

Susceptibility of Arsenic-Exposed ICR Mice to Buruli Ulcer Development

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ABSTRACT

Background: Buruli Ulcer is assuming public health importance in Ghana, prompting research into possible ways by which the disease can be managed. The study aimed at investigating the susceptibility of arsenic-exposed ICR mice to the development of Buruli Ulcer. **Methodology:** Upon continuous exposure of mice to variable concentrations of arsenic via drinking water, they were inoculated intraplantarly with approximately 15×10^8 CFU mL⁻¹ (5 McFarland standard) *Mycobacterium ulcerans*. Cage-side and clinical observations were carried out daily for post-exposure inoculation clinical manifestations. Hematological and histopathological studies were also performed and observations compared with controls. Tissue from developed lesion obtained was confirmed for the presence of *Mycobacterium ulcerans* by Polymerase Chain Reaction test. **Results:** Inoculated arsenic exposed mice developed erythema on day 25 which progressed to swelling of the footpad, foot oedema, thigh oedema and ulcer within 112 days. The onset and progression was directly related to the arsenic exposure dose. Within this period, there were no developments in the MU-only treated and the normal mice. *Mycobacterium ulcerans* positive lesions however started developing on the hind feet of this treatment group on day 122 (50 days after this manifestation had been observed in *Mycobacterium ulcerans* inoculated arsenic exposed mice). White blood cell numbers decreased significantly ($p \leq 0.01$) and dose-dependently in MU inoculated arsenic exposed mice as well as the arsenic-only treatment group. Histopathological reports revealed that inoculated arsenic exposed mice had dose-dependent liver and spleen damage after 112 days of the study similar to the *Mycobacterium ulcerans* only treatment. **Conclusion:** Results from the study revealed that, arsenic has an immunosuppressive potential that can hastens a possible MU infection in mice.

Key words: Ziehl-Neelsen staining, arsenic, polymerase chain reaction test, hepatocyte ballooning, multi-nucleated cells formation

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INTRODUCTION

Buruli Ulcer (BU) also known as the Bairnsdale Ulcer or Searle's Ulcer¹ is an infectious ulcerative disease of the skin, subcutaneous tissue and sometimes bone². This disease is caused by *Mycobacterium ulcerans* (MU) and

results in non-ulcerative (papules, nodules, plaques and oedematous form) as well as ulcerative dermatological disorders leading to restricted movement of affected limbs³. BU can affect any part of the body, but in about 90% of cases the lesions are on the limbs, with nearly 60% of all lesions on the lower limbs⁴. MU can thus be isolated from primary lesions of infected individuals after a 5-8 week incubation period although it could go up to 6 months⁵.

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MU infections occur mostly in tropical areas of the world i.e., in Australia, Uganda, and several countries in West Africa, Central and South America and Southeast Asia. BU is currently causing a serious health challenge, especially in West Africa more importantly with an unclear pathogenesis till date⁶. The disease is presently endemic in the Benin, Côte d'Ivoire, Guinea, Liberia, Nigeria, Sierra Leone, Togo and Ghana⁷.

The first probable case of *Mycobacterium ulcerans* infection in Ghana was reported in the Greater Accra Region in 1971; the presence of additional cases along the tributaries of the Densu River in the area was considered likely⁸. In Van der Werf *et al.*⁹ described 96 cases in the Asante Akim North District of Ashanti Region¹⁰. This report was followed by the description of a major endemic focus in the Amansie West District of Ashanti Region of Ghana¹¹.

BU is assuming public health importance worldwide with the focus on West Africa e.g., Ghana, prompting research into possibly ways by which this disease can be prevented and/or treated. Although, there has been an extensive study and research on BU in an attempt to elucidate the pathogenesis of this MU infection, these have been elusive¹¹. In 2004, samples of water from arsenic enriched domains and farmlands in the Amansie West District; part of which has a high prevalence of BU; was studied after which it was hypothesized that arsenic in drinking water indirectly could contribute positively to BU infection¹².

In a pilot study conducted by Gyasi *et al.*¹³ in the same District, revealed a positive correlation between environmental arsenic levels and BU. The research further reported that streams and soils in these BU endemic communities was generally high (exceeding WHO specifications) for arsenic contamination. A follow up research involving the clinical, hematological and histopathological responses to arsenic toxicity in ICR mice using arsenic levels synonymous to Buruli Ulcer endemic communities in the Amansie West District of Ghana also revealed that, high levels of arsenic in tissue (possibly from accumulation) caused inflammation, erythema and open ulcers on the skin, and has the potential to cause liver and spleen damage¹⁴. The study further showed a reduction of the immune system function as well as red blood cell microcytosis.

In this present study, an *in vivo* study in ICR mice linking the susceptibility of arsenic exposure to the development of BU is being investigated. The finding from this study therefore, could offer invaluable insight to the pathogenesis and a possible solution to Buruli Ulcer control.

MATERIALS AND METHODS

Research clearance and biosafety considerations: Institutional research clearance was obtained before the

study was undertaken. The study was carried out in a level 2 biosafety laboratory. Protocols for the study were approved by the Departmental Ethics Committee. All the technical team observed all institutional biosafety guidelines for protection of personnel and laboratory.

Study design and site: An experimental study was conducted in the Animal Experimentation Facility of the Department of Theoretical and Applied Biology, College of Sciences, KNUST, Kumasi, Ghana from December, 2011 to July, 2012.

Experimental animals: Six-week old Imprinting Control Region (ICR) mice obtained from the Animal Facility of the Department of Pharmacology, KNUST, Ghana, were used for the study. They were transferred to the Animal Experimentation Facility at the Department of Theoretical and Applied Biology, to acclimatize for 7 days. During this period, mice were observed (physical; in-life) daily and weighed. At initiation of treatment, animals were approximately 7 weeks old. The mean weight for the males was 20.5 g and the females 19.3 g. The females were nulliparous and non-pregnant. Individual weights of mice placed on test were within $\pm 20\%$ of the mean weight for each sex. Animals were kept in a plastic cages (diameter of 30 cm) with wire meshed cover, fed with normal pelleted mice chow (GHAFCO, Tema, Ghana) and kept under ambient conditions of temperature, light and dark cycle and relative humidity. All procedures and techniques used in these studies were in accordance with the National Institute of Health for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985).

Obtaining MU for preparation of inoculum

Diagnosing MU in human tissue: The presence of MU was diagnosed using Polymerase Chain Reaction (PCR) test carried out on tissue obtained from active lesions on BU patients sampled from the Asante Akim North District of the Ashanti Region of Ghana described as follows:

Tissue collection and transport: Elliptical biopsy samples obtained from the margin of active lesions or ulcers were quickly placed into 2 mL test tubes containing anaerobic transport medium, which is a modified Dubos medium (Fisher Scientific, Suwanee, Ga. USA) supplemented with oleic acid-albumin-dextrose-catalase (Remel, Lenexa, Kans. USA), PANTA™ (Becton Dickinson and Co., Franklin Lakes, N.J. USA) and 0.5% agar (Fisher Scientific, UK). The tissue was transported to the Kumasi Centre for Collaborative Research laboratory, Kumasi, Ghana for immediate analysis.

DNA isolation from tissue: The tissue was homogenized in a mortar with a sterile pestle in 500 μL of digestion buffer (30 mM Tris-HCl, 30 mM EDTA, 5% Tween 20, 0.5% Triton X-100, 800 mM guanidine hydrochloride), followed by the addition of 20 μL of proteinase K (20 mg mL^{-1} stock solution). The solution was vortexed and incubated with rotation at 60°C for 30 min. After incubation, the sample was sonicated for 8 min in a water bath sonicator (Elmasonic PH 750 EL, USA) at room temperature. The tissue homogenate was pelleted by centrifugation supernatant was transferred to a sterile eppendorf tube (Shanghai Aude Medical Science and Technology Development Co., Ltd., China) and 40 μL of diatomaceous earth (Bio-Rad, Hercules, Calif. USA) added. The sample was incubated in a water bath at 37°C for 1 h. Following incubation, the pellet was washed twice with 70% ethanol and once with acetone. The pellet was dried at 60°C for 5 min and resuspended in 100 μL of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Finally the solution was incubated at 65°C for 20 min and centrifuged at 2,000 g and the supernatant was transferred to a sterile eppendorf tube and used for PCR test.

DNA amplification: Ten microliters of the sample acquired after the DNA isolation procedure was amplified in a 50 μL reaction mixture containing 20 pmol of each primer (MU1 and MU2), 1U of Faststart *Taq* DNA polymerase (Roche, Indianapolis, Ind.), 200 μM (each) deoxyribonucleotide triphosphate, 1.5 mM MgCl_2 and PCR buffer. The primers used in this amplification were MU1 (5'-GGCAGGCTGCAGATGGCAT-3') and MU2 (5'-GGCAGTTACTTCACTGCACA-3'), directed at the IS2404 sequence and producing a 549-bp fragment in the presence of MU genomic DNA. The thermocycling profile was as follows: denaturation at 94°C for 5 min; 40 cycles of 94°C for 1 min, 66°C for 1 min and 72°C for 1 min and a final extension at 72°C for 7 min throughout the cycling time, the heated lid function was used at 108°C. Agarose gel electrophoresis was done to confirm the status of negative and positive controls.

If the controls for the first PCR were correct, the procedure continued with the following step of the nested PCR. One microliter of product from the first PCR was used in a 25 μL reaction mixture with primers PGP3 (5'-GGCGCAGATCAACTTCGCGGT-3') and PGP4 (5'-CTGCGTGGTGCTTTACGCGC-3'). These primers produce a 217-bp product. The buffers and other reaction components were added at concentrations identical to those in the first PCR step. The same thermocycling profile was used, except for a change of the annealing temperature, from 66 to 64°C.

Agarose gel electrophoresis: Agarose gels (1.6%) in Tris-borate-EDTA buffer were prepared with ethidium bromide (MWmarker VIII; Roche Biochemicals, Germany). Fifteen microliters of each PCR mixture was mixed with loading dye and added to the wells. The products from the first PCR, with primers MU1 and MU2, were loaded in the top row of wells while the products from the second PCR, with primers PGP3 and PGP4, were loaded in the bottom row of wells. One lane of each row on the agarose gel included molecular weight standards (MWmarker VIII; Roche Biochemicals, Germany). Gels were run at 90 v for 30-45 min. The DNA fragments were visualized with UV light and were documented on film (Polaroid). The protocol was designed for a nested PCR, and results were based on the presence of a 217 bp fragment resulting from the second PCR round with the PGP3 and PGP4 primers. A positive diagnosis of MU in the human tissue samples obtained necessitated the need to pick a swap sample of MU from the infected patient.

Picking MU from human tissue: Cotton wool swaps from lesions on BU patients was obtained from the Ashanti Akim North District of the Ashanti Region of Ghana. They were washed into sample tubes containing sterile phosphate buffer, kept in an ice chest and sent to the Kumasi Centre for Collaborative Research laboratory in Kumasi, Ghana, for storage.

Confirmation of the presence of MU in human samples picked: Confirmatory test for the presence of MU was carried out using the Ziehl-Neelsen staining and Bacterial Culture.

Ziehl-Neelsen (ZN) staining: Two drops of the prepare MU suspension was put onto a glass slide and dried at 60°C for 10 min over a benson flame, followed by heat-fixing at 90°C for 10 min. The slide was flooded with Carbol Fuchsin (Asia Pacific Speciality Chemicals Limited, Australia) after which it was held over the benson flame without letting it boil. The slide was allowed to sit for 5 min in a hood and rinsed with distilled water. It was then flood again with 30% hydrochloric acid in isopropyl alcohol and allowed to sit again for 1 min.

The slide was rinsed again with distilled water, flooded with Methylene Blue and allowed to sit for another 1 min, followed by rinsing with distilled water. The slide was blotted dry with a Whatman's No. 1 filter paper (Sigma-Aldrich, UK) and examined under a Ceti magnum-T/trinocular microscope for fluorescence (Medline Scientific limited, UK) at objective magnification X40 to detect the presence of acid fast bacilli; which was present.

Bacterial culture: The prepared MU suspension was inoculated on slants of Löwenstein-Jensen (L-J) media (Acumedia, Neogen Cooperation, USA) and kept in an incubator (Oaken, UK) at a temperature of 32°C for 8 weeks after which they were observed for growth. The growth observed was a confirmation of the presence of MU. The need for a pure strain of MU required that a sub-culture be made.

Preparation of MU Sub-culture: With a sterile standard loop, MU from the primary bacterial culture were harvested and inoculated on a newly prepared Löwenstein-Jensen media. They were placed in an incubator at a temperature of 32°C. Observation of the slants after 8 weeks revealed positive results for MU using AFB test and PCR.

Preparation of MU inoculum: A drop of sterile Phosphate Buffer Solution (PBS) (0.01M, pH 7.0) was added to a test tube containing 15-20 sterilized glass beads to wet them. A loop full of MU obtained from the MU sub-culture was added to the beads and drops of the sterile PBS were added intermittently and vortexed to break up the MU colonies and to adjust the turbidity of the suspension to a 5 McFarland Nephelometric standard.

Preparation of a 5 Mcfarland standard: A 5 McFarland standard¹⁵ was prepared by mixing 0.5 mL of 1.175% barium chloride dehydrate with 9.5 mL of 1% sulphuric acid. The 5 McFarland standard prepared had an approximate cell density of 15×10^8 CFU mL⁻¹.

Experimental conduct and design: Eighty healthy ICR mice were put into seven groups of ten and labelled A-H. Group A, (Control 1) was fed with normal drinking water and inoculated with only MU during the study. Groups B-F was given water containing 0.8, 1.6, 2.4, 3.6 or 4.8 mg L⁻¹ of arsenic (prepared from a stock solution of 10 mg L⁻¹ arsenic trichloride by serial dilution) in place of drinking water *ad libitum* through the study period and inoculated with 0.05 mL of MU inoculum intraplantarly fourteen (14) days after the onset of arsenic exposure. Group G, was given drinking water containing arsenic (4 mg L⁻¹) with No. MU inoculation (Control 2) while Group H was given normal drinking water and no inoculation (Control 3). Arsenic concentrations were selected in accordance with levels detected from soil and water samples collected from water and soil in Buruli Ulcer endemic communities of the Amansie West District of the Ashanti Region of Ghana.

Cage-Side and clinical evaluation: Cage-Side and clinical observations were carried out daily for

post-exposure clinical manifestations of MU. Mortality and emaciation were sought for morning and evening. Daily observations of the skin for hair loss, open or closed lesions, or abnormal masses, secretions and excretions from the eye, nose, oral cavity, anus and external genitalia, as well as pupil size and respiratory pattern and "chattering" were made. Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypy (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backward) were sought for.

Lethargy, aggression and hunch appearance was also noted once daily for any signs of toxicity. Pharmacologically toxic symptoms sought for included, constipation, hemorrhage, sedation, diarrhoea, polyuria, polydipsia, polyphagia, anorexia, rhinorrhoea/nasal congestion, loss of autonomic reflexes, decreased locomotory activity, neuromuscular inco-ordination and collapse, hyperesthesia, hypothermia, twitching, spasticity, writhing, and convulsion. Whenever any discharge (exudate, pus) or scab was available they were Ziehl-Neelsen stained for the detection of Acid Fast Bacilli (AFB).

Hematological studies: Prior to the study, whole blood samples were collected from normal mice into MediPlus K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan) for hematological analysis using the KX-21 N Automated Hematology Analyzer (Sysmex Corporation, Chuo-ku, Kobe, Japan) at the KNUST Hospital, Kumasi, Ghana. Parameters measured were White Blood Cell (WBC), Red Blood Cell (RBC) Hemoglobin Concentration (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Blood Cell Distribution Width (RDW-CV and RDW-SD), Platelet Count (PLT), Mean Platelet Volume (MPV) and Platelet Distribution Width (PDW).

Hematological analysis was performed again on development of erythematous lesion, swellings of paws and tail regions after 98 days of MU inoculation in arsenic exposed mice.

Histopathological studies: Three mice from each group were euthanized on developing either foot-thigh oedema or ulcer after 112 days. The heart, lung, liver, spleen and kidney were harvested from both experimental and control mice and then placed in 10% phosphate buffered formalin for 36 h. The samples were transferred to a histological cassette and then immersed in multiple baths of progressively more concentrated ethanol, to dehydrate the tissue followed by xylene for 16 h. The tissue was then finally immersed in extremely

hot paraffin. Additional paraffin was added to create a paraffin block which is attached to the outside of the cassette. With the help of a microtome, the tissues were cut into very thin (2-7 μm) sections. The microtome sliced tissue are ready for microscopic examination. The slices were layered on a glass slide ready for staining. The processed histological slides were stained with a combination of hematoxylin and eosin to provide contrast under the microscope. With the help of a specialist pathologist at the Pathology Department, Okomfo Anokye Teaching Hospital, the findings were interpreted.

Confirmation of MU in mice lesions: Tissue from lesion developed in mice were obtained by punch biopsy or fine needle aspiration and transferred into sterile eppendorf tubes and kept on ice. These were sent to the KCCR laboratory for detection of MU by PCR technique (described earlier).

RESULTS

Cage-side and clinical evaluation: Mice inoculated with *M. ulcerans* after exposure to different concentrations of arsenic developed similar clinical lesions which followed the same progression. The lesions progressed from erythema of the footpad to swelling of the footpad, through to development of foot oedema, to thigh oedema and ulcer of the foot. Though the animals developed similar lesions, the onset and progression of the lesions were directly related to the arsenic dose.

The study showed that, after day 24 of arsenic exposure followed by *M. ulcerans* inoculation, all mice (experimental and control) looked healthy with generally good body posture. Daily clinical observations recorded during the period were considered common findings in laboratory mice and unrelated to arsenic or *M. ulcerans* exposure. There were no secretions from the eye, ear, nose, anus and external genitalia, no "wasting", audible "chattering", alopecia and pallor in the eyes. The mice were not lethargic, they fed well and their stool showed no signs of diarrhoea. There were no ocular findings, decreased motor activity and neurological conditions. There was no significant test article effect on body weight in either sex. No mortality occurred.

However, on day 25, there were visible signs of erythema (inflammation) on the hind paws in mice exposed to 4.8 mg L^{-1} arsenic followed by MU inoculation. This clinical presentation was observed again in mice exposed to 3.6 and 1.6 mg L^{-1} arsenic (followed by MU inoculated) on day 28 and 36, respectively. The erythematous lesion was followed by swelling of hind paws and tail regions and the mice looked generally weak. Two deaths were recorded in the MU inoculated after exposure to 3.6 and 4.8 mg L^{-1}

arsenic in drinking water prior to the detection of *M. ulcerans* in the lesion. Similar lesions were seen in the arsenic-only treated group (Control 2). The *M. Ulcerans*-only treated (Control 1) and the normal mice (Control 3) however, did not show any developments.

All forms of ulceration and oedema were negative for *M. Ulcerans* until day 72 where there were other manifestations of erythema on the hind paws of mice exposure to 2.4 to 4.8 mgL^{-1} arsenic (Plate 1). This manifestation was again observed (with ulcerated genitals) in the 0.8 and 1.6 mgL^{-1} arsenic exposure groups on day 112 (Plate 2). PCR test proved positive for *M. Ulcerans*. However, PCR test was negative for the arsenic only-treated mice (Control 1). The *M. Ulcerans*-only treated (Control 2) and the normal mice (Control 3) still did not show any developments.

By day 112, all mice exposed to arsenic and *M. Ulcerans* had developed lesions on several parts of the hind limbs which had tested positive for *M. Ulcerans*



Plate 1: Inflammation and erythema in the right hind paw after 72 days of MU exposure

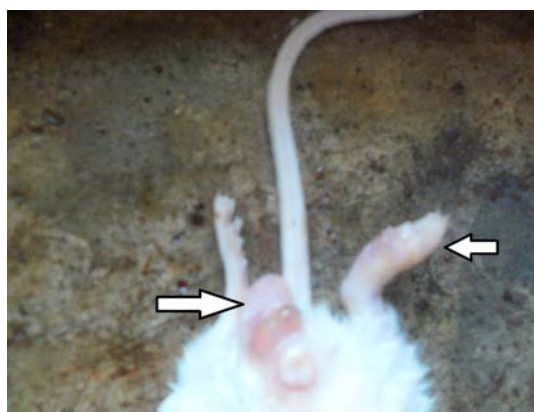


Plate 2: Lesions on hind paw with ulcerated genitals of mice after 112 days of MU exposure



Plate 3: Hind paw edema with ulcerated surface after 112 days MU exposure



Plate 4: A previously positive lesion that has amputated from the thigh after 112 days MU exposure

(Plate 3). There were however no lesion in Control 1 and 3. It was revealing to observe at this stage that, the study had one amputee mouse (i.e., 3.6 mg L⁻¹ of arsenic exposed MU inoculated), which had previously tested positive for *M. ulcerans* and had lost the entire limb from the thigh region (Plate 4) due to aggravation of the infection. Fine needle aspiration method extraction beneath the scar and PCR of the aspirated mass proved negative for *M. ulcerans*.

Lesions started developing on the hind foot of the *M. Ulcerans*-only treated group on day 122 (50 days after this manifestation had been observed in mice exposed to 4.8 mgL⁻¹ of arsenic and inoculated with *M. ulcerans*). These were PCR positive for *M. ulcerans*. The normal mice (Control 3) never developed any lesion in the entire study period. Plate 5 is a picture showing ultraviolet transillumination of PCR results of arsenic exposed and *M. ulcerans* inoculated as well as controls of ICR mice after 112 days.

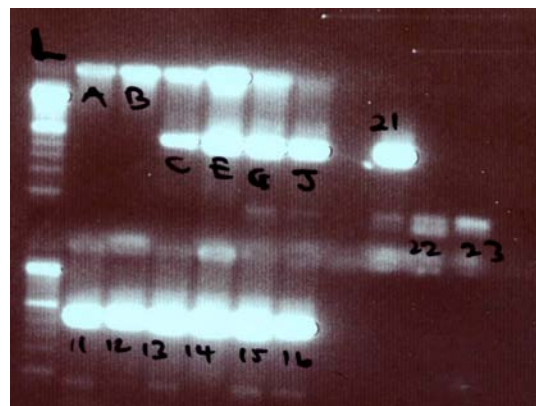


Plate 5: Ultraviolet transillumination of PCR results of arsenic exposed and *M. ulcerans* inoculated as well as controls of ICR mice after 112 days, L: Loading Dye (Gel electrophoresis process); A: Control, B: Amputee, C: Arsenic exposure 1.6 mg L⁻¹ with *M. ulcerans* injection after day 112; E: Arsenic exposure 2.4 mg L⁻¹ with *M. ulcerans* injection after day 112; G: Arsenic exposure 3.6 mg L⁻¹ with *M. ulcerans* injection after day 112; J: Arsenic exposure 4.8 mg L⁻¹ with *M. ulcerans* injection after day 112; 21: Positive control (For PCR process), 22: Negative control (For PCR process), 23: Negative extraction control (For PCR process), 11-16: Inhibition controls to the corresponding samples as shown in picture

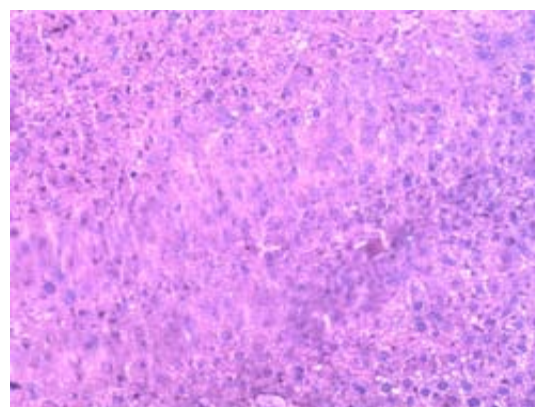


Plate 6: Photomicrograph of normal liver (control). Magnification X400; Stain: Haematoxylin and Eosin

Histopathological presentation: Histopathological reports revealed that mice exposed to arsenic, followed by injection with 0.05 mL of *M. ulcerans* inoculum had dose dependent liver and spleen damage after 112 days compared to the normal (Plate 6 and 7, respectively).

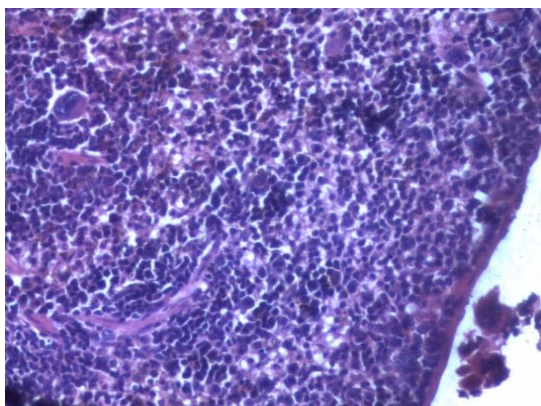


Plate 7: Photomicrograph of normal spleen (control). Magnification X400; Stain: Haematoxylin and Eosin

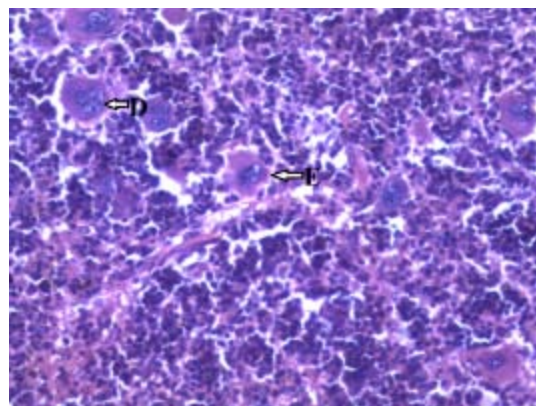


Plate 9: Photomicrograph of the spleen with lymphoid background and formation of multinucleate cells (D and E) on day 98 after arsenic and *M. ulcerans* exposure. Magnification X400; Stain: Haematoxylin and Eosin

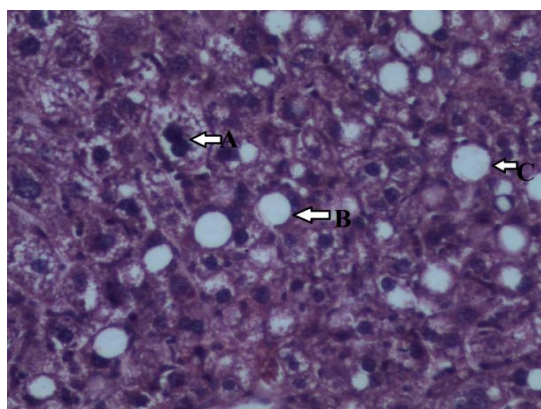


Plate 8: Photomicrograph of the liver showing (B and C) severe ballooning of hepatocyte, (A) necrosis with severe multinucleate formation on day 98 after arsenic and *M. ulcerans* exposure. Magnification X400; Stain: Haematoxylin and Eosin

These damages were more severe than the group exposed to only arsenic (3.6 mg L^{-1}). High power observation (400X) of hepatocytes for mice exposed to $2.4\text{-}4.8 \text{ mg L}^{-1}$ arsenic with *M. ulcerans* inoculation revealed that, in addition to severe hepatocyte ballooning with the loosening of its cell wall, there were also severe nuclei loss and multinucleation (Plate 8). Sinusoidal spaces had expanded severely due to severe shrinkage and necrosis. This indicates a gradual process of cell loss. However, there was neither steatosis nor fibrosis of the hepatocytes.

The spleen from these mice exposure group ($2.4\text{-}4.8 \text{ mg L}^{-1}$ arsenic) and MU inoculation also showed

a severe multi nucleated cells formation with a severe lymphoid background (Plate 9). The group however showed normal myocardial cells of the (heart) with normal alveoli (Lungs). The kidney tissue from mice in these groups presented a normal glomeruli and normal tubules with mild interstitial chronic inflammation.

Mice in 0.8 and 1.6 mg L^{-1} arsenic exposed and MU inoculated group showed similar histopathological trend but this was not as severe compared to mice in $2.4\text{-}4.8 \text{ mg L}^{-1}$. The liver of these mice exposure groups after 112 days showed a less severe ballooning of hepatocytes with a less severe nuclei loss. There was neither steatosis nor fibrosis of the hepatocytes. The spleen from this exposure group showed a less severe multi nucleated cells formation with a lymphoid background. The group showed normal myocardial cells as well as normal alveoli. The kidney tissue from mice in these groups presented a normal glomeruli and normal tubules with no interstitial chronic inflammation.

Mice exposed to *M. ulcerans* only (treated group, control 1) showed similar histopathological features to that of exposure groups 0.8 and 1.6 mg L^{-1} after day 112 but these were milder in their presentations. There was a mild degenerate changes with cells showing mild cytoplasmic vacuolation. There was mild ballooning of hepatocytes with a mild nuclei loss (Plate 10). The spleen from the "*M. Ulcerans* only" inoculated group showed a mild multi nucleated cells formation with a mild lymphoid background (Plate 11). The group also showed normal myocardial cells of with normal alveoli. The kidney tissue from mice in this group presented a normal glomeruli and normal tubules and interstitial chronic inflammation was absent.

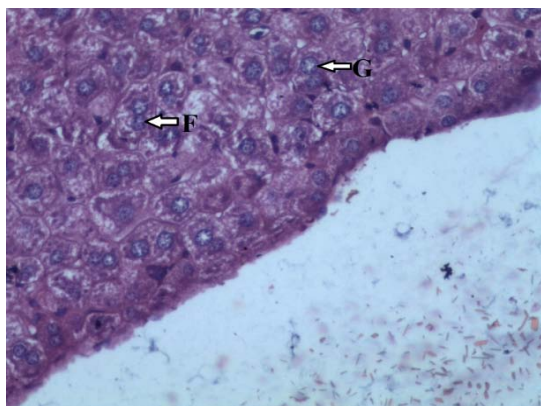


Plate 10: Photomicrograph of the liver showing (G and F) mild hepatocyte necrosis with associated mild multinucleate formation on day 98 after arsenic and *M. ulcerans* exposure. Magnification X400; Stain: Haematoxylin and Eosin

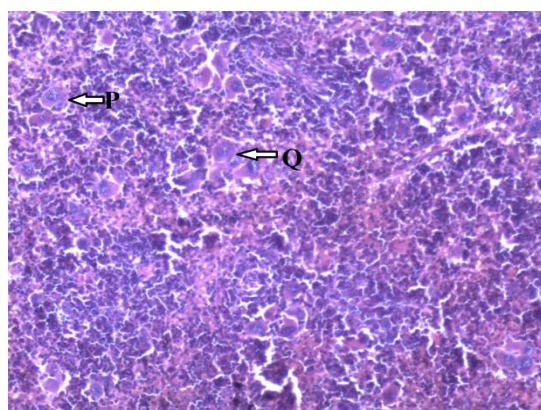


Plate 11: Photomicrograph of the spleen showing mild lymphoid background and relatively fewer formations of multinucleate cells P and Q on day 98 after arsenic and *M. ulcerans* exposure. Magnification X400; Stain: Haematoxylin and Eosin

Haematological analysis: Haematological examination showed that, RBC, HGB, MCH, MCHC, PLT and PDW in all treatment groups were not significantly different ($p > 0.05$) from that of the controls. However, WBC levels decreased significantly ($p \leq 0.01$) and dose-dependently in mice exposed to 3.6 and 4.8 mg L⁻¹ arsenic only (Control 1) as well as the arsenic and *M. ulcerans* groups. RDW (-CV and -SD) for mice inoculated with *M. ulcerans* only decreased significantly ($p \leq 0.05$) (Table 1).

DISCUSSION

The study has revealed that, arsenic exposed mice are susceptible to Buruli Ulcer infection with the degree of susceptibility depending on the arsenic dose. Arsenic can be toxic through its interaction with sulfhydryl groups of proteins and enzymes by denaturation process within cells¹⁶. Through an increase of reactive oxygen species in cells, arsenic consequently causes cell damage¹⁷. For this reason, oxidative stress induced by trivalent methylated arsenicals could inhibit glutathione (GSH) reductase¹⁸ and thioredoxin reductase¹⁹ with subsequent impairment of cellular protective mechanism against invasion by microorganism. These processes mostly depend on the dose and exposure time of arsenic and this was consistent with this study.

Lethargy and abnormal body posture with weakness observed for mice exposed to high doses of arsenic observed after day 25 could be attributed to energy deficit in their body²⁰. Arsenate has a similar structure as phosphate and as such, it can substitute for phosphorus there by leading to replacement of phosphorus in bones in the body of the mice for many days²¹. Since arsenate is hydrolyzed easily (in the cell) it prevents subsequent transfer of phosphate to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) (the energy currency of the cell) and thus depletes the cell of its energy²² making the mice weak.

Visible signs of erythema (inflammation) in arsenic treatment and MU inoculation (day 25 arsenic exposure; day 11 MU inoculation) demonstrates signs of reduced immunity after chronic arsenic exposure. A reduction in immune system function exposes the body tissue to microbial attack by MU. Microbial infection leads to inflammation. With the peaking of inflammation, there is swelling of the affected body part when the mycobacterium increases in number. Loss of connective tissue by the microbial action results in the tissue expansion and ruptures hence the ulceration²³. After inoculation into the skin, MU proliferates extracellularly and elaborates the toxin mycolactone, which enters the cells and causes necrosis of the dermis, panniculus and deep fascia²⁴.

The progression of *Mycobacterium ulcerans* infection is characterized not only by damage to skin, nerves and blood vessels but also by absence of inflammatory response during the early and acute phases of the disease¹¹. This confirms that the bacterium secretes an immunosuppressive toxin (Mycolactone) and this toxin is normally at or close to the site of infection²⁵. It is only when the production of interleukin-2 (IL-2) and Tumour Necrosis Factor (TNF), "T" helper-1 (Th1) (the pro-inflammatory immune response) is suppressed and down-regulated that the disease can develop⁶. Therefore, the development of *Mycobacterium ulcerans*

Table 1: Haematological analysis of mice exposed to *M. ulcerans* only (Control 1), arsenic only (3.6 mg L⁻¹, Control 2), No MU plus ordinary drinking water (control 3) and *M. ulcerans* plus various concentrations of arsenic in drinking water for 112 days

Haematological parameters	Control 1 (<i>M. ulcerans</i> only)	0.8 (AS) mg L ⁻¹ + <i>M. ulcerans</i>	1.6 (AS) mg L ⁻¹ + <i>M. ulcerans</i>	2.4 (AS) mg L ⁻¹ + <i>M. ulcerans</i>	3.6 (AS) mg L ⁻¹ + <i>M. ulcerans</i>	4.8 (AS) mg L ⁻¹ + <i>M. ulcerans</i>	Control 2 (Arsenic only, 3.6 mg L ⁻¹)	Control 3 (no Arsenic, no MU)
WBC ($\times 10^9/L$)	9.50 \pm 3.70ns	8.85 \pm 0.05ns	9.45 \pm 1.25ns	11.25 \pm 1.55ns	4.65 \pm 1.05*	6.55 \pm 1.35*	4.2 \pm 0.63*	26.00 \pm 4.00
RBC ($\times 10^6/L$)	7.15 \pm 0.25ns	9.57 \pm 0.27ns	8.40 \pm 0.61ns	8.315 \pm 0.56ns	7.89 \pm 0.13ns	8.08 \pm 0.16ns	8.31 \pm 1.25ns	7.495 \pm 1.34
HGB (g dL ⁻¹)	10.45 \pm 0.25ns	13.30 \pm 0.40ns	12.75 \pm 0.95ns	12.40 \pm 0.90ns	12.15 \pm 0.25ns	12.20 \pm 0.50ns	12.0 \pm 1.62ns	10.65 \pm 1.55
HCT (%)	38.00 \pm 0.80ns	48.05 \pm 0.85ns	44.45 \pm 3.35ns	44.00 \pm 2.50ns	43.20 \pm 0.50ns	43.00 \pm 1.30ns	36.8 \pm 3.56ns	40.30 \pm 6.70
MCV (fL)	53.10 \pm 0.70ns	52.80 \pm 2.00ns	52.95 \pm 0.15ns	52.95 \pm 0.55ns	54.70 \pm 1.50ns	53.25 \pm 0.45ns	48.6 \pm 4.21ns	53.85 \pm 0.65
MCH (pg)	14.60 \pm 0.10ns	14.60 \pm 0.70ns	15.15 \pm 0.05ns	14.90 \pm 0.10ns	15.40 \pm 0.60ns	15.10 \pm 0.30ns	14.4 \pm 1.88ns	14.30 \pm 0.50
MCHC (g dL ⁻¹)	27.50 \pm 0.10ns	27.65 \pm 0.35ns	28.65 \pm 0.05ns	28.15 \pm 0.45ns	28.15 \pm 0.25ns	28.40 \pm 0.30ns	32.6 \pm 3.30ns	26.55 \pm 0.55
RDW-CV (%)	22.50 \pm 0.60*	18.30 \pm 3.90ns	17.75 \pm 1.95ns	18.70 \pm 2.40ns	16.25 \pm 0.65ns	13.05 \pm 0.55ns	15.15 \pm 5.17ns	15.60 \pm 0.60
RDW-SD (fL)	39.65 \pm 1.25*	32.85 \pm 3.95ns	21.90 \pm 13.80ns	33.00 \pm 1.10ns	31.70 \pm 1.00ns	28.60 \pm 0.60ns	30.75 \pm 4.35ns	31.20 \pm 0.90
PLT ($\times 10^9/L$)	1.02 \pm 4.60ns	1.371 \pm 4.55ns	1.17 \pm 2.07ns	1.621 \pm 9.60ns	1.372 \pm 4.10ns	0.84 \pm 1.76ns	1.172 \pm 9.07ns	1.179 \pm 8.16
MPV (fL)	6.60 \pm 0.10ns	6.50 \pm 0.10ns	6.45 \pm 0.25ns	6.850 \pm 0.25ns	6.200 \pm 0.10ns	6.90 \pm 0.30ns	6.15 \pm 0.21ns	6.650 \pm 0.15
PDW	8.05 \pm 0.05ns	7.60 \pm 0.30ns	7.60 \pm 0.50ns	8.150 \pm 0.35ns	6.950 \pm 0.15ns	7.95 \pm 0.45ns	7.08 \pm 0.21ns	7.850 \pm 0.35

Values quoted are Means \pm SD (n = 3). White Blood Cells (WBC), Haemoglobin (HGB), Red Blood Cell (RBC), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Blood Cell Distribution Width (RDW-CV and RDW-SD), Platelet Count (PLT), Mean Platelet Volume (MPV) and Platelet Distribution width (PDW). Levels of significance between arsenic plus MU exposure groups and the control were compared using students one-way ANOVA followed by Dunnett's multiple comparisons test. ns: Not significant, *Significant at p < 0.01

infection has much to do with the state of the immune response of the mice²⁶.

The dose-dependent liver and spleen damage in experimental mice with MU inoculation, after 112 days of arsenic exposure was anticipated. Susceptibility to microbial infection studies involving acute arsenic toxicity studies have shown that, organ most affected are those involved with absorption, accumulation and/or excretion. These organs are the liver, kidney and the skin among many others and other organs are secondarily affected (e.g., heart)²⁷. In a bid to fight back the MU infection regulated by arsenic toxicity, these organs especially the liver have its hepatocytes ballooned with the loosening of its cell wall with occasional nuclei loss. This was consistent with the study.

The significant dose-dependent reduction in WBC in mice exposed to high levels of arsenic (3.6-4.8 mg L⁻¹) and inoculated with MU could be mediated by arsenic. Arsenic is known to cause a decrease of white blood cell during the arsenic antagonism in male mice making them prone to microbacterial infection²⁸. This supports the toxicity of arsenic treated mice to the development of Buruli Ulcer in the Amansie West District, Ghana¹⁴.

CONCLUSION

Arsenic in drinking water at sub-toxic levels has an immunosuppressive potential and enhanced susceptibility to infection. This can hastens a possible *Mycobacterium ulcerans* infection in mice. It is therefore concluded that arsenic in water and soil enhances susceptible to a possible Buruli Ulcer infection in an endemic area.

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