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Microcystin-leucine arginine (MC-LR) induces bone loss and impairs bone micro-architecture by modulating host immunity in mice: Implications for bone health☆

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ABSTRACT

Osteoporosis or enhanced bone loss is one of the most commonly occurring bone conditions in the world, responsible for higher incidence of fractures leading to increased morbidity and mortality in adults. Bone loss is affected by various environmental factors including diet, age, drugs, toxins etc. Microcystins are toxins produced by cyanobacteria with microcystin-LR being the most abundantly found around the world effecting both human and animal health. The present study demonstrates that MC-LR treatment induces bone loss and impairs both trabecular and cortical bone microarchitecture along with decreasing the mineral density and heterogeneity of bones in mice. This effect of MC-LR was found due to its immunomodulatory effects on the host immune system, wherein MC-LR skews both T cell ($CD4^+$ and $CD8^+$ T cells) and B cell populations in various lymphoid tissues. MC-LR further was found to significantly enhance the levels of osteoclastogenic cytokines (IL-6, IL-17 and TNF-α) along with simultaneously decreasing the levels of anti-osteoclastogenic cytokines (IL-10 and IFN-γ). Taken together, our study for the first time establishes a direct link between MC-LR intake and enhanced bone loss thereby giving a strong impetus to the naïve field of "osteo-toxicology", to delineate the effects of various toxins (including cyanotoxins) on bone health.

1. Introduction

Osteoporosis is a progressively common pathological condition of bones affecting more than 200 million individuals worldwide (Laird et al., 2017). Osteoporosis leads to reduced quality and density of bones which in long run results in weakened skeleton leading to higher risk of fractures the prime cause for increased morbidity and mortality (Dar et al., 2018a). In addition, osteoporosis will take a heavy toll on the economy with an estimated burden of USD 131.5 billion worldwide by 2050 (Dar et al., 2018a). Cyanobacteria (CB) represent group of photoautotrophic bacteria, occurring in fresh as well in brackish water bodies. Due to interference of severe anthropological activities including global warming they are now a menace, polluting waters across the world (Buratti et al., 2017; Funari et al., 2012; Paer and Otten, 2013). Different cyanobacterial toxins have been implicated with serious health issues worldwide due to the production of various bioactive and toxic metabolites especially microcystins (MCs) which are toxic to living organisms including humans (Dietrich and Hoeger, 2005). Drinking contaminated water or consumption of foods containing cyanotoxins (fresh water fish, vegetables etc.) are other sources through which humans get infested. The effect of CBs on human health is more dreadful as many edible coastal foods (fish, crustaceans and mussels etc.) are not routinely checked for these cyanotoxins thereby making their way directly to humans (Buratti et al., 2017). With the increasing accumulation of cyanotoxins at various environmental levels they now seem to be affecting both human and livestock health with serious implications on world economy. It is worth to mention here that chronic or repeated

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exposure to low cyanotoxin levels is one of most important factors affecting human health in general.

There are presently more than 90 microcystin isoforms and among them microcystin-leucine arginine (MC-LR) is the most potent and abundant variety of microcystin (Welker and Von Döhren, 2006; Lone et al., 2016). MCs are found in every part of the world except in Antarctica region and its toxicity has been reported in nearly 80 countries (Lone et al., 2016; Zurawell et al., 2005; Harke et al., 2016). MC-LR has been designated by International Agency for Research on Cancer as potent carcinogen leading to hyperphosphorylation of cellular proteins (Fan et al., 2014). According to World Health Organization (WHO) only 1 μg/L of MC-LR is admissible but the concentration of microcystins in present day water bodies is many times beyond the recommended guidelines (Lone et al., 2015). MC-LR is also reported to be a well-known hepatotoxin and is the main culprit for causing serious organ damage as evident by various MC-LR associated gastrointestinal disorders, reproductive toxicity, immune and kidney impairment (Zhang et al., 2008; Zhou et al., 2012). MCs have also been linked to reduced DNA repair property of cells (Kleppe et al., 2015) thereby leading to enhanced apoptosis (Liu et al., 2016).

The emergence of the novel field of "osteoimmunology", dealing with the intricate interplay between both immune and bone system is responsible for maintaining bone health (Dar et al., 2018b; Arron and Choi, 2000). Bone is a dynamic organ with continuous cycles of bone-remodeling due to active interaction between bone forming osteoblasts and bone eating osteoclasts (Dar et al., 2018b). Interestingly, immune system plays a pivotal role in this remodeling phenomenon with T cells playing a nodal role in regulating bone health (Grcevic et al., 2000). It has been observed that depletion of $CD4^+$ T lymphocytes in mice leads to anti-osteoclastogenic signals thereby enhancing bone health (Grcevic et al., 2000). In addition, an elevated level of $CD8^+$ T cells have been associated with anti-osteoclastogenic property (John et al., 1996). Furthermore, B cells also have been reported to suppress osteoclast formation, thereby inhibiting bone loss by stimulating osteoprotegerin (OPG) production (Weitzmann et al., 2000; Thirunavukkarasu et al., 2001; Klausen et al., 1989; Polanczyk et al., 2004). $CD4^+$ T cells are mainly responsible for inducing osteoclastogenesis through induction of elevated levels of IL-17, RANKL, TNF-α and lower levels of IFN-γ (Sato et al., 2006; Kelchtermans et al., 2008; Palikova et al., 2013; Dar et al., 2018b). Interestingly, MC-LR treatment in mice has been associated with significantly higher population of $CD4^+$ T cells along with reduced population of both $CD8^+$ T cells and B cells in different lymphoid tissues (Palikova et al., 2013). MC-LR also has been reported to enhance the production of pro-inflammatory cytokines such as TNF-α and IL-6 (Chen et al., 2017).

The present study for the first time reports that MC-LR due to its established immunomodulatory property induces bone loss and impairs bone-microarchitecture by tweaking the population of both T and B lymphocytes in mice. We report that MC-LR significantly enhances bone loss by increasing percentage of $CD4^+$ T cells along with simultaneously decreasing the population of both $CD8^+$ T cells and B cells *in vivo*. This effect of MC-LR is mediated by decreased levels of anti-osteoclastogenic cytokines (IL-10 and IFN-γ) with concomitant increase in the levels of osteoclastogenic cytokines (IL-6, IL-17, RANKL and TNF-α) thereby leading to the observed enhanced bone loss in MC-LR treated mice. The present study is pioneering in the field, which for the first time demonstrates the direct effect of MC-LR on bone health via affecting the host osteo-immune system. The study thus highlights the risk associated with intake of MC-LR contaminated water and food not only to our immune system but also to our bones.

2. Material and methods

2.1. Animals

Twenty male mice (BALB/c) of 10–12 weeks with an average body weight (mean ± standard error of the mean) of 30g ± 2g were selected and divided into two groups viz. normal and MC-LR treated. The MC-LR treated group received MC-LR (10 μg/kg bw/day, ip) and normal mice group was administered same volume of normal saline for 15 days (Fig. 1) (Sedan et al., 2013; Lone et al., 2017). Mice were maintained under specific pathogen-free conditions and fed sterilized food and autoclaved water *ad libitum*. At the end of experiment (15 days), animals were sacrificed and bones, lymphoid organs (thymus, spleen, lymph node and bone marrow) and serum were collected for further analysis. All the procedures involving animals were conducted according to the requirements and with the approval of the Institutional Animal Ethics Committee (SIPS/EC/2015/64).

2.2. Antibodies and reagents

MC-LR was purchased from Sigma-Aldrich Co., USA. The following antibodies/kits were bought from BD Biosciences (USA): PerCP-Cy-5.5 Rat Anti-Mouse CD4-(RM4-5) (550954), APC-Rat-Anti-Mouse-CD8a-(53–6.7) (561093), PE-Rat-Anti-Mouse-CD45R/B220-(RA3-6B2) (553089) and mouse Cytometric Bead Array-(CBA) kit. RBC lysis buffer (00-4300-54) was obtained from eBioscience (USA).

2.3. Scanning electron microscopy (SEM)

For SEM analysis, femur cortical bone samples were kept in 1% Triton for 48–72 h and later transferred to 1xPBS solution till final analysis is done. Bone slices were made and dried under incandescent bulb before SEM analysis and scanned in NOVA NANOSEM 450 microscope equipped with a tungsten filament gun operating at WD 10.6 mm and 20 kV. SEM images were digitally photographed at low (15x), intermediate (1000x), and high magnifications (10,000x) to picture the best cortical structure. Images were later processed and analyzed using Adobe Photoshop 6.3. The data was further analysed by MATLAB software (Mathwork, USA).

2.4. Atomic force microscopy (AFM)

Femur cortical bone samples were dried in dust free environment with 60 W lamps for 6 h followed by high vacuum drying and subsequently examined under atomic force microscope (AFM). (INNOVA, ICON Analytical Equipment, Bruker) operating under the Acoustic AC mode (AAC or Tapping mode), with the aid of a cantilever (NSC 12(c) from MikroMasch, Silicon Nitride Tip) by NanoDrive™ version 8 software. The force constant was 0.6 N/m, while the resonant frequency was 94–136 kHz. The images were taken in air at room temperature, with the scan speed of 1.5–2.2 lines/s. The AFM images were obtained with the help of Nanoscope software. The data was further analysed using MATLAB software (Mathwork, USA).

2.5. Micro computed tomography (μ-CT)

μ-CT of the femur/tibia (trabecular/cortical) and lumbar Vertebrae-V (trabecular) was performed using SkyScan 1076 scanner (SkyScan). Scanning was done at 50 kV, 201 mA using a 0.5-mm aluminium filter and exposure set to 590 ms. In total, 1800 projections were collected at a resolution of 6.93 μm/pixel. Reconstruction process was done using NRecon software. While