المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
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Jordan Journal of Biological Sciences (JJBS) (ISSN: 1995–6673): An International Peer- Reviewed Research Journal funded by the Scientific Research Support, Ministry of Higher Education and Scientific Research, Jordan and published quarterly by the Deanship of Research and Graduate Studies, The Hashemite University, Jordan.

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An International Peer-Reviewed Scientific Journal

ISSN 1995-6673

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JJBS

Volume 5, Number 2,June 2012
ISSN 1995-6673

Jordan Journal of Biological Sciences

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Jordan Journal of Biological Sciences

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 85 - 89

IgG, IgA and IgE Reactivities to Sperm Antigens in Infertile Women

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Received on August 29, 2011, Accepted on January 10, 2011

Abstract

Nowadays, World Health Organization (WHO) appoints infertility as a disease. Still more couples are diagnosed with unexplained infertility, it reaches almost 20 % of all cases. Immune system plays an important role: iso-immunization of women is possible by human sperm cells. For better understanding, it is necessary to detect and characterize antigens recognized by women immune system. Our study is focused on female infertility and is based on the ejaculate sample from four healthy donors. Antigens analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing (followed by IgG, IgA and IgE immunoblotting) were detected by forty-five sera from women who were diagnosed with fertility failure. Ten children's sera were used as negative controls. The immunodominant sperm antigens detected by IgG antibodies were 68 and 123 kDa proteins with pI from 6.60 to 6.62, respectively. Concerning mucosal immunity the most frequently recognized sperm IgA-binding protein reached 130 kDa and pI 6.9, 7.4, 9.5. This study did not prove the association of IgE antibodies with unexplained infertility in women.

Keywords: Human sperm, unexplained infertility, polyacrylamide gel electrophoresis, isoelectric focusing, immunoblotting.

1. Introduction

The prevalence of infertile couples significantly increases. The World Health Organization (WHO) appoints human infertility as a disease and defines it as an inability to conceive a child after a period of one year of unprotected intercourse with normal frequency.

The cause of infertility could be due to women as well as men. Pathological spermiogram represents 40 % of the cases; problems of women genital tract also 40 % and 20 % of the reasons are still unknown. The immune system dominates in the last mentioned case. Although the relative importance of immunological factors in human reproduction remains controversial, substantial evidence suggests that human leukocyte antigens (HLA), antisperm antibodies (ASA), integrins, cytokines, antiphospholipid antibodies, endometrial adhesion factors or e.g. mucins (MUC1) contribute to reproductive failure (Choudhury and Knapp, 2001). Some cases of infertility considered inexplicable were found to have an immunologic basis. Spermatozoa are highly antigenic cells. When the state of immune tolerance is interfered, iso-immunization in women can occur. Some studies have mentioned and shown that immune infertility could be caused by iso-immunization of female organism by antigens from human semen (Verpillat *et al*., 1995; Ulcova-Gallova, 2006; Ulcova- Gallova, 2003).

Semen proteins represent potential antigenic and immunogenic structures for female immune system. Iso-immunization evokes strong immune response that prevents successful pregnancy. This phenomenon could be seen in connection with natural tolerance failure or with possible consequence of recurrent inflammation of the genital tract or susceptibility to allergic reactions (Dimitrov *et al*., 1994; Ulcova-Gallova, 2006). ASA are significantly connected with immunological infertility and they can be detected in female blood serum, also in cervical mucus. The character of ASA is most apparently sperm-agglutinating, sperm-cytotoxic and sperm-immobilizing (Shetty *et al*., 2006; Sedlackova *et al*., 2010). Unfortunately, there is no definitive global treatment known. For successful treatment of unexplained infertility it is necessary to reveal and characterize particular antigens coming from sperm cells (Jones, 1991; Doherty and Clark, 2006).

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The aim of this study was to define sperm antigens implicated in unexplained infertility in association with IgG, IgA and IgE antibodies from sera of infertile women: IgG as a representative immune reaction, IgA as a potential mucosal immune response and IgE as a potential local allergic reaction. Collected data should be used as preliminary for thorough two-dimensional analysis and further identification.

2. Materials and Methods

2.1. Sample Preparation

Semen samples from four healthy donors with normal semen characteristics (WHO, 1992) were obtained by masturbation after 3 – 5 days abstinence under informed written consent. Ejaculates were let to liquefy and a mixture of protease inhibitors (0.05 M ε-aminocaproic acid, 5 mM ethylene glycol tetraacetic acid **(**EGTA) and 0.01 M benzamidine) was added.

1 ml of ejaculate from four individuals was centrifuged separately at 1075x g for 15 minutes at 4°C. The supernatant representing seminal fluid was removed. The pellet obtained after centrifugation of whole ejaculate represented predominantly sperm cells then small amount of epithelia. Each pellet was washed twice by PBS (0.01 M phosphate buffered saline with NaCl 0.15 M, pH 7.4) supplemented with protease inhibitors mixture and centrifuged at the same conditions. Lysis was proceeded using 1% Triton X-100 (Sigma, USA) in deionized water, containing a mixture of protease inhibitors (1:50) for 4 hours at 4 °C on ice. Insoluble parts were removed by final centrifugation at 3000x g for 10 minutes at 4°C. The supernatants of all samples were pooled.

Protein concentration of pooled samples was measured by the bicinchoninic acid protocol (BCA, Sigma, USA) (Smith *et al*., 1985) using bovine serum albumin (BSA) as standard. The unused samples were stored at -20°C until assayed.

2.2. Patients

Serum samples were obtained from forty-five women diagnosed with fertility failure and from ten eight-year-old girls. Sera were frozen immediately at -20°C until further use. Children's sera were selected as negative controls because these girls had no contacts to sperm antigens. All experiments were done under informed written consent.

2.3. One-Dimensional Polyacrylamide Electrophoresis (1D SDS-PAGE)

SDS-PAGE was performed at room temperature in Mini Protean Cell Bio-Rad (Bio-Rad, USA) according to Harlow and Lane modification (1988). Preparation required casting two different layers of acrylamide between clean glass plates, separating gel and stacking gel that includes the sample wells.

Aliquots of pooled samples were separated on 10% acrylamide gel (30% acrylamide mix, 1.5 M Tris-HCl pH 8.8, 10% SDS, 10% ammonium persulfate (APS), tetramethylethylenediamine (TEMED)–Serva, Heidelberg, Germany) with 5% acrylamide stacking gel (30% acrylamide mix, 0.5 M Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED). Slots were loaded at the protein concentration of 200 µg/ml. Sample was diluted 1:1 with nonreducing protein loading buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue). Samples were heated for two minutes in boiling water before the loading into the gel. Pre-stained protein standard was used as known marker (Pre-stained SDS-PAGE standard, broad rang, Bio-Rad, USA). After the electrophoresis, separated proteins were either transferred onto a nitrocellulose sheet (NC, Serva, Heidelberg, Germany) or silver-stained (SilverTM Plus Stain Kit, Sigma, USA).

2.4. Immunoblotting Analysis

Mini Trans-Blot Cell Bio-Rad (Mini Protean III system, Bio-Rad, USA) was used for Western blot. Electro-transfer was performed onto 0.45 µm pore size NC membrane under an electric field of 100 V for forty-five minutes at 4°C using Tris-glycin transfer buffer (48 mM Tris, 39 mM glycin, 10% v/v methanol). The membrane was cut to three-millimeter strips then saturated by incubation for two hours at room temperature in blocking solution (PBS-Tw, 0.3% v/v Tween 20, pH 7.4). Each sheet was incubated separately with 50-fold diluted female sera at 4 °C overnight under constant rocking. Binding of the primary antibody from sera to the target protein was followed by a complex with alkaline phosphatase (AP)-linked secondary antibody formation (Goat Anti-Human IgG, Promega, USA or Goat Anti-Human IgA, Sigma, USA or Goat Anti-Human IgE, Sigma, USA). The IgG conjugate was 10000-fold diluted, IgA and IgE conjugates 5000-fold diluted. The created immune complex was visualized by chromogenic substrate NBT/BCIP (IMMUNO NBT/BCIP, Liquid substrate plus, MP Biomedicals, USA) according to the manufacture's instruction. The blots were washed three-times for ten minutes with PBS-Tw 0.1% between each incubation step.

2.5. One-Dimensional Acrylamide Gel Isoelectric Focusing (1D IEF)

IEF separation of pooled samples was carried out in a polyacrylamide gel (CleanGel™ IEF, GE Healthcare) containing 5% v/v Servalyt® pH 3-10 (Serva Electrophoresis GmbH, Heidelberg, Germany) in Milli-Q water. Sample was loaded on the anode side. The flat bed electrophoretic chamber (Multiphor II, GE Healthcare) was cooled at 15 °C. After separation, the proteins were either transferred to cyanogen bromide (CNBr) activated nitrocellulose membrane (Demeulemester *et al*., 1987) for Western blotting or Coomassie Blue stained. Isoelectric point standards in the range from 3.5 to 10.65 (Serva, Heidelberg, Germany) were used as a reference.

Passive transfer was performed onto CNBr-activated NC membrane for 1 hour. The membrane was dried and then cut into three-millimeter wide strips. The following steps were identical for those described before *(2.4 Immunoblotting analysis)*.

2.6. Gel Scanning

Coomassie blue or silver stained gels were scanned with EPSON Perfection 4990 Photo equipment. The figures were drawn using Corel PHOTO PAINT 12.

3. Results

Pooled samples of ejaculate with normal characteristics from four healthy donors were used for sperm protein extract. The protein concentration was in the range from 0.8 to $3x10^3$ µg/ml. Antibodies-binding proteins were examined by antisperm antibodies coming from forty-five serum samples of women with fertility failure. Ten control samples from sexually inactive girls were negative in immunoblotting analysis.

Prepared sample of sperm proteins was separated by 1D SDS-PAGE (Fig. 1A) and 1D IEF (Fig. 1B). Figure 1 showed the 1D sperm protein profile and IgG-binding proteins using patient and control sera. The range of molecular masses (Mr) is wide but values of pI are very close. The Mr range of sperm antigens reached the scale from 31 to 200 kDa. Immunoblotting analysis allowed us to find out the most frequent antigens, in meaning of 68 and 123 kDa proteins (Fig. 1A). 68 kDa antigen was intensely recognized by 93 % of all sera and 123 kDa antigen by 84 % of them. IgGs from serum S1 interacted also with 50, 86 and 90 kDa antigens, serum S2 interacted with Mr 36 and 48 kDa, serum S3 recognized 50 kDa antigen and serum S4 recognized 43, 50, 64 and 86 kDa antigens. However, their presence was minor. No IgG-binding activity was detected using control sera.

IEF offers resolution of sperm proteins according to isoelectric points (pI). Collected data from IEF displayed IgG reactivity to protein of pI 6.6 and 6.62 (Fig. 1B). It was recognized by 82 % of all forty-five female sera. No other one was detected individually. No IgG-binding proteins were found by using controls.

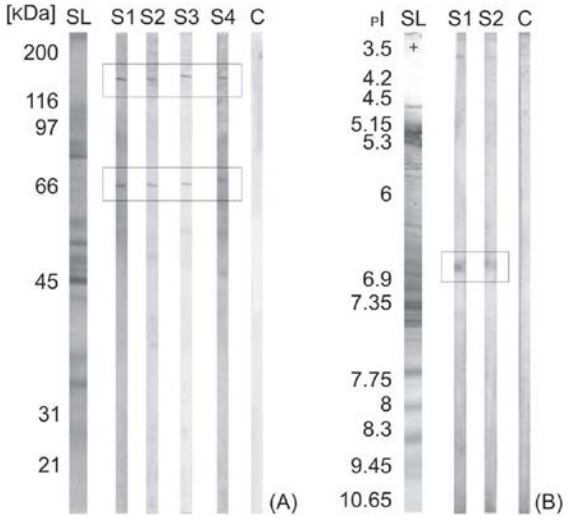

Figure 1. IgG reactivity of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: proteins of pooled sperm extract, silver and Coomassie blue stained; s1 – 4: screening of sera interacting with sperm antigens; C: negative control serum.

Another task of our study was to find out the potential mucosal immune response of IgA antibodies from female patient sera against sperm proteins which were separated by 1D SDS-PAGE (Fig. 2A) and 1D IEF (Fig. 2B). 130 kDa antigen turned out to be the dominant one and it was claimed in 71 % of all sera (Fig. 2A). Other antigens interacting with IgA antibodies were not detected. No IgA-binding at all was observed by using control sera.

Three antigens (pI 6.9, 7.4 and 9.5) were recognized by IgA antibodies in Western blot of 1D IEF gel (Fig. 2B). pI 6.9 was proved in 44 % of used sera, pI 7.4 in 51 % and 9.5 in 66 %. No IgA-binding was detected using control sera.

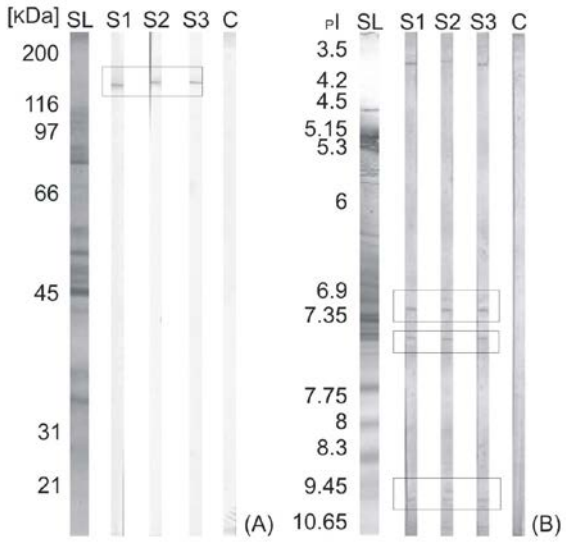

Figure 2. IgA detection of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: proteins of pooled sperm extract, silver and Coomassie blue stained; s1 – 3: screening of sera interacting with sperm antigens; C: negative control serum.

Figure 3 illustrates anti-human sperm reaction type IgE. Sperm proteins were not recognized by IgE antibodies from sera of women with fertility failure either after SDS-PAGE (Fig. 3A) or IEF (Fig. 3B).

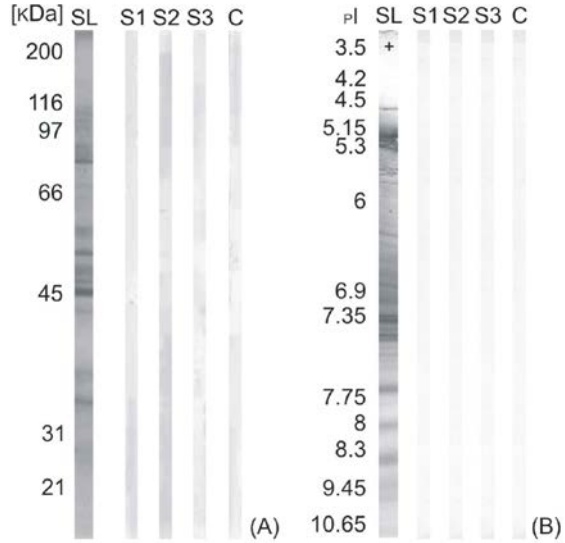

Figure 3. IgE detection of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: protein of pooled sperm extract, silver and Coomassie blue stained; s1 – 3: screening of sera interacting with sperm antigens; C: negative control serum.

4. Discussion

More and more couples with fertility failure are diagnosed with unexplained infertility. For better understanding, it is necessary to look upon sperm as a

potential immunogenic agent. The characterization of sperm antigens plays an important role in successful treatment of immunological infertility.

In this paper, we analyzed the reactivity of IgG, IgA and IgE present in the sera of women with fertility failure with human sperm antigens separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing. Our aim was to describe characteristics of immunodominant human sperm antigens in Western blot of 1D gel, in terms of the molecular weight and the isoelectric point of the most frequently recognized antigens. The emphasis of experimental work was on appropriate choice of the healthy donors with normal sperm parameters (WHO, 1992). Pooled sperm extract represented the antigen source. In the present study four donors of semen were chosen and forty-five sera of women with fertility failure were used for immunodetection and ten children's sera as negative control.

In general, higher levels of IgG antibodies in women with fertility failure represent the immunopathological response. Considering all results we showed that major proteins are rarely recognized as antigens, those are detected among the minor proteins in sperm protein extract.

Another study distinguished a panel of IgG-binding proteins of molecular weight (Mr) less than 70 kDa (Feng, 2008). Our results showed higher molecular masses. Feng (2008) reported immunoreactivity of sera from female infertile patients toward the 35, 40, 47, 65 kDa proteins extracted from donor sperm. 123 kDa antigen revealed in our Western blot analysis is not in an agreement with mentioned study. We explain this by using different protocol for sperm protein extract. It is possible that 1% SDS used in their study may dissolve another groups of antigens than 1% Triton-X100 used in our samples. They reported immunogenicity of antigens coming from sperm surface membrane. Potentially, antigen presenting Mr of 123 kDa could come in origin from inner part of sperm cells. Which protein it is exactly, membrane or plasmatic, and whether or not the similar Mr mean the same proteins can be judged only on the base of mass spectrometry analysis. The same applies to close values of Mr: 65 kDa reported by Feng and 68 kDa revealed in our study. IgG-binding proteins reached pI 6.6 and 6.62. It presents one or two antigens. Bohring (2001) showed approximately same isoelectric point (pI 6.5) only for one antigen but detected by male infertile patients and also not as the most frequently recognized one. It means that IgG-binding protein with pI close to 6 could be universal for both sexes. We would like to remark the sample deposit in the part of pI 3.7. Overall, collected data demonstrated that IgG antibodies presented in female patients interfere with infertility.

Kuttech (1995) determined the immunoglobulin A distribution of antisperm antibodies in the sera and cervical mucus of infertile women. Our results are in agreement with the presence of IgA in female patient sera. In comparison with study of Bohring (2001) involving characterization of IgA-binding proteins from sera of men subjects, our results differ from those described in the literature: Bohring (2001) found Mr not higher than 85 kDa and pI value more basic than 6 while we displayed Mr of 130 kDa and pI 6.9, 7.4, 9.5, maybe representing isoforms. Although, these three bands were not always detected simultaneously by patient sera therefore cannot be marked as isoforms. IgA reaction to possible protein of pI 3.7 means a sample deposit and to evaluate it as the real IgA-mediated antigen would be very doubtful. It is well-known that antibodies in the genital track are predominantly IgG and not IgA (Johansson and Lycke, 2003). Our findings indicate that mucosal immunity represented by IgA reactivity to sperm proteins and their local and circulating distribution provide important and valuable knowledge about association with infertility in women.

Our study in contrast with other (Marthur *et al*., 1981) did not prove anti-IgE reactivity from female sera. The absence of IgE-mediated response could be explained by the very low level of IgEs in patient sera, antibodies possibly bound antigens very faintly or shortly after immediate contact with sperm cells, or it is possible to explain this by the fact that no women suffered from the typical allergic reactions after sexual intercourse like itching, swelling, burning etc. even anaphylaxis to individual semen components. Our results did not document the occurrence of local allergic reaction mediated by IgE antibodies which was however published elsewhere (Ferre-Ybarz *et al*., 2006). It is also well-known that the allergic reaction to semen components is not always associated with infertility (Ebo *et al*., 1995; Weidinger *et al*., 2005).

Acknowledgement

The authors would like to gratefully thank semen donors. This work was supported by the grant projects from Czech Ministry of Education VZ MSMT 0021620812, VZ MSMT 6046137305 and from specific university research MSMT no.21/2011.

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JJBS

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 91 - 97

Jordan Journal of Biological Sciences

Acute Effects of Arsenic on the Regulation of Metabolic Activities in Liver of Fresh Water Fishes (Taki) during Cold Acclimation.

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Received on December 6, 2011, Accepted on January 14, 2012

Abstract

A variety of *Channa punctatus* fishes were exposed to low temperature for 30 min, 1h, 2h and 4h respectively, since these species of fishes are highly energetic and survive in the critical situations. The protein content in liver extract was not altered significantly with the extension of time and remains similar to the control. Cold exposure does not significantly affect inorganic phosphate (Pi), however, a significant increased level was observed after a 30 min exposure. The alkaline phosphatase (ALKP) activity was significantly enhanced by time dependency and was optimum at 1h of cold exposure. To clarify whether arsenic impairs the cold-induced metabolic functions, groups of fishes were exposed to 10 mM and 100 mM sodium arsenate (Na_2HAsO_4) as well as in cold. The protein content was significantly enhanced by Na_2HAsO_4, however, the effects were lower in response to 100 mM concentration. Similar increase in Pi level in liver in response to arsenic was observed and reduced in cold exposure. ALKP activity was increased by arsenic treatment; however, the higher dose appeared to reduce the potency by inducing the activity. To find the role of arsenic on liver only, arsenic treated and control livers were excluded completely and their weights recorded. A significant increased liver exposed with 10 mM Na_2HAsO_4 was found; and on the other hand, whenever the fishes were exposed to 100 mM Na_2HAsO_4, reduced liver weight was noted. The results demonstrate that cold exposure is critically involved in the regulation of certain metabolic activities in liver and arsenic might be involved in interaction of cold induced effects.

***Keywords*:** Arsenic exposure, low temperature, liver, metabolic regulation.

1. Introduction

Cold exposure has been recognized as a major environmental sympathetic stimulus (Saito, 1928 ; Leduc, 1961) and is a stressful event that elicits different thermogenic adaptive responses in endotherms and exotherms. In mammals, including humans, the physiological responses involve changes in energy expenditure, heat production and dissipation, physical activity and appetite (Lowel and Spiegelman, 2000). In rodents, shivering, activation of the sympathetic axis (Rayner and Trayhurn, 2001; Spiegelman. and Flier, 2001) with remarkable activity of mitochondrial uncoupling proteins (UCPs) (Boss *et al*., 2000; Golozoboubova *et al*., 2001) were reported as pivotal mechanisms. The greater the UCP concentration, the greater the capacity to uncouple mitochondrial oxidative phosphorylation so that heat is produced. Among the peripheral tissues, liver plays an important role in metabolic regulation. The metabolic activities in liver are modulated by both environmental and chemical stimuli. The enhanced nerve activity in response to cold is involved in regulation of metabolic activities in liver as well as in other peripheral tissues. The increased nerve activity in liver has been involved in changes of degradation of cellular ATP (Kennedy *et al*., 1997; Westfall *et al*.,2000). Moreover, liver glycogen metabolism is influenced in response to cold (Thomas and George, 1975). The deposition and degradation of cellular metabolite are biological processes in liver by which the organism is benefited and survives in the atmosphere. Higher degradation of liver glycogen releases energy available for doing mechanical work and survive in the critical circumstances and environment. The vertebrate organisms also utilize shivering and non-shivering thermogenesis for the survival process during energy deficiency. Higher expression of uncoupling protein (UCP-1) involved in non-shivering thermogenesis in peripheral tissue of rodents was found (Golozoboubova *et al*., 2001). UCP has been regarded to be the thermogenic protein and plays the critical role in the regulation of energy balance and its molecular weight is 32 Kdal. Ablation of UCP-1 results in a cold-sensitive phenotype (Ricquier and Bouillaud, 2000). Therefore, it is presumably assumed that UCP-1 plays a crucial role in the regulation of body temperature in rodents. Adaptive thermogenesis, the dissipation of energy in the form of heat in response to external stimuli, has been implicated in the regulation of energy balance and body temperature. In shivering thermogenesis, because of the higher oxidative

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process, generation of ATP rather than UCP is predominant and hydrolysis of ATP yields energy for living in the atmosphere. Therefore, it is generally accepted that the organisms survive in the critical environment by different mechanisms and varies species to species. However, the mechanism of the survival process is not clarified. It is assumed that other factors are also involved in the survival process.

Channa punctatus is generally found in fresh waters of haor, bil, river in Bangladesh. They are much energetic and survive in the critical circumstances for long time. They are the major sources of protein in the diet for human being. It is assumed that the higher energy content of this fish is caused by the increased activity of the sympathetic nerves. During environmental low temperature, it is assumed that liver might be involved critically on its regulation of metabolites to survive in the atmosphere. However, to survive in the atmosphere, the critical role of liver of these fish species on adaptive response involving the regulation of metabolic processes is not understood.

Arsenic is toxic to the living organisms. Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou *et al*., 2003) and causes different types of pathogenic syndromes in rodents, fishes and other organisms. Exposure of higher concentration of arsenic in water may also cause severe effects in fish and might be involved in producing cancer or other cellular effects. However, the mechanism underlying the effects of acute arsenic exposure on the regulation of oxidative and glycolytic processes in liver exposed to cold is not known. Arsenic is classified as a human carcinogen based on several epidemiological studies showing an association of arsenic exposure with cancers in lung, bladder, kidney and liver (Hughes, 2002; Tchounwou *et al*., 2003). Moreover, fish have long been used as sentinels for biomonitoring of aquatic environmental pollutants and are good indicators of arsenic toxicity (Abdel-Hameid, 2009). The regulation of metabolic activities in liver in response to changes of temperature is an important aspect in fish and to clarify the role of arsenic in cold-induced liver metabolic functions responsible for the survival of these species of fishes in the environment, the current protocol was designed for investigation.

2. Materials and Methods

2.1. Fishes

Taki fishes (*Channa punctatus*) weighing 50 g to 60 g were used and maintained in normal water with ambient temperature (25.0 ± 1.0^{o}C) . On the day of the experiment, cold exposure of between 4~8^{o}C was given to different groups of fishes in the cold chamber for 30 min, 1 h, 2 h and 4 h period with full aeration and with free access of water. After cold exposure treatment, fishes were quickly decapitated and liver carefully removed and weighed (Chyo, JL-180, China) and kept at –20 ^{o}C. Control fishes were similarly used for tissue sampling except not exposing to cold.

2.2. Arsenic Treatment

To examine the role of arsenic on the regulation of metabolic activities in liver, groups of fishes were exposed with different concentrations of arsenic compound (10 mM and 100 mM Na_2HAsO_4. $7H_2O$, BDH Chemical Ltd.) in cold for 1 h. Respective groups of fishes were treated with only 10 mM and 100 mM of arsenic compound (Na_2HAsO_4) for 1 h in the ambient temperature. The tissues were sampled after the treatment similarly as mentioned above.

2.3. *Assay of Tissue Metabolite*

Liver was homogenized with pre-cooled water and centrifuged at 8000 rpm for 10 min. The supernatant was used as crude extract for assay of protein, inorganic phosphate and alkaline phosphatase activity. Protein was determined by Lowry method (Lowry *et al*., 1951) using 50 μL crude extract. Inorganic phosphate (Pi) and alkaline phosphatase activity were determined by using 100~200 μL liver extract. For Pi estimation, 200 μL tissue extract was diluted to 5 mL with water and was mixed vigorously with 5 mL of 5% TCA (Trichloroacetic acid) and centrifuged at 6000 rpm for 10 min. 5 mL supernatant was transferred to another glass tube and kept on ice. 1 mL molybdate reagent (10 g of ammonium molybdate in 100 mL water was taken and 100 mL of 5N H_2SO_4 was added to prepare 200 mL solution) was added and mixed. The solution was mixed with 0.4 mL aminonaptholsulphonic acid reagent. 3.6 mL water was added and mixed. The tube was kept standing for 10 min for the complete development of color. For the blank, 5 mL of 5% TCA and 5 mL water were mixed only. Absorbance was taken at 690 nm against the blank. The Pi in tissue extract was calculated using a standard KH_2PO_4 solution. The enzyme activity was determined by the procedure of Ramnik (1999). 0.25 mL of *p*-nitrophenyl phosphate (PNPP) (1.2 mg/mL in glycine-NaOH buffer, pH 10.0) was added to 0.5 mL glycine-NaOH buffer (pH 10.0) and incubated for 5 min at 37^{o}C. 100~200 μL of tissue extract was taken to the solution and for blank the same volume of buffer was used in place of tissue extract and incubated for 30 min. After incubation, it was made up to 4 mL with 0.1N NaOH solution and absorbance was taken at 410 nm. The amount of PNP (*p*-nitrophenol) produced after hydrolysis of PNPP by the enzyme was measured from the standard PNP solution (500 μmol/L in buffer, pH 10.0). The enzyme activity is expressed as μmol of PNP/min/mg of tissue.

2.4. Statistical Analysis

Results of the experiments were expressed as mean and standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by paired t-test using SPSS software.

3. Results

3.1. Time Course Effect of Low Temperature on the Regulation of Protein Content in Liver

As shown in Fig. 1, the average protein content in liver of fishes exposed to cold for 30 min, 1 h, 2 h and 4 h were 10.87 ± 0.57 g, 10.20 ± 0.55 g, 10.15 ± 1.85 g and 10.41 ± 0.94 g respectively while for the control fish, the value was

10.63 ± 0.72 g/100 g of tissue weight. No significant changes in protein content in liver were recorded up to 4 h of cold exposure and almost similar to the control fishes. The results demonstrate that cold exposure is involved in the regulation of metabolic functions in the liver without alteration of tissue protein content.

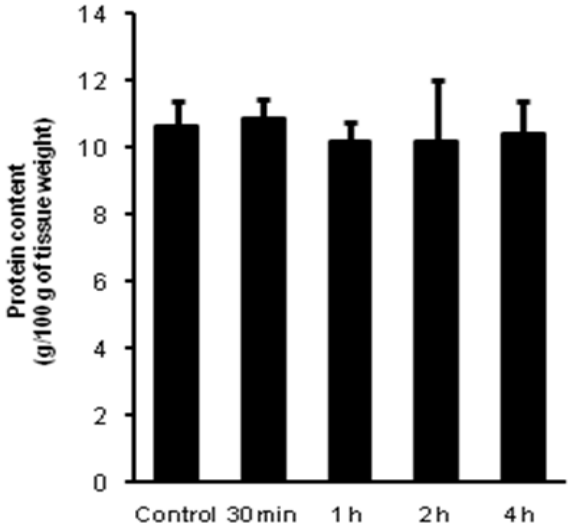

Figure 1. Changes of protein content in liver of fishes exposed to cold. The fishes were kept for 30 min, 1 h, 2 h and 4 h in the cold chamber. Control fishes were similarly used for sampling of tissue except giving cold exposure. The data are ± SEM for 4 fishes in each group.

3.2. Time Course Effect of Low Temperature on the Regulation of Inorganic Phosphate (Pi) in Liver

To examine whether cold exposure is involved in the regulation of liver Pi, groups of fishes were exposed to cold. The amount of inorganic phosphate (Pi) in liver of fishes for 30 min, 1 h, 2 h and 4 h in cold were 3.22 ± 0.18 mg, 2.31 ± 0.33 mg, 2.27 ± 0.17 mg and 1.93 ± 0.27 mg respectively while for the control fish, Pi content was 2.68 ± 0.19 mg/100 g of tissue weight. Although the Pi values decreased by 13.8%, 15.3% and 27.9% for 1 h, 2 h and 4 h respectively, at 30 min cold exposure, a significant (P<0.05) 20.14 % increased Pi was observed compared to the control (Fig. 2).

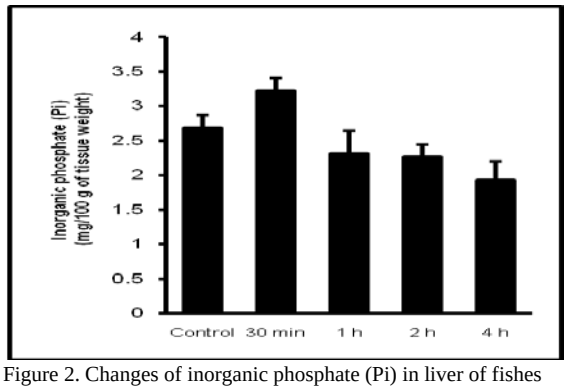

Figure 2. Changes of inorganic phosphate (Pi) in liver of fishes exposed to acute cold exposure. The fishes were exposed to cold for 30 min, 1 h, 2 h and 4 h in the cold chamber. Control fishes were similarly used for sampling of tissue except giving cold exposure. The data are ± SEM for 4~5 fishes in each group.

The results demonstrate that low temperature does not significantly affect Pi in liver however a higher value was found whenever the fishes were exposed to cold for 30 min. It could be interpreted as that after 30 min of cold exposure, the production of the inorganic phosphate is higher than the consumption, while after 1 h of exposure the consumption overcomes the production.

3.3. Time Course Effect of Low Temperature on the Regulation of Alkaline Phosphatase (ALKP) Activity in Liver

To clarify whether cold exposure is involved in the regulation of ALKP activity in liver, fishes were exposed to cold and the average ALKP activities in the extract of liver of fishes for 30 min, 1 h, 2 h and 4 h were 0.61 ± 0.02 μmol, 0.80 ± 0.07 μmol, 0.77 ± 0.06 μmol and 0.76 ± 0.10 μmol respectively, while for the control fish, the enzyme activity was 0.35 ± 0.08 μmol/min/g of tissue weight. A significance of 74.2% (P<0.001) and 128.5% (P<0.005) enhanced ALKP activities in liver were found after 30 min and 1 h respectively (Fig. 3) and 120.0% (P<0.005) and 117.1% (P<0.05) increased activities after 2 h and 4 h were found respectively compared to the liver of control fishes.

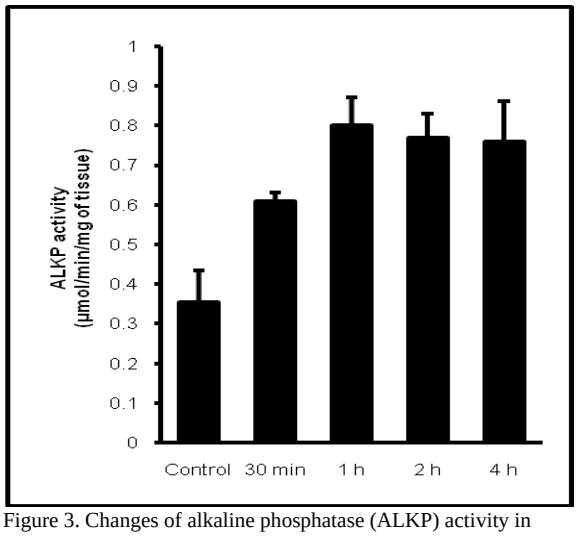

Figure 3. Changes of alkaline phosphatase (ALKP) activity in liver of fishes exposed to acute cold exposure. The fishes were exposed to cold for 30 min, 1 h, 2 h and 4 h in the cold chamber. Control fishes were similarly used for sampling of tissue except giving cold exposure. The data are ± SEM for 4~5 fishes in each group.

Higher alkaline activity was observed after 1 h of cold. Cold exposure stimulates enzyme activity time dependents up to 1 h. ALKP activity is proportional to the duration of the cold exposure until it does not exceed 1 hour. At 1 h of the cold exposure this stress response reaches its maximum and does not increase after further exposures, for 2 and 4 hours. The changes of liver ALKP in response to cold might be involved in the regulation of liver metabolic functions. The alteration of ALKP in liver is an index for the characterization of the sensitivity to the environmental temperature.

3.4. Role of 10 mM and 100 mM Na_2HAsO_4 on Protein Content in Liver

Groups of fishes were used to examine the role of arsenic on the changes of protein in liver. As shown in Fig. 4, the amount of protein in liver in response to arsenic (10 mM) was significantly increased. Protein content of

arsenic-treated fishes for 1 h was 31.2 ± 1.22 g where as for control fishes, was 10.63 ± 0.72 g/100 g of tissue weight. A significance of 193.5% (P<0.001) increased protein in liver was observed after 1 h when compared with control liver. Groups of fishes were exposed to cold with arsenic solution and the amount of protein in liver for 1 h was 20.04 ± 1.16 g. The results indicated that 88.5% (P<0.001) increased liver protein was found in response to 10 mM Na_2HAsO_4 in cold when compared to the control fishes and the value was reduced significantly (P<0.001) in cold exposure even with arsenic solution compared to the respective control.

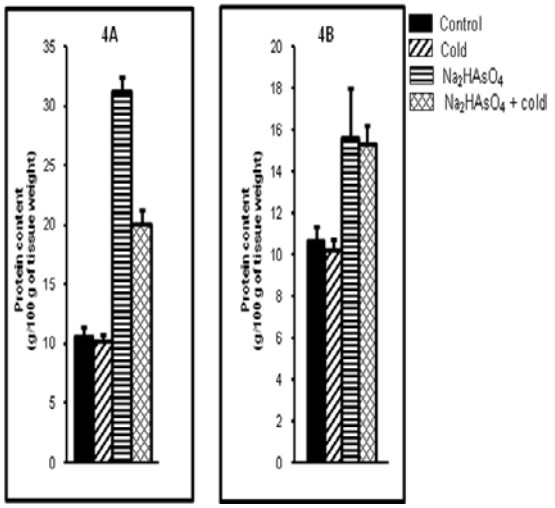

Figure 4. Effects of arsenic on protein content in liver of fishes. The groups of fishes were treated with arsenic solution and kept for 1 h in cold. The respective controls were treated with arsenic only while other fishes were exposed to cold for 1 h. After the treatment, the fishes were immediately decapitated and sampling of tissue was performed. Control fishes were similarly used except giving cold exposure. 4A and 4B represent 10 mM and 100 mM Na_2HAsO_4 respectively. The data are means ± SE for 4~5 fishes in each group.

Fishes exposed to 100 mM Na_2HAsO_4 for 1 h had 15.57 ± 2.42 g protein in their livers while for control; the value was 10.63 ± 0.72 g. The results show that 100 mM Na_2HAsO_4 also causes the increase (46.5%, P<0.1) liver protein compared to that of the control fishes. However, 10 mM Na_2HAsO_4 induces higher protein content than 100 mM concentration (Fig. 4A, Fig. 4B). In response to arsenic in cold, the amount of protein was 15.32 ± 0.90 g while for the respective control exposed to cold only, the value was 10.20 ± 0.55 g. The protein content was increased by 50.2% (P<0.001) and 44.1% (P<0.001) compared to that of cold exposed and control fishes respectively. The results also demonstrate that arsenic causes synthesis of protein in adverse environmental conditions.

3.5. Role of 10 mM and 100 mM Na_2HAsO_4 on Pi Level in Liver

To examine the role of arsenic on Pi level in liver, the fishes were exposed to 10 mM Na_2HAsO_4 solution for 1 h and the respective group was induced by cold exposure. As shown in Fig. 5, Pi content in liver of fishes exposed to arsenic was 9.26 ± 0.78 mg while for the control liver, the value was 2.68 ± 0.19 mg/100 g of tissue and for cold exposed liver, the value was 2.31 ± 0.33 mg per 100 g of tissue. The Pi content in livers of arsenic-treated fish was increased (245.5%) significantly (P<0.005) than in livers of control fish and also of cold exposed fishes (300.8%). Fishes exposed to cold with arsenic had 2.71 ± 0.27 mg Pi in their livers. The results indicate that the increased Pi in response to arsenic was reduced significantly (P<0.001) by cold treatment.

In separate examinations, groups of fishes exposed with 100 mM Na_2HAsO_4 had 3.59 ± 0.59 mg Pi after 1 h and 4.44 ± 0.98 mg in cold with arsenic while 2.31 ± 0.33 mg for cold only. The Pi content in liver was increased similarly in response to 100 mM Na_2HAsO_4 as well as in cold exposure compared to the respective cold-exposed fishes and also to the control. However, the release in Pi in response to higher concentration of arsenic was lower than that of the previous low dose (Fig. 5A, Fig. 5B). The results suggest that the increased Pi in liver might be due to the higher activity of some enzymes responsible for the degradation of the cellular organic compounds and could be considered as the survival factors for this species in critical environment.

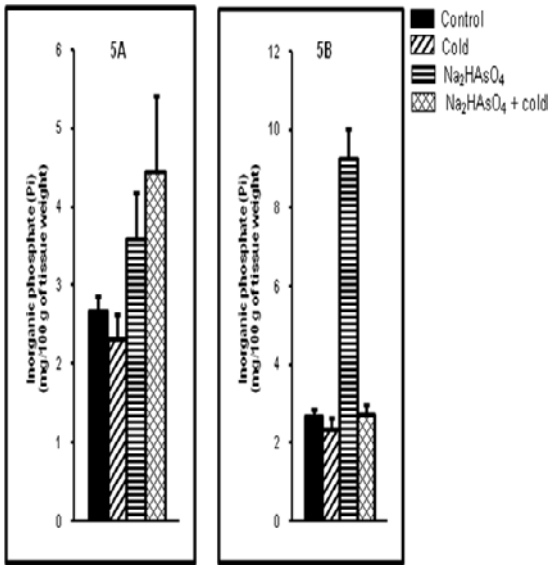

Figure 5. Effects of arsenic on inorganic phosphate (Pi) in liver of fishes. The fishes were exposed to cold with arsenic solution for 1 h. Other groups of fishes were treated with arsenic and exposed to cold for 1 h respectively. After the treatment, the fishes were immediately decapitated and sampling of tissue was performed. Control fishes were similarly used except giving cold exposure. 5A and 5B represent 10 mM and 100 mM Na_2HAsO_4 respectively. The data are means ± SE for 4 fishes in each group.

3.6. Role of 10 mM and 100 mM Na_2HAsO_4 on ALKP Activity in Liver

Liver ALKP is sensitive to toxic response and releases Pi from PNPP (p-nitro phenyl phosphate). To examine the role of arsenic on ALKP activity in liver, groups of fishes were treated with 10 mM and 100 mM Na_2HAsO_4 in cold for 1 h and the respective control livers were also examined with Na_2HAsO_4 only (Fig. 6A and Fig. 6B). The ALKP activity in response to 10 mM Na_2HAsO_4 were 1.28 ± 0.04 μmol and for arsenic treated in cold was 0.65 ± 0.02 μmol whereas for control and the cold exposed fishes, the

activities were 0.35 ± 0.08 µmol and 0.80 ± 0.07 µmol/min/g of tissue respectively. The results demonstrate that ALKP activities were significantly stimulated (265.7%, $P<0.05$) by arsenic compared to control, however, the activity was reduced ($P<0.001$) in cold when compared to arsenic treated group alone. The ALKP activities in livers of groups of fishes in response to 100 mM Na_2HAsO_4 and in cold were 0.93 ± 0.12 µmol and 1.01 ± 0.06 µmol respectively while for control and cold exposed livers, the activities were 0.35 ± 0.08 µmol and 0.80 ± 0.07 µmol respectively.

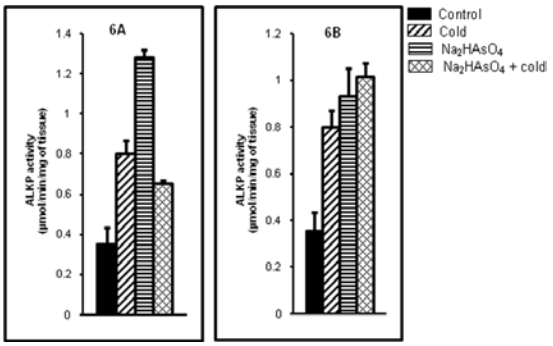

Figure 6. Effects of arsenic on ALKP activity in liver of fishes. The fishes were exposed to cold with arsenic solution for 1 h. Other groups of fishes were treated with arsenic and exposed to cold for 1 h respectively. After the treatment, the fishes were immediately decapitated and sampling of tissue was performed. Control fishes were similarly used except giving cold exposure. 6A and 6B represent 10 mM and 100 mM Na_2HAsO_4 respectively. The data are means ± SE for 4 fishes in each group.

A significant increased response on ALKP activity was observed for fishes exposed to arsenic (165.7%, $P<0.05$) and also even in cold (189.1%, $P<0.05$) when the groups were compared to control. The results would suggest that cold exposure did not overcome the stimulatory effect of higher concentration of arsenic compared to control fishes. The results also demonstrate that higher concentration of arsenic causes stimulation of ALKP activity in liver and the effects were lower than those of 10 mM Na_2HAsO_4.

3.7. Effect of 10 mM Na_2HAsO_4 on Weight of Fish Liver

Groups of fishes were used to examine the role of arsenic on the changes of liver weight. As shown in Table 1, the average liver weight in response to arsenic was significantly increased.

Table 1. Effects of arsenic (10 mM Na_2HAsO_4) on liver weight of respective groups of fishes. The fishes were exposed to cold with arsenic solution for 1 h and 2 h in the cold chamber. The respective controls were treated with arsenic only. After the treatment, the fishes were immediately decapitated and sampling of tissue was performed. Control fishes were similarly used except giving cold exposure.

	Control (n=6)	Na_2HAsO_4 (1 h) (n=5)	Na_2HAsO_4 + cold (1 h) (n=6)	Na_2HAsO_4 (2 h) (n=5)	Na_2HAsO_4 + cold (2 h) (n=6)
Liver weight (g)	0.407 ± 0.04	0.796 ± 0.04^A	0.515 ± 0.04^A	0.474 ± 0.04^B	0.447 ± 0.03^C

The data are means ± SE for 5~6 fishes in each group. $^AP<0.001$ versus control. $^BP<0.05$ versus control. $^CP<0.1$ versus control.

Liver weight of arsenic-treated fishes for 1 h and 2 h were 0.796 ± 0.04 g and 0.474 ± 0.04 g respectively whereas for control fishes, the average liver weight was 0.407 ± 0.04 g. A significant 95.5% ($P<0.001$) and 16.4% ($P<0.05$) increased liver weight were observed after 1 h and 2 h respectively when compared with control liver. Groups of fishes were exposed to cold with arsenic solution and the average liver weight of fishes for 1 h and 2 h were 0.515 ± 0.04 g and 0.447 ± 0.03 g respectively. The results indicated that 26.6% ($P<0.001$) and 9.8% increased liver weight were found in response to 10 mM Na_2HAsO_4 compared to the control fishes.

3.8. Effect of 100 mM Na_2HAsO_4 on Fish Liver Weight

The average liver weight in response to arsenic treatment for 1 h and 2 h were 0.313 ± 0.04 g and 0.347 ± 0.05 g respectively and for control the value was 0.407 ± 0.04 g. The results show that 100 mM Na_2HAsO_4 causes the reduced liver weight compared to that of the control fishes. However, the effects of higher concentration of arsenic are almost reciprocal to that of the low concentration of arsenic (Table 1 and Table 2). The results (shown in Table 2) demonstrate that the liver weights of fishes in response to arsenic were reduced significantly by 23.1% ($P<0.05$) and 14.7% ($P<0.1$) after 1 h and 2 h respectively compared to the control liver.

Table 2. Effects of arsenic (100 mM Na_2HAsO_4) on liver weight of respective groups of fishes. The fishes were exposed to cold with arsenic solution for 1 h and 2 h in the cold chamber. The respective controls were treated with arsenic only. After the treatment, the fishes were immediately decapitated and sampling of tissue was performed. Control fishes were similarly used except giving cold exposure.

	Control (n=6)	Na_2HAsO_4 (1 h) (n=6)	Na_2HAsO_4 + cold (1 h) (n=6)	Na_2HAsO_4 (2 h) (n=5)	Na_2HAsO_4 + cold (2 h) (n=5)
Liver weight (g)	0.407 ± 0.04	0.313 ± 0.04^A	0.334 ± 0.06^B	0.347 ± 0.05^B	0.328 ± 0.02^A

The data are means ± SE for 5~6 fishes in each group. $^AP<0.05$ versus control. $^BP<0.1$ versus control.

The average liver weight of other groups of fishes exposed to cold with arsenic solution for 1 h and 2 h were 0.334 ± 0.06 g and 0.328 ± 0.02 g respectively. The values indicate that arsenic treatment in this condition also reduced the liver weight by 17.8% ($P<0.1$) and 19.4% ($P<0.05$) compared to the control fishes.

4. Discussion

The results of the present study demonstrate the regulation of metabolic functions in liver of *Channa punctata* in response to environmental temperature and adverse effect of sodium arsenate (Na_2HAsO_4) in cold induced fishes. The fish are generally energetic and survive in critical environments, for instance, in water deficiency. Therefore, it is assumed to be it as a major source for characterization of the regulation of metabolic activities. Although the amount of protein in liver was not

changed up to 4 h of the study in cold, treatment with both 10 mM and 100 mM Na_2HAsO_4 resulted the significant increased protein during cold acclimation. Because cold exposure has been believed to have dynamic effect on cellular activity, therefore, during the first few hours of cold stimulation, the activation of a stress response may directly influence whole body metabolism; however, after adaptation, central input coordinate responses to warrant optimisation of the effects. Although variation of temperature causes the alteration of cell activity, effect of acute and prolonged exposure to cold is used to maintain the homeostasis of the species. The increased protein caused by arsenic exposure has been found in our study and appeared to be reduced in cold. Both 10 mM and 100 mM Na_2HAsO_4 produced the toxic environment where the fishes want to survive. Therefore, it is reasonable that an adaptive response by the species was created and some stress proteins are synthesized. However, cold exposure decreases the ability to respond to low arsenic exposure and is appreciable that increased sympathetic nerve activity induced by cold prevents the toxic effect of arsenic. Cold exposure was unable to reduce the toxic effect of arsenic whenever the fishes were exposed with 100 mM Na_2HAsO_4. The results clarify that this higher arsenic produces a severe effect on liver where the sympathetic nervous system does not play its role. The liver is the organ where most of the biotransformation of inorganic arsenic takes place (Del Razo *et al*., 2001). Up regulation of several genes in arsenic-induced adaptive response has been observed (Verma *et al*., 2002; Chelbi-alix *et al*., 2003). Their findings suggest that arsenic may induce the synthesis of molecules responsible for the survival process.

The liver is a major target organ of arsenic toxicity in both mice (Waalkes *et al*., 2003) and humans (Chen *et al*., 1992). Arsenic-induced liver injury in humans is a common phenomenon, typically manifesting initially as degenerative lesions with jaundice, progressing to noncirrhotic portal hypertension, fibrosis, cirrhosis, and neoplasia such as hepatocellular carcinoma (Mazumder *et al*.,1998; Lu *et al*., 2001; Centeno *et al*., 2002). Prolonged exposure of higher concentration of arsenic has been involved in liver injury and damage (Taylor *et al*., 1989; Chiou *et al*., 1995). Liver metabolism is a known potential target for the toxic action of chemicals (Hinton *et al*., 2001).

The released Pi in response to cold was enhanced after 30 min which might be due to the effect of degradation of cellular energy rich compound such as ATP. Since Na^+-K^+ ATPase activity has been demonstrated to be increased by cold exposure (Videla *et al*., 1975), therefore, the increassed Pi might be due to the higher activity of the enzyme Na^+-K^+ ATPase. Alkaline phosphatase (ALKP) might also be involved in this respect. Both 10 mM and 100 mM Na_2HasO_4 induce Pi release in liver, however, the increased Pi is significantly reduced in cold. It is assumed that the sympathetic nervous system is induced by cold and is directly involved in reducing the toxic effect of arsenic. Therefore, it is also assumed that the biotransformation of foreign toxic substances might be influenced by activation of the sympathetic nervous system. Allen et al. (2004) found that arsenic impairs the sympathetic nerve activity induced by cold. ALKP is predominantly found in liver and is an index of the characterization for liver pathogenesis. The increased activity was observed by cold and enhanced the time dependently. It is assumed that this enzyme is sensitive to cold or the activation of sympathetic nervous system is involved during cold acclimation. The released Pi might be influenced in response to low dose of arsenic, because ALKP activity is augmented however, the activity was appeared to be reduced in cold. The results would indicate that metabolic function involving higher ALKP activity in liver is sensitive to this dose. Recent investigation reveals that arsenic exposure stimulated ALKP in liver (Sharma *et al*., 2007). The higher activity demonstrates that increased Pi release would be a survival factor during energy deficiency. Liver weights of fishes exposed to higher concentration of arsenic were reduced significantly compared to control while low dose of arsenic significantly increases liver weight suggesting that higher dose of arsenic might be involved in liver damage, on the contrary, low arsenic is assumed to be involved in biosynthesis of lipids (Sharma *et al*., 2007; Chen and Chiout, 2001). 100 mM arsenic can cause liver glycogenolysis because of its severe effects and liver weight is reduced however, cold exposure could not prevent liver damage in presence of higher arsenic level. Up regulation of genes associated with carbohydrate catabolism such as glycolysis and downstream genes associated with generation of precursor metabolites and energy clearly indicate a continuous need for production of precursor metabolites and energy to sustain various homeostatic and adaptive responses in the liver of arsenic-treated fish compared with the liver of control (non-treated) fish.

5. Conclusion

In summary, as a peripheral tissue, liver is metabolically important for energy consumption and energy expenditure. Environmental low temperature is a major stimulus exerting its effect on metabolic changes. However, the regulatory process in response to cold could be prevented or modulated by the toxic effects of arsenic. As a major metabolic site, liver plays the critical role in the biotransformation of foreign toxic substances. The diverse metabolite regulation in response to low temperature is an index for survival of these species and is a useful biological process, however, arsenic probably takes part in modulation of the metabolic process.

Acknowledgement

This study was carried out in the Department of Biochemistry and Molecular Biology, Rajshahi University and was supported by the University Grant Commission (UGC), Bangladesh.

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JJBS

Volume 5, Number 2, March 2012
ISSN 1995-6673
Pages 99 - 103

Jordan Journal of Biological Sciences

Haematology and Erythrocyte Osmotic Fragility Indices in Domestic Chicken Following Exposure to a Polyvalent Iodophorous Disinfectant

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Received on November 15, 2011 , Accepted on January 21, 2012

Abstract

The effect of prolonged use of Iodosteryl, a polyvalent iodophorous compound, as water disinfectant, on the hematology and erythrocyte osmotic fragility of the domestic chicken was investigated. Twenty eight adult male domestic chickens of the Nera black strain were divided into four groups of seven birds per group. Birds in groups B-D were given potable water containing 1 ml, 2 ml and 4 ml/l Iodosteryl respectively for six weeks. Group A served as the control. Blood samples were collected from each bird after six weeks and analyzed immediately. No significant changes were observed in the packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet total and differential leucocytes values. However, red blood cells (RBC) were slightly lower while erythrocyte osmotic fragility and erythrocyte sedimentation rate (ESR) was higher in those birds exposed to Iodosteryl compared with control. This study confirms that prolonged use of Iodosteryl is stressful and may lead to intravascular haemaolysis as indicated by the higher erythrocyte fragility and ESR values, respectively. The damage observed may be due to peroxidation of erythrocyte membrane lipids, proteins or a generation of free radicals induced by iodine.

Keywords : Chicken, hematology, iodophorous compound, iodosteryl, osmotic fragility.

1. Introduction

Water, an essential component of all organic tissues without which organism cannot survive, is the major component of the earth. It constitutes about 75% of the Earth's surface in the form of ocean, lakes, rivers and streams. Despite the abundance of these water bodies however, only 1% is available as fresh water for human and animal consumption. This is principally because; the water is either salty or unwholesome for consumption. This therefore calls for water treatment before it is made available for use. In fact, more than one billion people lack access to safe drinking water, while more than 200, 000 children die of one form of waterborne disease or the other on a daily basis worldwide (World Health Organization, 2010).

The need for water disinfection for the production of wholesome and potable water has been recognized since the medieval period. In recent time however, several more sophisticated procedures are being used in water purification, these include physical methods such as irradiation, ultrasound, ultra filtration, reverse osmosis, heating, freezing, and the use of ionizing radiation (Mako *et al*., 2007; Lukhele *et al*., 2010; Fittipaldi *et al*., 2010). Other methods include coagulation-flocculation or precipitation prior to sedimentation or filtration, adsorption with activated charcoal, clay etc., and ion exchange processes (World Health Organization, 2010). The chemical methods are so called because of the abilities of the compounds to destroy or inhibit water borne pathogenic organisms that may be present in the impure or contaminated water (Thomas *et al*., 2009). This however, is not with its attendant consequences especially with prolonged or excessive use of these compounds (Morgans and Trotter, 1953).

One of the commonly used chemical agents in water sanitation and disinfection is Iodine and its compounds have been found to be an effective water disinfectant for short term use. According to WHO report on Water Sanitation and Health (World Health Organization, 2010), Iodine, either dissolved in water or in the form of an iodinated exchange resin, has been used for short-term water treatment by outdoor recreationists (campers, hikers, etc), field military personnel, and persons displaced by natural disasters and human conflicts in wars and other societal disruptions (Rogers and Vitaliano, 1977). The use of Iodine in short term water disinfection for military personnel with proven efficacy against viruses, bacteria and protozoan cysts has also been well documented (Clarke and Bettin, 2006). However, several iodine and

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iodine resin or iodophores are in circulation for use in water disinfection in forms of tablets, resins and solutions (Backer, 2002; Backer and Hollowell, 2000; Butkus *et al*., 2005; Clark *et al*., 2003; Crittenden *et al*., 2005).

The most widely used iodine based water disinfectant in Nigeria, especially; among livestock producers is Iodosteryl, a polyvalent iodophorous compound used in disease prevention in poultry (Mako *et al*., 2007). According to the manufacturer's claim and field reports from farmers, the compound has been reported to possess bacteriocidal and virucidal properties. It is also known to inhibit protozoan cysts especially coccidial oocyts. However, most of these claims are yet to be documented. This notwithstanding, the use of Iodosteryl is on the increase in disease prevention in poultry production in Nigeria (Mako *et al*., 2007).

The present study was designed to investigate the effects of prolonged use of Iodosteryl in water on the haematological parameters and erythrocyte osmotic fragility of the domestic chicken.

2. Materials and Methods

2.1. Animals

Adult male domestic chickens of the Nera black strain were used for the study. Twenty-eight, adult (16 weeks old), male Nera black chickens were purchased from a local farm in Ibadan. The birds were stabilized for two weeks, during which they were dewormed with Piperazine hydrochloride (Alfasan, Holland), while antibiotics, Doxygen®, containing gentamycin and doxycycline (Adamore, Nigeria Limited) was administered via their drinking water for 5 days.

2.2. Experimental design

The birds were randomly divided into 4 groups (A-D) consisting of seven birds per group. Group A, which served as the control received clean tap water while groups B-D received tap water with 1 part, 2 parts and 4 parts per million of Iodosteryl, which correspond to low, medium and high doses, respectively, incorporated into their water for a period of six weeks. All the birds were given grower mash and water *ad libitum* throughout the period of the experiment. 1 ml per litre of Iodosteryl is the manufacturer's recommendation for water disinfectant.

Iodosteryl was purchased from Crosley Sinbad & Co. Limited (Nigeria). Blood samples (5 ml) were collected from each bird (at the end of six weeks of exposure to Iodosteryl) into heparinized bottles for determination of haematological parameters and erythrocytes osmotic fragility. Packed cell volume (PCV) was determined by the micro haematocrit method, haemoglobin (Hb) by cyanmethaemoglobin method while red blood cell (RBC), white blood cell and platelet counts were determined using haemocytometer with the improved neubauer slide (Douglas and Harold, 2004). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were then calculated according to Jain (1986). Differential leucocyte count was also determined from blood smears stained with Giemsa. Erythrocyte sedimentation rate was determined from blood diluted with 4 parts citrate solution (3.3% sodium citrate) allowed to stand in Westergen tube.

Erythrocyte osmotic fragility was determined according to the method described by Oyewale (1992). Briefly, 0.02 ml of blood was added to tubes containing increasing concentration of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 (0, 0.1, 0.3, 0.5, 0.7, and 0.9% NaCl concentration).

The tubes were gently mixed and incubated at room temperature (29^{o}C) for 30 minutes. The content of each tube was then centrifuged at 3500rev/min for 10 minutes and the supernatant removed for measurement. Optical density of the supernatant was determined spectrophotometrically at 540 nm using SM22PC Spectrophotometer (Surgienfield Instruments, England). Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%.

2.3. Animal ethics

All of the animals received humane care according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy Science and published by the National Institute of Health. The ethic regulations have been followed in accordance with national and institutional guidelines for the protection of animals' welfare during experiments (Public Health Service, 1996). The experiment was carried out at Biochemistry Laboratory, Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria.

2.4. Statistical analysis

All values are expressed as mean ± SD. The test of significance between two groups was estimated by Student's t-test. One-way ANOVA with Dunnett's post-test was also performed using GraphPad Prism version 4.00.

3. Results

The effect of prolonged administration of Iodosteryl as water disinfectant on the haematological parameters is shown in Table 1.

Table 1. Haematological parameters of adult, male Nera black chicken after exposure to water treated with various concentrations of Iodosteryl

Parameters	Group A (Control, n = 7)	Group B (1 ppm, n = 7)	Group C (2 ppm, n = 7)	Group D (4 ppm, n = 7)
PCV (%)	26.00 ± 4.80	26.00 ± 2.00	25.70 ± 2.50	24.67 ± 0.87
Hb (g/dl)	8.15 ± 1.73	8.64 ± 0.07	8.53 ± 0.86	8.32 ± 0.87
RBC ($\times10^6$/µl)	3.78 ± 0.44	3.49 ± 0.38	2.63 ± 0.44	2.97 ± 0.10
MCV (fl)	60.25 ± 19.16	74.00 ± 8.41	99.14 ± 13.01	83.17 ± 7.91
MCH (µg)	19.75 ± 5.56	24.71 ±3.25	32.71 ± 4.54	27.50 ± 2.66
MCHC (g/dl)	33.17 ± 0.62	33.24 ± 0.28	33.22 ± 0.42	33.02 ± 0.45
Platelet ($\times10^3$/µl)	105.00 ± 19.10	104.30 ± 29.40	107.10 ± 27.50	131.70 ± 18.00
WBC ($\times10^3$/µl)	4.65 ± 1.59	3.63 ± 0.84	3.91 ± 0.94	4.78 ± 0.52
Lymph ($\times10^3$/µl)	2.94 ± 0.99	2.00 ± 0.46	2.82 ± 0.68	3.07 ± 0.33
Heter ($\times10^3$/µl)	1.67 ± 0.05	1.62 ± 0.38	1.06 ± 0.26	1.66 ± 0.18
ESR	4.00 ± 0.82	7.43 ± 2.94	8.86 ± 4.71*	8.71 ± 2.04*

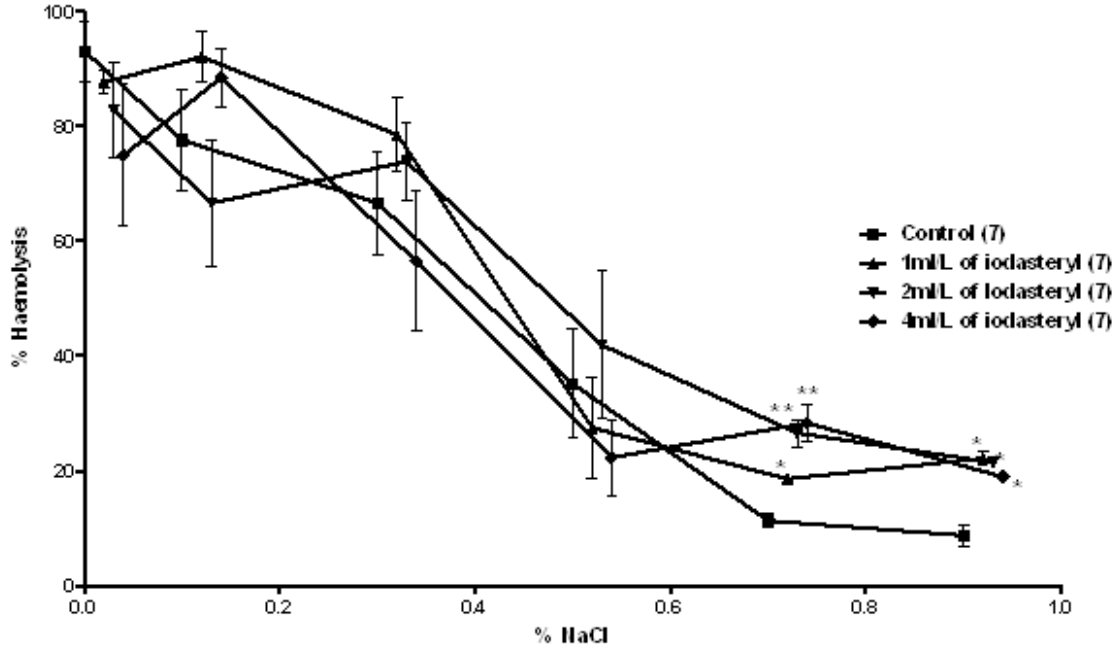
Data are presented as means ± SD, n = (numbers of birds) and values within the same row with asterisks are significant

compared with the control group at $p \leq 0.05$ using analysis of variance (ANOVA) test

The PCV, Hb concentration, WBC and platelet counts as well as the erythrocyte indices of the test groups (groups B to D) that received various concentrations of Iodosteryl were not significantly different from those of the control. Similarly, the differential leucocytes count obtained in the birds treated with Iodosteryl in water were not significantly different from the control. However, the erythrocyte sedimentation rate of the birds exposed to 2ppm and 4ppm of Iodosteryl per litre of drinking water were significantly higher ($p<0.05$) than those of the control. The RBC counts in those birds that received 2 and 4ppm of iodosteryl was lower, though non-significantly than that of the control

As shown in Figure 1, the erythrocyte osmotic fragility of the domestic chicken given 1ppm Iodosteryl (Group B) was significantly higher than that of the control at 0.7% NaCl ($p<0.05$) and 0.9% NaCl concentrations. Similarly, erythrocyte fragility of the chicken in Groups C and D was significantly higher than that of the control at 0.7% NaCl ($p<0.01$) and 0.9% NaCl ($p<0.05$).

Fig 1.0: Erythrocyte osmotic fragility of 24 weeks old Nera black cockerel after adminstration of Iodasteryl® as water sanitizer. Values are means ± SD. Number of birds in parenthesis.



Asterisks indicate significant difference from the control. *=P<0.05, **=P<0.01

4. Discussion

Iodine and several iodine products have been in use for ages in disinfection of wounds because of the biocidal properties of the halogen and its compounds. This has been due to the oxidant property of halogens, precipitated by the ability of halogens to react with biological molecules, thereby leading to oxidative damage to the molecules. Iodine also forms hypoiodous acid a potent oxidant when it reacts with water (Hollowell, 2000; Clarke and Bettin, 2006), a property that has been employed in the use of iodine products in water disinfection especially in the rural areas and third world countries or even during disaster and other emergencies (Hollowell, 2000).

The potency of iodine as water disinfectant notwithstanding, the problem of residue of halogens in drinking water and the effects on consumers have been an object of serious concern to health practitioners worldwide, especially in prolonged and excessive use of these water disinfectants. The evaluation of the effects of prolonged use of these water sanitizers or disinfectants is especially important because, the effectiveness of iodine as a disinfectant is a function of the contact time with microorganism, water pH and the temperature (Clarke and Bettin, 2006). This tends to promote prolonged and continuous use of the disinfectant and the attendant complications on the consumers.

From the result obtained in this study, prolonged use of Iodosteryl appeared quite safe because the PCV, Hb and RBC values were similar to those in the control especially at low dose of 1 part per million. At higher doses of 2 and 4 part per million however, there were marginal decreases in the RBC counts while the ESR was significantly higher than the control. This is an indication that the use of Iodosteryl at these concentrations must have resulted in intravascular hemolysis especially with the concurrent increase in the erythrocyte osmotic fragility at 0.7 and 0.9% NaCl concentrations. The MCV and MCH values in these groups of birds also appeared higher, (though non-significantly) than those of the control because the RBC counts in these two groups were slightly lower than that of the control (Table 1).

The total and differential leucocyte values were also not affected despite the prolonged use of the compound in drinking water. However, the erythrocyte osmotic fragility increased significantly irrespective of the dosage of Iodosteryl used as water disinfectant. The effect of Iodosteryl on erythrocyte fragility is not farfetched; this is because erythrocyte fragility has been reported to increase in conditions associated with oxidative stress and free radical release into the blood circulation. These conditions include exercise (Kurkcu *et al*., 2010), aging (Droge, 2002), diabetes mellitus (Baynes, 1991), neurodegenerative diseases and even HIV infection (Droge, 2002).

Increased erythrocyte fragility usually results when reactive oxygen or nitrogen radicals or other forms of oxidants react with integral and other proteins on the erythrocyte membrane leading to destruction of the membrane structure. They may also attack the membrane lipids, resulting in lipid peroxidation, membrane fluidity and ultimate destruction of the bilayer integrity of erythrocyte membrane (Girotti, 1985). Summarily, we can infer that stress, from whatever source increases erythrocyte osmotic fragility by lipid peroxidation and destruction of membrane proteins in erythrocytes. This shows that prolonged administration of Iodosteryl in this study is a form of stress associated with decreased osmotic resistance of the erythrocytes. This may also result in intravascular hemolysis as in the groups that received higher doses as evidenced by their lower RBC counts and higher ESR values. Of course, erythrocyte sedimentation rate is a measure of the rate of rouleaux formation by erythrocytes, a direct indicator of higher levels of acute phase protein especially fibrinogen and globulin during inflammatory conditions. Fibrinogen levels in the plasma increases progressively during inflammation and stress until the condition is resolved and can be measured directly or estimated by erythrocyte sedimentation (Barham *et al*., 1979). But the leucocytes parameters in this study did not indicate presence of any inflammation; therefore, the increased ESR may be associated with stress induced by iodine and the associated intravascular hemolysis. Increase in ESR may also occur concomitantly with increased erythrocyte fragility as previously reported by Barham *et al*. (1979).

Iodine and its product have been used successfully as a water disinfectant among soldiers in field conditions with minimal complications (Hollowell, 2000). It has several advantages over chlorine for field use, including: greater chemical stability of the product and less reactivity with organic nitrogenous wastes and contaminants in water, leaving higher free residual concentration in water and more acceptable taste in equipotent doses. This use of iodine based water disinfectants has also been associated with few isolated cases of goitre (Henjum *et al*., 2010; Kettel-Khan *et al*., 1998; TWAS, 2002; Zhao *et al*., 2000).

The present study showed that, prolonged use of Iodosteryl as water disinfectant is not entirely safe, especially when used indiscriminately. It could induce intravascular hemolysis at high doses as a result of decreased erythrocyte osmotic resistance associated with peroxidation of the lipid bilayer and damage to erythrocyte membrane proteins and lipids by iodine. We however, recommend further evaluation of the prolonged use of Iodosteryl as water disinfectant on endogenous antioxidants such as MDA, SOD, Catalase and glutathione as well as the assessment of the type of free radicals involved in the oxidative damage.

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JJBS

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 105 - 111

Jordan Journal of Biological Sciences

New Records of Twelve species of Oligochaeta (Naididae and Aeolosomatidae) from the Southern Iraqi Marshes, Iraq

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Received on December 1, 2011, Accepted on January 21, 2012

Abstract

Oligochaete worms were collected from 4 sites at Al-Swaib marshes, southern Iraq, during October 2010 to March 2011. A total of one aeolosomatid species (*Aeolosoma leidyi* Cragin, 1887) and 11 naidid species (*Pristina sima* (Marcus, 1944), *Pristina osborni* (Walton, 1906), *Dero* (*Aulophorus*) *furcatus* (Müller, 1773), *Dero* (*Dero*) *digitata* (Müller, 1773), *Dero* (*Dero*) *nivea* Aiyer, 1929, *Slavina appendiculata* d'Udekem, 1855, *Nais pardalis* Piguet, 1906, *Nais variabilis* Piguet, 1906, *Nais communis* Piguet, 1906, *Allonais gwaliorensis* (Stephenson, 1920), *Allonais pectinata*(Stephenson, 1910) were recorded for the first time from Iraq. The results indicate that there is high number of species of oligochaetes in the Iraqi limnic environment, in which these species are finding the favorable conditions for live.

Keywords: Oligochaeta, Naididae, Aeolosomatidae, Marshes, Southern Iraq.

1. Introduction

Oligochaetes classification depends on some external morphological features, among these; the color of the body, number of segment, size and body appendages or on the anatomy of digestive and reproduction systems as well as some features concerning their movement pattern and habitat nature (Harman, 1980). Brinkhurst and Jamieson (1971) distinguished the family Naididae from other Tubificoidea families based on reproduction, body length,shape of setae and site of male pores. Family Aeolosomatidae includes worms of small length; they possess a ventrally ciliated prostomium and small number of segments (Brinkhurst and Jamieson, 1971)

In Iraq, limited studies concerning the Oligochaeta have been conducted. Al-Lami *et al.* (1998) recorded the annual densities for some species of Aeolosomatidae, Lumbricidae, Enchytraeidea, Naididae, Tubificidae and Lumbriculidae. Jaweir (2011) recorded three new tubificid species (Aulodrilus pigueti; Embolacephalus velutinus; and Limnodrilus profundicola) from Al-Hawiezah Marsh southern Iraq.

Five naidid species were recorded Basrah marshes, southern Iraq (Al-Abbad, 2009; Al-Abbad, 2010; and Al-Abbad and Al-Mayah, 2010). Moreover, several publications described the Oligochaeta of Turkey (Balik *et al.*, 2004; Yildiz and Balik, 2006; Yildiz and Balik, 2010).

The objectives of the present study are to record species of Oligochaeta in southern Iraqi waters.

2. Materials and Methods

From October 2010 to March 2011, a total of 110 oligochaete worms were collected from 4 sites at Al-Swaib marshes: Sit 1 (55° 30' 68,18"N 29° 47' 43 27E); Site 2 (55° 30' 15,31"N 29° 47' 48,29E); Site 3 (56° 30' 13,10"N 29° 47' 43,06E) and Site 4 (56° 30' 01,18"N 29° 47' 55,02E). The oligochaetes were collected among bryophytes, other submerged plants and the surface layer of mud. In the field, the materials were passed through 250 and 75 µm mesh size sieves. In the laboratory, live samples were examined and illustrated by digital camera, then preserved in 4% formalin. Specimens were mounted for examination in a glycerin, covered with a cover slip and left in this fluid for several hours before examination. Identification was based on Brinkhurst and Jamieson (1971). Photographs for live and fixed specimens were taken by a digital camera mounted on Olympus microscope. The setae of fixed specimens were drawn by the aid of Camera Lucida.

3. Results

The present study made records of 12 species of oligochaetes for the first time in Iraq. These species were isolated from a total of 77 individuals of oligochaetes, five specimens were *Aeolosoma leidyi* Cragin, 1887, of the family Aeolosomatidae. The rest of specimens were belonging to the family Naididae (11 species): *Pristina sima* (Marcus, 1944), *Pristina osborni* (Walton, 1906), *Dero* (*Aulophorus*) *furcatus* (Müller, 1773), *Dero* (*Dero*)

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digitata (Müller, 1773), *Dero* (*Dero*) *nivea* Aiyer, 1929, *Slavina appendiculata* d'Udekem, 1855, *Nais pardalis* Piguet, 1906, *Nais variabilis* Piguet, 1906, *Nais communis* Piguet, 1906, *Allonais gwaliorensis* (Stephenson, 1920), *Allonais pectinata* (Stephenson, 1910), furthermore to the previously recorded species *Pristina longiseta* Ehrenberg, 1828 and *Pristina proboscidea* Baddard, 1896. Table (1) shows the occurrence of the species identified at Al-Swaib marshes during study period.

Table 1. The occurrence of Oligochaetes species at the study period of Al-Swaib marshes southern of Iraq

Family	species	Study period					
		Oct. 2010	Nov.	Dec.	Jan. 2011	Feb.	Mar.
Aeolosomatidae	*Aeolosoma leidyi*	+	-	-	-	+	-
Naididae	*Pristina longiseta*	+	+	+	-	+	+
	Pristina proboscidea	+	+	+	-	-	+
	Pristina sima	+	-	+	-	-	+
	Pristina osborni	+	+	+	-	-	-
	Dero (Aulophorus) furcatus	-	-	+	-	+	+
	Dero (Dero) digitata	-	+	+	-	-	+
	Dero (Dero) nivea	-		+	-	-	+
	Slavina appendiculata	-	+	-	-	+	-
	Nais pardalis	-	+	+	-	-	+
	Nais variabilis	-	+	+	-	+	-

Aeolosoma leidyi Cragin, 1887

*Diagnosis***:** Small olive worms. Body consists of about 11-13 segments. Prostomium is the widest region of the body (Fig. 1a). Septa are absent. Body segments bearing bundles of two types of smooth simple pointed tip setae, long flexible and short stiff sigmoid setae; each bundle have 2-4 setae. The sigmoid setae are slightly sickle shaped and present in all bundles (Fig. 1b, 5a).

*Measurements***:** Body length ranged from 1.03-1.5 mm in length, and 130 to 195 µm in width (n = 5). The long setae range in length between 162 and 200 µm. The sigmoid setae range in length from 50-68 µm.

*Remarks***:** Brinkhurst and Jamieson (1971) reported measurements of specimens as follow: length 1.5-3 mm, width 120-170 µm, length of setae 90-180 µm and length of sigmoid setae 45-70 µm. The Iraqi specimens seem to be shorter and wider than those recorded by Brinkhurst and Jamieson (1971). Members of the genus *Aeolosoma* are cosmopolitan, present in fresh water and brackish water, less often terrestrial, and the species *A. liedyi* reported from America, France and Netherlands (Brinkhurst and Jamieson, 1971).

Pristina sima (Marcus, 1944)

Diagnosis: The worms are naked, transparent, and the body consists of 31-51 segments. Prostomium without proboscis. Eyes are absent (Fig. 2c). Dorsal setal bundles beginning in segment II consisting of 1-2 smooth hairs and 1-2 pectinate needles (with 2-3 intermediate teeth) with distal nodulus (Fig. 2d, 5f). Ventral setae bifurcated, 3-4 per bundle, those of anterior segments are longer than those which follow. The upper tooth in the first five segments is equal or slightly longer and thinner than the lower ones compared with the upper and lower teeth of the rest of segments which are of equal length. Nodulus median in segment II, distal in the following segments (Fig. 5c-e).

Measurements: Body length is 2.9-3.9 mm long, 0.24-0.3 mm wide (n= 8). Hairs are 175-230 µm long, needles are 50-75 µm long. Ventral setae of segment II-V are 62-80 µm long while the following setae ranged between 50 and 60 µm in length.

*Remarks***:** Our specimens have length and number of hairs and needles per dorsal bundles more than those of specimens reported by Brinkhurst and Jamieson (1971). Species of the genus *Pristina* are cosmopolitan, and *Pristina sima* was recorded from different countries such as Brazil (Brinkhurst and Jamieson, 1971) and from Turkey (Yildiz *et al*., 2007).

Pristina osborni (Walton, 1906)

*Diagnosis***:** Bbody is naked, transparent and consists of 22-27 segments. Prostomium without proboscis. Eyes are absent (Fig. 1e). Dorsal setal bundles start in segments II, consisting of one smooth hair and one bifid needle. Needles with short diverging teeth and distal weak nodulus (Fig. 1f, 5i). Ventral setae are bifurcated, 4 per bundle in the anterior segments, 3 per bundle in the middle segments, and 1-2 per bundle in the posterior ones (in the last five segments), with upper and lower teeth of equal length, nodulus proximal in the anterior segments and distal in the middle and posterior segments (Fig. 5g-h).

Measurements: Body length 2.5-3 mm, width 0.2-0.3 mm (n= 3). Hairs 175-188 µm in length; needles 45-50 µm in length. Ventral setae 38-75 µm long.

*Remarks***:** The Iraqi specimens are larger than those reported by Brinkhurst and Jamieson (1971), but specimens from the two areas have distal nodulus in the needle setae, proximal in segment II, and distal in the follow segments. *Pristina osborni* was also recorded from India, Brazil, North America, Africa (Brinkhurst and Jamieson, 1971) and Turkey (Capraz and Arslan, 2005).

Dero (*Aulophorus*) *furcatus* (Müller, 1773)

Diagnosis: Body consists of 19-42 segments, yellowish to transparent in color. Prostomium short and conical (Fig. 2a). Bundles of dorsal setae starts in segment V and onwards and consists of one smooth hair and one curved bifid needle. Needles of the upper tooth slightly shorter than the lower one, nodulus distal (Fig. 2b, 5l).

Ventral setae begin in segment II, and consist of 4 per bundle, except in the last two segments, where 2 setae per bundle are present. Ventral setae of segments II-IV have a different shape from those of on rest of segments, with the upper tooth longer than the lower and of with equal thickness, from segment V the upper tooth slightly shorter and thinner. Nodulus median in segments II-V, and at 1/3 the distance from the distal end in the rest of segments (Fig. 5j-k). Anus opens into branchial fossa. Branchial fossa with parallel palps and 3 pairs of gills.

Measurements: Body length 1.3-6.7 mm long, 0.1- 0.17 mm wide (n= 14). Hairs are 110-137 µm long, needles are

20-81 µm long. Ventral setae are 30-150 µm long. Palps of branchial fossa are 0.2-0.36 mm long.

*Remarks***:** Brinkhurst and Jamieson (1971) reported a length of 6-20 mm; numbers of segments 35-82, ventral setae of segments II-IV were 2-5 per bundle with the upper tooth longer than the lower. Branchial fossa with 3 or 4 pairs of gills. The present specimens are shorter and have fewer segments than those reported by Brinkhurst and Jamieson (1971) . The characters of ventral setae and branchial fossa are apparently similar. The genus *Dero* is cosmopolitan (Brinkhurst and Jamieson, 1971), Capraz and Arslan (2005) recorded *Aulophorus furcatus* from Turkey.

Dero (*Dero*) *digitata* (Müller, 1773)

Diagnosis: Body consists of 34-39 segments, yellowish to transparent in colour. Eyes are absent. Prostomium short and conical (Fig. 2c). Dorsal setal bundles begin in segment V onwards, consisting of one smooth hair and one curved bifid needle. Needles with upper and lower fine teeth of equal length, nodulus at 1/3 the distance from the distal end or more proximally (Fig. 2d, 6d). Ventral setae are 4 per bundle, somewhat of bifurcated crotchets, and those of segments II-V have a different shape from those of the rest, with the upper tooth longer than the lower and of equal thickness, whereas in the followed segments they become equal in length and slightly thinner, while in the posterior segments they become slightly shorter and of equal thickness. Nodulus of ventral setae gradually shifted from proximal to distal in segments II-XX (Fig.6a-c). Anus opens into branchial fossa. Branchial fossa not prolonged, containing 4 pairs of gills.

Measurements : Body length 3.7-4.9 mm and about 0.2 mm in width (n= 5). Hairs are 100-130 µm long, needles are 25-40 µm long. Ventral setae are 60-70 µm long.

*Remarks***:** The length of the specimens reported by Brinkhurst and Jamieson (1971) are 6-32 mm, with 20-105 segments, dorsal setae from segment VI, needles with the upper tooth 1-2 times as long as the lower one, ventral setae in segments II-V are longer than the rest, with nodulus proximal, the rest of segments with 2-5 per bundle and have distal nodulus, and the upper tooth hardly longer than the lower. The present specimens are shorter, and differ from those of Brinkhurst and Jamieson (1971) in several features, such as the dorsal setae which are starting from segment V, needles with equal fine teeth, posterior ventral setae with upper tooth shorter than the lower. The other features are seemingly similar. *Dero digitata* is cosmopolitan (Brinkhurst and Jamieson, 1971) and recorded from different localities such Turkey (Capraz and Arslan, 2005; Yildiz *et al*., 2007).

Dero (*Dero*) *nivea* Aiyer, 1929

Diagnosis: Worms consist of 34-37 segments. Body is yellowish to transparent in colour. Eyes are absent. Prostomium is short and conical, the branchial fossa prolonged posteriorly (Fig 2e.). Dorsal setal bundles start in segment VI onwards, consisting of one smooth hair and one weakly curved bifid needle. The needles with fine teeth and of equal length (Fig. 2f, 6g). Ventral setal bundles consist of 4 bifurcated crotchet setae in segments II-V, compared with 3 per bundle in the posterior segments. Ventral setae in segments II-V are different from those of the rest of segments, with the upper tooth twice as long as the lower, while posteriorly the teeth are of about equal length and the lower teeth slightly thicker, or the upper teeth slightly shorter than the lower ones. Nodulus of ventral setae gradually shifted from proximal in segments II-V to distal region after segment VI (Fig. 6e-f). Anus opens into branchial fossa. Branchial fossa prolonged posteriorly, containing 2 or 3 pairs of gills (Fig. 2e).

Measurements : The range of length of the worms were 3.6-3.9 mm, and the width 0.35 mm (n= 9). Hairs 175-212 µm long, needles 50-67 µm long. The anterior ventral setae (II-V) are 88 µm long compared with 55-75 µm of posterior.

*Remarks***:** The present specimens have body length and number of segments agree with the ranges of length (2.5-10 mm) and number of segments (23-45) given for this species by Brinkhurst and Jamieson (1971). They reported 4 ventral setae per bundle as opposed to 4 per bundle in the anterior segments and 3 per bundle in the posterior segments in the present specimens. They also reported equal length of teeth in all segments after segment V as opposed to the present specimens in which the upper tooth is shorter than the lower one in some posterior segments. *Dero nivea* was recorded from Europe, Asia, America, Africa and Australia (Brinkhurst and Jamieson, 1971), Gorni and Alves (2008) reported the species from Brazil.

Slavina appendiculata ďUdekem, 1855

Diagnosis: Body is stout, consists of 30 segments. Eyes are present (Fig. 3a-b). Dorsal setal bundles of segment VI, consists of one smooth stout hair and one simple pointed needle. Hairs setae of segment VI are elongated (Fig. 3b). Ventral setae are bifurcated crotchets, beginning in segment II; 3 per bundle in segments II and VI, 2 per bundle in other segments. Ventral setae with upper and lower teeth of equal length, the lower tooth of segments II-V is thicker than the upper. Nodulus at 1/3 the distance from proximal end in segments II-V, and more proximal in the rest of segments (Fig. 6h-i).

Measurements: Body length 2.4 mm, 0.2-0.35 in width (n= 2). Hairs of segment VI are 388 µm long, compared with 184-296 µm in the rest of segments. Needles are about 50 µm long. Ventral setae of segments II-V are 100-125 µm long, compared with 90-100 µm in the following segments.

*Remarks***:** Body length, number of segments, number of ventral and dorsal setae per bundle and nodulus site on ventral setae is close to those reported by Brinkhurst and Jamieson (1971). The present specimens are characterized by the presence of upper and lower teeth of equal length in the ventral setae compared with slightly longer upper teeth than the lower ones in those of Brinkhurst and Jamieson (1971). *Slavina appendiculata* was reported from Europe, North and South America, South and East Asia, Africa, New Zealand (Brinkhurst and Jamieson, 1971) and Brazil (Alves *et al*., 2006).

Nais pardalis Piguet, 1906

Diagnosis: Worms are yellow, body consists of 18 segments, eyes are present (Fig. 3c). Dorsal setae beginning in segment VI onwards, the bundles consist of one smooth hair, and one needle with a fine, parallel and equal teeth and weak a median nodulus (Fig. 6m). Ventral setal bundles composed of 5 setae in segments II-III, 4 setae in the rest of segments. Ventral setae of segments II-V with median nodulus and upper tooth as long as the

lower one or slightly longer (Fig. 6k), whereas in the followed segments the nodulus at 1/3 the distance from the distal end and the upper tooth is slightly longer than the lower in the other ventral setae. Ventral setae of segment VI and several followed segments are thickened (Fig. 3d, 6l).

Measurements: Body length 1.9 mm, 0.2-0.28 mm in width (n= 3). Hairs are 100 µm in length, needles are 25 µm in length. Ventral setae of segment II are 80 µm long, compared to 63-75 µm long in the rest of segments. The ventral setae are 1.5 times thicker than the other setae.

*Remarks***:** Arslan and Sahin (2003) gave diagnostic features of *Nais pardalis* from Turkey. Examined specimens are with shorter setae (ventral and dorsal) and shorter upper teeth in the anterior and posterior ventral setae compared with the Turkish specimens, however, the nodulus of the anterior and posterior ventral setae have the same site in both the specimens of both localities. The genus *Nais* is cosmopolitan and the species *N. pardalis* is distribute in Europe, Asia, North and South America (Brinkhurst and Jamieson, 1971) and was reported from Turkey (Capraz and Arslan, 2005; and Yildiz *et al*., 2007).

Nais variabilis Piguet, 1906

Diagnosis: Yellow to transparent in colour. Eyes are present (Fig. 3e). Body consists of 28-40 segments. Dorsal setae begin in segment VI. Hairs and needles are 1 per bundle, needles are bifid with a median weak nodulus and fine teeth (Fig. 3f, 7c). Ventral setae are 4-5 per bundle, those of segments II-V with upper teeth longer than the lower, median nodulus and teeth are of equal length, nodulus at 1/3 the distance from distal end of the ventral setae in the following segments (Fig. 7a-b). Stomach abruptly widening.

Measurements: Body length 2.8-3.8 mm, and 0.2-0.28 mm in width (n= 3). Hairs 125-137 µm in length, the needles about 37 µm in length. Ventral setae 88-100 µm in length.

Remarks: The Iraqi specimens have longer body and shorter needles than the Turkish specimens (see Arslan and Sahin, 2003). However, Arslan and Sahin (2003) recorded higher number of setae per dorsal bundles (1-2 of each hair and needle) and higher distal nodulus in needles compared with 1:1 hair and needle per bundle and median nodulus in needles in the present specimens. Smith (1984) reported features of the specimens from Washington, such as the number of setae per dorsal bundle (1 needle and 1 hair) and the length of ventral setae in segments VI-posterior (88-110 µm) which resemble those of the Iraqi specimens. Also there are others differences such the presence of longer hairs (150-280 µm) and needles (54-60 µm) in the specimens from Washington. *N. variabilis* is cosmopolitan, found also in brackish water (Brinkhurst and Jamieson, 1971), and was reported from different region like Washington (Smith, 1984) and Turkey (Capraz and Arslan, 2005; and Yildiz *et al*., 2007).

Nais communis Piguet, 1906

Diagnosis: Worm are yellow, body consists of 15-27 segments, eyes are present (Fig. 4a). Dorsal setal bundle beginning in segment VI onwards, composed of hair and bifid needle (Fig. 4b, 7f), 1 per bundle. Needle setae are with divergent fine teeth. Ventral setae are 4 per bundle, the setae in segment II are longer than those in the rest of segments, with equal teeth or the upper teeth slightly longer than the lower, and always the upper tooth is thinner, with a median nodulus. Setae of the following segments with teeth of equal length, nodulus are distal (Fig. d-e). The stomach is slowly widening.

Measurements: Body length 1.4-2.8 mm, 0.18-0.2 mm in width (n= 6). Hairs 120-140 µm in length. Needles 37.5-45 µm in length. Ventral setae of segment II are 89 µm in length compared with 75 µm in the other segments.

*Remarks***:** Hairs and needles of the present specimens are shorter than those reported by Smith (1984). However, he noticed the presence of more ventral setae and more needles per bundle (4-6, and 2, respectively) than reported by the present study. The eyes are present in all Iraqi specimens, whereas in some of Washington's specimens the eyes are absent (Smith, 1984). *N. communis* is cosmopolitan, found also in brackish water (Brinkhurst and Jamieson, 1971), also reported from Washington (Smith, 1984), Brazil (Alves *et al*., 2006; and Gorni and Alves, 2008) and Turkey (Capraz and Arslan, 2005; and Yildiz *et al*., 2007).

Allonais gwaliorensis (Stephenson, 1920)

Diagnosis: Body consists of 24-49 segments. Eyes are absent. Prostomium with rounded tip, without a proboscis (Fig. 4c). Dorsal setae bundle starts from segment VI, with 1-2 smooth hair setae and 1-2 bifid needle setae. Needle teeth fairly narrow, upper tooth is slightly longer than the lower ones, nodulus slightly distal (Fig 4d, 7i). Ventral setae usually 3-5 per bundle. Anterior ventral setae of segments II-VI with upper tooth slightly longer than the lower or of equal length in some specimens, with median nodulus, median and posterior ventral setae with upper tooth as long as the lower one, with nodulus at 1/3 the distance from the distal end (Fig. 7g-h).

Measurements: Body length 2.4-4.3 mm, 0.15-0.17 mm in width (n= 14). Hairs 68-125 µm and needles 25-43 µm in length. Ventral setae of segment II 55-62 µm in length, while of segments III- posterior are 45-50 µm in length.

Remarks: Most of the present specimens are shorter than those reported by Brinkhurst and Jamieson (1971), but the latter had a wider range of ventral setae per bundle (3-5 in segments II-V, and 4-6 in the rest segments) than the present specimens (3-5 in every segment), and the nodulus of the ventral setae are at the same sites in the examined specimens. *A. gwaliorensis* was recorded from Asia and Africa (Brinkhurst and Jamieson, 1971) and Turkey (Yildiz *et al*., 2007).

Allonais pectinata (Stephenson, 1910)

Diagnosis: Body consists of 32-48 segments. Eyes are absent. Prostomium short and conical, without a proboscis (Fig. 4e). Dorsal setae bundle starts from segment VI onwards, with 1-2 smooth hair setae and 1-2 needle setae. No elongated hair seta present, needle teeth equal in length, with 2-3 intermediate teeth (pectinate), nodulus slightly distal (Fig. 4f, 7l). Ventral setae are bifurcated crotchets, 3-4 per bundle. Anterior ventral setae with the upper teeth slightly longer than the lower or equal in length, whereas the upper teeth of the posterior setae are of equal length. Nodulus is present in the median (Fig. 7j-k).

Measurements: Body length 2.2-4.2 mm, and 0.14-0.3 mm in width (n= 5). Hairs 163-175 µm and needles 25-38 µm in length. Ventral setae 25-53 µm in length.

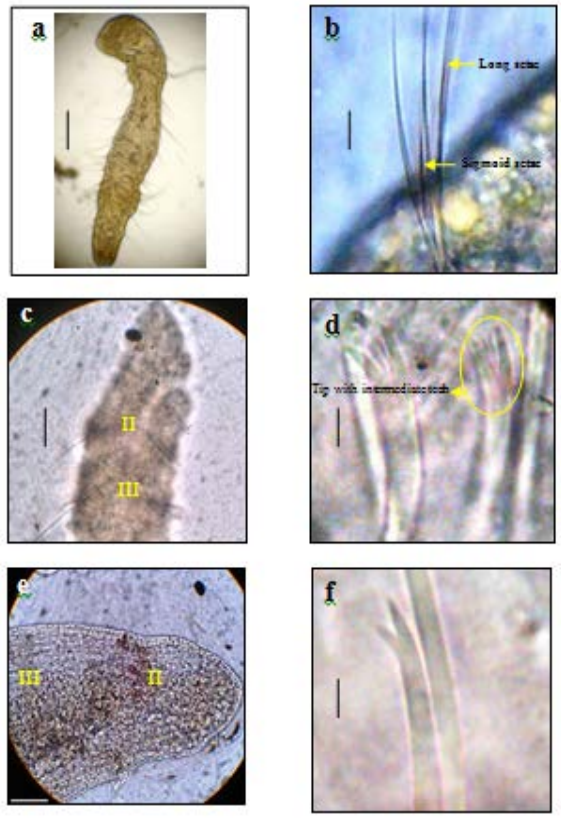

Figure: 1. ***Aeolosoma liedyi*** a- general view of the body, b- setal bundle**.** ***Pristina sima*** c-Anterior end of the body, d- dorsal bundle. ***Pristina osborni*** e- Anterior end of the body, f- dorsal bundle. Scale: a 160 µm; b 17 µm; c 90 µm; d 3.5 µm; e 57 µm; f 3.7 µm.

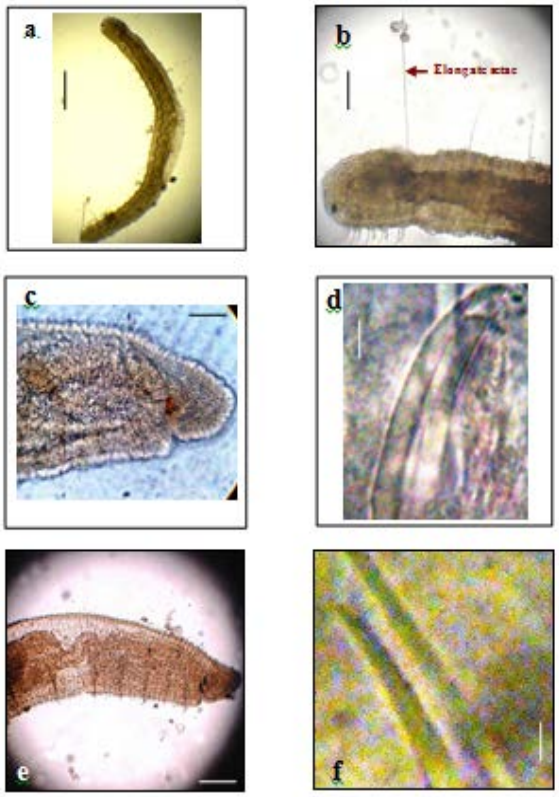

Figure: 3. *Slavina appendiculata* a- general view of the body, b- Anterior end of the body. *Nais pardalis* c- Anterior end of the body, d- ventral bundle of seg. VI. *Nais variabilis* e- Anterior end of the body, f- dorsal bundle. Scale: a 310 µm; b 97 µm; c 58 µm; d 4 µm; e 110 µm; f 2.2 µm.

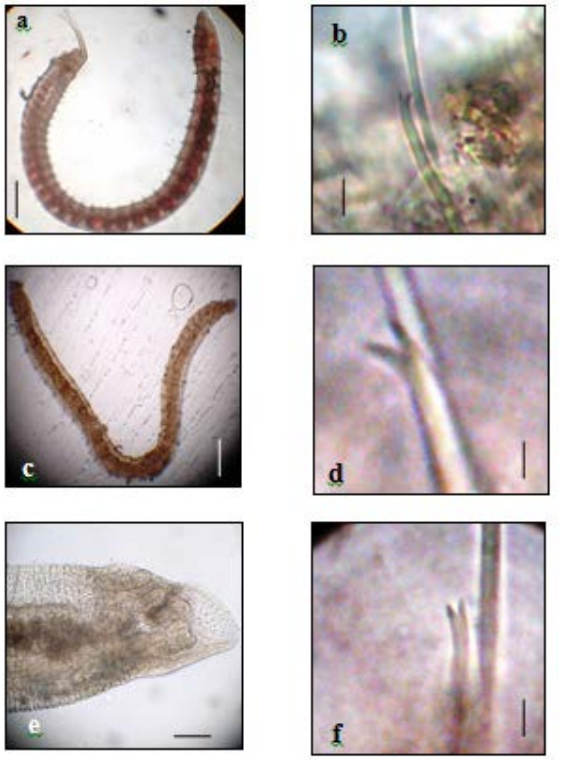

Figure: 2**.** ***Aulophorus furcatus*** a- general view of the body, b- dorsal bundle. ***Dero digitata*** c- general view of the body, d- dorsal bundle. ***Dero nevia*** e- posterior end of the body, f- dorsal bundle. Scale: a 200 µm; b 5 µm; c 270 µm; d 2.5 µm; e 87 µm; f 5 µm.

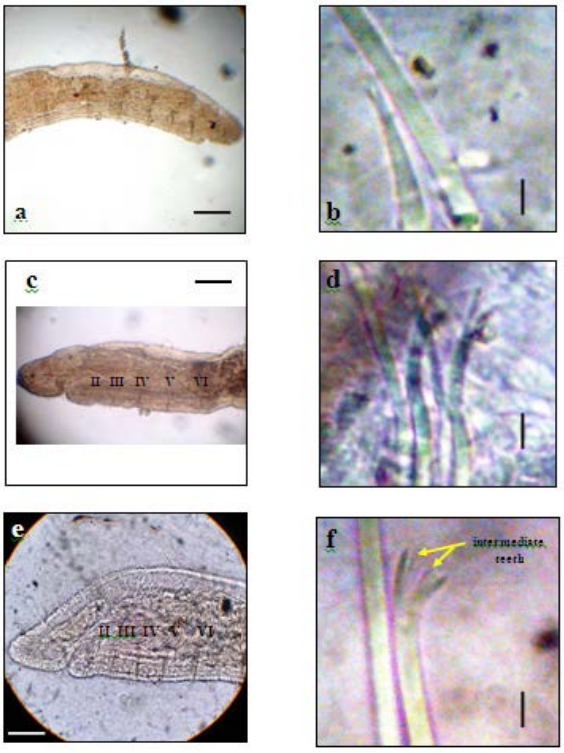

Figure: 4. ***Nais communis*** a- general view of the body, b- dorsal bundle. ***Allonais gwaliorensis*** c- Anterior end of the body, d- dorsal bundle. *Allonais pectinata* e- Anterior end of the body, f- dorsal bundle. Scale: a 110 µm; b 3.5 µm; c 84 µm; d 3 µm; e 59 µm; f 2.7 µm.

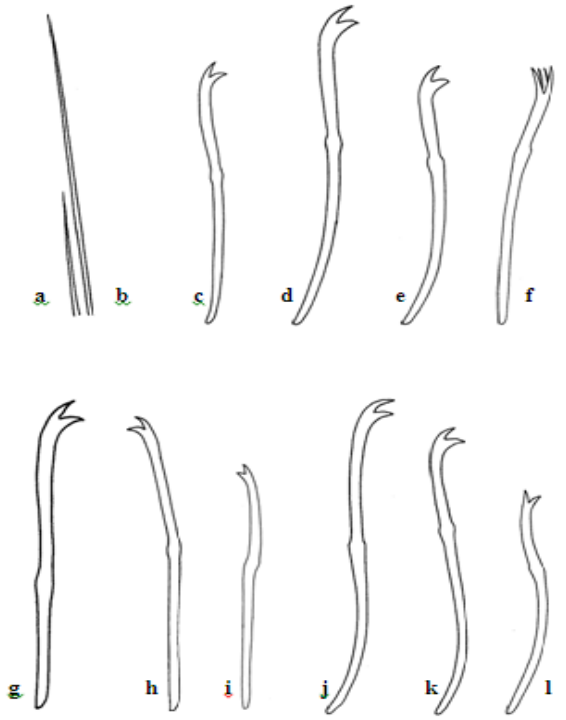

Figure: 5. *Aeolosoma liedyi*. a- sigmoid setae, b- long setae. *Pristina sima*. C- ventral setae in segment II, d- ventral setae in segment V, e- posterior ventral setae, f- needle setae. *Pristina osborni*. g- ventral setae in segment II, h- median ventral setae, i- needle setae. *Dero (Aulophorus) furcatus*. j- ventral setae in segment II, k- ventral setae in segment VI, l- needle setae. Scale: a, j, k 15 µm; b 20 µm; c, d, f 8.5 µm; e 7.7 µm; g 8.2 µm; h 7 µm; i 6.8 µm; l 12 µm..

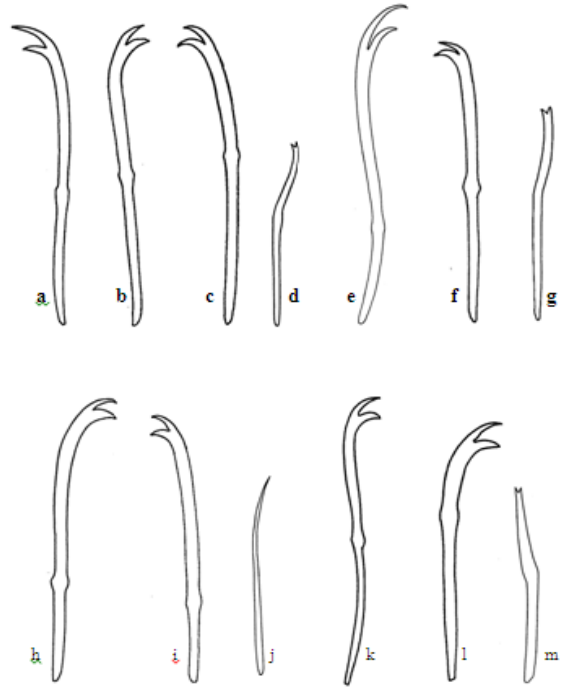

Figure: 6. *Dero (Dero) digitata*. a- ventral setae in segments II-V, b- median ventral setae, c- posterior ventral setae, d- needle setae. *Dero (Dero) nevia*. e- ventral setae in segment II, f- ventral setae in VI, g- needle setae. *Slavina appendiculata*. h- ventral setae in segments II-V, i- posterior ventral setae, j- needle setae. *Nais pardalis*. k- ventral setae in segment II, l- ventral setae in segment VI, m- needle setae. Scale: a-c 7.8 µm; d 7 µm; e, g, k, l 10.1 µm; f,j 9.1 µm; h 15 µm; i 13 µm; m 4.7 µm..

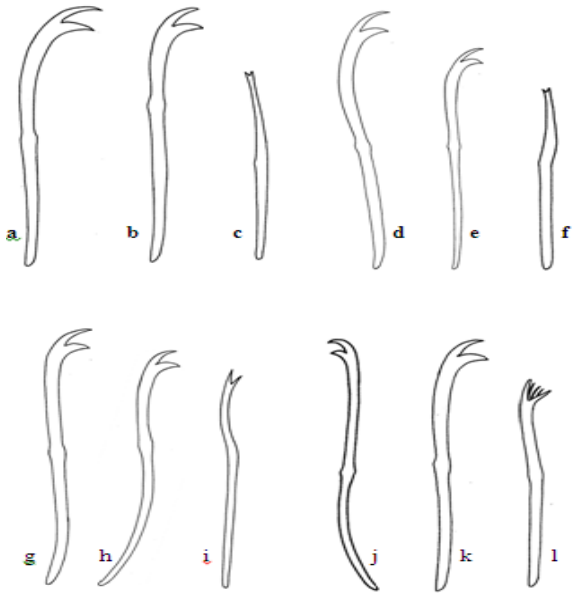

Figure: 7. *Nais variabilis*. a- ventral setae in segment II , b- posterior ventral setae, c- needle setae. *Nais communis*. d- ventral setae in segment II, e- posterior ventral setae, f- needle setae. *Allonais gwaliorensis*. g- anterior ventral setae, h- posterior ventral setae, i- needle setae. *Allonais pectinata*. j- anterior ventral setae, k- posterior ventral setae, l- needle setae. Scale: a 11.5 µm; b 11 µm; c, h, j 6.1 µm; d, e 10.6 µm; f, g 6.8 µm; i, k, l 4.5 µm.

Remarks: Brinkhurst and Jamieson (1971) reported measurements of the body length of 1.5-8 mm and intermediate teeth of needles (1-5) and ventral setae at segments V-posterior (2-7 per bundle) which are different from those in the present specimens. In both examined specimens, the length percentage of teeth of the ventral setae are resembling each other, but some of the anterior setae of the Iraqi specimens with the upper tooth are slightly longer than the lower ones. *A. pectinata* is present in Asia, Africa and Australia (Brinkhurst and Jamieson, 1971).

4. Discussion

Morphometric measurements (ex. the lengths of the body and seta and number of segments) of the Iraqi specimens have shown some differences from those measured elsewhere in the world. These variations are common in oligochaetes, and suspected to be due to locality differences. Harbe (1938) and Sperber (1948) emphasized that these variations in length and number of segments occurred even within the same species. Al-Abbad (2009) recorded varied number of body segments in *Chaetogaster limnaei* from Iraq. On the other hand, the setae of the Iraqi specimens have average length and numbers differ from those recorded by Smith (1984) from the United States. Smith (1984) also recorded varied measurements of setae of *C. limnaei* between specimens taken from Washington and those from Colorado. These variations are common in the family Naididae and there are many examples reflecting this matter. For instance, Sperber (1948) recorded 8-21 segments of the body of *C. longi*. Therefore, the identification of the same species depends on a limit of body length and number of segments; this indicates that these general variations may

have systematic importance (Brinkhurst and Jamieson, 1971).

There are many examples on the variations of the body features according to changes in the habitat, for instance Loden and Harman (1980) reported on morphological variations in genera like *Dero*, *Nais* and *Pristina*. They placed *P. aequiseta* in an artificial habitat, the giant seta of the posterior zooid was not formed, and hence should be recognized as *P. foreli*, but if it is returned to full strength habitat water, the posterior zooid under the new regimen produces the giant seta.

Acknowledgements

I am indebted to Prof. S. D. Salman (Department Marine Biology, Marine Science Center) for critical reading of the manuscript.

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JJBS

Jordan Journal of Biological Sciences

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 113 - 119

The Effect of Sustanon (Testosterone Derivatives) Taken by Athletes on the Testis of Rat

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Received on November 24, 2011, Accepted on January 28, 2012

Abstract

Intentional abuse of anabolic androgenic drugs (AASs) by athletes has been increased rapidly in many countries. Therefore, investigations are needed for studying the adverse effects of these drugs. Administration of three doses of Sustanon 250mg (3, 5 and 10mg/kg.B.wt) which includes four testosterone derivatives and taken by athletes as body builder androgenic drug has caused several biochemical, and histological alterations in the testes of rats. The biochemical changes included the increase of serum testosterone and malondialdehyde (MDA) levels while the histological effect included the degeneration of the germinal epithelium and the appearance of multinucleated giant cells and several variable sized vacuoles in a stage-like changes. The multinucleated giant cells appeared having a large number of apoptotic-like nuclei with marginated chromatin. This study has also showed a significant decrease of the epididymis sperm count. The later result has been confirmed by scanning electron micrographs. All these changes were observed in a dose related pattern.

Keywords: Sustanon, testosterone, testis.

1. Introduction

Testosterone plays an important role in male sexual differentiation (Hines, 2009), puberty (Mitchell *et al*., 2009), sexual behaviour (Yates *et al.*, 1999; Balthazart and Ball, 2010) and spermatogenesis (Sofikitis, 2008).

Anabolic-androgenic steroids (AASs) are the man-made derivatives of the male sex hormone testosterone (Hoffman and Ratamess, 2006). Testosterone is the main androgen (male sex hormone), cholesterol derived hormone (Saladin, 2004; Postlethwait and Hopson, 2006; Parr *et al*., 2010) that regulates male secondary sexual characteristics. Along with follicle-stimulating hormone (FSH), testosterone stimulates sperm production (Mader, 2010).

In recent years, the intentional abuse of anabolic androgenic drugs especially the testosterone derivatives by athletes have increased rapidly in many countries to become a serious negative phenomenon (Bin Bisher, 2009). Abusers and many athletes, especially in the power sports like bodybuilding and weight lifting, administer illegally high doses of these drugs during sport competitions (Hartgens and Kuipers, 2004). Unfortunately, according to recent report from Iraq, users of anabolic androgenic drugs gradually increased during the past decade (unpublished data).

The anabolic androgenic drugs are an important therapeutic target for the treatment of diseases such as hypogonadism (Mudali and Dobs, 2004; Seal, 2009), treat senile osteoporosis (Gooren, 2007), in conjunction with other hormones to promote skeletal growth in prepubertal boys with pituitary dwarfism (delay puberty) (Bagatell and Bremner, 1996; Yavari, 2009), and to treat some types of anemia such as Fanconi's anemia (Velazquez and Alter, 2004; Maravelias *et al*., 2005).Sustanon is a useful medical drug which possesses multiple clinical therapeutic benefits (Socas *et al.*, 2005). It consists of four different testosterone esters (testosterone propionate, testosterone phenylprpropionate, testosterone isocaproate and testosterone decanoate), which provides a continuous release of testosterone into the blood and producing a stable testosterone level for a long period of time extending from 3-4 weeks (Harvey *et al*., 2006).

The adverse effects caused by abusing anabolic androgenic drugs included cardiovascular disorders (Sader *et al*., 2001), liver dysfunction (Shahidi, 2001; Amsterdam *et al*., 2010), kidney disease (Mulronery *et al*., 1999), testicular problems (Feinberg *et al*., 1997; Socas *et al*., 2005), psychiatric and behavioural disorders in both sexes (Maravelias *et al.*, 2005) as well as other problems on human body (Mader, 2010). High testosterone rate induces oxidative stress by alteration of the balance between ROS production and antioxidant defences (Alonso-Alvarez *et al.*, 2007). The aim of the present work was to study the effect of sustanon 250 mg on the testis and sperm count.

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2. Materials and Methods

2.1. Experimental Animals

The present study was conducted using 28 mature male albino rats (*Rattus norvegicus*). All rats were healthy, weighing 200 - 270 gm. and 8-10 weeks old at the time when the experiment started. The animals were bred and housed in plastic cages (56 x 39 x 19 cm) bedded with wooden chips in groups of seven rats per cage in a room with controlled temperature of 24 ± 3 ^{o}C, in animal house of Biology Dept. /College of Science /Salahaddin University-Erbil-Iraq. The animals were kept in 12/12 hours light/dark schedule during the experimental study. The rats were fed with standard laboratory chow containing 0.5% NaCl, 22% protein and 4-6% dietary fat (Krinke, 2000) and allowed to drink water *ad libitum*.

2.2. Sustanon 250

Sustanon ampoules (manufactured by N.V. Organon Oss Inc. Holland) have been obtained from the local pharmacy in Erbil-Iraq. Each ampoule contains 1 mL of oily solution of Sustanon. According to the manufacturer, this 1 mL of Sustanon consists of four testosterone ester compounds which include testosterone propionate, testosterone phenylprpropionate, testosterone isocaproate and testosterone decanoate. During the present study, three doses of Sustanon have been selected which were 3, 5 and 10 mg/Kg of the animal body weight (b.wt.).

2.3. Experimental Design

The rats were divided randomly into four groups (first group served as control and the other groups as the treated groups). Each group consisted of seven rats per cage. Group 1: Control: was injected once a week with 0.1ml of corn oil intramuscularly (i.m.). Group 2: Sustanon (3 mg/kg b.wt. in corn oil: injected i.m., once a week with 0.1ml of 3 mg sustanon/kg b.wt. Group 3: Sustanon (5 mg/kg b.wt. in corn oil): were injected i.m., once a week with 0.1ml of 5 mg sustanon/kg b.wt. Group 4: Sustanon (10 mg/kg b.wt. in corn oil): was injected i.m., once a week with 0.1ml of 10 mg sustanon/kg b.w. The duration of the experiment was four weeks.

2.4. Biochemical Analysis

2.4.1. Determination of MDA

The level of serum MDA was determined spectrophotometrically by thiobarbituric acid (TBA) solution. In brief: 150µl of blood serum was added to the followings: 1ml trichloroacetic acid (TCA) 17.5%, 1ml of 0.66 % TBA, then mixed well by vortex, incubated in boiling water for 15 minutes, and then allowed to cool. One ml of 70% TCA was added and left to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, and the supernatant was taken out for scanning spectrophotometrically at 532nm (Guidet and shah, 1989).

2.4.2. Determination of Serum Testosterone

Serum testosterone was determined by using Mini VIDAS (Biomerieux, Italy). The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (Enzyme Linked Fluorescent Assay). The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone), fluorescence was then measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of testosterone present in the sample. At the end of the assay, results were automatically calculated by the instrument in relation to the calibration curve stored in memory and then printed out.

2.5. Sperm Count

Sperms from the right cauda epididymis were released by cutting into 10 ml of normal saline in small petri dish and then minced by using manual glass homogenizer, using a light microscope (Axiostar plus microscope), and the number of sperms measured by using a hemocytometer. Sperm count was expressed as a number of sperm per milliliter (Elbetieha *et al*., 2008).

2.6. Histological Preparation

2.6.1. Paraffin Method

Testes were removed from the anesthetized animals, they immediately fixed in Bouin's fluid for 24 hours, followed by a dehydration using a series of graded ethanol in ascending concentrations (50%, 70%, 95%, and 100%), immersed in xylene for clearing, infiltrated in paraffin wax, and finally embedded in paraffin wax. Four micrometer thick paraffin sections were obtained by using rotary microtome (Bright, MIC) and stained by hematoxylin and eosin (H&E) (Bancroft *et al*., 1977). The specimens were examined and photographed under light microscope (digital binocular compound microscope 40x-2000x, built-in 3MP USB camera).

2.6.2. Plastic Method

Samples of testes ($\leq$1mm^3) were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2 - 7.4 for 24 hours, washed by cacodylate buffer 0.1M, postfixed in 1% Osmium tetroxide for one hour, dehydrated through a graded series of ethanol (50%, 70%, 95%, and 100%), and then cleared in acetone for 15 minutes (twice), infiltrated with acetone plus resin mixture (1:1) for 1hours, then with acetone plus resin mixture (1:3) for 12 hours, and finally embedded in resin.

2.7. Scanning Electron Microscopy

Testes were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2-7.4 for 24 hours. After washing by cacodylate buffer 0.1M, they postfixed in 1% Osmium tetroxide for two hours, and dehydrated in ethanol (50%, 70%, 85%, 100% and 100%), the samples were put in desiccator for air drying, after mounting they coated with gold by coating system (E5200 AUTO SPUTTER COATER) and then examined by SEM in Tehran University-Tehran-Iran (CamScan MV2300) and in Malaysia (ZEISS, super A, 55VP).

2.8. Statistical Analysis

All data were expressed as means ± standard error of mean (M ± SE) and statistical analyses were carried out using statistically available software of statistical package for social science (SPSS) version 11.5. One-way analysis of variance (ANOVA) was performed to test for significance followed by Duncan's multiple range comparison tests for comparisons between the groups. *P*

values ≤ 0.05 and 0.01 were considered statistically significant.

3. Results

The level of MDA was increased (p<0.05) dose dependently in the sustanon injected groups. In the lower dose of sustanon group, MDA (1.560 ± 0.1997 µmol/L) increased non-significantly while in the last two doses, it increased significantly (1.964 ± 0.4404 and 3.172 ± 0.3180 µmol/L respectively) when compared with control group (0.6367 ± 0.1289 µmol/L) (Fig 1).

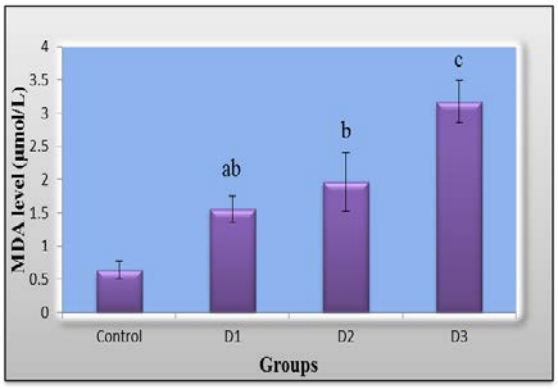

Figure 1. Effect of sustanon doses on MDA concentration.

The current study has shown that both last doses of sustanon caused significant increase (p<0.05) in the level of serum testosterone (3.434 ± 0.3401 and 4.540 ± 0.3835 ng/ml respectively) when compared with control group (1.640 ± 0.2261 ng/ml), while in the first dose, the level of serum testosterone (1.940 ± 0.2338 ng/ml) increased (but non-significantly) when compared with control group (Fig 2).

As shown in (Fig 3), Sustanon injected doses have caused significant decrease (p<0.05) of sperm count (2.142 ± 0.2743, 1.128 ± 0.2191 and 1.100 ± 0.2895 million/ml respectively) in comparison to control group (3.584 ± 0.1578 million/ml). Beside this, in both last two doses of sustanon injected rats, the numbers of multinucleated giant cells (1.813 ± 0.2277 and 3.375 ± 0.2869/seminiferous tubule respectively) were increased significantly (p<0.01) compared to control group (in which no such cells were detected) , while in the first dose, they (0.5000 ± 0.1291 / seminiferous tubule) increased non-significantly.

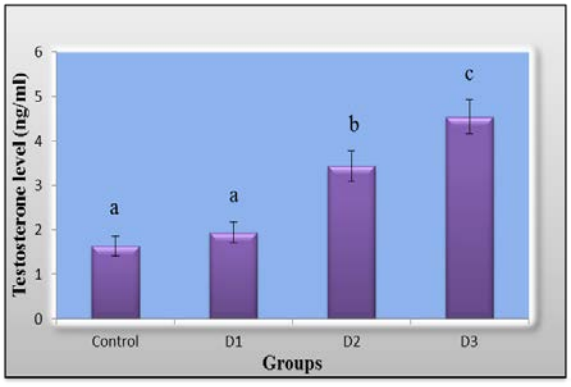

Figure 2. Effect of sustanon doses on serum testosterone level.

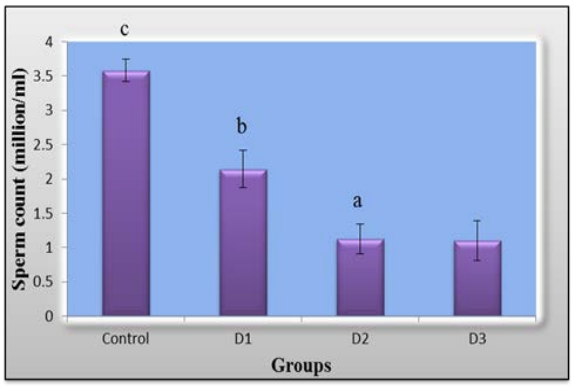

Figure 3. Effect of sustanon doses on sperm count

The histological structure of the testes of the control group showed normal germinal epithelium with well-formed spermatids (Fig 4A-D). In the lower sustanon dose treated group (3mg/kg wt), the most important histological changes were the absence of spermatids in the seminiferous tubules and the appearance of multinucleated giant cells and few vacuoles (Fig 4E and F).

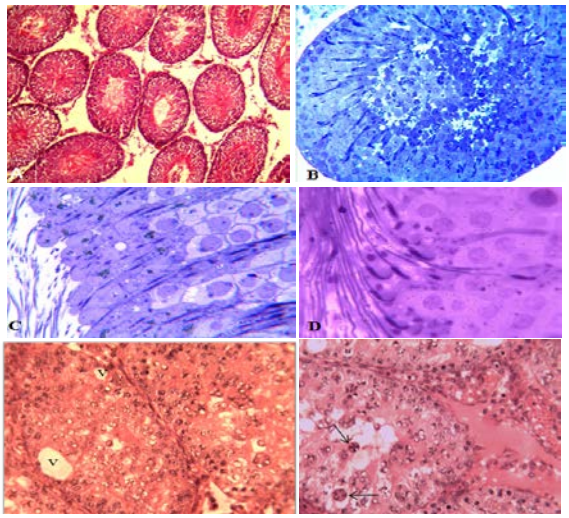

Figure 4. Sections through rat testes: A) control 100X,H&E, B) control 400X, Toluidine blue, C) and D) high magnification of the seminiferous tubule control 1000X, Toluidine blue, E) 3mg sustanon/kg b.wt showing few vacuoles (V), notice the large number of gonocytes and the absence of spermatids, 400X, H&E, F) same previous dose showing the appearance of multinucleated giant cells (arrows), 400X, H&E.

The number and size of vacuoles were increased in the second dose of sustanon (5mg/kg b.wt), while the number of gonocytes in the seminiferous tubules was decreased (Fig 5A & B). Higher numbers of multinucleated giant cells were seen in the testes of the last group (10mg/kg b.wt) (Fig 5C). The germinal epithelium in the seminiferous tubules appeared very thin (Fig 5D). The multinucleated giant cells appeared having a large number of apoptotic-like nuclei with marginated chromatin (Fig 5E & F). There was also a stage-like alteration in the higher dose of sustanon with respect to numbers and sizes of vacuoles and multinucleated giant cells and the thickness of the germinal layer.

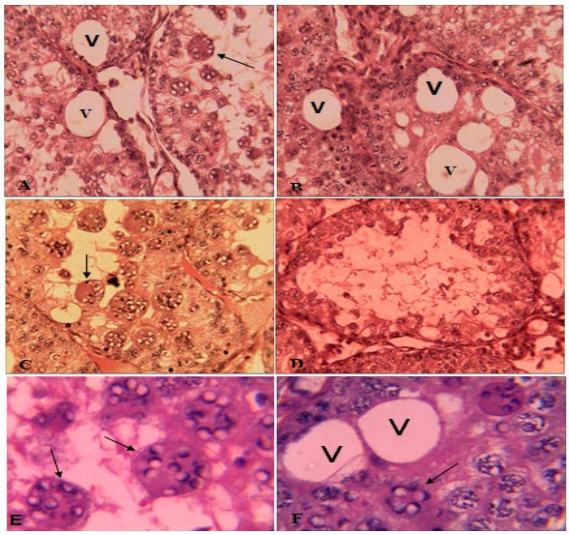

Figure 5. Sections through rat testes treated with sustanon: A) 5mg sustanon/kg b.wt treated rat testis showing increasing number of vacuoles (V), few multinucleated giant cells (arrows) are also seen, 400X, H&E, B) same dose with more vacuoles, 400X, (C-F): Section through the testes of 10mg/kg b.wt treated rats, H&E, C) high number of multinucleated cells, 400X, D) Little vacuoles and a thin layer germinal layer are seen, 400X, E) Number of multinucleated cells, notice they that contain apoptotic nuclei, 1000X, F) Number of multinucleated cells and few large sized vacuoles, 1000X.

The scanning electron micrographs of the testes of control rats showed seminiferous tubules with a large number of spermatids in the lumen (Fig 6A &B), while the seminiferous tubules of sustanon treated rats showed absolutely no spermatids, instead they contained large number of germinal epithelial cells, some tubules appeared having a thin germinal epithelium with empty lumen and a number of vacuoles (Fig 6C&D).

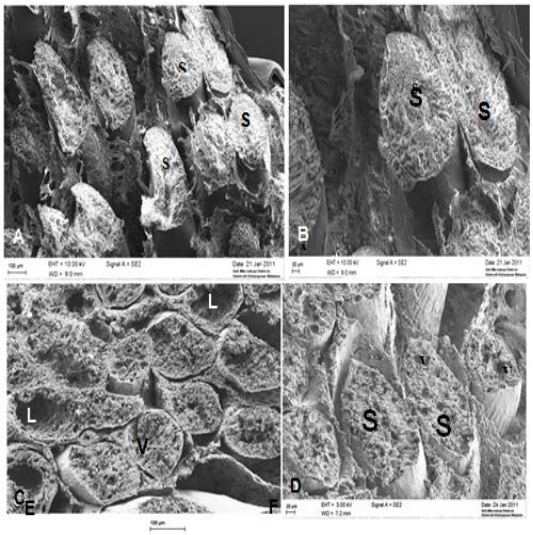

Figure 6. Scanning Electron micrograph sections through rat testes: A) & B) Control rat testis showing seminiferous tubules (S) containing large number of spermatids in the lumen, bar=100µm & 20µm respectively, C) & D) 10mg of sustanon/kg b.wt treated rat testis showing no spermatids and the presence of vacuoles, notice some seminiferous tubules appear empty (L), bar=100µm & 20µm respectively.

4. Discussion

Three different doses of sustanon, which have been chosen regarding the doses used by athletes, were administered to the rats in the current investigation. This study revealed that the higher doses of sustanon which used in this research caused a significant increase in the concentration of serum testosterone, while in the lower dose, the level of serum testosterone increased, but non-significantly when compared with control group. Increase or decrease of the concentration of serum testosterone in animals who received anabolic steroids depends on the period of AASs exposure, dosage use and type of AASs. This result is correlated with the findings of other investigators (Sanchis *et al.*, 1998; Muraoka, 2001; Shiono, 2001) who confirmed that taking testosterone and its derivatives caused elevation of the serum level of testosterone in rats. Not only testosterone derivatives could increase the level of serum testosterone, but giving other anabolic androgenic steroids which are used to increase blood testosterone levels for the purposes of increasing strength, lean body mass and sexual performance such as androstenedione and dehydroepiandrosterone were found to elevate testosterone levels (Bahrke and Yesalis, 2004). In contrast, Tahtamouni *et al*., (2010) showed that measuring total testosterone level in the serum of the control and treated groups indicated that injection of nandrolone decanoate caused a significant decrease in testosterone level in the treated animals compared with control group. Exogenous administered testosterone and its metabolite estrogen will suppress both GnRH productions by the hypothalamus and LH production by the pituitary gland and subsequently suppress testicular testosterone production (Dohle et al., 2003; Thabet et al., 2010). Also, high level of testosterone is needed inside the testis and this can never be accomplished by oral or parenteral administration of androgens. Suppression of testosterone production by Leydig cells results in a deficient spermatogenesis, as can be seen in men taking AASs (Dohle *et al*., 2003).

In the present study, the dose-dependent decrease in the cauda epididymal sperm count was observed in rats exposed to sustanon and indicated decreased spermatogenesis. As shown in the results, the sperm count decreased significantly in sustanon injected rats when compared with control group and decreasing the number of sperm was dose related. Sperm count decrease may occur due to increase free radical formation and undergoing apoptosis of somatic cells (Leydig and Sertoli cells) and the germ cells as a result of injection of AASs. Administration of testosterone propionate led to a significant elevation of oxidative stress (Aydilek *et al*., 2004). Subsequently, the targeted molecule becomes a free radical itself and initiates a cascade of events that can ultimately lead to cellular damage. The pathological roles of free radicals include lipid peroxidation, DNA damage and apoptosis (Kothari *et al*., 2010). Free radicals have the ability to directly damage sperm DNA by attacking the purine, pyrimidine bases and deoxyribose backbone as well as they can damage the sperm membrane (Tremellen, 2008). This supports the present results in which the level of serum MDA was increased dose dependently after sustanon administration, accompanied by dose dependent

biochemical and histological alterations. Testosterone administration has been found to cause oxidative stress through increasing the level of serum MDA level, thereby enhancing lipid peroxidation dose dependently (Sadowska-Krępa *et al*., 2011).

Decrease in the sperm count may be due to decreased level of intratesticular testosterone, because testosterone level is directly linked to spermatogenesis (Zirkin *et al*., 1989). Elkington, (1970) reported that testosterone converted in peripheral tissues to estradiol, subsequently, estradiol strongly suppresses the spermatogenesis.

It is also possible that the Sertoli cells might have been affected due to steroid injection and the other possibility might be due to its effect on the epididymal function (Bairy *et al*., 2010). The reduction in Sertoli cell number in treated rats with anabolic steroids could have resulted in a subsequent reduction in the number of spermatogonia leading eventually to a decrease in sperm count and testicular atrophy (Tahtamouni *et al*., 2010). Within the testes, the main target cells for toxicants that disrupt spermatogenesis are the somatic cells (Leydig and Sertoli cells) and the germ cells. In animal models, each of these cell types can be selectively targeted by specific toxicants, resulting in apoptosis (Reddy *et al*., 2009). Anabolic steroids such as nandrolone decanoate may cause loss of AR activity from Sertoli cells would lead to spermatogenic failure resulting in incomplete meiosis and collapse transition of spermatocytes to haploid round spermatids (Holdcraft and Braun, 2004).

O'donnell *et al*., (2001) revealed that exogenous testosterone administration caused significant decrease in sperm count and intratesticular testosterone concentration. Studies have reported that AASs use is strongly related to decreased sperm count, decreased sperm motility, abnormal sperm morphology (Ciocca, 2005; Brown, 2005; Doust *et al*., 2007).

The current work showed that multinucleated giant cells formed as a result of sustanon injection and they increased in number in dose dependent way, but the testes of control rats did not develop them. In the second and third doses of the present study, the multinucleated giant cells were increased significantly, while in the lower dose they increased non-significantly when compared with control group. Thabet *et al*., (2010) have also demonstrated that anabolic steroids caused formation of giant cells in the lumen of seminiferous tubules of rabbit. The multinucleated giant cells appeared to be one of the ultimate of testicular atrophy (Khattab, 2007). The findings suggested that the giant cells are formed as a result of the fusion of spermatids due to alterations in the intercellular bridges (Singh and Abe 1987) or failure of cytokinesis (Abdu, 2008).The most characteristics of these cells were their large size and the numerous nuclei they contain. The most important feature of the nuclei is the marginated chromatin material, a characteristic apoptosis feature (Goldsworthy *et al*.,1996).

Apoptosis which occurs in testis for regulation of germ cell population (Woolveridge *et al*.,1998; Giampietri *et al*.,2005) may be related to lowering of the level of testicular testosterone (Nandi *et al*.,1999; Richburg *et al*., 2000).The suppressing of testicular testosterone production, as mentioned previously, may be due to administration of exogenous testosterone (Thabet *et al*., 2010) and this may lead to the formation of more apoptotic germ cells. This may explain the dose dependent appearance of the multinucleated giant cells.

Depending on histological and ultrastructural study, the present work reported that intramuscular injection of male rats with different doses of sustanon for four weeks is deleterious to the structure of rat testes. After analyzing different sections of testes of control and treated animals by light microscope, clear differences were noted. The testes of sustanon injected groups showed histological and cytological changes including severe damage of the seminiferous tubules, degeneration of germinal epithelia, appearance of apoptotic nuclei, presence of multinucleated giant cells having an apoptotic nuclei, lack of spermatids in the seminiferous tubules and appearance of vacuoles which increase in size and number with elevated doses of sustanon. The scanning EM has confirmed these results. Other recent works have supported the present results (Naraghi *et al*., 2010; Thabet *et al*., 2010).

The present investigation showed a step-like stages of seminiferous tubules cells degeneration starting with the appearance of few apoptotic nuclei and vacuoles with small size, then followed by the appearance of multinucleated giant cells containing these apoptotic nuclei accompanied by larger size and number vacuoles and depletion of the germinal epithelium. Approximately similar stages were suggested by Anton (2003) after ligation of the efferent duct of the rat testis and he suggested a time dependence for this stepwise changes. Since the present work has a fixed duration for all groups, we propose a dose relation rather than time relation for these stages.

5. Conclusion

The present work illustrates that sustanon which contains testosterone derivatives taken by athletes has affected rat fertility through arresting spermatogenesis and degenerating the germinal epithelium in dose dependent stage-like pattern.

Acknowledgement

The authors would like to appreciate Miss Chnar Najmadden, University of Kebangseen in Malaysia for her assistance in electron micrographs photography.

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JJBS

Jordan Journal of Biological Sciences

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 121 - 124

Prevalence of Unilocular Hydatidosis in Slaughtered Animals in Aden Governorate-Yemen

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Received on December 4, 2011, Accepted on January 30, 2012

Abstract

The prevalence of cystic echinococcosis (CE) or unilocular hydatidosis caused by *Echinococcus granulosus* was investigated in four central abattoirs in Aden Governorate, Yemen during the period from October 2008- March, 2009. An overall infection rate with hydatid cysts in carcasses of 7507 livestock (2576 sheep, 4809 goats, 117 cattle and 5 camels) was 0.7% with 1.1%, O.5%, and 2.6% in sheep, goats and cattle respectively. None of the camels was found infected. The infection rates were greater in >two years old animals and while the infection was greater among female sheep and goats than males, only male cattle were found infected. Liver cysts were found in 59.3% of infected animals and 37% had multiple cysts involving both liver and lungs. In contrast, 3.7% of infected animals showed cysts in the lungs only. While fertile cysts were found in 78% of infected animals, sterile and calcified cysts were found in 12.7% and 9.3%, respectively.

Keyword: *Echinococcus granulosus;* cystic echinococcosis; hydatidosis;Yemen.

1. Introduction

Cystic echinococcosis (CE) or unilocular hydatidosis is an important parasitic disease that constitutes a major public health problem in many countries around the world including the Mediterranean zone (Eckert and Deplazes, 2004; Sadjjadi, 2006). Being a zoonotic disease, CE causes many health problems which threaten human life and livestock. Several human case reports and surgical resurrection of hydatid cysts from Yemani patients have indicated that CE is endemic and of major public health problem (Al-Hureibi *et al*., 1992; Azazy and Abdelhamid, 2000; Ghallab and Al-Sabahi, 2008; Alghoury *et al*., 2010). The prevalence of CE in domestic livestock has not been addressed adequately in Yemen. However, the prevalence was studied in several Yemeni Provinces (Ali *et al.*, 2003 (in Arabic); Baswaid, 2007; Al-Salami, 2007). In order to understand the magnitude of the disease incidence and its transmission dynamics, further studies that determine the infection rates in various slaughtered livestock in various Yemani Provinces is still needed. Therefore, the present study aimed to determine the prevalence of hydatidosis, types of organs affected and nature of cysts in livestock animals slaughtered in abattoirs in Aden Governorate.

2. Material and Methods

2.1. Animals samples

A total of 7576 livestock animals (2576 sheep, 4809 goats, 177 cattle and 5 camels) slaughtered in four official abattoirs in Aden Governorate, Yemen were examined for hydatid cysts during the period between October 2008-March 2009. Three visits were made to each abattoir weekly and the various organs of both male and female carcasses were carefully inspected for hydatid cysts. Animals were sex and age categorized into four age groups (<one year, 1-<2 years, 2-3 year and <3 years). The age was confirmed by a veterinarian. The organs of infected animals were sent to research laboratory at the Faculty of Science and Education-Aden University for further examination.

2.2. Laboratory work

Infected organs were processed as described by Abdel-Hafez *et al*. (1986) as follows: After washing infected organs with tap water, hydatid cysts with minimal surrounding tissue were individually separated. The hydatid fluid from each cyst was aspirated using ?? ml syringe fitted with ?? gauge needle. The cyst was opened by scissors and the remaining fluid was withdrawn using a micropipette and the total volume of hydatid fluid was measured using a graduated cylinder. The germinal layer of each cyst was transferred to a Petri dish, cut into small

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pieces and washed three times in normal saline to isolate protoscolices from underlining membrane.

The fluid was withdrawn after refining it from the parts of the germinal layer and was added to the previously collected fluid. After sedmentation of the fluid for 30 minutes, supernatant fluid was separated from sedimenting protoscolices which were tested for viability using 0.1% eosin dye. Viable protoscolices exclude the dye and active flame cells are detected under light microscopy.

2.3. Statistical analysis

Simple percentage and Chi-square test were applied for data analysis.

3. Results

An overall infection rate with hydatid cysts in carcasses of 7507 livestock (2576 sheep, 4809 goats, 117 cattle and 5 camels) was 0.7%. CE was detected in 28 (1.1%) sheep, 23(0.5%) goats, and 3 (2.6%) cattle. None of the five camels was found infected (Table 1).

Hydatid cyst was recorded in both sexes in sheep and goats, but females showed higher infection rates than males at 2.7% and 0.4% in sheep and 1.5% and 0.2% in goats respectively. Most of the cattle slaughtered were males and none of the three female cattle that were slaughtered were infected (Table 1).

Table1. CE infection rates in various livestock slaughtered in four central abattoirs in Aden Governorate, Yemen (2008/2009 survey).

Animal type	No. examined			No. infected			Infection Rate (%)		
	M	F	Both	M	F	Both	M	F	Both
Sheep	1803	773	2576	7	21	28	0.4	2.7	1.1
Goats	3900	909	4809	9	14	23	0.2	1.5	0.5
Cattle	114	3*	117	3	0	3	2.6	0.0	2.6
Camels*	4	1	5	0	0	0	0.0	0.0	0.0
All	5821	1686	7505	19	35	54	0.3	2.1	0.7

*Numbers are too low to draw any conclusions.

Sheep, goats whose ages were 2-3 years showed higher infection rates than younger ages, but animals older than 3 years were negative for hydatid cysts. In contrast, hydatid cysts were detected only in cattle over 2 years old (Table 2).

Table 2. CE infection rates among different age group of livestock animals slaughtered in central abattoirs in Aden Governorate-Yemen (2008/2009 survey).

Age group (Yrs)	Sheep		Goats		Cattle	
	No. examined	No. & (%) infected	No. examined	No. & (%) infected	No. examined	No. & (%) infected
<1	151	2 (1.3)	705	0 (0.0)	7	0 (0.0)
1- <2	1285	3 (0.2)	1899	6 (0.3)	88	0 (0.0)
2-3	1116	23 (2.1)	2135	17 (0.8)	14	2 (14.3)
>3	24	0 (0.0)	70	0 (0.0)	8	1 (12.5)
All	**2576**	**28 (1.1)**	**4809**	**23 (0.5)**	**117**	**3 (2.6)**

Most of the infected sheep, goats and cattle had cysts in the liver and over one third of them showed multiple infections in both liver and lungs (Table 3). None of the infected sheep had cysts in the lungs only although 42.9% of the infected sheep had multiple infections in both liver and lungs. In cattle one of the three infected bulls had cysts in the lungs only. In goats only one (4.3%) of the infected animals had hydatid cysts in the lungs only (Table 3).

Table 3. Organ distribution of hydatid cysts found in livestock ani mals slaughtered in central abattoirs in Aden Governorate-Yemen (2008/2009 survey).

Animal Type	No. & (%) of infected animals having hydatid cysts in following organs		
	Liver	Lung	Both
Sheep	16 (57.1)	0 (0.0)	12 (42.9)
Goats	14 (60.9)	1 (4.3)	8 (34.8)
Cattle	2 (66.7)	1 (33.3)	0 (0.0)
All	32 (59.3)	2 (3.7)	20 (37.0)

While most of the cysts found in sheep and goats were fertile (82 % and 83.3% respectively), all cysts in cattle were found to be sterile. Sterile and calcified cysts accounted for 6.2% and 11.1% of cysts collected from sheep and 10% and 6.7% of cysts from goats respectively (Table 4).

Table 4. Number and type of hydatid cysts found in livestock animals slaughtered in central abattoirs in Aden Governorate, Yemen (2008/2009 survey).

Animal type	Total no. of cysts examined	Cyst type					
		Fertile		Sterile		Calcified	
		No.	%	No.	%	No.	%
Sheep	81	67	82.7	5	6.2	9	11.1
Goats	30	25	83.3	3	10	2	6.7
Cattle	7	0	0.0	7	100	0	0.0
All	**118**	**92**	**78.0**	**15**	**12.7**	**11**	**9.3**

4. Discussion

The present study revealed that CE or unilocular hydatidosis is prevalent among the main domestic animals in Aden Governorate of Yemen. Prevalence recorded here is higher than previously recorded by Ali *et al*. (2003) who studied infection rates in sheep and cattle in one abattoir only. In contrast, higher infection rates were recorded in sheep and goats (3.2% and 11%, respectively) in Hadramaut Province by Baswaid (2007). Although the infection rate in cattle was found to be significantly higher than that in sheep and gaots, epidemiologically the latter animals are more significant than cattle. This is because most of the slaughtered animals in Yemen are sheep and goats and due to the fact that all cysts found in cattle were sterile. In contrast, sheep and goat cysts were mostly fertile. The role of camels in supporting the overall transmission dynamics in Aden cannot be determined from this study because the number of camels inspected was too small to draw any conclusions.

The infection rates reported in the present study fit within the framework of prevalence of CE in livestock in the Middle East including other Arab countries although much higher rates have been recorded in most other countries (Abdel-Hafez and Kamhawi, 1997; Nourian, *et al*., 1997; Sobeih *et al*., 1998; Saeed *et al*., 2000; Sadjjadi, 2006; Daryani *et al*., 2006; Haridy *et al*., 2006; Arbabi and Hooshyar, 2006; Goz *et al*., 2007). This difference is attributed, perhaps, to the variability in the origin of animals, mode of grazing and other environmental factors and attributes pertaining to the dog definitive host. Al-Abbassy (1980) attributed low rates of infection to different factors such as periodical destruction of dogs,

improved standards of meat inspection and overall improvement in socioeconomic conditions.

The higher infection rates in female sheep and goats compared to males can be explained to older ages of slaughtered females than males. In practice, female sheep and goats are usually maintained for longer periods than males to give offspring several times before slaughtering. In contrast, most male sheep and goats are slaughtered at young ages of six months to less than two years. In younger animals, either hydatid cysts have not developed into detectable size or are too small and easy to miss. Indeed, the present study as well as many other studies elsewhere (Al-Yaman *et al*., 1985; Pandey *et al*., 1988; Baswaid, 2007) has shown higher infection rates in older animals. This attributed to two factors: Firstly, higher age reflects a much longer period of exposure to infective egg stage in the pasture, and secondly, the chances of detecting cysts at meat inspection are higher in aged animals due to their bigger size. It must be stated, however, that the lack of infection in sheep and goats over 3 years of age is unexpected and thus unexplainable. As for cattle, the number of female cattle slaughtered was too small to draw any conclusion.

The present study revealed that liver was the most affected organ in infected sheep and goats, but multiple infections in both liver and lungs was found in more than one third of infected animals. No sheep showed infection in the lungs only. This indicates that the liver is the primary site for cyst development and lung involvement comes as a secondary consequence. This was primarily true for hydatidosis in goats as only one infected goat showed cysts in the lungs only.

Similar observations were made by Farah *et al.* (1984), Al-Khalidi (1998), Azlaf & Dakkak (2006), and Baswaid (2007).

The majority of cysts in sheep and goats were fertile indicating that these animals are the major intermediate hosts responsible for the perpetuation of the life cycle. This is consistent with other previous finding reported in other Middle Eastern countries (Singh and Dhar, 1988; Al-Khalidi, 1998). Cattle do not seem to be very important in transmission dynamics as all cysts removed from cattle were sterile.

In conclusion, this preliminary study indicates the existence of CE in main slaughtered livestock in Aden Province, Yemen. Further studies are needed to determine infection rates in the dog definitive host and determination of *E. granulosus* strain/s in Yemen. It is mandatory to carry out a cross sectional prevalence study in other abattoirs, especially rural ones and involving enough animals especially camels in order to understand the transmission dynamics of the disease in Yemen. Human surgical incidence and seroepidemiology in various Yemeni provinces must be determined to understand the magnitude of the disease as a problem in the country.

Acknowledgment

We are highly grateful to the workers in the abattoirs in Aden, and to Dr. Yahia Mohammed Mosaed and Mr. Wahid Ali Al- Saqir for their help during the present study.

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JJBS

Jordan Journal of Biological Sciences

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 125 - 128

Explanation of the Decrease in Alkaline phosphatase (ALP) Activity in Hemolysed Blood Samples from the Clinical Point of View: *In vitro* study

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Received on December 25, 2011, Accepted on January 30, 2012

Abstract

Hemolysis is still the most common reason for rejecting samples, while reobtaining a new sample is an important problem. The aim of this study was to explain the decrease in the activity of Alkaline phosphatase (ALP) enzyme after hemolysis of blood samples and whether conversion of zinc and magnesium ions to inhibit alkaline phosphatase activity after they released from red blood cells. Twenty healthy volunteers were enrolled in the study. Four hemolysis levels were constituted according to hemoglobin concentrations (0.02, 0.27 ,0.75 and 3.34 g/L). Non–hemolysed samples was obtained from each volunteer and considered as control. Hemolysis was achieved by mechanical trauma. Alkaline phosphatase activity and the concentrations of zinc and magnesium ions were measured in the hemolysed and non-hemolysed samples. Ten non-hemolysed serum samples (Hb concentration was < 0.02 g/L) were divided into two groups samples A and B. ALP activity was measured in these samples. In vitro study was carried out including the addition 68.3 mg/dL of magnesium chloride to group A and 5.1 µg/dL of zinc chloride to group B. ALP activity was measured in the sera of the two groups. The significant decrease (p<0.001) in ALP activity was observed at moderate (13.2 ± 7.2IU/L), and severe hemolysis (5.5± 2.3IU/L) compared with that in non hemolysed samples. In these levels of hemolysis the concentrations of Zn^{+2}ions (5.1 ±1.1µg/dL) and Mg^{+2}ions (68.3± 8.6mg/dL) were significantly increased (p<0.01) compared with their concentrations in non-hemolysed samples. Alkaline phosphatase activity was inversely proportional with the increase in the hemoglobin concentrations in the hemolysed samples. A significant decrease (p<0.005) in the activity of ALP was observed after the addition of 68.3.0 mg/dl of magnesium chloride to group A. There was no significant decrease (p>0.1) in activity of ALP in the samples of group B. The findings of this study indicate that blood cell hemolysis reduces the activity of ALP which is directly proportional to the level of hemolysis. Released Mg^{+2} ions were found to inhibit ALP activity in the blood hemolysed samples.

Keywords: Alkaline phosphatase activity, hemolysed blood samples, Mg^{+2} Ions, *in vitro* study.

1. Introduction

Hemolysis is the most common preanalytical source of error in clinical laboratories and responsible for nearly 60% of rejected samples (Plebani *et al*., 1997; Bonini *et al*., 2002). Blood hemolysis may occur *in vivo* or *in vitro*. The ratio of *in vivo* hemolysis is only 3.2% of all the hemolyzed specimens (Carraro *et al*., 2000). In vitro hemolysis occurs more often and it is caused by improper sample drawing, handling or centrifugation. Especially hardly collected samples, or stored and/or transported, have increased risks for hemolysis.

Most of the hemolyzed samples are being rejected on pre-analysis stage according to the visual detection of serum interferences, even if the requested tests may not be interfered with hemolysis. Besides, according to the reports, visual assessment of sample hemolysis showed little agreement with the actual concentration of hemoglobin interferent (Hinckley, 1997; Plebani, 2007; Simundic *et al*., 2009). Conversely, even if the hemolysis is not visible, there is also a discharge of the cell constituents into serum or plasma (Thomas, 2010). So invisible hemolysis is an important cause of false results and has to be detected before the investigation procedure.

Alkaline phosphatase enzyme has an important diagnostic value in liver diseases and bone diseases. The effect of hemolysis on the activity of ALP is less understood. Some studies (Yucel and Dalva, 1992; Lippi *et al*., 2006) revealed a decrease in the activity of Alkaline phosphatase in hemolysed blood samples; other studies (Grafmeyer *et al*., 1995**;** Arise *et al***.,** 2008) have not found any change in the activity of ALP in hemolysed blood samples.

Alkaline phosphatase is a metallo-enzyme that is activated by magnesium and zinc ions (Mehmet *et al*., 2011). Hemolysis causes a release of intracellular ions in the serum among these are the magnesium and zinc ions which are usually found in large concentration in hemolysed samples. The present study aims, therefore, at

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investigating the possible inhibitory role of Zn^{+2} and Mg^{+2} ions on the activity of ALP in hemolysed blood samples.

2. Materials and Methods

2.1. Preparation of Blood Samples

All reagents, Medical Kits and were purchased from Agencies Med. Lab. Trading Com. (L.L.C) Amman /Jordan. Twenty healthy volunteers (23.0± 4.0 years old) were tested for their fitness and health by measuring their levels of ALP before they are enrolled in the study. Venous blood samples were collected from twenty healthy volunteers. Hb concentration was measured to make sure that these sample were free from hemolysis.

To study the effect of *in vitro* hemolysis, procedure of Mehmet *et al*., (2011) was used where four samples were drawn from the healthy volunteers through the needles of 5 mL syringe (1.5 inch, 21 gauge) speedily for 2, 4, 6 and 8 times respectively to lyse the cells by mechanical trauma to obtain slightly, mildly, moderately and severely hemolyzed samples. They were all centrifuged at 1000 x g for 15 minutes and sera were collected.

2.2. Determination of Hemoglobin (Hb) Concentration

Free Hb of all samples were measured spectrophotometrically (Shimadzu Corporation; Kyoto, Japan) with Na_2CO_3 solution (10 mg/100 mL) as a reagent (Fairbanks and Klee, 1999). Absorbencies were measured at 415, 450 and 700 nm for non-hemolysed and hemolysed blood samples.

Total serum hemoglobin was calculated according to the formula:

Hb = 154.7 × (A425) – 130.7 × (A450) – 123.9 × (A700) (9). The Reference range was from 0 - 0.1 g/L for serum free Hb.

2.3. Determination of Alkaline Phosphatase (ALP) Activity Non-Hemolysed and Hemolysed Samples

The activity of ALP was determined according to the procedure of Matsushita and Komoda (2011). 50 µl of the each serum sample was mixed with 1ml of the reagent of alkaline phosphatase enzyme. The mixture was incubated for 1minute.The absorbance of the each sample was measured for 3 consecutive minutes at 550 nm. The activity of ALP (IU/L) was calculated according to the following formula:

(IU/L) = ΔAbs./min. x 2187

Normal level of ALP = 40 - 140 IU/L.

2.4. Determination Zn^{+2} ions in non-Hemolysed and Hemolysed Samples

The determination of Zn^{+2} ions was based on the procedure of Knoell *et al* (2009). 200µL standard, sample and sample blank (200 µL Sample + 8 µL EDTA) were transferred to appropriately labeled tubes. 800µL working reagent were added, mixed and Incubated for 30 min and the light absorbance were read at 425 nm. Concentration of Zn in the samples was determined from a standard curve prepared for Zinc standard concentrations (data are not shown).Normal zinc ion concentration (µg/dL) = 45.2 ± 4.4.

2.5. Determination of Mg^{+2} ions

Procedure of Whang (1987) was used for the determination of Mg^{+2} ions**.** 25 µL diluted standard and samples were transferred to appropriately labeled tubes.1000 µL working reagent was added and mixed. After incubation for, the absorbance was read at 500nm. 50 µL EDTA solution was added, mixed well, incubated for 2 minutes and read at 500 nm.

Absorbance of Mg^{+2}(Abs. $_{Mg+2}$) and Absorbance of Mg blank (Abs. $_{Mg+2blank}$) are Absorbance values at 500 nm of the standard(2 mg/dL) before and after the addition of EDTA.

$$\text{Concentration of } Mg^{+2} = \frac{\text{Abs. }_{\text{Sample}} - \text{Abs. }_{\text{Blank}}}{\text{(mg/dl) Abs. }_{\text{Mg}} - \text{ Abs. }_{\text{Mg Blank}}}$$

Normal Magnesium ion concentration (mg/dL) = 1.77 ± 0.02

2.6. Alkaline Phosphatase Activity in non Hemolysed Blood Samples to Which Zinc and Magnesium are Added

Ten non-hemolysed serum samples (free Hb concentration was < 0.02 g/L) were divided in to two groups samples (named as Group A and B). ALP activity was measured in these samples. In vitro study was carried out including the addition 68.3 mg/dL of magnesium chloride to group A and 5.1 µg/dL of Zinc chloride to group B. All samples were incubated for 10 minutes. ALP activity was measured again in the sera of the two groups.

3. Results

As seen in table 1, Alkaline phosphatase activity was significantly decreased in hemolysed samples compared with that in non hemolysed samples (83.3 ±10.6 IU/L). The significant decrease ($p<0.001$) in the activity of the enzyme was observed at moderate (13.2 ± 7.2IU/L), and severe hemolysis (5.5±2.3IU/L) compared with that in non hemolysed samples (83.3 ±10.6 IU/L). The activity of ALP at slight hemolysis (81.4±18.4 IU/L) did not show any significant decrease ($p<0.5$), however. In these level of hemolysis, the concentrations of Zn^{+2} (5.1± 1.1µg/dL) and Mg^{+2} (68.3± 8.6mg/dL) were significantly increased ($p<0.01$) compared with their concentrations in non-hemolysed samples (2.75 ± 0.82µg/dL and 17.66 ± 2.3mg/dL, respectively) as observed in table 1.

The decrease in the activity of ALP was inversely proportional with the increase in the hemoglobin concentrations in the hemolysed samples (Table 1).

Significant decrease of ALP activity started at moderate hemolysis where the concentrations of Mg^{+2} and Zn^{+2} ions were 68.3 ± 8.6 mg/dl and 5.1± 1.1 µg/dl, respectively.

Table1. The activity of Alkaline phosphatase(ALP), the concentrations of Zn+2 and Mg+2 and the Hemoglobin (Hb) concentrations in blood samples hemolysed to different levels.

Levels of hemolysis	Hb concentration (g/L)	Mg^{+2} (±SD)[a] (mg/dL)	Zn^{+2} (±SD)[a] (µg/dL)	ALP activity(IU/L) (± SD)[a]
No hemolysis	0.012	17.66† (2.3)	2.75 (0.82)	83.3•(10.6)
Slight	0.02	18.2 (1.8)	2.8 (0.31)	81.4• (18.4)
Mild	0.27	23.3 (5.3)	3.5 (1.2)	78.5•(9.5)
Moderate	0.75	68.3† (8.6)	5.1 (1.1)	13.2*(7.2)
Severe	3.34	71.6 (11.3)	6.3 (2.3)	5.5*(2.3)

a Mean +SD,*p< 0.01; •p> 0.1 ; †p < 0.01

Slight and mild hemolysis did not affect the ALP activity where the concentration of Hb was 0.02 and 0.27 respectively (Table 1).A significant decrease (p<0.005) in the activity of ALP from 93.7± 10.2 to 47.4 ±10.7IU/L was recorded after the addition of 68.3.0 mg/dl of magnesium chloride to group A (Table 2).The activity of ALP in group B (85.1± 8.6IU/L), before the addition of 5.1 µg/dl of Zinc chloride, was not significantly decreased (p>0.1)with that activity (77.2± 12.3 IU/L) after the addition (Table 2).

Table2. Activity of ALP in non hemolysed serum samples before and after the addition of $MgCl_2$ and $ZnCl_2$.

	Before addition of $MgCl_2$; $ZnCl_2$		After addition of $MgCl_2$	After addition of $ZnCl_2$
	Group A(±SD)	group B (±SD)[a]	Group A(±SD)[a]	Group B(±SD)[a]
ALP activity (IU/L)	93.7• (10.2)	85.1*(8.6)	47.4•(10.7)	77.2*(12.3)

[a] Mean ±SD, p•<0.005; p*>0.1

4. Discussion

Most studies (Carraro *et al*., 2000; Bonini *et al*., 2002; Lippi *et al*., 2006; Plebani, 2007; Simundic *et al*., 2009) have unanimously agreed on the effect of hemolysis on the activity of ALP. Some studies (Plebani, 2007; Simundic *et al*., 2009) attributed the significant decrease in the ALP activity to the dilution factor as a possible effect where the leakage of intracellular components into the surrounding fluid especially in severe hemolysis may cause decreased ALP activity. Other studies (Carraro *et al*., 2000; Lippi *et al*., 2006) attributed such a decrease to the direct impact of some of the contents of blood cells on the activity of ALP without specifying the nature of these contents.

As found in the current study hemolysis significantly decreased the activity of ALP and the data suggests a progressive inhibition of ALP when exposed to increasing level of hemolysis. This method of cell lyses was chosen because blood transferring into a tube by pushing forcedly down on the syringe plunger is analogous to the mechanical disruption of erythrocytes that frequently occurs during blood collection. In this method, there is no standardization way of the force applied while transferring the blood by syringe. Besides, every patient's fragility of red blood cell is different, so free Hb concentrations of all samples were not correlated with the force.

The significant decrease in ALP activity started at moderate hemolysis where the concentrations of Mg^{+2} ions was four times greater than at normal level. The increase in concentration of Zn^{+2} ions was twice greater than the normal at this hemolysis level. It seemed that the effect of the increase in the concentration of Mg^{+2}ions in the hemolysed samples resulted in a significant decrease in ALP activity.

In vitro experiment same concentration of Mg^{+2} ions which was measured at moderate hemolysis was prepared as $MgCl_2$ and was added to non hemolysed sample as a result of this addition there was a significant decrease in the activity of ALP. However this finding was not observed in the ALP activity in non-hemolysed sample to which $ZnCl_2$ was added.

From these observations in table 2, it is very obvious that elevated level of Mg^{+2} ions play an inhibitory effect on the ALP activity. This explanation seems to be reasonable since ALP is a metallo-enzyme which depends on Mg^{+2} and Zn^{+2}ions as cofactors (Arise *et al*., 2008). In the current study, the increase in Mg^{+2} ions resulted in a feedback inhibition on ALP activity.

Hemoglobin concentration is recommended to be measured before carrying out ALP activity. Slight and mild hemolysis as the concentration of Hb is ≤ 0.27 g/L did not affect the ALP activity as manifested in the present study. For ALP measurement, grossly hemolysed samples should be rejected and new samples should be requested. It is recommended to determine free Hb level in serum or plasma, to detect the degree of hemolysis.

5. Conclusion

Inhibition of ALP activity was found due to releasing of large amount of Mg^{+2} during blood hemolysis which confirms the feedback inhibition exerted by the ion on the enzyme action.

Acknowledgement

We wish to thank Taif University represented by the Deanship of higher studies and scientific research for their financial support (Grant No. 1\432\1190). Special thank to our colleagues in the Medical Biochemistry Department/ College of Medicine for their technical support and valuable discussions. Also special thanks to Dr. Aymen Biomy and Mr. Yehya Al-Hazaai for their help to complete this study

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JJBS

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 129 - 133

Jordan Journal of Biological Sciences

Alterations in Antioxidant Defense System in the Plasma of Female Khat Chewers of Thamar City, Yemen

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Received on December 28, 2011, Accepted on February 5, 2012

Abstract

Chewing Khat leaves (*Catha edulis*) is highly prevalent in Yemen and East African countries. Unfortunately, farmers use to apply pesticide for the better product of Khat. The present study has been designed to investigate the activity of plasma butyrylcholinesterase (BChE) and to assess the antioxidant defense system in the plasma of female Khat chewers in Thamar city, Yemen. Plasma of twenty female Khat chewers and twenty controls (non Khat chewers) were prepared and the activities of BChE and catalase (CAT) were estimated along with the measuring levels of reduced glutathione, total thiols and cholesterol. At biochemical level a significant decrease in the activities of BChE and CAT were observed in the plasma of female Khat chewers ($P < 0.05$) concomitant with reductions in the levels of reduced glutathione and total thiols ($P < 0.05$) as compared to non Khat chewers. This alterations on the antioxidants resulted in decrease of plasma cholesterol in Khat chewers group ($P < 0.05$). The present data show that the production of oxidants which are responsible for reduction in antioxidant defense system might be due to chewing Khat plant with more attention to the pesticide applied to the plant.

Keywords: Khat; organophosphates; butyrylcholinesterase; antioxidant; oxidative stress.

1. Introduction

Chewing Khat leaves (*Catha edulis*) is highly prevalent in Yemen and East African countries (Manghi *et al.*, 2009). The three main alkaloids present in Khat leaves are cathinone, cathine and norephedrine (Kalix, 1992). There are also small amounts of sterols and triterpenes, together with 5% protein and ascorbic acid. Khat leaves also contains tannin and minute amount of thiamin, niacin, riboflavin, iron and amino acids. Thus, only freshly picked leaves have the full efficacy (Lugman and Danowski, 1976).

Taken in excess, Khat causes extreme thirst, a sense of exhilaration, talkativeness, hyperactivity, wakefulness, and loss of appetite. It also can cause damage to the nervous, respiratory, circulatory, and digestive systems. Khat is reported to produce constipation and antispasmodic action (Makonnen, 2000). Chewing Khat has been linked with increased oxidative stress (Aleryani *et al.*, 2011). Oral administration of total aqueous Khat extract or of its alkaloid fraction exacerbated the oxidative stress in restrained rats due to the decreased activity of antioxidant enzymes, superoxide dismutase, catalase, glutathione-S-transferase (Kalix *et al.*, 1990). Similarly, Khat induced an increase in reactive oxygen species (ROS) and a depletion of intracellular glutathione in the cell cultures of human keratinocytes and fibroblasts, the reactions that could be opposed by addition of exogenous antioxidants (Lukandu *et al.*, 2008). But on the other hand, the flavonoid fraction of the Khat enhanced the activity of the antioxidant enzymes in rats and thus could provide a protection against the oxidative stress (Al-Qirim *et al.*, 2002). It was estimated by world health organization (WHO) that 30–50% of adult females consume Khat on a regular basis (WHO 2007). Unfortunately, farmers use to apply pesticide for the better product of Khat. The pesticides have two actions; it help the humans to remove harmful insect from the plants; on the other hand, it have negative effect for the human body especially for human's body enzymes . The fundamental toxicological activity of organophosphorus compounds (OPs) in human is due to the inhibition of esterases. Butyrylcholinesterases (BChEs) are enzymes belonging to a group of hydrolases classified by Aldridge (1953) as B-type esterases, they are inhibited by OPs and carbamate pesticides. As well as being present in the plasma, BChE is found in central nervous system, liver and other organs. As a result, along with acetylcholinesterase, BChE inhibition has been used as an indicator of exposure in biomonitoring programs of pesticide contamination. The exact physiological function of BChE is not yet clear, they appear to have a protective function by sequestering

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* Abbreviations used: BChE, Butyrylcholinesterase; GSH, Reduced glutathione; T-SH, Total thiols; OPs, Organophosphate compounds; CAT, Catalase

circulating OPs, thereby decreasing the toxic effect of these compounds on brain AChE (Russell and Overstreet, 1987). It has been reported that exposure to OPs induces oxidative stress by enhancing generation of ROS and/or by alterations in antioxidant defense system (Banerjee *et al.*, 2001). Oxidative stress occurs when the production of ROS overrides the antioxidant capability of the target cell (Klaunig *et al.*, 1998). ROS are potentially very damaging to cells, leading to oxidation of essential cellular constituents including proteins, lipids and DNA (Paradies *et al.*, 2002). The present study has been designed to investigate the pesticide effect of Khat via measuring plasma BChE and to assess the antioxidant defense system in the plasma of female Khat chewers in Thamar city, Yemen.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of highest grade commercial products. Buryrylthiocholine iodide and DTNB (Ellman reagent) were from HiMedia, India. Kits of other tests were purchased from Spinreact, Spain.

2.2. Population and grouping

Thamar city female populations were divided into two groups each having n=20. Both group participants included in the present study fulfill the following criteria: healthy, non diabetic volunteers and aged between 20 and 35 years those excluded are suffering from hepatitis, carcinoma and diabetes:

1. Khat chewers group: local females with habit of chewing Khat.
2. Non-Khat chewers (control) group: local females never chew Khat.

Non-Khat chewers are those who never chew Khat, whereas, chewers are those who chew Khat daily. The study was performed in accordance with the Helsinki Declarations and was approved by Institution.

2.3. Sample collection

Blood samples of 40 individuals (20 each group) were collected between 8-10 A.M. 12 hours after end of Khat session, plasma of all samples were separated and were stored at -4 C° till the day of analysis.

2.4. Butyrylcholinesterase assay

Plasma BChE activity was determined colorimetrically by the method of Ellman *et al.* (1961). Briefly, plasma was pre-incubated with 10 mM of 5, 5-dithiobis-2-nitrobenzoic acid (DTNB or Ellman reagent) in 0.1 M phosphate buffer (pH 8.0) before the substrate butyrylthiocholine iodide was added (10 mM). Variations in optical density were recorded at 412 nm for 2 min at 25 C° using a spectrophotometer. Plasma BChE activity was expressed as nano-moles of substrate hydrolyzed per minute per mg protein using an extinction coefficient of DTNB (13,600 $cm^{-1}M^{-1}$).

2.5. Total thiols

Total thiol groups were quantified in the plasma according to the method of Ellman (1959) as modified by Sedlak and Lindsay (1968). Briefly, reaction mixture containing 0.2 M Tris-HCl and 0.02 M EDTA buffer (pH 8.2), plasma and 0.01 M DTNB (in methanol) was incubated for 15 minutes at room temperature then was centrifuged at 1,200 x g for 5 minutes. The supernatant was collected and the absorbance was read at 412 nm. Results were expressed as nmoles of T-SH/mg protein using molar extension coefficient of DTNB (13,600 $cm^{-1}M^{-1}$).

2.6. Low molecular weight thiols

Low molecular weight thiols, LMW-SH (primarily GSH) were measured in the plasma according to the method of Ellman (1959). Briefly, proteins in the plasma were precipitated by 4 % (w/v) sulphosalicylic acid followed by centrifugation at 1200 x g for 5 minutes. To the supernatant, 0.1 mM DTNB in 0.1 M phosphate buffer (pH 8.0) was added and the absorbance was read at 412 nm after 2 minutes. Results were expressed as nmoles of LMW-SH /mg protein using molar extension coefficient of DTNB (13,600 $cm^{-1}M^{-1}$).

2.7. Catalase activity

Catalase activity was assayed in the plasma following the method of Luck (1971). Appropriate amount of plasma was added to 12.5 mM H_2O_2 in 0.067M phosphate buffer (pH 7.0). The decrease in absorbance was followed at 240 nm for 3 minutes. Results were expressed as μmoles of H_2O_2 decomposed/min/mg protein using molar extinction coefficient of H_2O_2 (71 M-1 cm-1).

2.8. Cholesterol level

Cholesterol level was measured in the plasma according to the protocol provided by commercial kit, Spinreact, Spain. Results were expressed as mg/dl.

2.9. Protein content

Protein content was measured in the plasma according to the protocol provided by commercial kit, Spinreact, Spain. Results were expressed as g/dl.

2.10. Statistical analysis

Data were expressed as mean ± S.D. and were analyzed by student t-test. Differences between groups were considered significant when $P < 0.05$. All analyses were performed using the sigma-stat software (version 3.5).

3. Results

A decrease of 30.87% in the activity of BChE of Khat chewers group was observed as compared to the non Khat chewers group (Fig. 1), whereas, catalase activity was inhibited by 11.62% in the Khat chewers group (Fig.2). The thiol contents of Khat chewers group were also decreased as compared to non Khat chewers. It was observed that 14.42% of T-SH levels were less in Khat chewers concomitant with 49.12% decrease in the level of LMW-SH of Khat chewers group (Table 1). The increase in oxidative stress which is marked by the above results was affect the cholesterol level which was found to be reduced by 47.26% in Khat chewers group as compared to non Khat chewers group (Fig.3).

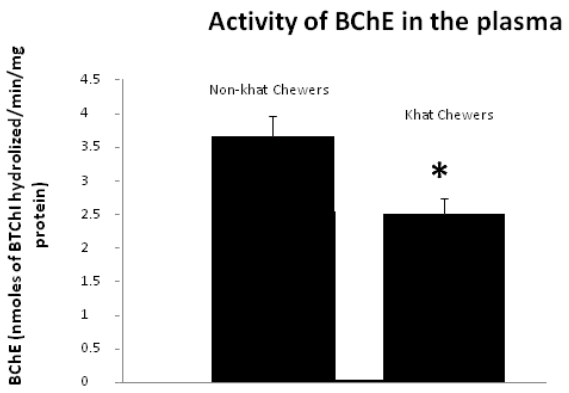

Figure 1. Activity of butyrylcholinesterase in the plasma of non-Khat chewers and Khat chewers. Results are expressed as mean ± S.D.; n= 20. Data were analyzed by student-t- test. *p<0.05 was considered significant from control group.

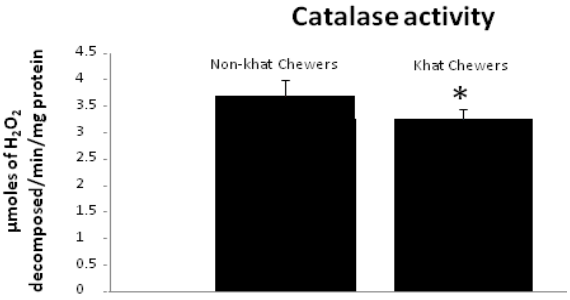

Figure 2. Activity of catalase in the plasma of non-Khat chewers and Khat chewers. Results are expressed as mean ± S.D.; n= 20. Data were analyzed by student-t- test. *p<0.05 was considered significant from control group.

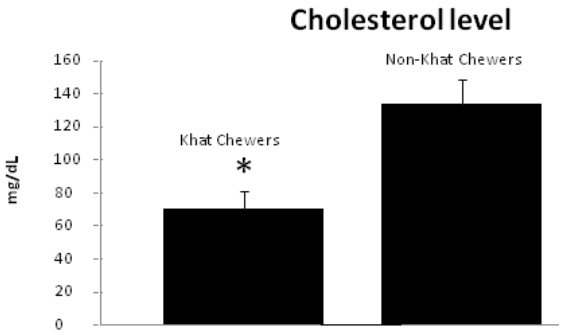

Figure 3. Levels of cholesterol in the plasma of non-Khat chewers and Khat chewers. Results are expressed as mean ± S.D.; n= 20. Data were analyzed by student-t- test. *p<0.05 was considered significant from control group.

Table 1. Effect of Khat chewing on thiol contents

	nmoles/mg protein	
	Non Khat Chewers	**Khat Chewers**
Total Thiols (T-SH)	256.73 ± 15.04	219.69±18.72*
LMW-SH (GSH)	122.23 ± 10.26	62.19±6.77*

Results are expressed as mean ± S.D; n= 20. Data were analyzed by Student-t- test.
*p<0.05 significantly different from control

4. Discussion

Khat is now widely chewed in Yemen and East African countries, however, only the immigrant communities from these countries are Khat chewers in Western countries (Manghi *et al.,* 2009). BChE and antioxidants CAT, GSH and TSH were decreased concomitant with decrease in cholesterol level in the present study in the plasma of female Khat chewers. BChE inhibition has been used as an indicator of exposure to pesticide. These findings are in agreements with those of Al-Akwa *et al.* (2009) who reported that chewing Khat increases the capacity of oxidant production and they also reported inhibition of AChE in the plasma of Khat chewers. ROS and the end products of LPO are believed to be largely responsible for the cytotoxic effects observed during oxidative stress (Cassarino and Bennett 1999). Moreover, cellular oxidative stress and cancer have been linked with pesticide exposure, particularly exposure to carbamate and OPs (Kassie *et al.,* 2001; Ranjbar *et al.,* 2002). The cell detoxifies free radicals via its antioxidant defense system, which includes non-enzymatic antioxidants like GSH and antioxidant enzymes; superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Cassarino and Bennett, 1999). Thiols are organic sulfur derivatives characterized by the presence of sulfhydryl groups (-SH). Thiols are classified as large molecular weight (protein) thiols and low molecular weight thiols (GSH, cysteine and homocysteine). GSH is an important water soluble antioxidant that is central to cellular defense against oxidative stress and potentially toxic chemicals (Meister and Anderson 1983). It directly quenches reactive hydroxyl radicals and other oxygen-centered free radicals, and conjugates to the xenobiotics to water soluble products (Kidd, 1997). Low levels of GSH could be due to enhanced generation of ROS which are scavenged by GSH or decreased activity of GR enzyme, which converts oxidized glutathione (GSSG) to its reduced form. The relationship between the reduced and oxidized state of glutathione, the GSH/GSSG ratio or glutathione redox status, is considered as an index of the cellular redox status and a biomarker of oxidative damage, because glutathione maintains the thiol-disulphide status of proteins, acting as a redox buffer. Glutathione depletion was reported to induce apoptotic cell death which occurs through the upregulation of novel protein kinase C and activator protein-I (Domenicotti *et al.,* 2000). Therefore, disruption of the GSH redox status by OPs can alter transcriptional responses to induce programmed cell death. CAT is an antioxidant enzyme that appears to be less significant as it has relatively low affinity for H_2O_2 but is an important enzyme at higher H_2O_2 concentration (Chance *et al.,* 1979). The activity of CAT was decreased in the plasma of Khat chewers as compared to control group this will lead to accumulation of H_2O_2 that will contribute to high hydroxyl radical. Cholesterol is involved in the clustering of particular lipids within the membrane bilayer to form rafts, which in turn influences the distribution of proteins within the membrane. Cholesterol might exert its affect on membrane proteins viz. adenylate cyclase, GABA uptake transporters and the nicotinic acetylcholine receptor (Fong and McNamee, 1986; North and Fleischer, 1983). Alterations in cholesterol contents might alter the activity

of various membrane proteins. Inhibition of critical antioxidant enzymes and scavenger proteins may be the mechanism by which free radicals reduce antioxidant capacity (Grzelak *et al.*, 2000). It has been reported that Khat leaves reduced significantly the cholesterol levels in rabbit adrenal (Ahmed and el-Qirbi, 1993) and rabbits blood (Al-Habori and Al-Mamary, 2004). This decrease is attributed to the increase level of cAMP and increase adrenocorticotrophic hormone which is believed to be mediated by the activation of adenylyl cyclase, hence, increase cAMP level. The increase in cAMP concentration has an inhibitory effect on cholesterol synthesis (Mayes, 2000). However, the present finding are in contrast with those reported by Al-Zubairi *et al.* (2003) were the total cholesterol and LDL- cholesterol were non-significantly affected by Khat chewers as well as Khat chewers who also smoke. Our results are in agreement with the findings of some researchers, Al-Akwa *et al.* (2009) have reported an increase on the levels of free radicals of serum of male Khat chewers. Al-Qirim *et al.* (2002) have reported that Khat consumption increases circulating free radicals in rats. Lukandu *et al.* (2008) showed similar observations in keratinocytes and fibroblasts. Oral administration of Khat extract to rats induced lipid peroxidation and oxidative stress in hepatic and renal tissues as shown by significant increases in lipid peroxidation and significant decreases in levels of CAT and GSH (Al-Hashem *et al.*, 2011).

It is concluded that applying pesticides for better Khat production might be responsible for some of the changes in the antioxidant defense system which needs to be eliminated in future studies by using appropriate animal model and chemical-free Khat.

5. Conflict of Interest

No financial, personal, or other conflict of interest to be mentioned.

Acknowledgement

The authors are greatly acknowledged Mr. Ahmad Alkhalaqi, lab technician for his valuable assistant.

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JJBS

Jordan Journal of Biological Sciences

Volume 5, Number 2, June 2012
ISSN 1995-6673
Pages 135 - 139

Attachment of Embryonic Stem Cells-derived Cardiomyocytes in CultiSpher-S Microcarriers by using Spinner Flask

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Received on December 30, 2011, Accepted on February 10, 2012

Abstract

Embryonic Stem (ES) cells have the ability to differentiate under *in vitro* conditions into cardiomyocytes. A transgenic α-myosin heavy chain (α-MHC$^+$) ES cell line was generated, exhibiting puromycin resistance and expressing enhanced green fluorescent protein (EGFP) under control of the α-MHC$^+$ promoter. A puromycin-resistant, EGFP-positive α-MHC$^+$ cardiomyocyte population was isolated with over 92% purity. The cultivation of these cardiomyocytes, in macroporous gelatine microsphere beads in a spinner flask bioreactor has been studied. After we specified the most suitable agitation conditions and the optimal timeframes of cultivation, the average number of cultivated cells per microsphere was optimised. Our study shows that 80 % of microspheres were colonised by cardiomyocytes under optimal conditions. The results of the scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) showed that the population of the beads was not limited to the microcarrier surface, but also invaded the inner surfaces of the microspheres. The present findings demonstrate the successful culture of α-MHC$^+$ cardiomyocytes in macroporous biodegradable microcarriers while maintaining the typical morphological and electrophysiological properties of cardiomyocytes. These findings suggest significant improvement in survival of grafted cardiomyocytes, thus helping overcome current limitations of cell replacement approaches.

Keywords: Cardiomyocyte; embryoid bodies; fluorescence; stem cell; SEM; microcarrier, Cultispher-S.

1. Introduction

The generation of functional cardiomyocytes from embryonic stem cells has several potential applications. Myocardial diseases resulting from damage of cardiac tissue effect millions of people, who may benefit from transplantation of cardiomyocytes. The feasibility of such an approach has already been demonstrated in animal models using several sources of cells (Li *et al.,* 2000, Liechty *et al.,* 2000). Congestive heart failure (CHF) is the resulting condition from any structural or functional cardiac disorder that impairs the ability of the heart to fill with or pump a sufficient amount of blood throughout the body. Cardiac transplantation studies have been conducted using ES cell-derived cardiomyocytes from grafted EBs, although these contained a mixture of different cardiac cell types (Klug *et al.,* 1996; Min *et al.,* 2002). Therefore, many strategies were developed to differentiate early cardiomyocytes from embryonic stem cells. The *in vitro* differentiation of embryonic stem cells and gene expression studies, that result in generation of tissue-specific somatic cells, may represent a powerful tool for general understanding of cellular differentiation and development *in vivo* (Gissel *et al.,* 2005). So far, specific investigations on the generation of cardiomyocytes with high purity and the characterisation of their gene expression signatures have been conducted from a transgenic α-MHC (α-myosin heavy chain) ES cell line (CGR8) exhibiting puromycin resistance enhanced green fluorescent protein (EGFP), under control of α-MHC promoter (Doss *et al.,* 2007). The microcarrier technique is commonly used to grow anchorage-dependent cell lines in suspension cultures. Microcarriers are inert materials with surface characteristics suitable for promoting cell growth including charge, polarity, hydrophobicity and functional groups. An advantage of microcarrier culture systems is the ability to scale up into large volume production units. Microcarrier culture has been used to grow a wide variety of cell types. A wide range of biodegradable materials has been used for preparing microcarriers employed in cell culture, including Gelatin (e.g. Cultispher-S, G & GL) (DelGuerra *et al.,* 2001), D, L polylactic-coglycolic acid (PLGA) (Newman and McBurney, 2004), Cellulose and others (Yang *et al.,* 2007).

In the present study, we cultivated α-MHC$^+$ cardiomyocytes within CultiSpher-S microcarriers by using spinner flask bioreactor and tested their survival. The use of biodegradable microcarriers (CultiSpher-S) as a potential system to increase the survival and to achieve a new protocol can help attach these cells in the biodegradable microspheres.

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2. Materials and Methods

2.1. Generation of ES-derived α-MHC$^+$ Cardiomyocytes

Murine CGR8 ES cells (strain 129; Ampegic 7) were cultured in the absence of feeder cells in Glasgow minimum essential medium (GMEM) (Invitrogen, UK) supplemented with: 10% fetal calf serum (FCS) (Invitrogen, UK), 2 mM L-glutamine (Invitrogen, UK), 100 units/ml leukaemia inhibitory factor (LIF) (Chemicon), 50 μM β-mercaptoethanol (MRE) (Invitrogen, UK) and 100 mg/ml Neomycin (Invitrogen, Germany) in gelatine coated flasks (0.2% gelatine in Phosphate buffer saline), and maintained at 37C° and 5% CO2 (Gissel *et al.*, 2005).

ES cells were passaged four times before induced their differentiation to EBs. To assess this differentiation, the ES cell suspension of 2.5 x 10^5 cells/ml cultivated in Iscove's modified Dulbecco's Medium (IMDM) (Invitrogen, UK) supplemented with 20% FCS, 2 mM/l L-glutamine, 100 µM/l β-MRE, and 1% non-essential amino acids (Invitrogen, UK) (vol/vol). The preparation of EBs was carried out according to the (Gissel *et al.*, 2005) by hanging drops (20 µl/ drop) was made on the lid of 10 cm bacteriological dish containing 5 ml calcium and magnesium-free phosphate-buffered saline (PBS). Hanging drops of EBs were incubated in the corresponding growth medium at 37^{0}C in an atmosphere containing 5% CO_2 for two days. Then, these hanging drops were washed with 20 % IMDM medium and re-seeded in a new sterile dish to get 2 days old EBs and incubated at 37^{0}C for 5 more days to generate 7 days old EBs.

On day 8 the beating clusters were observed and α-MHC$^+$ cardiomyocytes were formed. These cells were treated with 4µg/ ml puromycin (Sigma Aldrich, Germany) as described by (Doss *et al.*, 2007) and cultivated in the same conditions for 6 days. The α-MHC/EGFP$^+$ beating cardiomyocytes were examined under a fluorescence microscope and documented through the videos and pictures (Axiovert 10, Zeiss, Germany) (Fig. 1, left).

2.2. Preparation of CultiSpher-S Mircrocarriers

CultiSpher-S microcarriers beads (diameter 130 – 380 µm) were obtained from Percell Biolytica (Astrop, Sweden) and were prepared according to the manufacturer's instructions. They represent macroporous gelatine beads in which the cardiomyocytes have the ability to use the interior surface. Briefly, for their preparation dry microcarriers (100 mg dry CultiSpher-S/5 ml) were swollen and hydrated in calcium and magnesium-free PBS for 2 hours at room temperature. Without removing the PBS, the microcarriers were sterilized by autoclaving at 121^{0}C, 15 psi (about 1 bar) for 15 min. Consequently, PBS was removed and the microcarriers were washed twice in IMDM medium. The sterile CultiSpher-S were counted by haematocytometer and divided in aliquots over small bacteriological dish (3 cm) where each one contained 200 microspheres prior to use, as shown in Figure 2 C.

2.3. In Vitro Expansion of α-MHC$^+$ Cardiomyocytes in CultiSphere-S

The 14 days old of beating α-MHC$^+$ cardiomyocytes were dissociated with collagenase B (Roche, Mannheim, Germany) (1 mg/ml calcium and magnesium free PBS), and incubated at 37^{0}C in 5%CO_2 for 30-45 minutes (Figure 2.d). After dissociation the cells suspension was centrifuged (1000 rpm for 7 min.) Then the supernatant was removed and the single dissociated cardiomyocytes were re-suspended in the 1 ml culture medium IMDM supplemented with 20% FCS, 1% non-essential amino acids (vol/vol), 2 mM/l L-glutamine, and 100 µM/l β-MRE (Figure 1, left). Dissociated cells were mixed with known numbers of hydrated CultiSpher-S microcarriers in siliconized sterile spinner flask filled with IMDM medium plus 20 % FCS, and incubated for four days at 37 °C, 5 % CO_2, over the magnetic stirrer at stirring rate of 40 rpm for 4 days. The entrapment of the cells was observed before and after the stirring of the cells and Cultispher-S microcarriers. Cell entrapment was monitored using a fluorescence microscope (Axiovert 10, Zeiss, Germany).

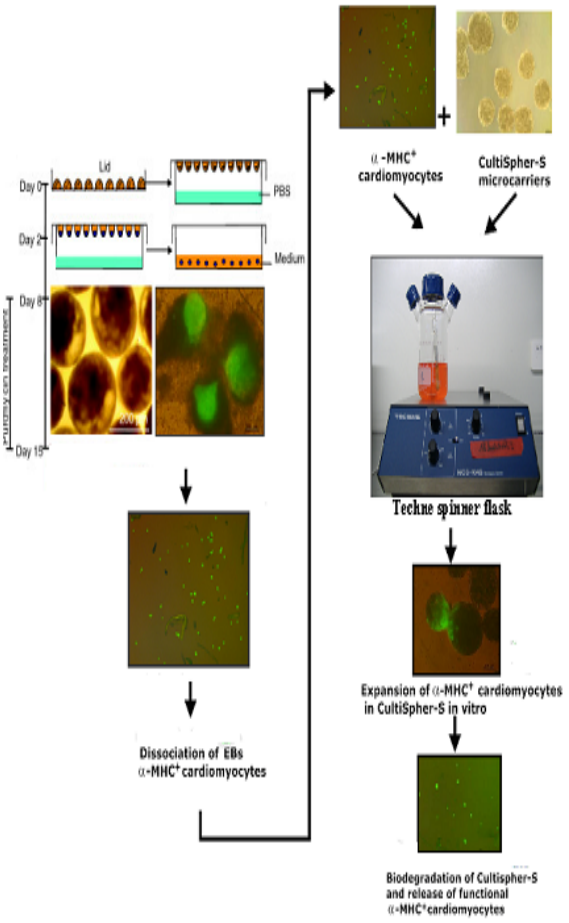

Figure1. Diagrammatic illustration of Techne spinner flask over magnetic Stirrer rotating the suspension of CultiSpher-S and cardiomyocytes at rate 30 rpm. Protocol used for generating ES cell-derived cardiomyocytes (left) and expansion of α-MHC+ cardiomyocytes in CultiSpher-S beads *in vitro* (right).

The 7 days old α-MHC$^+$ cardiomyocytes entrapped in CultiSpher-S were transferred into 15 ml Falkon tubes and were washed with calcium and magnesium-free PBS without centrifugation. The CultiSpher-S microspheres carrying α-MHC$^+$ cardiomyocytes were incubated in the presence of collagenase B (2 mg/ml) for 2 hours until the complete dissociation of the microcarriers, the cells were counted by using both the haemocytometer (Thoma Optic Labor, Germany) and vital stain the trypan blue 0.4% in normal saline (Figure 1, right).

2.4. Scanning Electron Microscopy (SEM) Of Cultispher-S Entrapped With Cardiomyocytes

CultiSpher-S microcarriers containing entrapped α-MHC cardiomyocytes were washed with 0.25 M HEPES buffer (Sigma-Aldrich, Germany) and were fixed with glutaraldehyde, which was added to the suspension in a final concentration of 2% (v/v). The suspension was washed with sodium cacodylate buffer, pH 7.2, and was embedded in 2% low melting agarose (Sigma-Aldrich & Taufkirchen, Germany). The post fixation was performed by the treatment of the microcarriers suspension in three steps using 2% osmium tetroxide, 1% tannic acid and 1% uranyl acetate in water as previously described (Katsen *et al.,* 1998). Dehydration of the suspension was carried out in increasing series of ethanol, while the last dehydrating step was performed in acetone.

The CultiSpher-S microcarriers entrapped with the cells were prepared for block-face scanning electron microscopy (SEM) (Philips, USA). Briefly the microspheres entrapped with the cells were embedded in epoxy resin, cross-sectioned with ultra-microtome, coated with carbon in SCD-030 (Balzers, Lichtenstein) and examined in a field emission scanning electron microscope (FESEM) XL30 (Philips, USA) using Back Scattered electron (BSE) modes with 12 KV accelerating voltage and 10 mm working distance (Katsen *et al.,* 1998).

2.5. Confocal Laser Scanning Microscopy (CLSM)

Detection of α-MHC+ cardiomyocytes on CultiSpher-S microcarries was done by using the confocal laser scanning microscopy (Eclipse TE2000-U microscopy, D-Eclipse C1 CLSM, Nikon, Japan). The entrapped microspheres with α-MHC+ cardiomyocytes were cultured in 20% IMDM medium, and placed into a micro-dish (IBID GmbH, München, Germany). A Z-stack was performed with 5 µm step size. Images were taken at room temperature, in separated channels for red and green emission. A three dimensional image was constructed afterwards by merging single Z-stack images by software EZ-CE1 (Nikon, Japan)**.**

3. Results and Discussion

3.1. Isolation of Highly Purified A-MHC$^+$ Cardiomyocytes from Transgenic Embryonic Stem Cell Line

In this study the cardiomyocytes were generated with high purity from transgenic α-MHC+ ES cells line and the puromycin resistance and EGFP under the control of the α-MHC$^+$ promoter was detected microscopically within the EBs after treatment with puromycin. These puromycin-resistant, EGFP-positive α-MHC $^+$ cardiomyocyte populations were isolated with over 92% purity.

The transcriptomic analysis of the α-MHC$^+$ cardiomyocytes in comparison to undifferentiated transgenic ES cells and 15 day old control EBs identified transcripts differentially regulated in the cardiomyocyte population (Figure 2.a,b,c). The specific gene expression pattern of the α-MHC$^+$ cardiomyocytes reflected the biological, physiological, and functional processes that take place in mature cardiomyocytes.

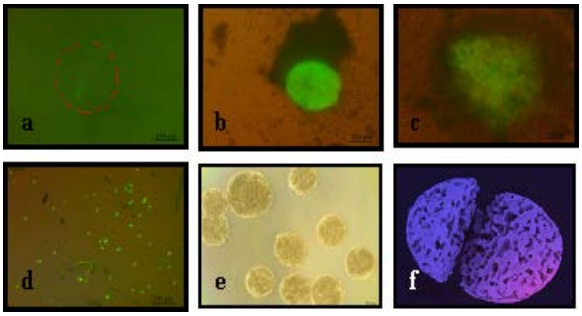

Figure 2. (a) Control group: the attachment of the cardiomyocytes aggregates without puromycin treatment. (b,c) Progressive purification of α-MHC$^+$ cardiomyocytes aggregates after treatment of the 8days EBs with puromycin for 7days. (d) Single cardiomyocytes after dissociation of the EBs with collagenase-B (e) CultiSpher-S microspheres (f) External features of CultiSpher-S. microspheres .

*3.2. In vitro Expansion of A-MHC+ Cardiomyocytes in Cultispher-S Microspheres Using Spinner Flask Bioreactor***.**

CultiSpher-S gelatine microspheres exhibited less porosity than other commercial microcarriers of (20µm in diameter) with a larger matrix component.

Transplantation of ES cells-derived cardiomyocytes is currently limited by the low survival rate of the transplanted cells. The loss of transplanted cells might be partially due to mechanical stress and could be avoided by delivering cells embedded in carriers offering a protected environment for cells. We therefore investigated whether cardiomyocytes can be colonised on CultiSpher-S microbeads in spin cultures. EGFP$^+$ cardiomyocytes were co-incubated with the beads, with and without stirring for four days in culture and when examined by fluorescence microscopy, the entrapment was more efficient in the case of stirred cultures, and less efficient in the case of without stirring. These cells were adherent, spread and occupied the CultiSpher-S gelatin microspheres (Figure 3.A-F). From this result it's appeared that stirring conditions such as spinning is important for enhancing attachment of the cells to the microspheres. Fernandes *et al.,* (2007) reported that a stirred culture system (spinner flask) and CultiSpher-S microspheres was successfully used to scale up mouse ES cells expansion.

Medium replacement in the stirred microcarrier culture system allowed the supply of nutrients to the cells and the removal of waste products inhibiting cell growth. Notably, the cardiac cell expansion in the CultiSpher-S beads led to the maintenance of the cultures in a steady state from several days up to four weeks, indicating efficient supply of nutrients to the cells.

The macroporous microcarriers allowed cardiomyocytes to attach to both the external and internal surfaces of the matrix, thus increasing the usable surface area of the matrix and providing cells with protection from mechanical stress. For a multitude of cell types, the three-dimensional macroporous collagen surface had been previously demonstrated to contribute to increased cellular attachment, resulting in high cell density (Ohlson *et al.*, 1994; Werner *et al*., 2000). A variety of microcarriers including Cytodex 3 (Abranches *et al*., 2007; Fernandes *et al*., 2007) and CultiSpher-S microcarriers (Tielens *et al*., 2007) have already been investigated in mouse ES cells with potential applications for tissue engineering. Alternatively, polystyrene-based cationic trimethyl ammonium-coated microcarriers in spinner flasks were used to expand human fibroblasts enabling extended self-renewal and expansion while retaining full differentiation potential (Phillips *et al*., 2008).

Surface area, bead size, cell adhesion and cell spreading as well as spinning condition are all critical factors that may modulate cell attachment, and ultimately the cellular expansion potential (Koller and Papoutsakis, 1995; Hammond and Hammond, 2001). Notably, $EGFP^+$ cardiomyocytes growing on gelatine beads exhibited similar growth kinetics and beating behaviour to control $EGFP^+$ cardiomyocyte cultures (Figure 2.b,c).

In order to determine the optimum conditions for obtaining entrapment cardiomyocytes, the microbeads colonisation profiles were analysed and the average number of $EGFP^+$ cardiomyocytes that can be seeded per bead were estimated, we found that about 1400 cardiac cells were entrapped by the CultiSpher-S beads. Under these conditions, 80% of total microcarries were populated by cardiomyocytes. From this result its appeared that for optimum entrapment of cardiomyocytes, a large excess of cells to microbeads was needed (Figure 3A-F, and Table.1).

Table 1. Correlation between the number of cardiomyocytes and entrapment

	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**
Number of Cells	50000	100000	300000	500000
% Entrapment	10 %	20 %	40 %	80 %

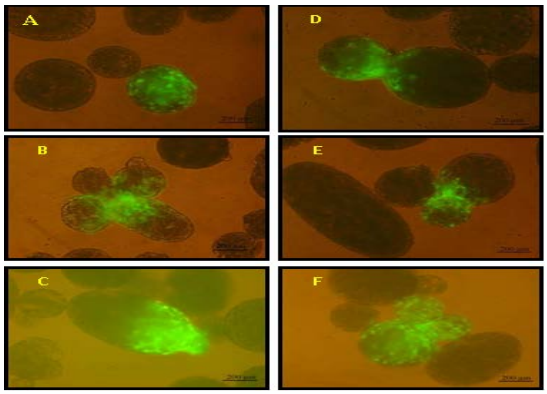

Figure 3.A-F Progressive entrapment of the 7 days old α-MHC^+ cardiomyocytes in CultiSpher-S microspheres by using the Techne spinner flask for four days at 50 rpm. Magnification 10 x, scale bar = 200 µm.

Characterization of Endogenous and Exogenous Expansion of Cardiomycytes on Cultispher-S Microspheres

The highly cross linked matrix of the CultiSpher-S beads permits the entrapment of a high cell numbers. In this study we estimate that about 1400 cardiac cells can be entrapped per microcarrier in both interior and exterior surfaces. Microscopical investigations by block face SEM and CLSM showed the attachment of cardiomyocytes in pore channels with cells not being restricted to the outer surface but also populating the inner surfaces of the gelatine beads after 7 days (Figure 4 A-F and Figure 5.A-H). The ability of ES cells-derived cardiomyocytes to attached in different sites of CultiSpher-S gelatine beads and survives for extended periods of time, this results agreement with the results of DelGuerra *et al.*, (2001), who reported that, the endogenous and exogenous colonisation of microspheres. The successful entrapment of cardiomyocytes on gelatine beads as well as migration of the cells deep into the macroporous matrix as demonstrated by SEM and CLSM is due to the macroporous structure of gelatine beads, which have closely mimicked a (required microenviromental niche) (Yang *et al.*,2007).

Identification features representative of the cardiac cytoskeleton, including myofilaments and muscle Z discs, were well defined in the cardiomyocytes populating the CultiSpher-S microcarriers. The lateral boundaries of the sarcomeres defined by the Z disc constitute the anchoring site for actin, titin, and nebulin filaments and are the primary conduits of the force generated by contraction (Guan *et al*., 1999). Crosslinked cardiac actinin and titin are organised into repeated sarcomeres along the length of the myofibril and give skeletal and cardiac muscles their striated appearance (Figure 5 E,F)

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The presence of these cellular characteristics in the cardiomyocytes cultured on the microcarriers as well as their contactile behaviour and electrophysiological properties demonstrate that the present microcarrier culture technique is suitable to maintain ESC-derived cardiomyocytes in culture.

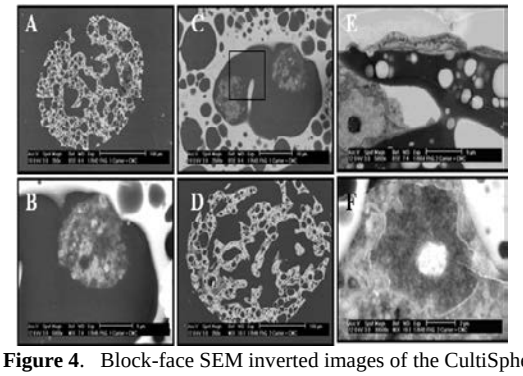

Figure 4. Block-face SEM inverted images of the CultiSpher-S microspheres colonised with α-MHC^+ cardiomyocytes. (A-D) Block-face SEM images showing the entrapment of cardiomyocytes in the pore channel of CultiSpher-S gelatine beads; B- view of area squared in A, rotation 90°. (E,F) block-face SEM images demonstrating the microcarrier - cardiomyocyte contact and typical features of cardiomyocytes.

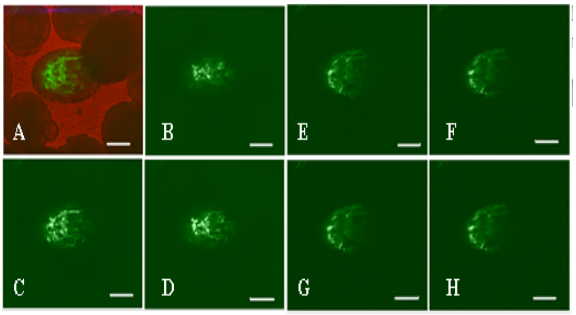

Figure 5. CLSM pictures of an entrapped CultiSpher-S microspheres with α-MHC+ cardiomyocytes (7 days old). A three dimensional image of merged Z-stack images. (A-H) Different sections of the scanned slides. Magnification 20 x. Scale bar = 100 µm.

4. Conclusion

In conclusion, α-MHC^{+} cardiomyocytes derived from ES cells attached efficiently both endogenously and exogenously to CultiSpher-S microspheres in agitated cultivation system and successfully expand *in vitro*. The cardiomyocytes entrapped within the CultiSpher-S microcarriers remain functional as demonstrated by the observation of intact action potentials and cytoskeletal features. The present study thus offers a perspective for transplantation strategies.

Acknowledgement

I would like to acknowledge and thank Professor Dr. Agapios Sachinidis and Prof. Dr. Jürgen Hescheler, Institute of Neurophysiology, Faculty of Medicine, University of Cologne, Cologne-Germany, for providing facilities for this study.

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JJBS

Volume 5, Number 2, March 2012
ISSN 1995-6673
Pages 141 - 146

Jordan Journal of Biological Sciences **Short Communication**

A Sub-Chronic Toxicity Study of Mercuric Chloride in the Rat

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Received on December 1, 2011, Accepted on February 3, 2012

Abstract

Heavy metal accumulates mostly in mammalian liver and kidney as these organs involved in the detoxification and excretion of foreign materials. Chronic exposure of heavy metals leads to intoxication of these organs. In the present study sub-chronic effect of mercury (as mercuric chloride at a dose of 3.75 mg/kg body weight) on biochemical parameter was studied on the experimental animals. Results revealed that mercury increased the aspartate transpeptidase (AST), alanine transpeptidase (ALT), lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) activities significantly than control group. This study also indicates that mercury increases the level of urea and creatinine; and decreases the iron level. Bioaccumulation of mercury was higher in kidney as compared to other organs.

Keywords: Albino rats, mercuric chloride, sub-chronic toxicity, LD50, AST, ALT, LDH, CPK.

1. Introduction

There is a growing problem of worldwide contamination of the environment with mercury. The fate and behavior of mercury in the environment depends on its chemical form. Inorganic mercury compounds enter water bodies by different ways and undergo a process of methylation (Gilmore and Henry, 1991). Mercury poisoning can result from inhalation, ingestion, or absorption through the skin and may be highly toxic and corrosive once absorbed into blood stream. High exposures to inorganic mercury may result in damage to the gastrointestinal tract, the nervous system, and the kidneys (US Environmental protection agency, 2010). Both inorganic and organic mercury compounds are absorbed through the gastrointestinal tract and affect other systems via this route. However, organic mercury compounds are more readily absorbed via ingestion than inorganic mercury compounds (US Environmental protection agency, 2010). Symptoms of high exposures to inorganic mercury include: skin rashes and dermatitis; mood swings; memory loss; mental disturbances; and muscle weakness (US Environmental protection agency, 2010).

Methyl mercury accumulates in lower organisms, and is enriched along the food chain (UNEP, 2002). Methyl and ethyl mercury compounds have been recognized as the cause of mercury poisoning and fatalities as a consequence of consuming contaminated foods. The toxicity signs and symptoms are non-specific at first; including parasthesias, malaise and blurred vision. These may develop later into visual field defects, deafness, dysarthria and ataxia followed by coma and death (Health Canada, 2008; Institoris *et al.*, 2002; and Fontaine *et al.*, 2008). Furthermore, mercury combines with proteins in the plasma or enters the red blood cells but does not readily pass into the brain or fetus and instead, may enter other body organs. The liver is a major site of metabolism for mercury and it can accumulate in the liver, resulting in severe hepatic damages (Wadaan, 2009). Studies have revealed that mercuric chloride caused histopathological and ultrastructural lesions in the liver evidenced by periportal fatty degeneration and cell necrosis (El-Shenawy and Hassan, 2008). Consumption of high quantity of mercury contaminated fish changes the blood pressure and cardiac autonomic activity (Valera *et al.*, 2011).

On the other hand, chronic exposure by inhalation, even at low concentrations in the range 0.7–42 μg/m^3, has been shown in case control studies to cause effects such as tremors, impaired cognitive skills, and sleep disturbance in workers. The serious consequences of chronic mercury toxicity make it important to understand their nature, in order to be able to design the most effective treatment modality (Ngim *et al*., 1992; Liang *et al*., 1993).

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The aim of the present work was to study the accumulation and the sub-chronic toxic effects of mercuric chloride on some biochemical parameters in different body organs, using the rat as the experimental model.

2. Materials and Methods

2.1. Animals

Male albino rats of a locally bred strain, each weighing 125-150 g, were used as the experimental animal model. Rats were obtained from the animal house of the Faculty of Medicine, Assiut University, Egypt. They were housed under constant environmental conditions of temperature (22 + 2 °C) and humidity, with a 12 hour light/dark cycle. All animals were fed *ad libitum* on a commercially available balanced ration.

2.2. Experimental Studies

2.2.1 Determination of The Median Lethal Dose (LD_{50})

These experiments were carried out according to Weil, (1952). One group of 5 animals received saline by gavages and served as control. Twenty rats were used for quantization of the LD_{50} of mercuric chloride. The rats used in this experiment were divided into four groups of 5 animals each. The four dosage levels used for the mercuric chloride experiment were 25, 50, 100 and 200 mg/kg body weight. All dosages were administered orally. The number of dead animals for each dose was recorded after 24 hours.

The oral LD_{50} for mercuric chloride was calculated to be 75 mg/kg. This value was taken as the basis for estimating the dosages used in further experiments where the animals used for the subchronic study for mercury received one twentieth ($1/20^{th}$) of the calculated LD_{50} [3.75 mg/kg] by the same route of administration.

2.2.2. Subchronic Toxicity Study of Mercuric Chloride

Each of twenty male albino rats received oral doses of 3.75 mg mercuric chloride/kg body weight by gavages twice weekly for 12 weeks. Each of a group of 10 rats received 1 ml normal saline twice weekly for the same duration by the same route and served as controls.

Every four weeks, blood samples were collected by orbital sinus bleeding from each rat for the duration of the experiment. The collected samples were left to clot at room temperature then centrifuged at 3000 rpm and used for the assays of the biochemical parameters.

At the end of the experimental period of 12 weeks, blood samples were obtained and the animals were then sacrificed by decapitation. Different internal organs and a sample of skeletal muscles were quickly dissected. The organs or tissues were washed with ice-cold normal saline and stored at -20 °C until assayed for mercury residue.

2.2.3. Biochemical Analyses

An autoanalyzer (Express Plus; Ciba Corning Diagnostics, Palo Alto, CA) was used for analysis of the biochemical parameters determined in the present study. Renal functions were assessed by urea and creatinine serum levels. Alanine transpeptidase (ALT), aspartate transpeptidase (AST) and lactate dehydrogenase (LH) were used to determine the extent of liver affection.

Other biochemical parameters assayed were uric acid, creatinine phosphokinase and alkaline phosphatase. Serum calcium, inorganic phosphate and iron were also assayed. Mercury levels in serum and in homogenized tissues were estimated using Thermo Scientific Graphite Furnace Atomic Absorption Spectrometer equipped with a vapor generation accessory (M-series Atomic absorption Spectrometry, Thermo scientific, USA) (Burger, 2004).

2.2.4. Statistical Analysis

All data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was performed on each variable and the Bonferroni statistics employed to compare the mean values of the control and experimental groups. Differences were considered significant at $P<0.05$. All statistical analyses were performed using SPSS statistical software (version 10).

Results

The renal function of the rats (treated with mercury) was significantly inhibited following administration of mercuric chloride. The increases in urea and creatinine were apparent in the four week of the experimental period and increased steadily afterwards until week 12 (Table 1).

Table 1. Changes in renal function in rats treated with mercuric chloride (3.75 mg/kg) twice weekly for 12 weeks.
Data presented as mean ± standard error (n=10, each group).

Weeks	Urea mg/dl			Creatinine mg/dl		
	Control	Mercury treated	*P* value	Control	Mercury Treated	*P* value
4	20.9 ± 2.4	34.6 ± 2.1 (+65.6%)	0.001	0.45±0.01	0,63±0.01 (+40.0%)	0.001
8	21.4 ± 0.8	35.7 ± 2.7 (+66.8%)	0.001	0.44±0.01	0.66±0.04 (+50.0%)	0.001
12	22.7 ± 1.0	39.7 ± 2.6 (+74.9%)	0.001	0.45±0.01	0.73±0.11 (+62.2%)	0.02

*Number in parentheses represents percentage difference from the corresponding control value.
All values for treated animals were significantly higher than corresponding control values

Urea in the serum was elevated by 65.6% (34.6±2.1 mg/dl) in week 4 and reached 74.9% (39.7±2.6 mg/dl) above control value by week 12, while the corresponding increases in creatinine were 40.0% (0.63±0.01 mg/dl) and 62.2% (0.73±0.11 mg/dl) during the same period.

The liver functions were also affected by the administration of mercury. Liver affection was more apparent in the elevated activities of the serum enzymes which reflect the functions of hepatocytes. The activities of all three enzymes assessed; AST, ALT and LDH were all significantly elevated at week four. The level of AST, ALT and LDH were 223%, 282% and 108% higher than the respective control group after 4^{th} week. The statistical analysis indicates that there were no significant difference in the AST level in control and treated group after week 4 (table 2).

Table 2. Changes in liver functions in rats treated with mercuric chloride (3.75 mg/kg) twice weekly for 12 weeks

Time Weeks	AST U/L		ALT U/L		LDH U/L	
	Control	Mercury treated	Control	Mercury treated	Control	Mercury treated
4	73.1 ± 8.84	236.3 ± 30.77 (+223.3%, $p \leq 0.001$)	66.5 ± 10.08	254.0 ± 30.69 (+282.0%, $p \leq 0.001$)	85.5 ± 5.58	177.7 ±5.79 (+107.8%, $p \leq 0.001$)
8	73.7 ± 9.90	173.3 ± 67.37 (+135.1%, p=0.171, NS)	68.9 ± 10.14	160.7 ± 36.99 (+133.2%, $p \leq 0.038$)	82.8 ± 4.81	160.6 ± 8.02 (+107.1%, $p \leq 0.001$)
12	73.6 ± 7.65	197.9 ± 63.97 (+168.9%, p=0.082, NS)	65.6 ± 0.71	169.0 ±50.76 (+157.6%, p=0.074, NS)	83.3 ± 4.07	160.3 ± 6.98 (+92.4%, $p \leq 0.001$)

Data presented as mean ± standard error (n=10, each group).
*Number in parentheses represents percentage difference from the corresponding control value and p values. (NS, not significant)
AST: Aspartate transpeptidase, ALT: Alanine transpeptidase, LDH: Lactate dehydrogenase

The ALT level was significantly higher (133%) up to week 8. However the results indicate there were no significant differences between the ALT levels after 8th week. The LDH level was 92% (160.3 units/l) higher than the corresponding control group after 12th week.

Moreover, there was practically no change in the activity of ALP. The fluctuations in the activity of this enzyme did not exceed 6.9% (Table 3). The concentration of uric acid in the serum approximately followed the same pattern, with statistically insignificant increases between 5.8% (2.29 mg/dl) and 17.8% (2.36 mg/dl) above the corresponding controls. In the mean time, the activity of CPK was highest at the 4-week point reaching 203.7% (434.6±79.5 units/l) above control value, and started a steady, but slight decline afterwards reaching a mean value of 145% (367.2±50.5 units/l) above that of the corresponding control by 12th week. The result indicates that there was no significant difference in CPK level at 8th week.

Table 3. Changes in alkaline phosphatase, creatine phosphokinase and uric acid in the blood of rats treated with mercuric chloride (3.75 mg/kg) twice weekly for 12 weeks

Time Weeks	ALP (U/l)		CPK (U/l)		Uric Acid (mg/dl)	
	Control	Mercury treated	Control	Mercury treated	Control	Mercury treated
4	163.7 ± 8.41	156.7 ±6.26 (- 4.3%, p=0.522, NS)	143.1 ± 31.64	434.6 ± 79.50 (+203.7%, $p \leq 0.003$)	2.09 ± 0.12	2.29 ± .10 (+9.6%, p=0.221NS)
8	166.6 ± 7.53	158.6 ± 4.74 (- 4.8%, $p \leq 0.001$)	143.1 ± 30.45	326.1 ± 105.52 (+127.9%, p=0.115 NS)	2.09 ± o.12	2.46 ± 0.15 (+17.2%, p=0.404 NS)
12	167.8 ± 7.65	156.3 ± 5.16 (- 6.9% $p \leq 0.031$)	149.9 ± 33.52	367.2 ± 50.49 (+145.0%, $p \leq 0.002$)	2.02 ± 0.11	2.36 ± 0.16 (+16.8%, p=0.75 NS)

Data presented as mean ± standard error (n=10, each group).
*Number in parentheses represents percentage difference from the corresponding control value and P values (NS, no significant difference).
ALP: Alkaline phosphatase, CPK: Creatine phosphokinase

The changes in serum components extended to some inorganic elements (Table 4). The concentration of calcium increased in the early time points of the experimental period, reaching its highest values of 45.2% (14.51±0.23 mg/dl) above control in week 4. This was followed by fluctuations and tendency toward decline reaching a mean value higher by 13% (11.21±0.17 mg/dl) at 12th week. The changes in calcium concentration were not accompanied by changes in inorganic phosphorus, which showed no statistically significant changes throughout the treatment period. Iron in the sera of treated rats showed a moderate decrease of 29.0% (138.5±5.64 mg/dl) after four weeks and such decrease persisted throughout the treatment period, ranging between 25.1% and 29.0% below corresponding control values.

Table 4. Changes in the concentrations of calcium, inorganic phosphorus and iron in the sera and mercury in whole blood of rats treated with mercuric chloride (3.75 mg/kg, p.o) twice weekly for 12 weeks

Time Weeks	Calcium mg/dl		Inorganic Phosphorus mg/dl		Iron mg/dl		Mercury µg/dl	
	Control	Mercury Treated	Control	Mercury treated	Control	Mercury Treated	Control	Mercury treated
4	9.99 ± 0.20	14.51 ± 0.23 (+45.2%, ($p \leq 0.001$)	8.22 ±0.21	7,76 ±0.19 (- 5.6%, p=0.63NS)	195.2 ±2.65	138.5 ±5.64 (- 29.0%, $p \leq 0.001$)	0.99 ±0.06	5.21 ±0.23 (+426.3%, $p \leq 0.001$)
8	9.96 ± 0.21	12.49 ± 0.15 (+25.4%, p≤0.001)	8.38 ±0.21	8.22 ±0.21 (- 1.9%, p=0.60NS)	195.5 ±7.07	146.4 ±2.91 (- 25.1%, $p \leq 0.001$)	1.04 ±0.5	5.85 ±0.30 (+462.5%, $p \leq 0.001$)
12	9.91 ± 0.24	11.21 ± 0.17 (+13.1% $p \leq 0.001$)	8.24 ±0.21	7.80 ±0.12 (- 5.3%, p=0.095 NS)	196.2 ±6.27	145.3 ±2.29 (- 25.9%, $p \leq 0.001$)	1.04 ±0.06	6.85 ±0.26 (+558.6%, $p \leq 0.001$)

Data presented as mean ± standard error (n=10, each group).
*Number in parentheses represents percentage difference from the corresponding control value and p values (NS, no significant difference)

As a result of mercury administration, the concentration of mercury itself in whole blood of treated rats was highly elevated to a level 426.3% (5.21±0.23 µg/dl) above control within 4 weeks. The concentration of mercury steadily increased with further treatment to reach a final concentration of 558.6% (6.85±0.26 µg/dl) above control by the end of the experimental period (Table 4 and Figure 1).

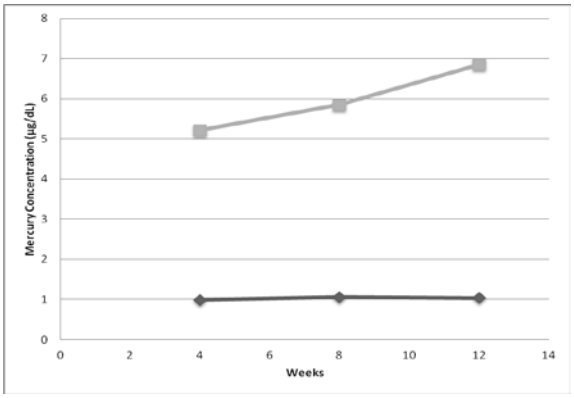
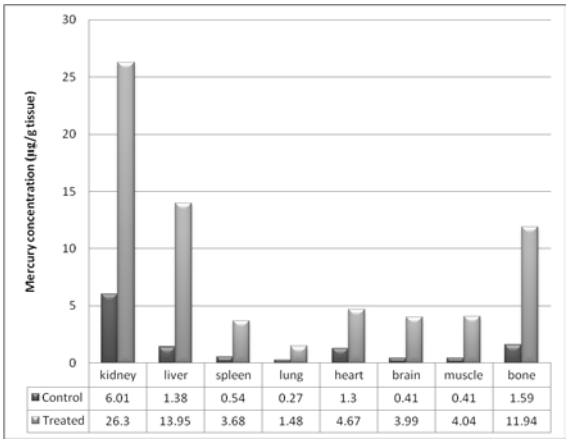


Figure 1. Changes in the concentration of mercury in the blood of rats treated with mercuric chloride (3.75 mg/kg, p.o.) twice weekly for 12 weeks (□- Treated, • - Control)

The distribution of mercury (µg/g) residues in different tissues and organs of treated rats is presented in Figure 2. The highest concentrations were found in the kidney, reaching a mean of 26.3 ± 4.93 µg/g tissues, followed by the liver, which contained an average of 13.95 ± 3.00 µg/g tissues. The level of mercury in the bone was unexpectedly high (11.94 ± 1.10 µg/g). Mercury concentrations in heart, muscle, brain and spleen were similar ranging from 4.67 to 3.68 µg/g tissue. The lowest concentration of mercury was found in lungs (1.48 ± 0.28 µg/g).



	kidney	liver	spleen	lung	heart	brain	muscle	bone
Control	6.01	1.38	0.54	0.27	1.3	0.41	0.41	1.59
Treated	26.3	13.95	3.68	1.48	4.67	3.99	4.04	11.94

Figure 2. Distribution of mercury in different organs and tissues of rats treated with mercuric chloride (3.75 mg/kg, p.o.) twice weekly for 12 weeks.

3. Discussion

Mercury is a widely used industrial chemical with serious health hazards resulting mainly from environmental pollution by industrial waste or accidental exposure. The serious nature of mercury toxicity necessitates full understanding of the underlying mechanisms involved in producing its harmful effects. Mercury toxicity is known to affect the redox status of the victims' tissues through increased production of free radicals leading to oxidative stress (Ercal *et al*., 2001). This causes disturbances in the functions of many body organs.

In the present study, the higher levels of urea and, in particular, creatinine clearly reflected progressing renal insufficiency in rats treated with mercuric chloride. Novelli *et al*. (1998) and Mahmoud (1999) reported higher urea and creatinine levels in rats administered mercury either in the form of inorganic salts or as a complex with metallothioneine. Such functional impairment probably resulted from both vasoconstriction and a direct cytotoxic effect of mercury (Girardi and Elias, 1993; and Barregard *et al*., 2010). Besides, the detrimental effect may be attributed mainly to the accumulation of this toxic metal in kidney. In the present work, renal concentration of mercury was highest among all tissues and organs tested, probably indicating the most serious organ affection.

Hepatic functions were also impaired by administration of mercury. Both the synthetic ability and the integrity of hepatocytes were affected. The deleterious effects of mercury on hepatocytes were clearly reflected in elevated levels of serum enzymes taken as indices for liver functions. Treatment with mercury significantly increased both serum ALT and AST activities. Mohmoud and Manal (1999) reported elevation in the activities of these enzymes following both acute and chronic exposure to mercury, which was attributed to its pathological effect in hepatic tissue. Moreover, the activity of LDH was also elevated as a result of mercury administration. The release of LDH into the serum is taken as an indication of hepatocyte damage and death.

The activity of CPK in the serum was significantly elevated, probably indicating multi-organ damage. Significant accumulation of mercury was shown in the present work to occur in all three organs, indicating widespread deleterious toxic effect of mercury in a multitude of body systems.

These findings are in agreement with those of Kuliczkowski *et al*. (2004) and Lim *et al*. (2010) who found marked elevation of serum CPK after mercury poisoning

The observed insignificant hyperuricemia associated with mercury toxicity may be attributed to its degenerative and necrotic effect upon many tissues including liver and kidney. As a result of necrosis, the broken down tissues caused accelerated catabolism of purines with subsequent increase in uric acid formation.

The degree of hyperuricaemia showed attenuation during the last four weeks, probably due to insufficiency of renal reabsorption of uric acid through degenerated renal tubules. The elevation of urea, creatinine and uric acid levels were reported to be proportionate with the severity of renal insufficiency (Kumar, 1994; Long *et al*., 1998; and Cid *et al*., 2009).

The disturbance in serum composition as a result of mercury toxicity was not limited to organic biochemicals, but extended to inorganic elements.

Serum calcium showed a significant increase starting early in the experiment. The accumulation of mercury in the bones may have affected the balance between the activities of osteoblasts and osteoclasts, leading to mobilization of calcium from the bones into the blood. Other mechanisms may have been involved, including effects on the parathyroid gland, calcitonin from the thyroid gland or disturbance in renal excretion of calcium as a result of renal damage (Shull *et al*., 1981). Contrary to the changes on calcium, inorganic phosphorus was not affected by administration of mercury. Plasma levels of phosphates are partly under the control of the parathyroid hormone, which controls its excretion by the kidney. The net effect is to increase the concentration of calcium and lower that of phosphate. This may explain the slight hypophosphatemia observed in the present study. The concentration of iron was also decreased, probably indicating iron deficiency anemia in mercury treated rats. Such deleterious effect of mercury started early after mercury administration and persisted throughout the whole experimental period. Lecavalier *et al*. (1994) and Institoris *et al*. (2002) reported that there was marked iron deficiency anemia in female rats after mercury administration, where as Grosicki and Kossakowski (1990), reported that mercuric chloride reduces the absorption of radiolabelled ferric chloride ($^{59}FeCl_3$) from stomach and intestine.

As may be expected, the concentration of mercury in the blood increased with the progress of the experiment. This means increased distribution of mercury into vital

organs with stronger toxic effects and metabolic disturbances. The same results were obtained by other researchers (Guglick *et al*., 1995; Harnly *et al*., 1997; and Schiawicke *et al*., 2008). Mercury residue was detected in all organs tested. The distribution of mercury into tissues and organs was not uniform. The kidney and liver showed the highest levels of mercury, which could be responsible for the abnormal functions of these organs. An unexpected result was the relatively high concentration of mercury in the bones. The combined effects in these organs probably represented a significant factor in the observed disturbance in calcium and phosphate homeostasis. Similar results were obtained by Pathak & Bhowmik (1998), Sundberg *et al*. (1999), and Yang *et al*. (1997) who found that mercury concentrations in liver, kidney and brain of young animals.

It is clear from the present work that mercury distributes in different body organs and tissues causing metabolic disturbances and deleterious effects.

The concentration of mercury residues depends on the particular organ and the extent of damage probably depends on both its concentration and the response and sensitivity of the organ.

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مجلة علمية عالمية محكمة

ISSN 1995-6673

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مجلة علمية عالمية محكمة

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