Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **3 (3):** 178-188 (2015)

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Research Article

OPEN ACCESS

Bone Tissue Alteration in Guinea Pig Due to Refinery Sludge Insultion

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ABSTRACT

This study focused on to evaluate effects of oral as well as intraperitoneal administration of various doses of refinery sludge on the bone histology in a guinea pig model. Adult guinea pigs were randomly selected to which refinery sludge was administrated in a dose range of 1.5-6.0mg/kg body wt. for a period of 3 weeks. The bone of interest from this animal model were fixed and decalcified. After being dehydrated with ascending grades of ethanol and xylene,the specimens were embedded in paraffin. These specimens were cut into serial sections 5 μm thick each in the frontal plane passing through the midline between the medial and lateral menisci. These sections were subjected to haematoxylin and,safranin-O-fast green, Masson's trichrome and toluidine blue staining. Histopathological analyses showed that refinery sludge insulted guinea pigs showed decrease in bone cell population density and cartilage degeneration and necrosis. Dose dependent and time dependant refinery sludge insulted might have developed lesion in the bone of guinea pigs,could well be inferred from this study,however demands further evaluation.

Key words: *sludge,guinea pig,histopathological,bone,cartilage*

INTRODUCTION

Refinery sludge is made up of thick, viscous mixture of sediments, water, oil, hydrocarbon concentration and considerable quantities of solid particles; which are very complex in nature and complexity depends on the source[1,2,3]. The sludge is produced by encountered during croude oil refining, cleaning of oil storage vessels and refining-waste water treatment. The processing activities of one kilogram of crude oil can generate 10-20 grams of oily sludge[4]. India, US EPA and OECD countries designated oily wastes as hazardous wastes[5,6]. Oil sludge is mainly composed of alkanes, asphaltenes, resin, sulphides, and polycyclic aromatic hydrocarbons (PAHs) of 4, 5, 6 and more rings, in over 10-20 fold concentration[7,8,9,10].

PAHs represent a serious environmental hazard due to the fact that they have high persistence in the environment, low biodegradability and high lipophility[11]. The BTEX (benzene, toluene, ethyl benzene and xylene) are the main components of the refinery sludge which are of serious concern due to their toxicity and as carcinogenic compounds[12,13]. This refinery sludge contaminant enters the environment as a result of human activities, which includes deliberate dumping, improper treatments and managements,storage,transportation and landfill disposal.

The environmental impact of oil sludge contamination includes physical and chemical alteration of natural habitats,lethal and sub-lethal toxic effects on aquatics and terrestrial ecosystem;which over the years have been reported as being genotoxic[14,15,16].

The aims of this study were to describe the histopathologic features as well as examined the alterations of structural cellular architecture to the guinea pigs bone caused by long term low-dose refinery sludge treatment and to identify the effects of refinery sludge on choreographic image of the bone.

The guinea pig is a small,stocky,tailless rodent most extensively used in biomedical research and diagonosis of infectious diseases[17,18]. Previous workers suggest that the components of refinery sludge caused a significant reduction of the haematocrit values of guinea pigs.The crude oil influence the unscheduled mitochondrion DNA synthesis and alteration of Ca^{2+} concentration gradient and finally inhibition of Ca^{2+} influx into the cytosol[19,20]. The acute toxicity of conventional crude oil results reduced fetal weight, decreased litter size, decreased fetal weight, incomplete ossification of nasal bones and caudal centra and increased incidence of pup mortality during lactation with respect to dose increase[21].

From the histological and cellular point of view, bone is a highly vascularized and innervated, mineralized conjunctive tissue, which is structured in lamellae of calcified osteoid matrix. The arrangement of these lamellae determines whether the bone is cortical or cancellous;which are composed of osteons. Cortical or compact bone containing osteocytes, is arranged concentrically around Haversian Canals. Cancellous or trabecular bone is formed by a network of bone lamellae, delimiting areolar cavities inside which the bone marrow is found[22]. Long bones of guinea pig are composed of an outer layer of dense compact bone and an inner meshwork of trabecular bone, which is particularly abundant in the epiphyses, and bone marrow[23].

Histologically, the osteoporosis like bone disease is characterized by chondrocyte and proteoglycan loss,extensive degeneration of the articular cartilage,fibrillation,chondrocyte cloning and osteophyte formation[24]. This multifactorial skeletal disorder also characterized by decreased bone mass,deteriorated microarchitectur that led to increase risk of fracture is generally viewed as resulting from a combination of age-related,hormonal,dietary,life style and genetic factors,all of which can lead to reduced bone mass[25]. Some researchers suggested that microdamage to subchondral bone by mechanical overload was led to bone sclerosis and influence the bone turnover;resulted in the deterioration of subchondral bone[26]. The abnormality of the subchondral bone influence the cartilage degeneration due to their cushion-and-chair analogy[27]. This cartilage degeneration features were analysed using the scoring system developed by a group of researchers. The following eight parameters were graded: loss of superficial layer, erosion of cartilage, fibrillation and/or fissures, loss of stainable proteoglycan, disorganization of chondrocytes, loss of chondrocytes, cluster formation and exposure of subchondral bone[28,29].

MATERIALS AND METHODS

Experimental animal and design:

Twenty four adult male and female guinea pigs (*Cavia porcellus*, L.), of 2 – 3 months old with body weight between 250 to 350 g were obtained from North Eastern Hill University, Shillong were selected and randomly divided into four different groups, 6 in each group . All animal experiments were conducted according to the research protocols approved by Gauhati University institutional Animal Ethics Committee(certificate number IAEC/PER/2012-13/173). The animals were housed in polypropylene cages at 22^{o}C on a 12 h light/dark cycle and provided with standard pelleted feed.

Food and water were provided ad libitum. Animals of Group I (controls) were normal fed with distilled water. Animals of Group II were oral as well as intraperitoneally injected 1.5mg/kgbw while Group III and Group IV animals had injected 3.0 mg/kgbw and 6.0 mg/kgbw of test chemical respectively. The procedure was repeated daily between 9.00-10.00 am for 21 consecutive days.

Toxicity study:

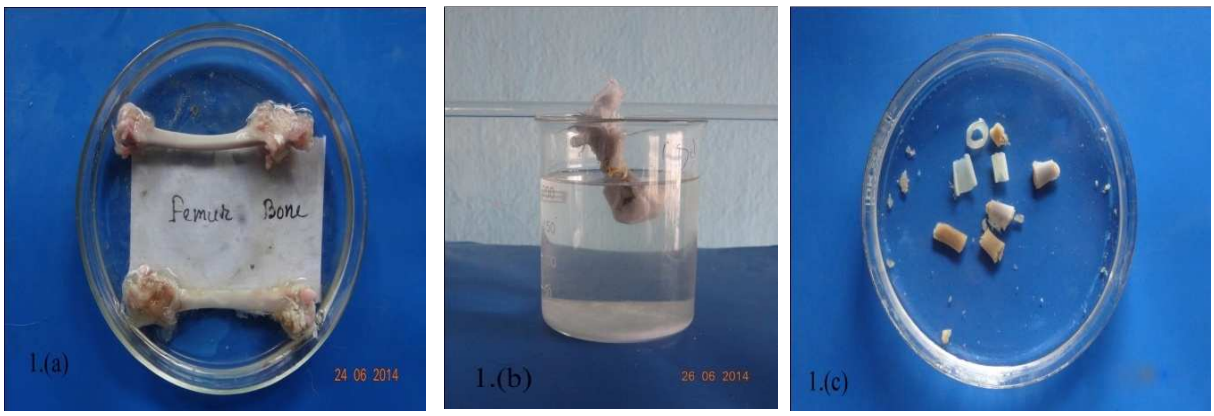
For toxicity studies, a group of 18 adult healthy normal guinea pig were treated with a dose of 20 mg/ kgbw the LD50 was determined(by Probit methods). The test chemical did not show any sign of acute toxicity or mortality over the observation period of 21 days below the dose level of 20 mg/bd wt when administered orally as well as intraperitoneal administration to adult guinea pig. Therefore, it was assumed that the chemical was devoid of any acute toxic effect proving their wide margin of safety up to the dose level used in the present study.

Tissue preparation for Histological Analysis :

Methods of fixation, decalcification and embedding:

On day twenty two, 24 hrs after last dose of sludge administration, the guinea pigs were anaesthetized with ether than sacrificed. The target specimens (The left and right femur and tibia were stripped of tendon and musculature) and bilateral knee joints were excised for histopathologic evaluation. The knee joints at 80-90^{o} of flexion was fixed in 10% neutral buffered formalin solution for 3 days and decalcified at 4 ^{o}C for 5 days, after neutralizing with 5% $NaSO_4$ at room temperature for 1 day, the sample was dehydrated and embedded in paraffin by the standard method. For bone histologic analysis slightly different protocol was followed. The femur and tibia were fixed in a neutral-buffered 10% formalin for 24 hrs, decalcification with either a solution of 10% EDTA, cut into the medial and lateral plateaus and embedded in paraffin wax at 56 ^{o}C and finally cast in paraffin blocks. Division of the samples was made with a microtome (Leica Biosystems, Germany) at a thickness of 5 μm with randomly prepared 4-6 histological sections were cut through each central portion of the plateau with a constant interval of 100-120 μm and these sections were displayed on a glass slab that was previously coated with a thin layer of Mayer albumin. These paraffin section stained with haematoxylin-eosin and Safranin O(0.04% in 0.1 M sodium acetate buffer, pH 4) staining for the general architecture of the bone[30]. Another histological staining procedures for toluidine blue(0.04% in 0.1 M sodium acetate buffer ,pH 4);stain utilized for optimal demonstration of mineralized bone,osteoid seams,osteoblast and osteoclasts. Masson trichrome method stains differently a mineralized bone (green/blue) and an osteoid (red/orange)[31]. Osteoclasts were identified as multinucleated cells [32]. All of the samples were subjected to optic microscopic (Olympus BX-60) examination at 21 days to evaluate differences in the bone structure.

Fig.1 (a,b,c): Photographs of Femur bone of Guinea pig during decalcification time



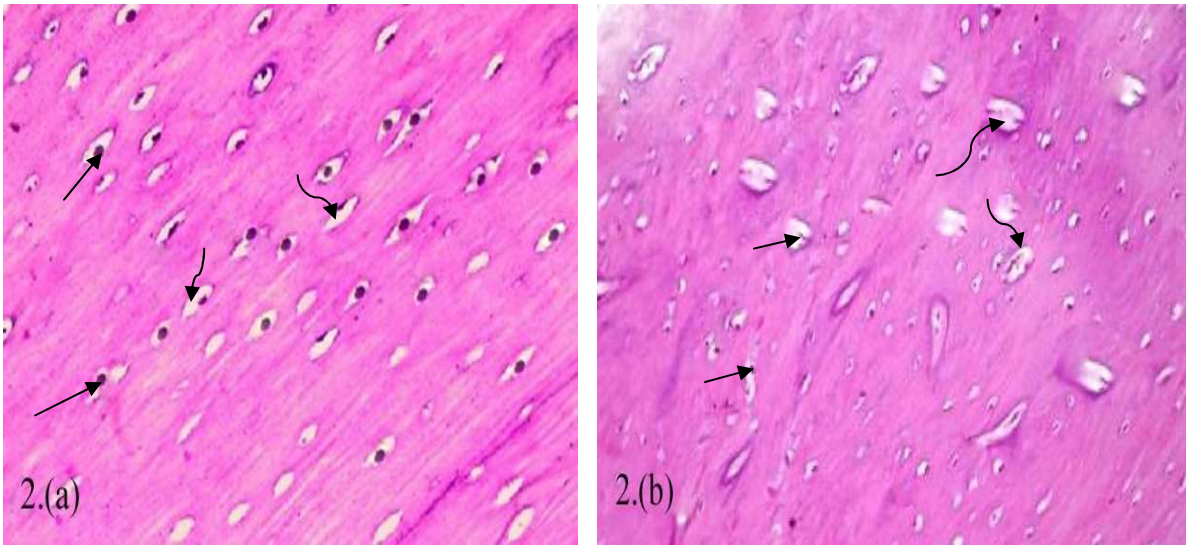
RESULTS

Macroscopic images of surfaces of guinea pig knee joins, were examined grossly in control as well as treated group animals.The control group animal having normal surface fissuring and increasing severity with surface ulcerations, pitting lesions on the surface (medium dose treated groups) to severe full depth very badly erosion to bone surface with respect to the dose increases.

Histological examination of haematoxylin and eosin staining, the sludge treated group showed histological characteristics of the necrotic bone. It showed pyknotic nuclei of osteocytes and empty lacunae. The numbers and size of empty lacunae were markedly increased compared to control group.

Fig.2 (a): Histologic photographs of the section of a control adult guinea pig tibial diaphysis showing normal bone architecture with osteocytes (⟶) resided in their lacunae (↗) [H&E;original magnification x400]

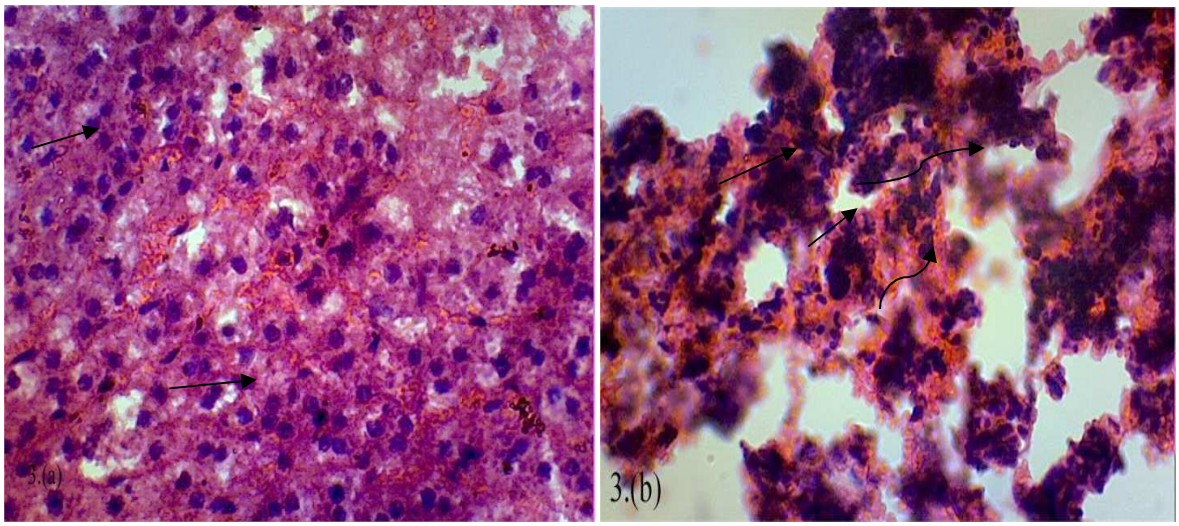
Fig.2 (b): Histologic photographs of the section of a refinery sludge treated adult guinea pig tibial diaphysis showing necrotic bone architecture with pyknotic nuclei of osteocytes (→) and increased number of lacunae (↗) [H&E;original magnification x400]



Bone cells are heavily populated in control whereas in treated condition these were less and osteoclast (multinucleated cells) like cells were clumped together.Bone trabeculae appeared very thin and discontinuous. Resorption cavities were observed in the cortical bone in treated group animals.Widening of bone marrow spaces was also seen. Erosion cavities and faint staining of bone trabeculae were frequently seen.

Fig.3(a):Images of light micrograph of section of a control adult guinea pig of femur hypophysis showing normal bone cell population (⟶) (H&E;original magnification x400)

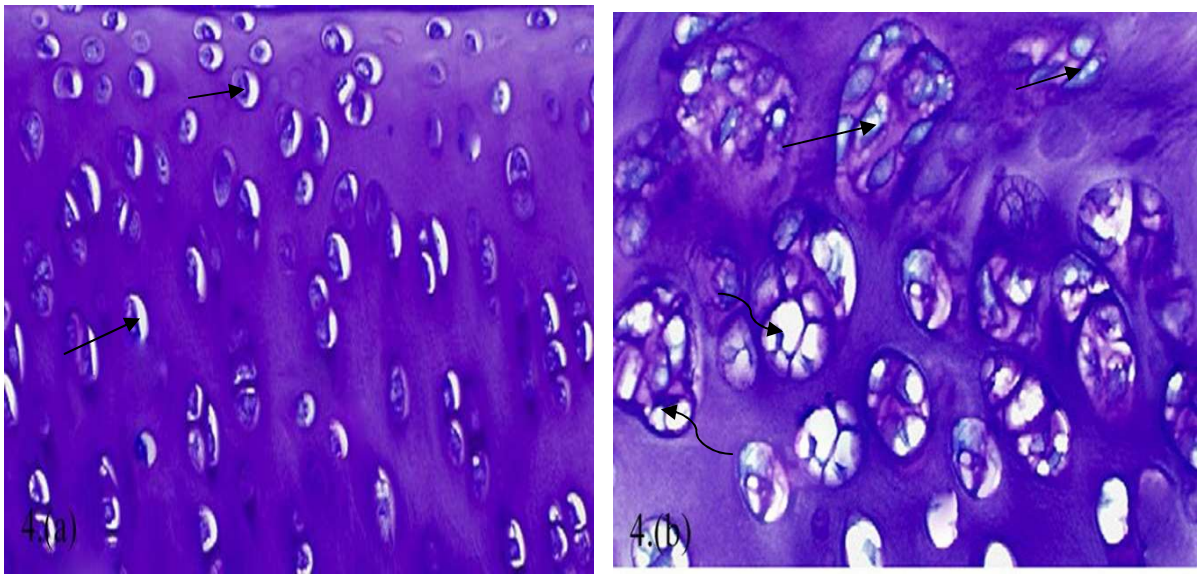
Fig.3(b): Images of light micrograph of section of a refinery sludge treated adult guinea pig represents cellular alterations recorded in bone cell population,clumped together,discontinuous and widely separated bone trabeculae (⟶) and bone marrow (↗) spaces is also seen. (H&E;original magnification x400)



Histological as well as cellular scoring for knee joint by toulidine blue staining shows that in control guinea pig animals present normal (1/2 cells/lacuna) cells but in treated group animal possess some regions of hypercellularity as well as clustering with respect to the doses of test chemical increases.

Fig .4(a): A photomicrograph of control adult guinea pig knees demonstrating the normal cellular level with (1/2 cells/lacuna) (→) [Toulidine blue stain;original magnification x250]

Fig.4(b): A photomicrograph of refinery sludge treated(6.0mg/kgbdwt) adult guinea pig knees demonstrating the abnormal cellular architecture with hypercellularity (→) and clustering each other (↗) [Toulidine blue stain;original magnification x250]



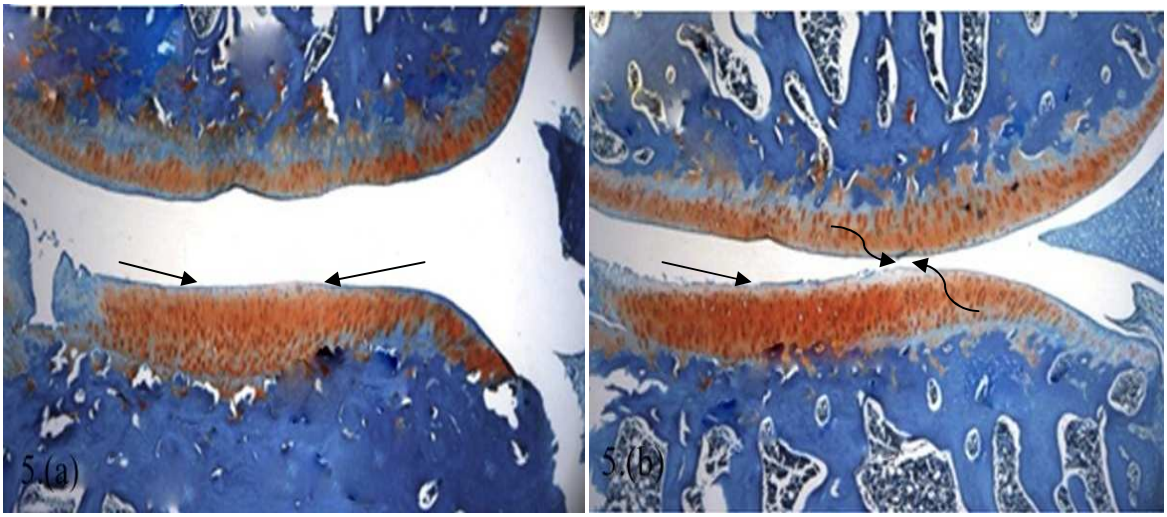
Cartilage destruction :

In control groups, the medial tibial plateau articular cartilage not covered with the meniscus had a smooth surface, and no abnormality was noted in chondrocytes or extracellular matrix.In sludge treated guinea pigs, number of chondrocytes was diffusely decreased in the tangential zone, and fibrillation expanded to the radial zone. In control group animals normal smooth, uninterrupted surface was seen.

In treated group animals irregular surface, 1-2 superficial clefts (fissures) and/or loss of cartilage extending into the deep zone. The distance between the osteochondral junction and the bone marrow cavity was decrease in treated group animals compare with the control sample.Histologically subchondral bone plate thikness and chondrocyte loss extending into the upper middle zone,fibrillation and proteoglycan loss was significantly varies from control to the treated animals.(Safranin-O/fast/hematoxylin)

Fig.5(a): Histologic findings demonstrating the levels of cartilage structure in control adult guinea pig medial tibial plateau articular cartilage not covered with the meniscus has smooth surface (→) [Safranin-o/fast green/haematoxylin stain;original magnification x20]

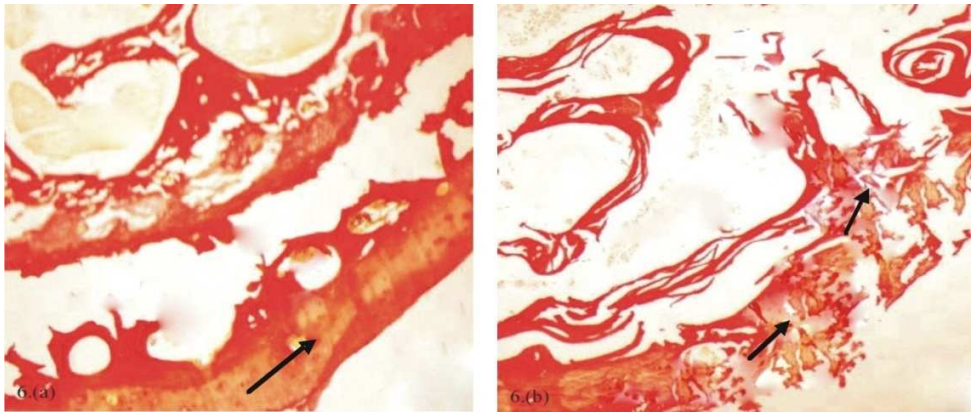
Fig.5(b): Histologic findings demonstrating the levels of cartilage structure in refinery sludge treated guinea pig(6.0mg/kgbdwt) having irregular surface (→with extending into the deep zone (↗) [Safranin-o/fast green/haematoxylin stain;original magnification x20]



Actually cartilage degeneration is characterized by the breakdown of collagen fiber and loss of proteoglycan. In our study showed that the arrangements of collagen fiber in extracellular matrix of bones. In treated group animals collagen fiber were less in exposed in compare with control sample. The less collagen in cortex and very thin collagen fiber formation in exposed bones.

Fig.6(a): A photomicrograph of collagen of femur cortex in control adult guinea pig showing normal and thick collagen fiber (→) [H&E;original magnificationx40]

Fig.6(b): A photomicrograph of collagen of femur cortex in refinery sludge treated adult guinea pig showing abnormal and very thin, discontinuous collagen fiber (→[H&E;original magnificationx40]

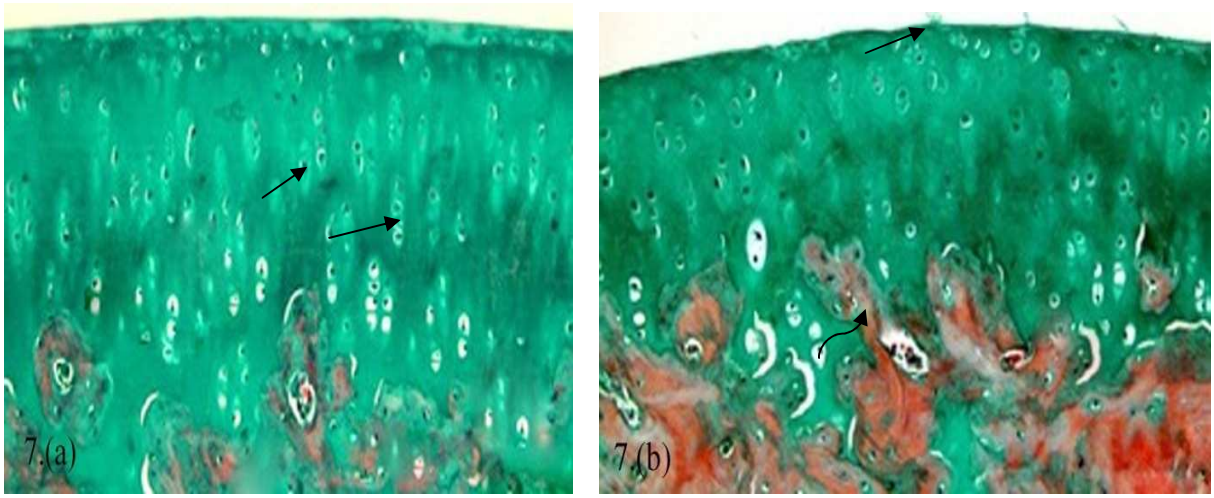


Histological evaluation of structure of articular cartilage from femoral condyle in control guinea pigs stained with Masson's trichrome showed no histological changes and uniform throughout articular cartilage ,a normal cellularity and extracellular matrix. In treated group animals (high dose) displayed loss of superficial layer,erosion of cartilage,lesion extends into the deep zone of uncalcified cartilage,chondrocyte hypertrophy and more obvious chondrocyte loss observed.

Fig.7(a): Histologic photographs of articular cartilage of control adult guinea pig showing normal and uniform cellularity and external matrix (→) [Masson's trichrome stain;original magnification x250]

Fig.7(b): Histologic photographs of articular cartilage of refinery sludge treated adult guinea pig showing abnormal superficial layer (→) with chondrocyte hypertrophy (↷).

[Masson's trichrome stain;original magnification x250]



DISCUSSION

In the present work, bone affection was found at a macroscopic or gross anatomic assessment in refinery sludge treated animals. The treated animal shows that the abnormal surface fissuring and increasing severity with ulcerations, badly erosion to the bone surface with respect to the test chemical increases. This finding precisely agree with the findings of the previous workers whose has reported that the refinery sludge causes birth defects, nervous disorders, and liver disease, depression, irregular heartbeats etc[33,34,35,36]. Some worker also explain toxicological affects of refinery sludge by its metabolic results produce epoxide compounds with mutagenic and carcinogenic that affects the skin, blood, immune system, liver, spleen, kidney, lungs, developing foetus, it also causes weight loss[37,38,39].

In our study, bone alteration was found at a cellular level in refinery sludge treated animals by microscopic observations. The bone sections revealed marked and significant thinning of the outer shell of compact bone. Histological characteristics of the necrotic bone was shown as apoptotic osteocytes with pyknotic nuclei and empty lacunae. The numbers and size of empty lacunae relatively increased compared with control group animals. Many researchers and investigators reported that, components of refinery sludge have been found to be cytotoxic and mutagenic effects into the cellular level, which is dominated by apoptosis.

In this current study, we found that bone cells are heavily populated and having normal cellular architecture in control group animals; where as in treated condition these were less and osteoclast like cells were clumped together and also seen very thinned bone trabeculae and discontinuous in some areas with erosion cavities and dimly stained bone marrow due to the widening of bone marrow.

This abnormal bone marrow spaces is the clinical characteristics of osteopenia. These findings can be attributed to agree with other workers already examined that osteopenia as a complication for childhood malignancies which is characterized by reduced bone marrow density and increased fracture risks[40,41]. This osteopenia would lead to the osteoporosis characterized by cancellous bone became manifested as thinning and focal disruption of the trabeculae followed by progressive enlargement of the perforations and total loss[42,43,44] .

In our experiment, it is found that osteochondral junction and the bone marrow cavity was decrease in treated group animals followed by irregular surface, superficial clefts and loss of cartilage extending into the deep zone. This thinner subchondral bone plate indicating less bone involvement and severe destruction of the articular surface.

This finding coincided with previous workers who has reported that cartilage destruction and subchondral bone sclerosis is most pronounced in the central medial condyle which is most commonly affected by osteoporosis[45].

In this study we found that the arrangements of collagen fiber in extracellular matrix of bones in treated group animals were less and very thin collagen fiber formation with respect to the control group animals. This findings to consent with previous workers has suggested that calcium binding receptor (glycoprotein) of plasma membrane plays important role in calcium influx which helps for the increase in osteoblast number and its biosynthetic product collagen[46]. Other workers explained that reduction of collagen fibers in bone cortex may be due to reduction of osteocytes and chondrocyte proliferation[47].

In our present study, these findings provide direct evidence that the histological changes observed by Masson trichrome stain, cellular changes in particular are mainly caused by chondrocyte death and it influences to the decreases to the number of osteoblast and osteoclast population. Our findings supported by other researchers who has reported that differentiation of osteoblast is one of the key events of bone formation[48,49] .

CONCLUSION

In conclusion, from the foregoing, it is observed that an increase in oxidative trauma by refinery sludge pathologically will result in the destruction of the macromolecular structure as well as cellular architecture of the bone histology. Dose dependent and time dependant refinery sludge insulted might have developed lesion in the histology of guinea pigs, could well be inferred from this study, however, demands further evaluation.

Acknowledgement

We would like to thank the authors, publishers, government and non- governmental agencies whose works had been cited.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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