

White blood cells (WBCs) total count and differential count

Fig. 2 shows the number of WBCs in blood of different groups of mice post administrating with treatment dose of PHB (1mg/ kg), PHB nanoparticles (1 mg/kg), PHB nanoparticles plus cefotaxime (0.5 mg/kg) and 0.5 mg/ kg of cefotaxime. The results showed no significant difference ($P<0.05$) between any experimental groups with control. This finding proves that the administration of treatment dose of above materials do not effect on the number of WBCs *in vivo*.

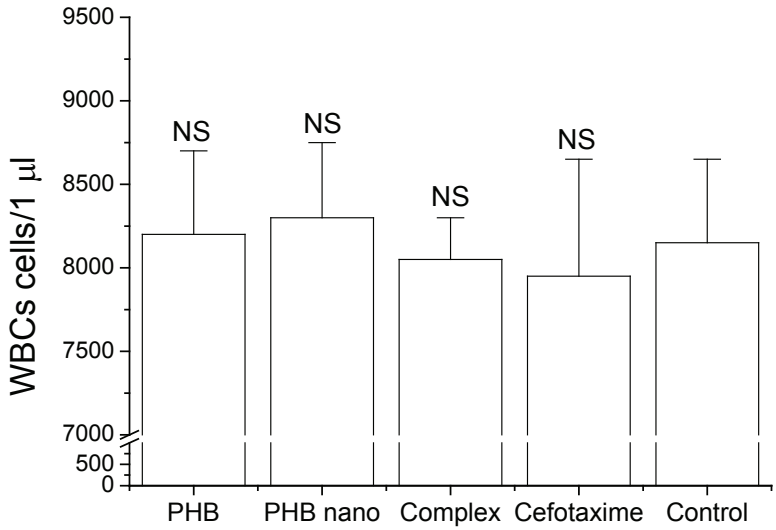

Figure 2. White blood cells (WBCs) count that collected from retro-orbital sinus of mice eyes post administrating orally with treated dose of PHB (1 mg/kg), PHB nanoparticles (1 mg/kg), PHB nanoparticles plus cefotaxime (0.5 mg/kg) and cefotaxime (0.5 mg/kg). NS, no significant difference from control group (mice administrated orally with normal saline).

After calculating the total number of leukocytes (WBCs) in peripheral blood of different groups of mice, the percentages of polymorphonuclear cells (neutrophil, eosinophil and basophil) and mononuclear cells (monocytes and lymphocytes) were calculated in the peripheral blood taken from retro-orbital sinus of mice eyes. The percentages of polymorphonuclear cells and mononuclear cells were calculated in blood samples of groups of mice administrated orally with treatment dose of PHB (1 mg/ kg), PHB nanoparticles (1 mg/ kg), PHB nanoparticles plus cefotaxime (1 mg/ kg), and cefotaxime (1 mg/ kg). **Fig. 3** shows no significant difference ($P<0.05$) in percentages of all types of leukocytes [polymorphonuclear cells (neutrophil, eosinophil and basophil) and mononuclear cells (monocytes and lymphocytes)] that obtained from all peripheral blood of experimental groups of mice as compared with percentages of all types of leukocytes that obtained from blood of control group (mice administered orally with normal saline).

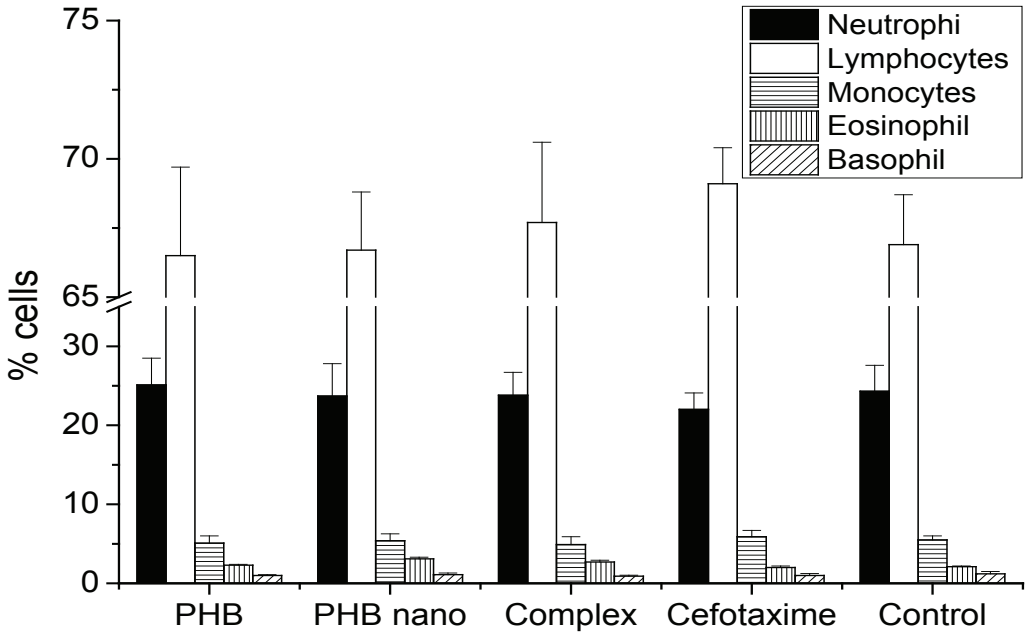

Figure 3. Differential count of leukocytes that collected from retro-orbital sinus of mice eyes and smeared on slides and stained with leishman stain. There is no significant difference in percentages of all types of leukocytes that obtained from different groups of mice that administrated with treatment dose of PHB, PHB nanoparticles and PHB nanoparticles plus cefotaxime (complex) and cefotaxime. The results were compared with corresponding cells in blood samples that collected from control group.

Discussion

During almost 40 years of use, the micronucleus assay (MN) has become one of the most popular methods to assess genotoxicity of different chemical and physical factors, including ionizing radiation-induced DNA damage [7]. In a modern world, humans are exposed to different genotoxic agents present in the polluted environment. Hence, tests are needed to determine the level of exposure and health risk. Although many tests classified as "in vivo biomonitoring" are available, a micronucleus test (MN) is one of the best and the most popular [12]. The assay is also widely used to test genotoxicity in vitro (7). Many previous studies used micronucleus assay (MN) to detect the toxicity of some of the materials used in different field such as therapeutic purpose such as drugs or as a food [13].

There are very few studies assessed the genotoxicity of PHB in vitro or in vivo. It was found only one study focused on this issue. de Sousa *et al.* (2016) checked the toxixcity of membranePHB/Norbixin/Ethyleneglycol by micronucleus test and they found that there is no toxicity in the natural material of membranePHB/Norbixin/ Ethyleneglycol and they can be used for biological purposes and these will contribute to future studies on the effects of the membrane on the healing of skin wounds[8]. This finding is matching with what we got in our study about the safety of PHB that is why we also suggest the possibility of using this material as a medicine for treatment in future.

Estimating the number of white blood cells in the blood is one of the indicators of the state of the immune system, since these cells represent the cellular arm of the immune response. Therefore, estimating its levels gives an indication of the body's response to an external stimulus, as well as a non-specialized picture of the state of the immune system. Estimating the number and percentage of Polymorphonuclear cells such as neutrophil, eosinophils and basophil and mononuclear cells such as lymphocytes and monocytes gives a more specialized picture of the state of the immune response after the occurrence of infection. However, these two indicators represents non-specific indicators through which it is not possible to determine the nature of the response and for which disease factor, even these to indicators will not give accuracy answer to the causative of the disease but they give general picture about the status of immune response. In present study the total number of WBCs and percentages of these cells were evaluated post PHB, PHB nanoparticles and PHB nanoparticles plus cefotaxime administration to mice orally.

Through searching the literature on the effect of PHB (nanoparticles and non nanoparticles) on the total number of leukocytes (WBCs) in laboratory animals, no article was found dealing with this topic, which supports that the present study is the first study that deals with the effect of PHB, PHB nanoparticles, PHB nanoparticles plus cefotaxime on the total number of leukocytes in peripheral blood of laboratory animals (mice). The finding of no effect of PHB in different form on the total numbers of white blood cells in laboratory animals confirms the safety of using these materials in treatment, and this opens the door for new research dealing with the possibility of using these materials in treatments, especially with regard to infectious diseases.

According to our knowledge there is no published literature covered the effect of PHB (nanoparticles and non nanoparticles) on the percentages of leukocytes [polymorphonuclear cells (neutrophil, eosinophil and basophil) and mononuclear cells (monocytes and lymphocytes)] in laboratory animals. That supports the present study as the first study that deals with the effect of PHB, PHB nanoparticles, PHB nanoparticles plus cefotaxime on the differential count of leukocytes in peripheral blood of laboratory animals (mice).

Several previous studies focused on the effect treatment doses of different medicine especially antibiotics on the number and percentages of polymorphonuclear. Shuman et al. (2012) have identified one category of medications that may cause decreased white blood cell/absolute neutrophil counts when combined with clozapine[14]. Their study supported the use of either ciprofloxacin or moxifloxacin as agents that may have less risk of reductions in white blood cell/ absolute neutrophil counts than are seen with penicillins, cephalosporins, and other antibiotics that may ultimately require interruption or discontinuation of clozapine therapy. Similar finding was reported by Nguyen et al (2016) they found that using antibiotics increase the maturation of neutrophil and that reflect on the total count of leukocytes as well[15].Previous study support strongly that the total count of leukocytes and differential count of all types of leukocytes in blood gives a good indication about the safety of the substances such as drugs or food to be administrated by human and animal [16, 17]. That is why; in this study it was focused on the differential count of leukocytes in peripheral blood of mice post administration with PHB to help in identifying the safety of these substances.

The present study proved that oral administrated of with treatment dose of PHB (1 mg/ kg), PHB nanoparticles (1 mg/ kg), PHB nanoparticles plus cefotaxime (1 mg/ kg), and cefotaxime (1 mg/ kg) do not effect on the total count of leukocytes and percentage of all types of leukocytes. That give another evidence about the safety of these substances (PHB, PHB nanoparticles, PHB nanoparticles plus cefotaxime and cefotaxime) to be using as a treatment substance and that attract us to go further in using of these substances in treating the burn mice model prepared in our laboratory.

Conflict of Interest: The authors declare that there is no conflict of interest regarding this study.

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Ethical committee approval: This work was approval by the ethical committee of Department of Biology, College of Science, University of Baghdad.

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