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HAMATOPROTECTIVE EFFECT OF BIOENCAPSULATED CHIRONOMOUS LARVAE WITH SHALAJIT ON ZEBRA FISH (*DANIO RERIO)* EXPOSED TO IONIZING RADIATION

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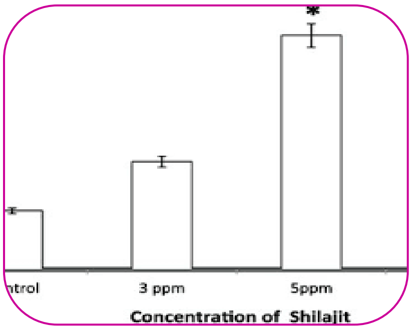
ABSTRACT

The present study aims to evaluate the radio-protective effects of bio-encapsulated chironomous larvae with shilajit on Zebra fish (Danio rerio). The Zebra fish were divided into three experimental groups and control group, each group containing ten fish. The three experimental fish groups, group I, group II, group III were fed with 3,5, and 7ppm shilajit encapsulated chironomous larvae and group IV served as a control fed with non-enriched diet. After 30 days of feeding trial, fish were irradiated with X-Ray at single acute dose of 1 Gy. Haematological parameters were analysed 72 hours of post-irradiation. Among the experimental groups 7ppm shilajit fed fish group shows the most significant radio-protective effects compared with control and other experimental fish groups. The present study indicates that shilajit have exemplary radio-protective capability.



KEYWORDS: *Radio – protective, Bio-encapsulated chironomous larvae, Shilajit.*

INTRODUCTION

This work relates with the application of Zebra fish (*Danio rerio*) to the study of radio biological effects of low doses of ionizing radiation. In recent years, the use of Zebra fish has grown considerably, pointing more and more as a very interesting model in biomedical research, essentially because of the level of homology shared with the human genome, complemented by an easy and reasonably affordable practical side. Haematology is the science of studying the anatomical, physiological and pathological aspects of blood. Blood is fluid tissue contained within the cardiovascular system. The fluid element of blood is plasma and the formed elements of the blood are the erythrocytes, leukocytes, and thrombocytes. The primary functions of blood are (i) oxygenation of tissues, (ii) nutrition of tissues, (iii) maintenance of acid-base balance. (iv) removal of metabolic waste products from tissues. Thus, any dysfunction of blood can have severe effects on the physiological activities of the entire body. Also, certain physiological dysfunctions in the body are reflected as alterations in the blood constituents, which can be used as diagnostic indicators. The knowledge of the haematological characteristics is an important tool that can be used as an effective and sensitive index to monitor physiological and pathological changes due to toxicants or pathogens. There is a world-wide concern about the use of ionization radiations (IR) in the medical field such as X rays, gamma (γ) rays and particles: α-particle, β-particle, protons and neutrons. Radiations are being extensively used in diagnosis as well as in therapeutic use for cancer patients. Therefore, the frequent use of machines by technicians such as X-ray diagnostic units as well as therapy units, CT-scans, PET, (Positron Emission Tomography), SPECT (Single Photon Emission Computed Tomography), gamma cameras, brachy therapy units, 60Cotele therapy units, linear accelerators, dose calibrators, radioimmunoassay counters and different radiopharmaceuticals pose health risks to occupational workers[1,2].Ionization radiations influence human health as they break chemical bonds of the molecules and damage DNA by the production of free radicals and hence proliferative cells can undergo apoptosis [3]. Stem cells are found radiosensitive and their damage by radiations can result into non-performance of functional cells. Long term exposure to even low doses of radiation can affect proliferating cells[4]. Ionizing radiation exposure is sensitive for those tissues (bone marrow, gastrointestinal tract and skin which turnover rapidly as well as to those tissues (central nervous system, lung, heart, liver, kidney and gonads) which turnover slowly[5]. Hematopoietic system is one of the most radiosensitive systems because its functional cells transport oxygen in the blood, which prevent immune system against viruses, bacteria, etc. This system also provides blood coagulation to safe intact blood vessels[6]. Ionizing radiations (IR) can damage stem cells of hematopoietic system which is radiosensitive and as a result alteration in the production of bone marrow, stromal cells is observed which are important to regulate this system. An exposure to IR can reduce bone marrow production where blood forming cells both immature and rapidly dividing cells locate. Dividing cells of the hematopoietic system are highly radiosensitive which divide rapidly and may show effects at even at low doses of long term ionizing radiations such as X-rays or γ-rays[6]. For the last 30 years, the fluoroscopically guided interventional procedures have increased X-ray exposure. These occupational health concerns are quite important to the cardiologists, radiologists, surgeon and other technicians working with the fluoroscopy techniques[7]. Silver nanoparticles (AgNP) have gained attention over the years due to the antimicrobial function of silver, which has been exploited industrially to produce consumer goods that vary in type and application. Undoubtedly the increase of production and consumption of these silver-containing products will lead to the entry of silver compounds into the environment[8]. Contaminant-induced feeding inhibition has direct and immediate consequences at higher levels of biological organization, by depressing the population consumption and thus hampering ecosystem functioning (e.g. grazing, organic matter decomposition). Thus, similarly to lethality and avoidance, feeding is mechanistically linked to ecosystem processes and is therefore an unequivocal ecologically meaningful response. The objective of the present study was to develop a short-term assay with the small freshwater fish *Danio rerio*, based on feeding [9]. The novel object recognition, or novel-object preference (NOP) test is employed to assess recognition memory in a variety of organisms. The subject is exposed to two identical objects, then after a delay, it is placed back in the original environment containing one of the original objects and a novel object. If the subject spends more time exploring one object, this can be interpreted as memory retention. To date, this test has not been fully explored in zebrafish (*Danio rerio*)[10].Sewage effluents contain pharmaceuticals, personal care products and industrial chemicals, exposing aquatic organisms to complex mixtures. The consequences of exposure to combinations of different classes of drugs in fish are largely unknown [11] .Aquatic ecotoxicity assays used to assess ecological risk assume that organisms living in a contaminated habitat are forcedly exposed to the contamination. This assumption neglects the ability of organisms to detect and avoid contamination by moving towards less disturbed habitats, if connectivity exists. In fluvial systems, many environmental parameters vary spatially and thus condition organisms' habitat selection[12] .

MATERIALS AND METHODS

Collection and Care of Experimental fish

The fresh water Zebra fish (*Danio rerio*). Adults were procured from a commercial fish farm and transported to the laboratory in oxygenated bags and released into 10L aquarium tank filled with dechlorinated tap water. The water quality parameters were analysed and maintained within the normal range (pH -7.5, dissolved oxygen -8.2mg/L, temp-25+1̊ C and hardness in terms of CaCo3-220mg/L) and the aeration was continuously provided in all aquaria to maintain the optimum dissolved oxygen by an oil free blower through plastic pipe via air stone regulators attached to each aquarium to adjust pressure of air.

Cleaning and Siphoning

To maintain hygienic condition and prevent pollution caused by remaining feed and faeces, the aquarium was cleaned every day prior to feeding time in morning by siphoning out the excreta and 80% of the water was exchanged to prevent sudden increase in water temperature as the experiment was conducted in summer months. The dead fingerlings, if any, were removed and recorded for calculating the survival rate.

Enrichment of Chironomous larvae with Shilajit

Shilajit (mineral pitch) is white in colour dried matrix purchased from Annai Aravindh Herbals, Chennai, India. Shilajit was dispersed in purified water and used without any further purification. Three different concentrations of shilajit solutions [3ppm (group I),5ppm (group II),7ppm (groupIII), and 0ppm (control)] were prepared in 1liter glass beaker. In each beaker 50 numbers of Chironomous larvae were introduced and allowed for 24 hours for proper enrichment. The same procedure was repeated for 30 days.

Experimental design and feeding schedule

Shilajit enriched Chironomous larvae were introduced into the experimental aquarium tanks (Ten Zebra fish for each experimental group) and control fish group fed with un-enriched Chironomous larvae, for two times a day at (7 h and 16 h). The unfed dead individuals were removed from the experimental tanks. The experimental duration was restricted to 30 days. At the end of the experiment, both control and experimental fish were subjected to irradiated with single acute dose of X-ray.

X-ray irradiation

After 30th day of experiment, fishes were irradiated with a single dose of 1.0 Gy X-ray from a Model Vision 100 X-ray system (100Ma Mobile X-ray Machine Serial No: V14152479). This is a non –filtered X-ray source and this dose was delivered by 100 keV over a 5-min period (a dose rate of 0.2Gy/min). The X-ray irradiation facility utilized at The Excellent Clinical Research Laboratory, Chennai, recognized by Atomic Energy Regulatory Board (AERB, Govt of India; AERB Authorization No: AERB/18/02).

Haematological parameters

Blood from fish was drawn through caudal venous puncture on each individual fish using a heparinized micro-haematocrit capillary tube of 2ml. To count the peripheral blood leukocytes, blood smears were prepared on clean glass slides. Blood smears were air dried, fixed in cold methanol for 5minutes and stained with Leishman stain (Monocytes. Lymphocytes and Neutrophils were identified and counted based on size and staining characteristics. Using Natt and Herick□s stain solution, the red and white blood cells (RBC and WBC) were counted under a Neubaur-hematocytometer. Haemoglobin was assessed by cyanmethemoglobin at 540 nm. Haematocrit (HCT) was measured by micro-centrifuge procedure and heparinized tubes. Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and Mean Corpuscular Volume (MCV) was calculated according to the following equations. The following parameters RBC, WBC, HCT, HB, MCV, MCH, MCHC, Monocytes, Lymphocytes, Neutrophils. Were analysed in the fish fed with bio-encapsulated Chironomous larvae with different concentrations of Shilajit and the results were compared.

Results

Hematoprotective efficacy of shilajit was evaluated by feeding Zebra fish with shilajit enriched chironomous larvae Fishes were fed with three different concentrations of shilajit (3,5 and 7 ppm) enriched chironomous larvae for 30 days, control group was fed with chironomous larvae without enrichment. Experimental animals fed with enriched diet were subjected to X-ray irradiation. Haematological parameters showed prominent hemato-protective efficacy of shilajit with the highest concentration (7ppm) tested. There was a significant increase with respect to monocyte count, neutrophil count, MCH& MCV with fish group fed with 3ppm shilajit enriched Chironomous larvae, whereas the lymphocyte count decreased in the same group. The values of parameters tested with higher concentration of shilajit were closer to the values of concentration (3ppm) tested. The present study confirms the role of shilajit in delivery hemato-protective effects against the X-ray irradiation.

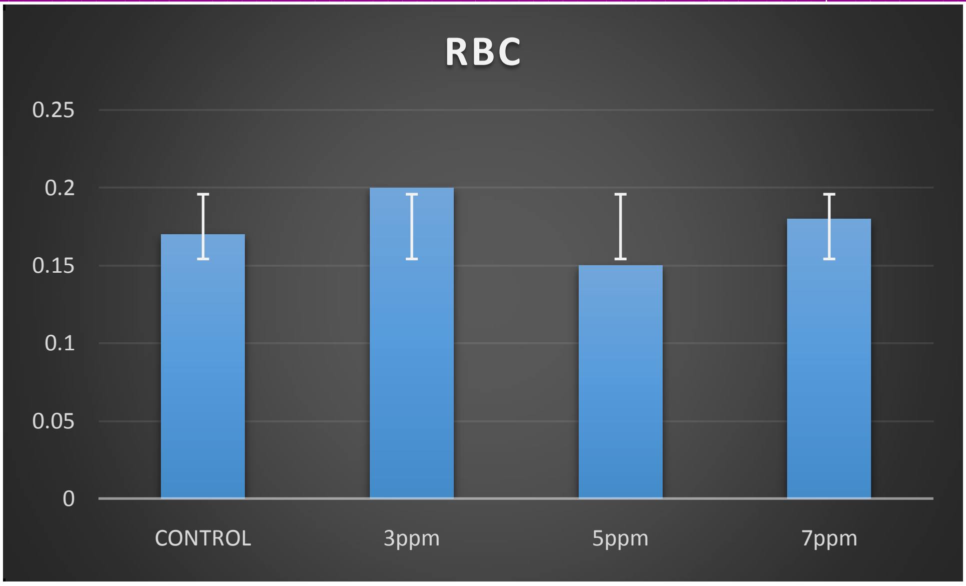


Figure 1. Red blood cells parameters of X-ray Zebrafish fed with bio encapsulated Chironomous larvae with Shilajit

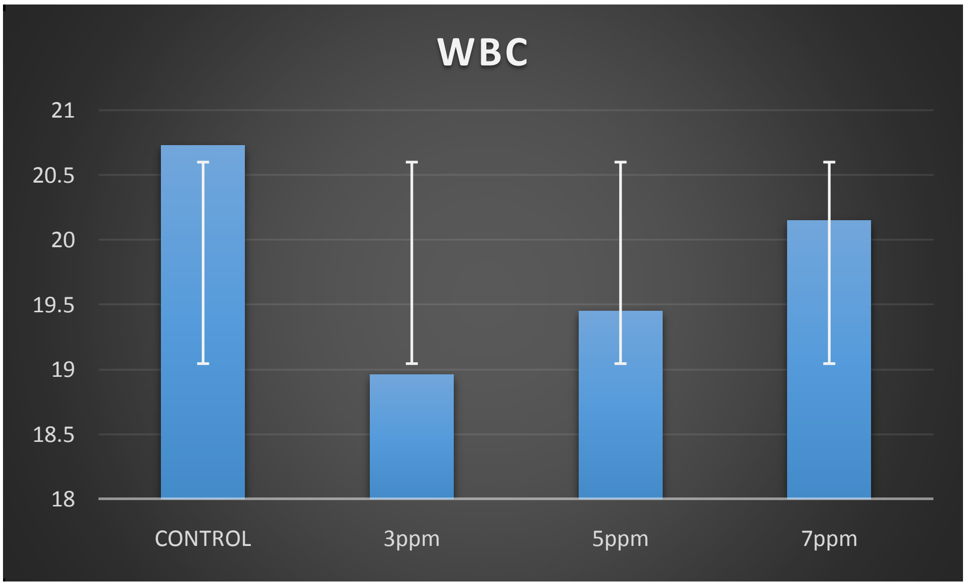


Figure 2. White blood cells parameters of X-ray Zebrafish fed with bio encapsulated *chironomous* larvae with Shilajit

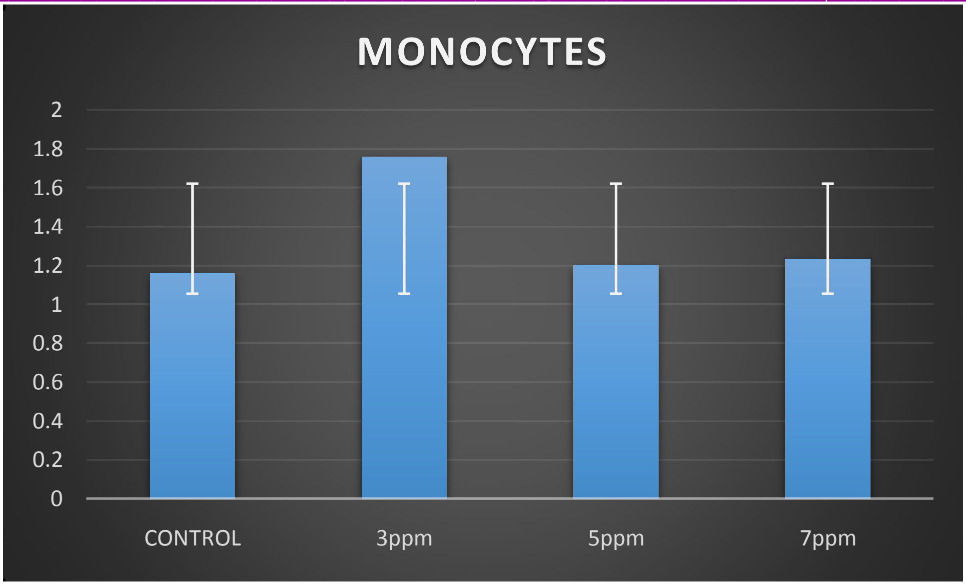


Figure 3. Monocytes parameters of X-ray Zebrafish fed with bio encapsulated *Chironomous* larvae with Shilajit

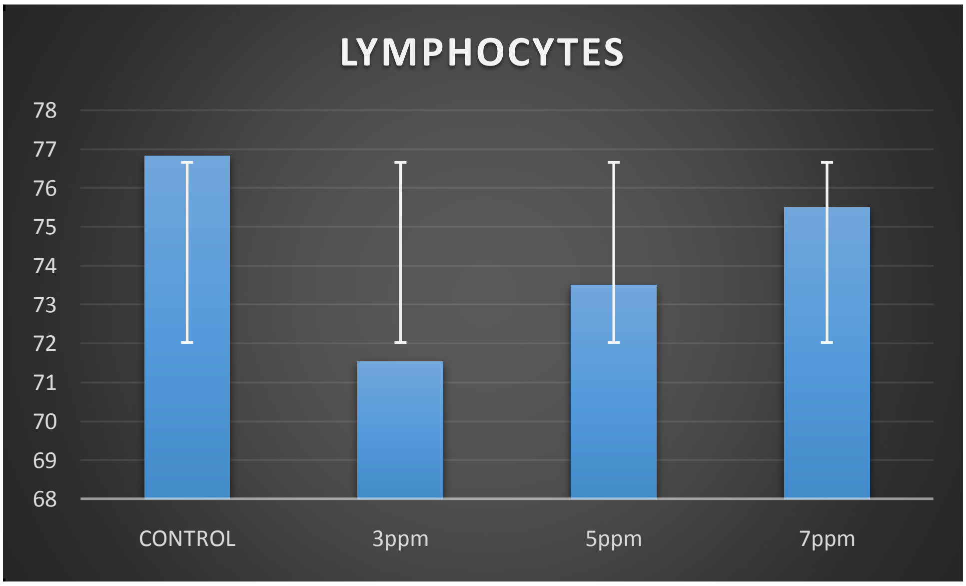


Figure 4. Lymphocytes Parameters of X-ray Zebrafish fed with bio encapsulated *Chironomous* larvae with Shilajit.

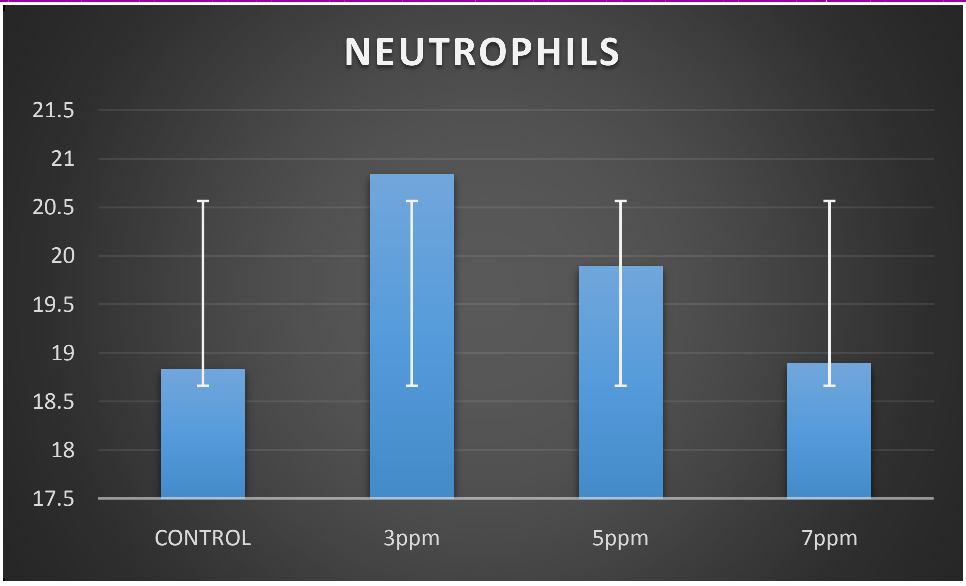


Figure 5. Neutrophils parameters of X-ray Zebra fish fed with bio encapsulated *Chironomous* larvae with Shilajit

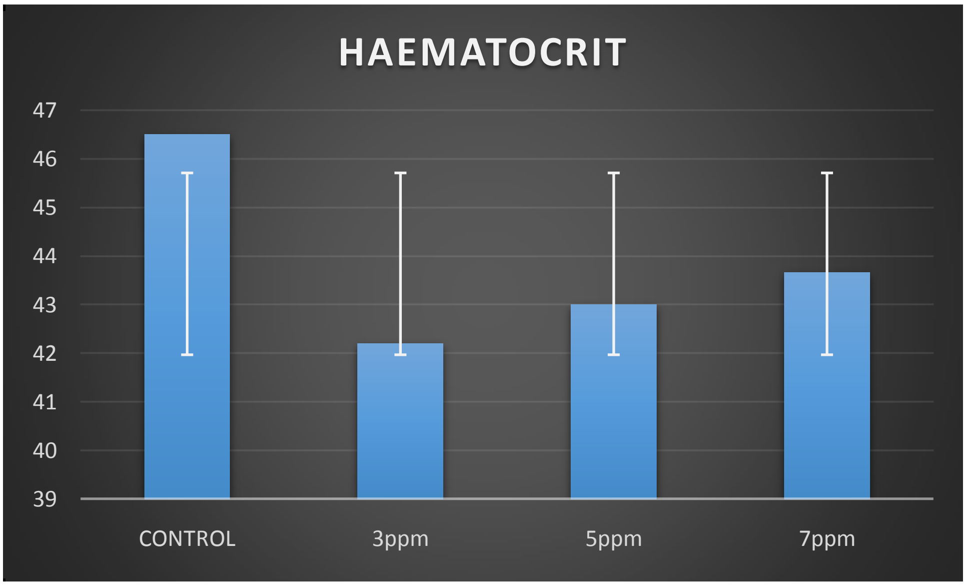


Figure 6. Haematocrit parameters of X-ray Zebrafish fed with bio encapsulated *Chironomous* larvae with Shilajit.

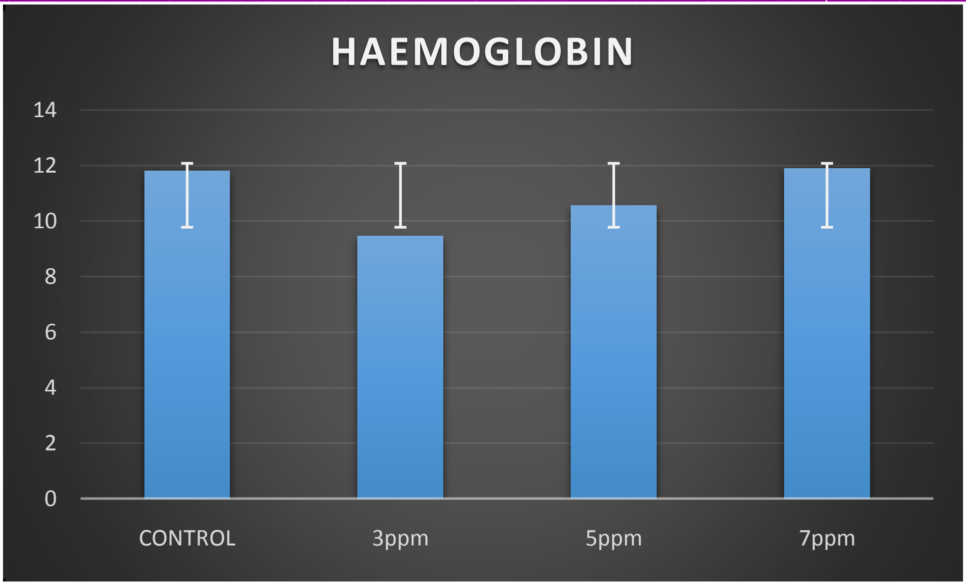


Figure 7. Haemoglobin parameters of X-ray Zebra fish fed with bio encapsulated *Chironomous* larvae with Shilajit

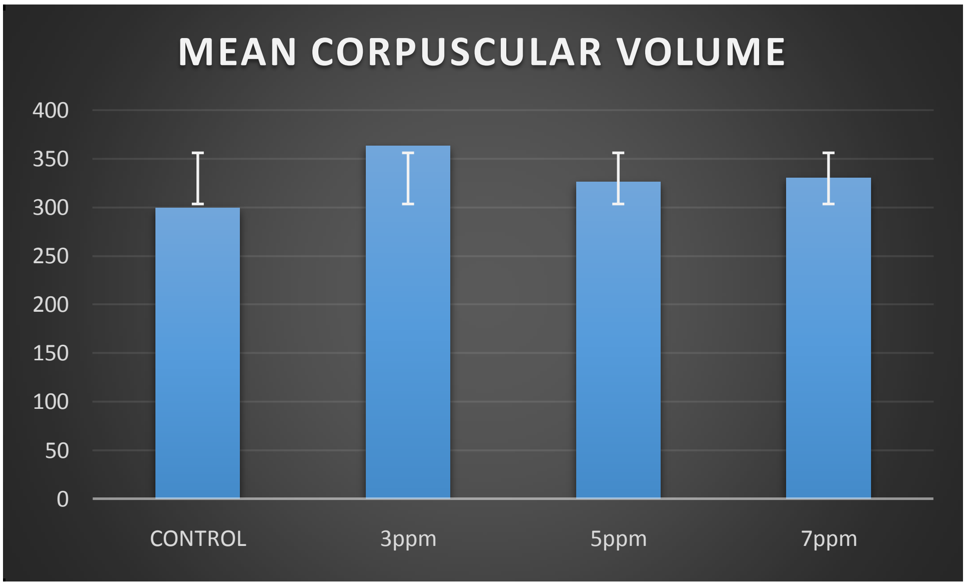


Figure 8. Mean Corpuscular Volume parameters of X-ray Zebra fish fed with bio encapsulated *Chironomous* larvae with Shilajit

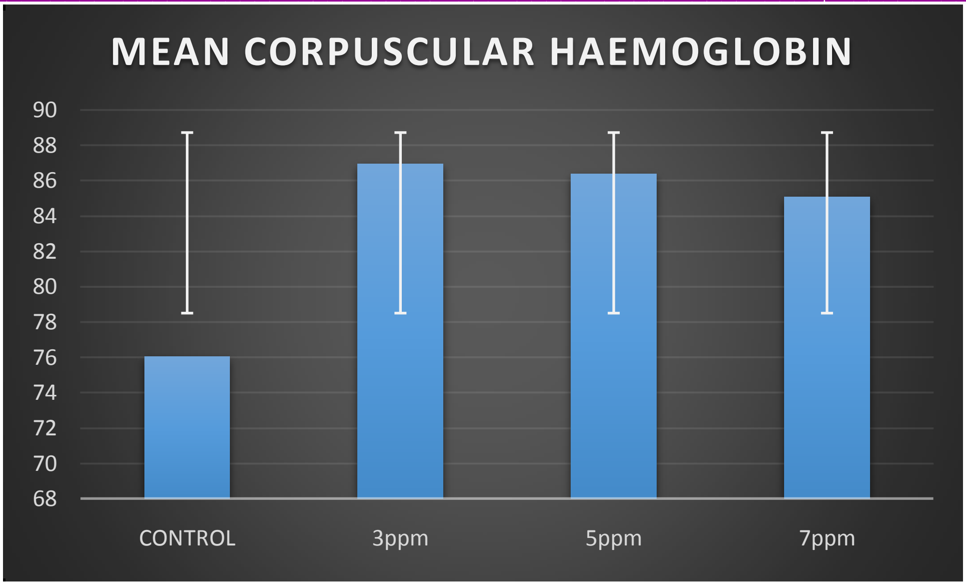


Figure 9. Mean Corpuscular Haemoglobin parameters of X-ray Zebra fish fed with bio encapsulated *Chironomous* larvae with Shilajit

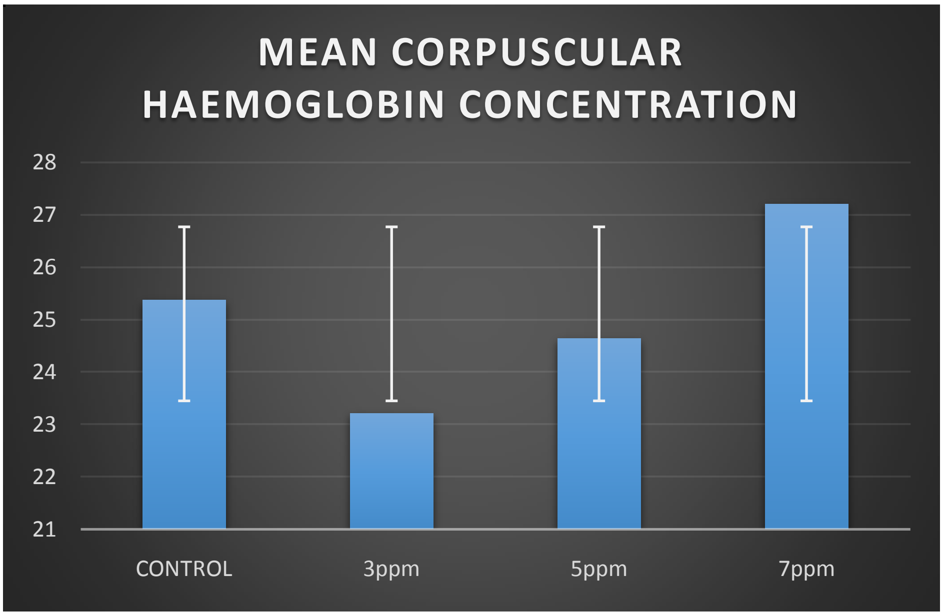


Figure 10. Mean Corpuscular Haemoglobin Concentration parameters of X-ray Zebra fish fed with bio encapsulated Chironomous larvae with Shilajit.

DISCUSSION

There is a world-wide concern about the use of Ionizing Radiations (IR) in the medical field such as X-rays, gamma (γ) rays and particles: α-particle, β-particle, protons and neutrons. Radiation are being extensively used in diagnosis as well as in therapeutic use for cancer patients. Therefore, the frequent use of machines by technicians such as X-ray diagnostic units as well as therapy units, CT-scans, PET (Positron Emission Tomography), SPECT (Single Photon Emission Computed Tomography), gamma cameras, brachy-therapy units, $60C^{o}$ tele therapy units, linear accelerators, dose calibrators, radio immunoassay counters and different radio pharmaceuticals pose health risks to occupational workers [1,2]. Ionizing Radiations influence human health as they break chemical bonds of the molecules and damage DNA by the production of Free radicals and hence proliferative cells can undergo apoptosis[3]. Stem cells are found radiosensitive and their damage by radiations can result into non-performance of the functional cells. Long term exposure to even low doses of radiation can affect proliferating cells[4]. Ionizing Radiation exposure is sensitive for those tissues which turn over rapidly as well as to those tissues which turn over slowly[5]. Ionizing Radiations (IR) can damage stem cells of hematopoietic system which is radio sensitive and as a result alteration in the production of bone marrow stromal cells is observed which are important to regulate this system. An exposure to IR can reduce bone marrow production where blood forming cells both immature and rapidly dividing cells locate. Dividing cells of the hematopoietic system are highly radiosensitive which rapidly and may show effects at even at low doses of long term ionizing radiations such as X-rays or γ-rays [6]. It has been observed that long term use of ionizing radiations of radiation workers can affect their immune system even at low doses of radiations as Leukocytes and lymphocytes are highly radiosensitive. Granulocytes may show an initial increase before they deplete and there would a gradual decline in the HCT and red cell count occurs after the exposure of moderate doses. It is known that ionizing radiations affects bone marrow and lymphocytes can decline along with a temporary sharp increase in granulocytes. This suggests that radiations can disturb immunity of those workers who are long term exposed to low doses of ionizing radiation. Because white blood cells, lymphocytes are the most sensitive to X-ray radiations therefore, lymphopenia can be happening depends on the absorbed dose. It was reported in some studies, that lymphocytes of radiation workers were slightly lower than the control group although the difference was not statistically significant. Radiological workers were found under the influence of the gradual decline of neutrophils, leukopenia and lymphopenia due to the modulations or alterations in leukocytes. Another investigation also indicated that the absolute number of peripheral blood lymphocytes was slightly lower than for radiology department workers as compared to the control group but there was no statistical significance found. It has been discussed that the decreased number of circulating lymphocytes and granulocytes by radiations can lead to infections and long-term work on radiations can induce anaemia. Therefore, long-term damage caused by IR can induce various haematological diseases[13,14,15]. Silver nanoparticles (AgNP) have gained attention over the years due to the antimicrobial function of silver, which has been exploited industrially to produce consumer goods that vary in type and application. Undoubtedly the increase of production and consumption of these silver-containing products will lead to the entry of silver compounds into the environment[8]. Contaminant-induced feeding inhibition has direct and immediate consequences at higher levels of biological organization, by depressing the population consumption and thus hampering ecosystem functioning (e.g. grazing, organic matter decomposition). Thus, similarly to lethality and avoidance, feeding is mechanistically linked to ecosystem processes and is therefore an unequivocal ecologically meaningful response. The objective of the present study was to develop a short-term assay with the small freshwater fish *Danio rerio*, based on feeding[9]. The novel object recognition, or novel-object preference (NOP) test is employed to assess recognition memory in a variety of organisms. 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In fluvial systems, many environmental parameters vary spatially and thus condition organisms' habitat selection [12].

CONCLUSION

Ionizing radiation passing through the living tissues generates reactive free radicals. These free radicals can interact with critical macromolecules, such as DNA, proteins or membranes, and can induce cell damage and potentially, cell dysfunction and death. Radio protective agents are synthetic compounds or natural products that are immediately administrated before irradiation to reduce injuries caused by ionizing radiation. So far, there is no study reported on the impact of Shilajit on innate immune response and radio-protective efficacy against ionizing radiation. The investigation was performed to elucidate the radio-protective effects of the traditional Indian medicine Shilajit exposed by X-ray using Zebra fish (*Danio rerio*) as a model organism. Among the experimental groups 7ppm Shilajit enriched diet fed fish group shows the most significant radio-protective effects compared with control and other experimental fish groups. The present study indicates that Shilajit have exemplary radio-protective capability. Thus, Shilajit could serve as a good source of radio-protective agent for patients who undergo treatment using ionizing radiations.

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