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Probiotics and Traditional Fermented Foods: The Eternal Connection (Mini-Review)

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Abstract

Interactions of probiotic bacteria with the host and the impact of this interaction continue to fascinate microbial biotechnologists. Highlights of these influences of bacteria on human systems have been obtained from comparisons using germ free and conventional experimental animals. Where applied, outcomes and therapeutic uses are involved.There is a need to diversify the sources of isolation of probiotic bacteria to include fermented traditional foods of different societies. This approach invites more comprehensive research on different models to prove probiotics efficacy in a very well-defined patient groups pertaining to varied geographical locations, different ethnic communities and cultural values. Such invitation, no doubt, is motivated by the pressing need to develop and design alternatives to drugs causing bacterial resistance and risky side effects as well as complimentary treatments for other non-microbial diseases.

الملخص

إن تفاعل المحسنات الحيوية البكتيرية مع العائل وأثر ذلك كان وما زال يثير إعجاب علماء التقنيات الحيوية للأحياء الدقيقة وإن الآثار المميزة لهذه البكتيريا قد تم توثيقها من خلال دراسات مقارنة على حيوانات مخبرية. وأشارت نتائج هذه المقارنات لضرورة تنويع مصادر عزل هذه المحسنات الحيوية البكتيرية لتشمل أغذية مخمرة تقليدية لمجتمعات متباينة. ومن المؤمل أن تؤدي هذه المقاربة لدراسات شاملة على نماذج مختلفة لإثبات فعالية هذه المحسنات على مجموعات المرضى المحددة مكانياً وعرقياً ومع قيمها الحضارية. ولا شك بأن هذا التوجه سيتعزز بالحاجة الماسة لتطوير وتصميم بدائل لعقارات تؤدي لظهور المقاومة في البكتيريا إضافة للأعراض الجانبية كما يمكن أن تكون هذه المحسنات البكتيرية مكملة في معالجة أمراض غير بكتيرية.

Keywords: Probiotics, Traditional foods, Microbiota, Host-probiotic interactions.

1. Introduction

"Let food be thy medicine and medicine be thy food" as Hippocrates said, is the principle of today (Suvarna and Boby, 2005). Probiotics are one of the functional foods that link diet and health. Probiotics "For Life" are living, health-promoting microbial food ingredients that have a beneficial effect on humans (Chuayana *et al*., 2003).

The concept of probiotics have been first proposed by Nobel Prize winner Russian scientist Elie Metchnikoff, who suggested that the long life of Bulgarian peasants resulted from their utilization of fermented milk products (Mercenier *et al*., 2002; Chuayana *et al*., 2003; Tannock, 2003). Metchnikoff thought that when the fermented milk products were consumed, the fermenting *Lactobacillus* positively influenced the microflora of the gut, decreasing toxic microbial activities there (Mercenier *et al*., 2002; Chuayana *et al*., 2003).

As a result of more investigations in the probiotic field, its concept has been expanded to include bacteria from intestinal origin beside those bacteria isolated from fermented dairy products (Zeng *et al*., 2010). And we believe now that traditionally fermented foods are the untapped source for a wide variety of beneficial probiotic microorganisms.

Lactic acid bacteria (LAB) are the most common type of microorganisms used as probiotics. Strains of the genera *Lactobacillus*, *Bifidobacterium* (Yateem *et al*., 2008) and *Enterococcus* (Ljungh and Wadström, 2006) are the most widely used and commonly studied probiotic bacteria. The yeast *Saccharomyces boulardii* has also been studied as probiotics (Ljungh and Wadström, 2006).

All of these microorganisms have been considered as probiotics according to several criteria such as their total beneficial effect on the host, being non-pathogenic (Suvarna and Boby, 2005), and their ability to survive transit through the gastrointestinal (GI) tract (Saito, 2004; Crittenden *et al*., 2005; Liong and Shah, 2005).

Probiotic bacteria actions include: adherence and colonization of the host gut (Sanders, 2003), suppression of growth and invasion by pathogenic bacteria (Reid and

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Burton, 2002), production of antimicrobial substances such as bacteriocins (Nowroozi *et al*., 2004), improvement of intestinal barrier function, and stimulation of host immunity (Tannock, 2003).

Evidence from *in vitro* systems, animal models and humans suggests that the therapeutic use of probiotics has been considered very successful in the cases of lactose intolerance (Suvarna and Boby, 2005), inflammatory bowel disease (Daniel *et al*., 2006), colon cancer, *Helicobacter pylori* infection (Ouwehand *et al*., 2002), reduction of allergy, irritable bowel syndrome (Ljungh and Wadström, 2006), and certain diarrheal disease including antibiotic-associated diarrhea in adults, travelers' diarrhea, and diarrheal diseases in young children (Reyed, 2007). Additionally, probiotics may improve intestinal mobility and relieve constipation, especially in seniors (Ouwehand *et al*., 2002; Crittenden *et al*., 2005). They also reduce the cholesterol levels in serum (Liong and Shah, 2005) and no doubt many other benefits are there to be found by deligent research efforts.

Nowadays, probiotics are available in a variety of food products, dietary supplements (Parvez *et al*., 2006) and drugs (Sanders, 2003). In the United States, food products containing probiotics are almost exclusively dairy products - fluid milk and yoghurt- due to the historical association of lactic acid bacteria with fermented milk (Schillinger, 1999). The most frequently used bacteria in these products include *Lactobacillus* and *Bifidobacterium* (Sanders, 2003).

Probiotics are commonly not long-term colonizers of the GI tract, although they can adhere temporarily to the epithelium (Bezkorovainy, 2001). Instead, they may divide very slowly in the intestine, while remaining metabolically active (Marco *et al*., 2006). Therefore, daily consumption of these bacteria is probably the best way to maintain their effectiveness (Champs *et al*., 2003).

Isolation and characterization of novel strains of lactic acid bacteria from uninvestigated niche could have the dual advantage of revealing taxonomic characteristics and obtaining strains with interesting new functional traits that may be useful for probiotic application (Ortu *et al*., 2007). Among resources of probiotic bacteria are traditional fermented foods which vary according to societies and social habits of dieting (Reddy *et al*., 2007).

2. Present status of the art

Searching literature demonstrates the rapid growth of probiotics research with most published articles dates after 2003 (Reid *et al*., 2006). The type of research is being extended to include discovering events taking place at the interface between mucosal lining and microbiota, links between probiotics use and effects at distant body sites. Applications in the fields of allergy, inflammation, cardiovascular diseases and cancers are top priority targets hoping to benefit mankind, the emphasis being upon therapy rather than health improvements only. Recent research results were successful in using certain probiotic bacteria as a delivery system of endostatin for cancer gene therapy (Hu *et al*., 2009). The biological activity of some probiotics is attributed to fermentation end products including among others organic acids, CO_2, H_2O_2, diacetyl, and bacteriocins (Müller *et al*., 2009; Sanahan, 2010). In the majority of cases, this biological activity is a combination of all these factors (Todorov and Dicks, 2005). Probiotic induced-pathogen inhibitor may provide significant protection against pathogen spread in the gastrointestinal tract leading to enhanced human health (Collado *et al*., 2007a) through inhibition, displacement or competition with the pathogen (Gueimonde *et al*., 2006). Among the most studied bacteria as a probiotic included mainly isolates of the genera *Lactobacillus* and *Bifidobacterium*. Other bacteria of less potential as probiotics are isolates of the genus *Bacillus* such as *Bacillus subtilis*, *Bacillus clausii*, *Bacillus pumilus* and *Bacillus coagulans* (Hong *et al*., 2005; Patel *et al*., 2009). Reports claiming that probiotics use strengthened immune system, and helped in combating allergies, excessive alcohol intake and other diseases (Nichols, 2007; Sanders, 2003) encouraged researchers to search for novel probiotic bacteria in traditionally fermented foods which is usually linked to good health of people who consume such foods regularly (Salminen *et al*., 1998).

According to Reid, *et al.,* 2006, the understanding of probiotic microorganisms has expanded in the last 20 years after incepting the term in research circles. The term now implicates exact speciation of the microorganism, safe and effective use of probiotic formulations, exhibition of health and or therapeutic benefits on experimental models and probably volunteers and finally safe manufacture and retail. Unfortunately, these requirements are not fully observed worldwide, resulting in marketing too many of the so-called probiotics that do not meeting the proper criteria of a true probiotic. Table 1 presents few examples of bacterial probiotic strains which belong mainly to species of the genera *Lactobacillus* and *Bifidobacterium* with exceptions pertaining to *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. Such information provide us with substantial evidence that in the last decade probiotics are beginning to be a convincing complimentary rout to traditional drug-based therapies. This trend, will with no doubt pave the way for more sophisticated approaches to develop genetically modified microorganisms which harbour new traits in constructed probiotics for special therapeutic purposes; such thoughts were entertained by Lartigue *et al*., (2007) in his article about genome transplantation in bacteria.

Table 1. Probiotic microorganisms exhibiting positive clinical responses.

Strain	Probiotic clinical response
Lactobacillus rhamnosus GG	Adhesion displacement of several pathogens (Collade *et al*., 2007b).
Bifidobacterium animalis BB12	Prevention and treatment of diarrhea (Weizman *et al*., 2005).
Bifidobacterium infantis 35624	Relieve abdominal pain (O'Mahony *et al*., 2005).
Lactobacillus paracasei LP-33	Relieve of allergic rhinitis (Wang *et al*., 2004a).
Lactobacillus acidophilus La5	*Helicobacter pylori* inhibition (Wang *et al*., 2004b).
Lactobacillus reuteri ATCC 55730	Production of CD4- positive T- lymphocytes (Valeur *et al*., 2004).
Bifidobacterium longum BL1	Serum cholesterol reduction (Xiao *et al*., 2003).
Saccharomyces cerevisiae Lyo	Reduced diarrhea (Kurugol and Koturoglu, 2005).
Escherichia coli Nissle 1917	Colitis treatment (Tromm *et al*., 2004)
Lactobacillus plantarum LP31	Inhibition of food pathogens (Müller *et al*., 2009).
Bifidobacterium longum	Antitumor effect (Hu *et al*., 2009).
Lactobacillus salivarius UCC118	Colon cancer reduction in mice (O'Mahony *et al*., 2001).
Lactobacillus buchneri P2	Serum cholesterol reduction (Zeng *et al*., 2010).
Lactobacillus casei	Reduction of bladder cancer (Ohashi *et al*., 2002).

3. Bioprospecting for probiotics in traditional fermented foods

The majority of infectious diseases caused by pathogenic bacteria and fungi represent a real challenge for current efforts to combat it through chemical and pharmacological research (Sanders *et al*., 2003). Rapid emergence of antibiotic resistance by many pathogens, along with increased toxicity of in field antibiotics prompts the continuous search and development of new anti-infective and antipathogenic materials (Sleator and Hill, 2006). Antipathogenic and probiotic bacteria would be the corner-stone in the search for new and effective alternative to traditional prophylactic means in a variety of clinical cases and settings (Sleator and Hill, 2008). In the last decade, it was observed that the selection of probiotics is based mainly on their ability to adhere and colonize the gastrointestinal epithelia and to compete with pathogens for binding sites and nutrients (Collado *et al*., 2007a; Ouwehand and Salminen, 2003). The functional activity of probiotics have been associated with managing the diarrheal and gastrointestinal infections by probiotics strains of different species of Lactics isolated mainly from fermented dairy products and related sources available in the western hemisphere (D'Souza *et al*., 2002). In addition, there is ample evidence suggesting the use of probiotics to treat and prevent urinary tract infection (Velraeds *et al*., 1998), rotavirus diarrhea (Szajewska *et al*., 2001), recurrence of certain cancers (Ohashi *et al*., 2002)), and reduction of allergies (Gill and Guarner, 2004)and recently, probiotic prophylaxis against asthma and eczema is being entertained (Sleator and Hill, 2008).

Most research articles pertaining to the aforementioned observations were dependent upon bacterial probiotics originating mainly from fermented dairy products and to less extent from human body indigenous microflora such as vaginal isolates or infants faeces (Reid, 2005). While the probiotic market is growing rapidly, it becomes eminent to diversify our sources in the search for new and novel probiotic microorganisms. This trend is encouraged by remarks stating that using different strains from different sources present interesting situations since differences between strains of the same species do exist (Reid, 2005; Weizman *et al*., 2005).

4. Oriental sources for probiotic strain selection

With the increased data about benefits of probiotics for human health and treatment and since most isolated and patented strains are of western origin, it is greatly inviting to try and isolate such probiotics from the untapped source examplified by a wide variety of different fermented foods of the orient (Table 2). For example, Keshik which is a Jordanian traditional fermented food made up of barboiled dried wheat and butter milk is of interest (Tamime and O'Connor, 1995). The product is similar to Tarhana (Turkish traditional fermented food) which proved to be a rich source of probiotic Lactic acid bacteria (Sengun, 2009). Among other potential foods also is the fermented eggplant (locally named Makdoos) made up of baby Aubergine stuffed with ground wall-nut, garlic, parsley and fermented in olive oil. Jameed which is solar-dried curd of sheep or goat naturally-fermented milk prepared and used traditionally by Jordanian Beduins (the old desert dwellers) is another unique source of probiotic bacteria. In a preliminary screen (un-published data) we were able to isolate a wide selection of lactic acid bacteria from these foods to be tested further for their suitability and unique probiotic properties. Recently, probiotic lactic acid bacteria have been isolated from unpasturized natural camel milk with superior probiotic characteristics (Yateem *et al*., 2008; Khedid *et al*., 2009). These are just few examples of traditional foods in Jordan as part of the Middle East and many other countries of the orient would probably be richer especially traditional fermented foods of South East Asia and India, which invites more research in this direction. The long shelf-life of such traditional foods could probably indicate the presence of

antimicrobial compounds among other characteristics exhibited by the indigenous bacteria which makes it a good probiotic candidate. Linking this to the increased consumer demand for natural and additive free foods would definitely maximize the interest in search for new sources of probiotic strains (Müller *et al*., 2009).

Table 2. Some oriental traditional foods of East Mediterranean countries including Jordan.

Traditional food	Ingredients/ natural fermentation
Aubergine Makdoos	Aubergine, wall-nuts, garlic, parsley, salt fermented in olive oil.
Green pepper Makdoos	Sweet green pepper, shredded tomato, parsley, salt garlic fermented in water or olive oil.
Pickled Green or Black Olives	Olives, salt, water fermented or pickled in olive oil.
Vine leaves	Vine leaves, pickled or packed to ferment naturally.
Keshik	Boiled wheat, thick curd of goat or sheep yoghurt or fermented milk, salt subjected to solar drying where slow fermentation takes place.
Jameed	Goat or sheep thick yoghurt curd, salt and then solar dried where slow fermentation improve the taste.
Tarhana	Dried mixture of cracked wheat yoghurt or fermented milk.
Turkish Tarhana	Cracked wheat or flour, yoghurt and vegetables fermented and then dried.
Cyprus Tarhana	Cracked wheat and fermented milk flavoured with bay leaf, thyme and fennel seed.
Shatta	Ground dried miniature chilies fermented in salt water and vegetable oil.

5. Probiotic delivery and persistence:

In order to secure full functionality of a probiotic, an efficient delivery system, in vivo survival and clear clinical efficacy must be met (Doleyres and Lacroix, 2005). In this context, it is implicated that increasing the stresses' tolerance level of the probiotic microorganism is of utmost importance (Sheehan *et al*., 2007). The novel thoughts of improving probiotic functions may be seen through modifying the strain tolerance to different stresses on the genetic level. This thought has been tested using some probiotic strains of *Bifidobacterium breve* and *Lactobacillus salivarius* (Sheehan *et al*., 2006), but further investigations are needed such as interference with the ability of a probiotic microorganism to enhance its compatible solutes productivity which is another approach of arming probiotics against different stresses (Termont *et al*., 2006), an area which also needs further exploration in the quest for the best probiotic formulations. The third prospective approach involves what is referred to as designer probiotics i.e. which are basically tailored-probiotics that would be very specific in targeting pathogens or intoxications where by doing this a pronounced prophylactic and therapeutic effects are realized (Laurel and Berger, 2005; Paton *et al*., 2005). Genetic engineering has been thought of as a useful approach for the design of probiotic bacteria that counteract the symptoms of genetic and age related diseases (Saier and Mansour, 2005). In combination therapy, components of pathogenic and nonpathogenic food-related bacteria are currently being evaluated as candidates for oral vaccines (Amdekar *et al*., 2009; Gill and Prasad, 2008; Collado *et al*., 2007b). It is believed now that as our knowledge of microbial variations is refined, tailoring of human associated microorganisms is rational and may lead to remodeling of their functions (Predis and Versalovic, 2009). Roos *et al*., (2010) reported improved humoral immune response against *E. coli* and bovine herpes vaccines in lambs through feeding with probiotic *B. cereus* and *Saccharomyces boulardii* strains.

6. Side effects and risks:

Some microorganisms have a long history of use as probiotics without causing illnesses in consumers. However, some probiotics safety has not been fully studied and understood scientifically. More information is especially needed on how safe they are for infants, children, old people and those with compromised immune systems.

Probiotics side effects, if they exist, would probably be mild digestive disturbances including gas and bloating. More serious contraindications have been observed in some incidences. Probiotics might in theory cause infections that need antibiotic treatments especially with people underlying health problems. They may also cause unhealthy metabolic activities i.e. too much stimulation of the immune system or even gene transfer among cells (Nerstedt, 2007).

Side effects and unsubstantiated claims are possible with prophylactic agents including probiotics. Probiotics are microorganisms, and we know the unpredictability of microorganisms in terms of how it will react to the existing or indigenous microbiota located in the target host. Gould and Shrot (2008) pointed out that particular illnesses such as post-antibiotic diarrheae are very responsive to probiotic treatment. However, they emphasize that many studies on humans have been disappointing although so

many of therapeutic potentials of probiotics still need great deal of substantiation before being recommended for medicinal applications. With these in mind, probiotics might be considered as one facet in a patient's health maintenance strategy. To conclude, we would say that since each human family and each individual in that family harbour a different combination of gut microflora, there may be some genetic propensities for and against certain probiotic strains. More investigations are needed to find out which group may benefit from which probiotic candidates and probably the source-effect of that probiotic microorganism. Further along the same concept, it is usually accepted to say that many studies of probiotics are carried on experimental animals rather than human subjects where lab results and animal studies cannot always be generalized to results in humans. In this context, it is observed that a single bacterial strain colonizing the gut of a gnotobiotic usually reaches a much higher counts than it does in a conventional animal, where the bacteria is faced with competition from others (Tannock, 2004). It is also noted that in the complex conventional ecosystem, the process of up and down regulation of host genes expression induced by different bacterial species vary tremendously with the presence of varying species and even strains (Hooper *et al*., 2001).

7. Conclusion

Probiotics are receiving great deal of attention in different fields of microbial biotechnology applications including health improvement and therapeutic purposes. This necessitates further diversification of sources of isolation to include traditional fermented foods of different cultures and geographical settings. Further works are needed to elucidate the functional characteristics of probiotic microorganisms. Designer probiotic for specific treatment is also an emerging field of research which would strengthen the drive of using probiotics for the treatment of different ailments and human health improvements.

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Effect of Selenium and its Compounds on Oxygen Uptake in Freshwater Fish *Gambusia affinis* after Exposure to Lethal Doses

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Abstract

The purpose of this study was to investigate the change in oxygen consumption of *Gambusia affinis* after exposure to selenium and its compounds. Male and female fish used in this study were exposed to known concentration of selenium and its compounds. All the forms of selenium showed concentration and exposure dependent inhibition. At the higher concentration of selenium and selenite male fish showed decrease in consumption, which later recovered, with increase in exposure period. Selenate showed relative decrease in uptake of oxygen. In female fish, selenium at lower concentration showed uniform decrease, at higher concentration after initial decrease recovery in uptake was observed. In selenate at lower concentration there was increase in oxygen uptake and at higher concentration there was decrease in uptake. All forms of selenium showed inhibitory action with decline at lower concentration and fluctuations at higher concentration of metals, with increase in exposure period there was slight recovery in oxygen uptake.

Keywords: Selenium, Selenite, Selenate, Oxygen uptake, *Gambusia affinis* and Fish.

1. Introduction

Freshwater are highly vulnerable to pollutants since they act as immediate sink for the consequences of human activity always associated with danger of accidental discharges or criminal negligence (Vutukuru, 2003). Heavy metals constitute a core group of aquatic pollutants and additional concentrations of these metals accumulate in the aquatic ecosystem as a result of land based activities. Fish mostly have the tendency to bioaccumulate heavy metals and human might be at great risk some time even lethal, through contamination of food chain (Ui, 1972). Selenium widely used in glass manufacturing industries chemical and pigment factories; it is also released from municipal waste, combustion of fossil fuels and industrial loses. Selenium is a naturally occurring element required in trace amounts for plants and animals. It is found in four oxidation states, selenate and selenite are highly soluble in water and are know to be toxic to biological system at relatively low concentration. Selenate and selenite predominate in aquatic environment because of their high solubility in water (Massecheleyn et al 1990). Studies have indicated that selenite was found to be more toxic then selenite (Maier *et al.,* 1988 b). Selenium is essential metal for number of domestic animals, the optimal concentration ranges for fish growth and reproduction are narrow and both excess and deficiency are harmful to the fish.

Information on lethal exposure of selenium and its compounds on the physiology of fish are limited and its effect on *Gambusia affinis* is not known. Knowledge of acute toxicity of a xenobiotic often can be very helpful in preventing and predicting acute damage to aquatic life in receiving water and as well as in regulating toxic waste discharges (APHA,1998). In view of this oxygen uptake by both male and female *Gambusia affinis* were studied after exposing it to lethal dose of selenium and its compounds. The corresponding results is being discussed in this paper and compared with other fishes exposed to various other metallic and environmental stresses.

Gambusia affinis is freshwater member of poecilidae is a diminutive fish rarely exceeding 46 mm in standard length, fish of this genus are well know for their consumption of insect larvae. This fish is used as biological control in the infestation of mosquito larva and is commonly known as mosquito fish. It is used world wide in control of mosquito larvae, native of southeastern United States and northeastern Mexico. Now it is one of the most widely distributed freshwater fish (Krumholz, 1948).

2. Materials and methods.

Fish collected from the local pond were transferred to fish tank in laboratory with continuous flow of dechlorinated tap water, fish were fed every alternate day

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with Shalimar fish feed. Male and female fish were separated based on their size (female being larger), coloration and presence of gonopodial hook in male and gravid spot in female fish. Prior to the study all the glassware were washed thoroughly and both male and female fish of approximately equal size transferred to rectangular glass for experimentation, each trial was conducted with parallel control, feed was not given during the study and 24 hr prior to the study. Water quality characteristic in each aquarium were determined at the initial stage by following APHA 1998.

2.1. Preparation of stock solution

Selenium: Metallic selenium (Se purity – 99.5%) was digested with 2.5 ml concentrated nitric acid in a beaker; the digested solution was transferred to 100 ml volumetric flask and diluted with distilled water upto the mark. Oxygen uptake was studied using this stock solution with a parallel control (with same amount of concentrated nitric acid).

Selenite: Sodium selenite ($NaSeo_3$ $5H_2O$- purity 99.0%) was weighed and transferred to volumetric flask, dissolved with distilled water and volume made upto 100ml, the concentration of selenite in salt was 0.478 g and Na and H_2O and impurities is 0.527 g per 100ml of solution. Therefore, each ml of stock solution contains 4.78 mg selenite. Oxygen uptake was calculated on the basis of amount of selenite in solution using distilled water as control.

Selenate: Sodium selenate 1.0 g ($NaSeo_4 10H_2O$ purity 97.0%) was weighed and transferred to 100 ml volumetric flask, selenate was dissolved in distilled water and the volume was made upto 100 ml. Selenate is 0.375 g and sodium and other impurities are 0.625 g per 100ml of solution. Therefore each ml of stock solution contains 75 mg of selenate. Oxygen consumption was calculated on the basis of selenate in the solution and using distilled water as control.

2.2. Oxygen uptake

For oxygen uptake Winkler's Azide modification method (APHA, 1998) was employed. In this method one molecule of O_2 = 2 molecules of iodine produced at the end of reaction in bottle.

Therefore 1 ml of standard sodiumthiosulphate (0.025 N) = 0.2 mg of dissolved oxygen as mg/l = 0.2×5 = 1 mg/l. Hence, burette reading directly gives amount in weight of oxygen dissolved. Choubey and Pandey (1993) method was adapted for measurement of oxygen consumption with surfacing prevented and surfacing allowed to the experimental fish, fishes were confined to fixed volume of water in respiratory jar for particular length of time, volume of water was in proportion to size and weight of fish, for each set of experiment a parallel control with fish and without toxicant and a blank was set. Blank was used to detect oxygen consumption of microorganism and other oxidizing materials. After a know interval of time dissolved oxygen of water in jar was estimated, from dissolved oxygen of blank dissolve oxygen of water in which control fish were put and dissolved oxygen of water in which experimental fish, the amount of oxygen consumed by test fishes was calculated, weight of control and exposed fish was noted and oxygen consumed was expressed as mg of O_2 $h^{-1}g$ body weight, the consumption rate of control was taken as 100% (Normal rate) around 10 samples were taken.

3. Results

Male and female fish were exposed to three different concentrations of selenium, selenite and selenate for 24, 48, 72, and 96 hrs and oxygen uptake of the stressed fish was measured at intervals of 24, 48, 72, 96 hr of exposure

At lower concentration of 5 mg selenium, male fish showed an initial increase (24 hr) of 36.70% in percent utilization of oxygen which dropped to 15.745 at 48 hr, there after a rapid increase in uptake was observed from 50.21 to 59.39% at 72 and 96 hr exposure. At 6 mg/l, small amount of 5.06% of oxygen was consumed followed by marked depletion in uptake at 48 hr (-62.96%) which later increased to 21.81%. Higher concentration of 10 mg /l showed an initial enhancement of 20.88% followed by sudden drop in uptake of -76.85% at 48 hr, which recovered from 72 hr with 3.43% and 12.04% at 96 hr.

On exposure to different concentration of selenite, it was found to be time and concentration dependent. Lower concentration of selenite (8 mg /l) showed steady increase of 28% which raised to 59.76% at 96 hr. Percent utilization of oxygen ranged from 23.40 at 24 hr to 55.85 at 96 hr on exposure to 9.5 mg/l. Oxygen consumption was reduced at 13 mg/l to 7.09% which thereafter increased gradually to 53.12%. It is evident from the data that percent uptake of oxygen declined with increasing concentration in the test medium.

Selenate a less toxic compound when compared to other two forms also induced fish to consume more oxygen available in test medium. At 65 mg/l selenate, a constant uptake of 32.74% in consumption of oxygen was observed upto 48 hr of exposure, thereafter a sudden increase in uptake of 53.19% was observed at 72 hr which further rose to 61.81% at 96 hr. At 75 mg/l, percent oxygen uptake ranged from 15.20 at 24 hr to 31.03 at 96 hr, high concentration of 85 mg/l selenate lowered rate of uptake was recorded, with the values that varied between 2.92 at 24 hr and 29.88% at 96 hr.

Consumption of oxygen in male fish was found to be concentration and time dependent, the rate of oxygen uptake reduced with increase in concentration of selenate, however with increase in exposure period there was increase in uptake of oxygen. The results were statistically analyzed with two way ANOVA and was found to be significant at $P<0.001$. Results are presented in Table 1 and Fig 1.

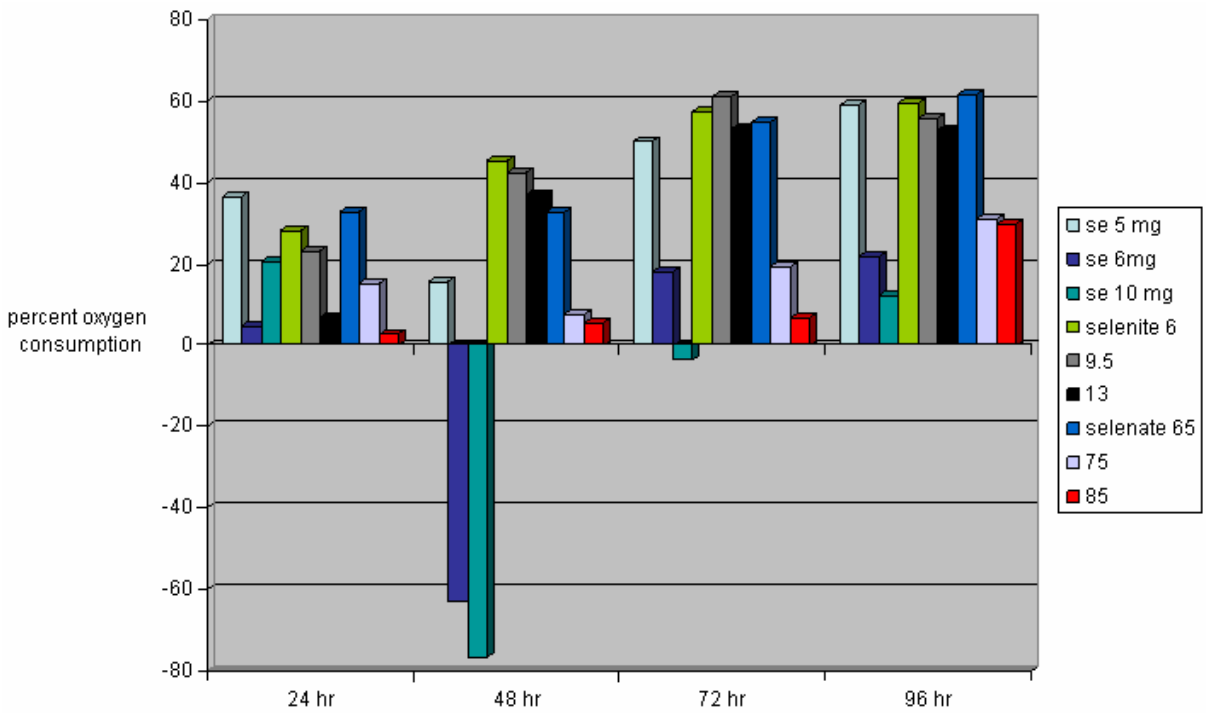

Figure 1. Oxygen consumption in male fish.

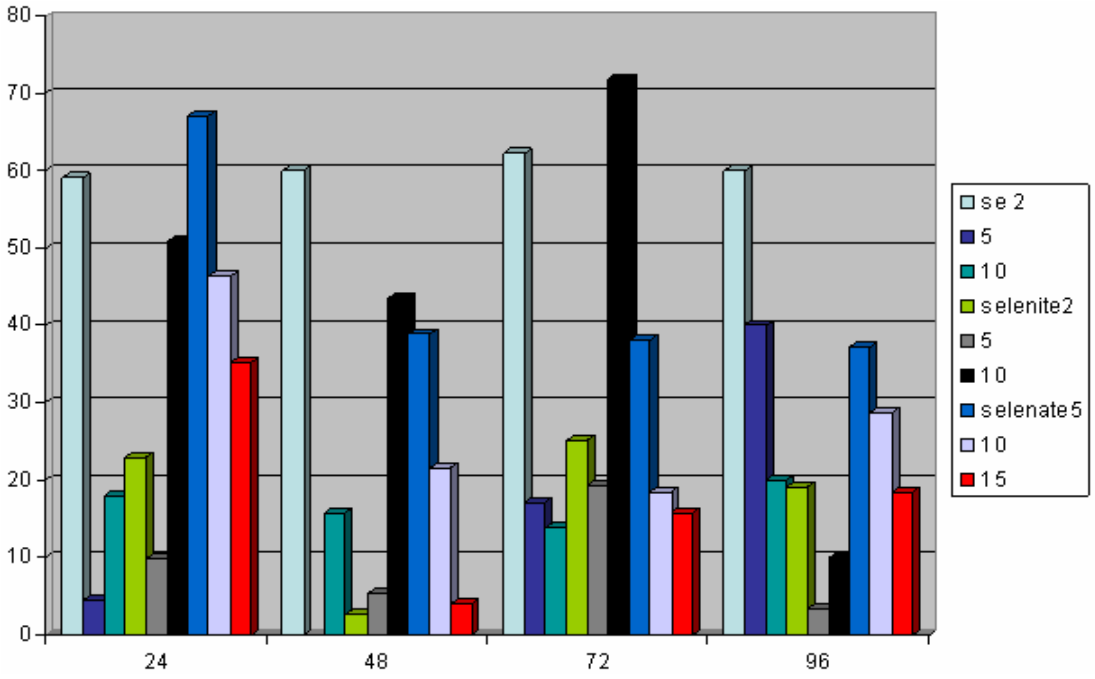

Figure 2. Oxygen consumption in female fish.

Female fish was exposed to three lethal concentrations of selenium and its compounds. The three lethal concentration of selenium chosen were 2, 5 and 10 mg/l and the response to oxygen uptake was studied. There was a uniform decrease in oxygen utilization at the lowest dose of 2 mg/l, percent utilization fluctuated between 59.01% and 62.24%. At 5 mg/l, the rate of uptake was drastically affected and a slow recovery in uptake with increase in exposure period was observed. Fish after exposure to 10 mg/l showed initial increase in uptake, which decreased gradually from 18.03% to 13.69% (at 72 hr) and later recovery in uptake of 40% was observed at 96 hr of exposure. Data revealed that first two days of exposure, percent uptake was not found to be concentration – dependent and decrease in last two days of uptake was concentration – dependent.

In response to selenite exposure (2, 5 and 10 mg/l), fish did not show uniform consumption of oxygen however the uptake rate fluctuated with time of exposure and concentration selenite concentration. Lower concentration of selenite showed initial increase in uptake fallowed by sharp decline to 2.61% and in subsequent observation an enhancement fallowed by decline in uptake was noticed. Similar trend in uptake was observed at 5 and 10 mg/l of selenite. The results were statistically analyzed with Two way ANOVA and the results were statistically significant at $P<0.001$ and $p>0.005$ at exposure period.

The pattern of variation in oxygen uptake in fish on exposure to three different concentrations of 5, 10 and 15 mg/l selenate showed a different trend. Fish dosed with 5 mg/l showed rapid increase in uptake at 24 hr. Thereafter there was a steep decline and later uniform pattern in uptake was observed through out the experimental period. Oxygen uptake reduced from 46.67% to 18.42% at 72 hr, followed by recovery in uptake of 28.81% at 96 hr. At higher concentration of 15 mg/l fish consumed 35.29% oxygen at 24 hr, which dropped to 3.88%, thereafter steady increase in uptake was observed. Results were statistically analyzed by two way ANOVA and were found to be significant at $P<0.001$, except at exposure period $P>0.0054$. Results are presented in table 2 and Fig 2.

4. Discussion

The results of the present study show that fish under toxic stress to different forms of selenium altered oxygen uptake. In general, initial decrease of oxygen uptake followed by an increase in its consumption was observed. All forms of Se have shown inhibitory action on rate of oxygen utilization and at time irregular uptake was noticed. However, as exposure period advanced a slight recovery was found in both male and female fish (Koti, 1996) has shown that Cu, Zn and Ni during lethal and sub lethal exposure (Individual and mixture of metals) altered oxygen consumption in both sexes of *G.affinis*. It was observed that fish in response to metal exposure secreted mucus in the test medium and red patches appeared at gill region and basal part of pectoral fins. Environmental factors such as temperature, pH, and hardness of the test medium, have shown cumulative effect on oxygen uptake of *G.affinis* in the presence of Cu, Zn and Ni (Kallangoudar and Patil, 1997). Lemly (1993) demonstrated metabolic stress in bluegills *Lepomis macrochirus* due to elevated concentration of selenium. He also showed reduced respiratory activity with increase in respiratory demand and oxygen consumption due to gill damage. According to (Tovell *et al.*, 1975) metals mainly enter fish through respiratory system. The mechanism for metal uptake through gill probably occur through pores by simple diffusion (Bryan, 1979) these metals are then absorbed through cell membranes (Opperhuizen *et al.*,1985) Metals then coagulate in protoplasm after absorption into the bodies of aquatic animals (Skidmore, 1964). The decrease in oxygen uptake observed following exposure to selenium, selenite and selenate was possibly due to mucus precipitation on gills, during the present study mucus was present on the gills which appeared as reddish patches and was possibly secreted in response to the irritation caused by metalloids (Carpenter, 1930) investigated lethal action of dissolved metals salts on fish leading to death. According to him death resulted from interaction between metallic ions and mucus secreted by the gills and not from internal poisoning. A layer of coagulated mucus is formed on the gill surface which impairs respiratory efficiency to such an extent that fish becomes asphyxiated (Rani and Ramamurthi, 1987; Gosh and Chakraborti, 1990). Increased utilization of oxygen during later part of lethal exposure to selenium and its forms indicate that under stress fish might have used more oxygen to meet metabolic demand. Similar effect of metal stress on respiratory activity in other fish has been reported, (Davis, 1975; Van Resburg1989; Shivraj, 1990; Koti, 1996; Vijayamohanan *et al.*, 2000). Other possible alternate reason of inhibitory action of pollutants include gill damage (Natarajan, 1981; Koti, 1996) and internal action of pollutants (Natarajan, 1981; Tuurala and Soivio, 1982)

Selenium and its compounds has shown to affect oxygen uptake in both male and female fish after exposure to lethal dose, which is evident from the gradual decline in uptake at lower concentration and fluctuations at higher concentration. Which may be due to over secretion of mucus resulting in blocking of gills or due to onset of severe hyoxia, which alter the metabolic pathways or due to damage caused my metals to gill. Increase in uptake may be due to increase in demand under toxicant stress.

Table 1. Percent oxygen uptake in male fish *G.affinis* after exposure to lethal concentration of selenium and its salts.

Toxicant	Dose(mg/l)	Exposure period (hours)				F-Ratio	P-value
		24	48	72	96		
Control	-	39.57±0.86 (100%)	35.25±0.96 (100%)	22.62±1.16 (100%)	18.69±1.28 (100%)	-	-
Se	5	36.70±0.48	15.74±0.44	50.21±0.57	59.39±0.53	0.530	0.001
	6	5.06±0.73	-62 .96±1.53	18.02±0.93	21.80±1.02	6.630	0.001
	10	20.88±0.61	-76.85±0.93	-3.43±1.18	12.40±1.14	5.570	0.001
selenite	8	28.36±0.51	45.59±0.53	57.44±0.50	59.76±0.52	70.260	0.001
	9.5	23.40±0.56	42.40±0.57	61.27±0.45	55.85±0.59	7.610	0.001
	13	7.09±0.65	37.17±0.60	53.19±0.57	53.12±0.61	52.220	0.001
selenate	65	32.74±0.60	32.96±0.65	55.08±0.55	61.68±0.50	186.854	0.001
	75	15.20±0.73	7.85±0.87	19.49±0.94	31.03±0.89	25.504	0.001
	85	2.92±0.81	5.75±0.90	6.77±1.08	29.88±0.90	12.914	0.001

Results are Significant at $P<0.001$

Table 2**.** Percent oxygen uptake of female fish *G.affinis* after exposure to lethal concentration of selenium and its salts.

Toxicant	Dose (mg/l)	Exposure period (hours)				F-Ratio	P- value
		24	48	72	96		
Control	-	39.92±0.83 (100%)	38.98±0.96 (100%)	33.77±1.13 (100%)	27.41±1.16 (100%)	-	-
Se	2	59.01±0.36	60.09±0.40	62.24±0.44	60.00±0.48	31.250	0.001
	5	4.37±0.85	3 .84±0.97	17.01±0.97	40.00±0.73	155.850	0.001
	10	18.03±0.73	15.86±1.50	13.69±1.02	20.00±0.97	3.460	0.001
selenite	2	22.85±0.61	2.61±0.92	25.10±0.92	19.12±1.01	73.260	0.001
	5	9.71±0.79	5.23±0.89	19.27±0.93	3.18±1.20	2.650	0.054*
	10	50.85±0.43	43.45±0.56	71.86±0.37	9.96±1.12	43.760	0.001
selenate	5	67.05±0.13	38.88±0.57	38.15±0.70	37.28±0.74	43.760	0.001
	10	46.47±0.45	21.66±0.70	18.42±0.92	28.81±0.82	176.260	0.001
	15	35.29±0.81	3.88±0.85	15.78±1.89	18.45±0.95	4.258	0.001

Result are significant at $P<0.001$, except the exposure period $P>0.054$

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Curative Effect of Garlic on Alcoholic Liver Diseased Patients

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Abstract

Alcohol is currently recognized as the most prevalent known cause of abnormal human development. Our aim was to investigate the effect of raw garlic on patients suffering from alcoholic liver disease. 20 alcoholic patients and 20 healthy individuals were selected. Both patients and normal individuals were subjected to detailed clinical examination and laboratory investigations. Blood samples were collected and the liver disease was assessed by measuring the activities of liver marker enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) which were elevated in alcoholic patients. Increased lipid peroxidation in alcoholic patients was accompanied by decreased activities of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx). Oral supplementation of 2 small sized raw garlic cloves (1 clove = 1.2g) to alcoholic patients for 45 days, significantly lowered the activities of liver marker enzymes, decreased the levels of lipid peroxidation and enhanced the antioxidant status to near normal. Thus, the data of the present study suggest that raw garlic offers protection against oxidative stress and antioxidant activities in alcoholic liver disease patients.

Keywords: Alcoholic liver disease, Antioxidants, Garlic, Lipid peroxidation, Liver marker enzymes, Oxidative stress.

1. Introduction

Alcoholic liver disease is one of the most serious consequences of chronic alcohol abuse. The disease is often progressive and is considered to be a major cause of morbidity and mortality (Sherlock, 1995). Free radicals and oxidative stress have been implicated in the pathogenesis of ethanol induced liver injury in humans and experimental animals (Lin *et al.,* 1998; Zima *et al.,* 2001). Basically, ethanol is metabolized into cytotoxic acetaldehyde by alcohol dehydrogenase in the liver and acetaldehyde is oxidized to acetate by aldehyde oxidase or xanthine oxidase giving rise to Reactive oxygen species (ROS) via Cytp450 (Fridovich, 1989; Nordmann *et al.,* 1992). Thus, excess intake of alcohol resulted in the production of oxygen radicals which leads to lowering the body's normal defense mechanism thereby altered enzyme activity, decreased DNA repair and impaired utilization of oxygen, lipid peroxidation and protein oxidation. Some of these alterations induced by oxidative stress can eventually cause necrosis and subsequently leads to oxygen damage (Kurose *et al.,* 1996). In recent years, the popularity of native medicine has increased for various reasons. Since there is no reliable hepatoprotective drug available in modern medicine, alcohol researchers have focused on developing phytotherapeutic medicines which can provide many invaluable drugs to treat alcoholic liver disease. Thus, the research conducted on several natural plant products used as hepatoprotective agents is well-documented (Saravanan *et al.,* 2006).

Allium sativum commonly known as garlic is a bulb-forming herb of lilliaceae family. Garlic is the oldest cultivated plant and has been used as a spice, food and folklore medicine for over 4000 years. It has been used as a traditional medicine in the treatment of heart diseases, tumors and headaches and exhibits medicinal properties including immunomodulation, hepatoprotection, antioxidant, antimutagenic, antibacterial and anticarcinogenic effects (Agarwal, 1996). Moreover, it has also been reported to possess antifungal (Halliwell *et al.,* 1992), hypoglycemic (Yoshida *et al.,* 1987), hyperglycemic (Nadkarni, 1976), hypolipidemic (Pushpendran *et al.,* 1982), anti-atherosclerotic properties (Bordia, 1981) and has been claimed to be effective against a number of diseases (Block *et al.,* 1984). The active principle present in garlic is organosulphur compound such as allicin, allin, alliase, S-allyl cystein, diallyl disulphide and allyl methyl trisulphide (Augusti, 1996). These active compounds are mainly responsible for protecting from tissue damage and various disorders. Among many supplements, aged garlic extract has a reproducible array of compounds which have been analyzed and studied extensively for their high antioxidant content and health protective potential. However, the inhibition of lipid peroxidation and free radical scavenging activity has been suggested as a possible mechanism of

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hepatoprotective action. Thus, the present study was under-taken to establish the hepatoprotective effect of raw garlic on alcohol liver disease patients.

2. Patients and Methods

The present study is comprised of 20 newly diagnosed alcoholic patients from Rajah Muthiah Medical College and Hospital, Annamalai University, Tamilnadu, India. An equal number of healthy subjects (volunteers) were also investigated. The subjects were all males with the ages ranging from 48-55years. Patients suffering from any other diseases other than alcohol intake were excluded from the study. The selected patients were alcoholic for the past 5-6 years and during the treatment, the patients stopped consuming alcohol. Both patients and normal individuals were subjected to detailed clinical examination and laboratory investigations. The ethical committee of Rajah Muthiah Medical College and Hospital Annamalai University, Tamilnadu, India, approved the study protocol in the year 2008.

Alcoholic patients received 2 small sized raw garlic cloves (1 clove = 1.2 g) daily morning under fasting conditions (12-24 hrs) for 45 days. Blood samples were collected from various arm puncture into plain tubes from healthy individuals and alcoholic patients before and after the treatment with raw garlic. Heparnised blood samples containing serum and plasma were separated by centrifugation at 3000rpm for 5min and buffy coat was removed and packed cell washed three times with physiological saline. Biochemical estimations were done in serum and in erythrocyte membranes of alcoholic patients before and after treatment with raw garlic. The results obtained were compared with normal individuals.

2.1. Biochemical Analysis

2.1.1. Estimation of liver marker enzymes

The activities of serum aspartate aminotransferase (AST, E.C.2.6.1.1) and serum alanine aminotransferase (ALT, E.C.2.6.1.2) were assayed by the method of Reitman's and Frankel (1957). Serum alkaline phosphatase (ALP, E.C.3.1.2.3.1) was estimated using Kind & King's method (1954), King (1965). The serum gamma glutamyl tranferase (GGT, E.C.2.3.2.2) was assayed according to the method of Rosalki and Rau (1972). The activity of lactate dehydrogenase (LDH, E.C1.1.27) was estimated by the method of King (1965). Serum total protein, albumin were estimated by Biuret method Reinhold (1953).

2.1.2. Lipid peroxidation and enzyme assays

Lipid peroxidation was measured by estimating the levels of malondialdehyde (MDA) using Thiobarbituric acid reaction method. Thiobarbituric acid (TBARS) in plasma was estimated by the method of Yagi (1978) and TBARS in erythrocyte membrane was estimated by the method of Donnan (1950). The activities of enzymatic antioxidants SOD (E.C.1.15.1.1) was assayed by the method of Kakkar *et al.,* (1984). The activity of CAT (E.C.1.11.1.6) was assayed by the method of Sinha (1972). The activity of GPx was assayed by the method of Rotruck *et al.,* (1973).

2.2. Statistical analysis

The values were expressed as mean ±S.D. Statistical evaluation was done using one way analysis of variance (ANNOVA) which is followed by Duncan's multiple range test (DMRT). The level of statistical significance was set at $p< 0.05$.

3. Results

Table 1 show that alcoholic patients have severe liver damage which was indicated by the increase in marker enzymes such as AST, ALT, ALP, GGT and LDH. However, administration of raw garlic significantly decreased the activity of these enzymes which was compared to that before treatment.

The levels of serum total protein was increased and the albumin levels were decreased in alcoholic patients, while on treatment with raw garlic it significantly improved both protein levels and albumin levels to near normal which was also compared to that of the normal individuals.

Table 2 shows that the levels of lipid peroxidation indicated by TBARS were significantly higher in plasma and erythrocytes of alcoholic patients as compared with normal subjects. TBARS level was lowered significantly in the plasma and erythrocytes of patients treated with garlic.

Further, the activities of SOD, CAT and GPx in erythrocytes were observed in normal and alcoholic patients. In alcoholic patients, the activity of SOD, CAT and GPx were significantly lower than the normal subjects. Treatment of alcoholic patients with garlic significantly elevated the antioxidant defense activity compared with that before treatment.

Table 1. Effect of raw garlic treatment on hepatic marker enzymes and serum proteins in alcoholic patients.

Parameters	**Groups**		
	I Normal individuals	**II Alcoholic patients before garlic treatment**	**III Alcoholic patients after garlic treatment**
AST (IU/L)	19.4±4.07[a]	80±21[b]	45±12[ac]
ALT (IU/L)	43.4±7.3[a]	124.2±15.7[b]	55±8.5[ac]
ALP (IU/L)	89.9±13.3[a]	165.9±21.9[b]	92±13.5[ac]
GGT (IU/L)	41.7±5.1[a]	226.7±28.8[b]	65±10.5[ac]
LDH (IU/L)	88.3±20.8[a]	349.8±32[b]	105±15.2[ac]
Total protein (g/dl)	7.2±0.2[a]	8.5±0.5[b]	7.0±0.2[ac]
Albumin (g/dl)	4.1±0.2[a]	3.4±0.7[b]	4.0±3.0[ac]

Values are expressed as mean ± SD; n=20. Values not sharing a common superscript letter are significantly different at $p<0.05$ (DMRT).

Table 2. Effect of raw garlic on lipid peroxidation and enzymatic antioxidants in alcoholic patients.

Parameters	Groups		
	I normal individuals	II alcoholic patients before garlic treatment	III alcoholic patients after garlic treatment
TBARS in plasma (nmol/ml)	2.17±0.15[a]	3.9±0.28[b]	2.42±0.20[ac]
TBARS in erythrocytes (nmol/ml)	0.30±0.02[a]	1.53±0.11[b]	0.31±0.03[ac]
SOD (U*/mg Hb)	1.54±0.16[a]	1.01±0.16[b]	1.42±0.15[ac]
CAT (U*/mg Hb)	17.2±1.1[a]	10.3±0.8[b]	16.07±1.4[ac]
GPx (U*/mg Hb)	2.01±0.11[a]	1.24±0.11[b]	1.91±0.14[ac]

Values are expressed as mean ± SD; n=20. Values not sharing a common superscript letter are significantly different at $p<0.05$ (DMRT).

*μ moles of H_2O_2 utilized per minute.

Enzymes required for 50% inhibition of nitroblue tetrazolium (NBT) reduction per minute.

4. Discussion

Free radical mediated damage to macromolecule plays a crucial role in the pathophysiology of atherosclerosis, inflammation, carcinogenesis, aging, drug reaction and toxicity (Jose *et al.,* 1999). When the liver gets damaged after consumption of alcohol, it leads to leakage of cellular enzymes into the plasma (Baldi *et al.,* 1993). The increased levels of serum enzymes such as (AST), (ALT), (ALP), (GGT) and (LDH) observed in alcoholic patients, resulted in liver damage, increased permeability and necrosis of hepatocytes (Goldberg and Watts, 1965). In our study, administration of raw garlic to alcoholic patients alleviates the increased activities of serum enzymes AST, ALT and ALP to near normal. Serum GGT is a sensitive marker enzyme widely used as a laboratory test for the hepatobillary diseases especially alcoholic liver disease and alcohol induced liver damage (Nakanishi *et al.,* 2006). In the present study, we observed that GGT has invariably elevated while AST and ALP are slightly increased in alcoholic patients. Garlic supplementation significantly lowered the activities of GGT demonstrating reduced liver damage following garlic administration.

Albumins and globulins are two key components of serum proteins. As albumin is synthesized in the liver, it can be used as a biomarker to monitor liver function (Friedman *et al.,* 1980). In serum total proteins, albumin contents were reduced in alcoholic patients. Hence a significant decrease in the serum total protein and increase in serum albumin was observed in alcoholic patients treated with raw garlic. This stabilization of serum protein level is a clear indication of garlic being related to an improvement in the functional status of the liver cells.

Lipid peroxidation mediated by free radicals is considered to play a pivotal role in the mechanism by which ethanol may exert its toxic effects on the liver and other extra hepatic tissues (Nordmann, 1994). Increase in the levels of TBARS indicates enhanced lipid peroxidation leading to tissue injury and failure of the antioxidant defense mechanism to prevent the formation of excess free radicals (Comporti, 1985). In our study we observed an increase in TBARS and a decline in antioxidant status in plasma and erythrocytes of alcoholic patients. However, treatment with garlic significantly decreased the levels of lipid peroxidation.

Free radical scavenging enzymes such as SOD, CAT, and GPx are the major defence enzymes against oxidative injury. SOD is a ubiquitous chain breaking antioxidant, plays an important role in protection against deleterious effects of lipid peroxidation (Dinkova–Kostova and Talalay, 1999). It converts the highly reactive superoxide radical to hydrogen peroxide, which in turn either metabolized by catalase or by glutathione peroxidase.

The primary role of catalase is to scavange H_2O_2 and convert it into H_2O. It plays an important role in the acquisition of tolerance to oxidative stress in adaptive response of cells. Studies have shown that decrease in catalase during alcohol consumption may be due to the decreased protein synthesis. Thus, there is an increased utilization of CAT during alcohol consumption.

Gpx is a selenium dependent enzyme found primarily in the cytoplasm and also found in the mitochondria. It catalyses the detoxification of endogenous metabolic peroxides and hydroperoxides that leads to the oxidation of GSH. It has a high potency in scavenging reactive free radicals in response to oxidative stress.

The antioxidant defense systems SOD, CAT and GPx activity is significantly decreased in alcoholic patients. This decrease could be due to a feedback inhibition or oxidative inactivation of enzyme protein because of excess ROS generation. The generation of α-hydroxyethyl radical may lead to inactivation of these enzymes (Pigeolot *et al.,* 1990) and accumulation of highly reactive free radicals also lead to deleterious effects such as loss of cell membrane integrity & membrane function (Krishnakanth and Lokesh, 1993).

There was a significant increase in the activity of these enzymes after raw garlic administration. It is reported that garlic suppresses the formation of superoxide anion and hydrogen peroxide by increasing the activity of SOD, CAT and GPx (Borek 2001). Therefore, garlic increases antioxidant action by scavenging ROS, enhancing the cellular antioxidant enzymes and increasing glutathione in the cells. Moreover, it has also been reported that garlic modulates the levels of lipid peroxidation (Hussein *et al.,* 2007). Although multiple actions may take place during hepatoprotective activity, modulation of lipid peroxidation and antioxidant status may be one of the important mechanisms by which garlic exerts its toxic inhibitory effect.

Thus, our results suggest that, oral administration of raw garlic protects tissue damage by increasing the antioxidant status against oxidative stress. Hence, garlic plays a promising role in antioxidant and it can be considered as a potent drug for the treatment of alcoholic disorders. Further studies are needed to unravel the mechanism of action of garlic and its active components.

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Seasonal Variation of Copepoda in Chabahar Bay-Gulf of Oman

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Abstract

Zooplankton was collected with vertical plankton tows using 100 µm mesh nets during August to November 2007 and February to May 2008 from five stations through the Chabahar Bay. Totally, 75 copepods species were identified. Post-monsoon was characterized by the highest numerous of copepod species and diversity. Pre-monsoon showed the lowest number of copepod species and diversity index. The results showed that seasonal variation of chlorophyll-a concentration associated phosphate concentration is a major factor controlling abundance of copepod after a time lag. Abundance of copepod was significantly higher during the pre-monsoon as compared to other seasons.

الملخص

جمعت الهوائم الحيوانية خلال الفترة (أب الى تشرين الثاني 2007 و شباط الى ايار 2008) من خمسة مواقع في خليج شهبهار. تم تحديد 75 نوعا من المجافيات. اظهرت النتائج تميزا لظهور المجدافيات بعد هطول الامطار الموسمية و كذالك اقل ظهورا قبل هبوب الرياح الموسمية. الاختلاف الجوهري للمجدافيات كان واضحا خلال فترة ما قبل هبوب الرياح الموسمية مقارنة بالمواسم الاخرى. اظهرة النتائج ان التغيرات الفصلية لظهور المجدافيات ارتبط ارتباطا وثيقا بتراكيز اليخضور و الفوسفات.

Keywords: Copepoda, Abundance, Diversity, Monsoon, Chabahar Bay, Species richness.

1. Introduction

Chabahar Bay is a sub-tropical and semi-enclosed bay with high biological production, providing an ideal breeding ground for many economically important fishes and shell fishes (Fazeli, 2008). Information on the marine zooplankton of the Chabahar Bay is scanty, except for the projects of Sanjarani, 2007 and Zareei, 1994, who worked in the Goater Bay. Further, many projects existed at Oman Sea such as Seraji, 2007.

The ecological importance of zooplankton, especially its main component such as copepods, in the pelagic food webs of the world's oceans has long been recognized. These organisms often constitute a significant component of the plankton community in many marine environments (Relevante *et al.,* 1985; Burkill *et al.*, 1987; Al-Najjar, 2002); and play a central role in the transfer of nutrients and energy through the marine food webs (Poulet and Williams, 1991; Williams *et al.,* 1994; Kiorboe, 1997) and have an important role in the trophy food web (Thiriot, 1978). Copepods support energy transfer between primary producers (phytoplankton) and the final consumer of highly valuable fish and crustacean species (Faure, 1951). Despite abundant evidence of the importance of zooplankton in marine pelagic food webs, little is known about their density, composition, stratification and distribution in Chabahar Bay. The main objective of the present study is to examine the composition of the main planktonic taxes, temporal and spatial variation of the abundance and distribution of copepods and the impact of environmental factors on copepod's abundance.

2. 2. Materials and Methods

2.1. Study Area

Chabahar Bay is a small semi-enclosed bay on the southeastern coasts of Iran (from 25° 17' 45"N- 60° 37' 45" E). This Bay is connected to the Indian Ocean by the Oman Sea. The Bay surface area is 290 km^2 with 14 km wide located between of Chabahar and Konarak (Fig.1). The average depth of this Bay is 12 m (ranges from 8-22m).

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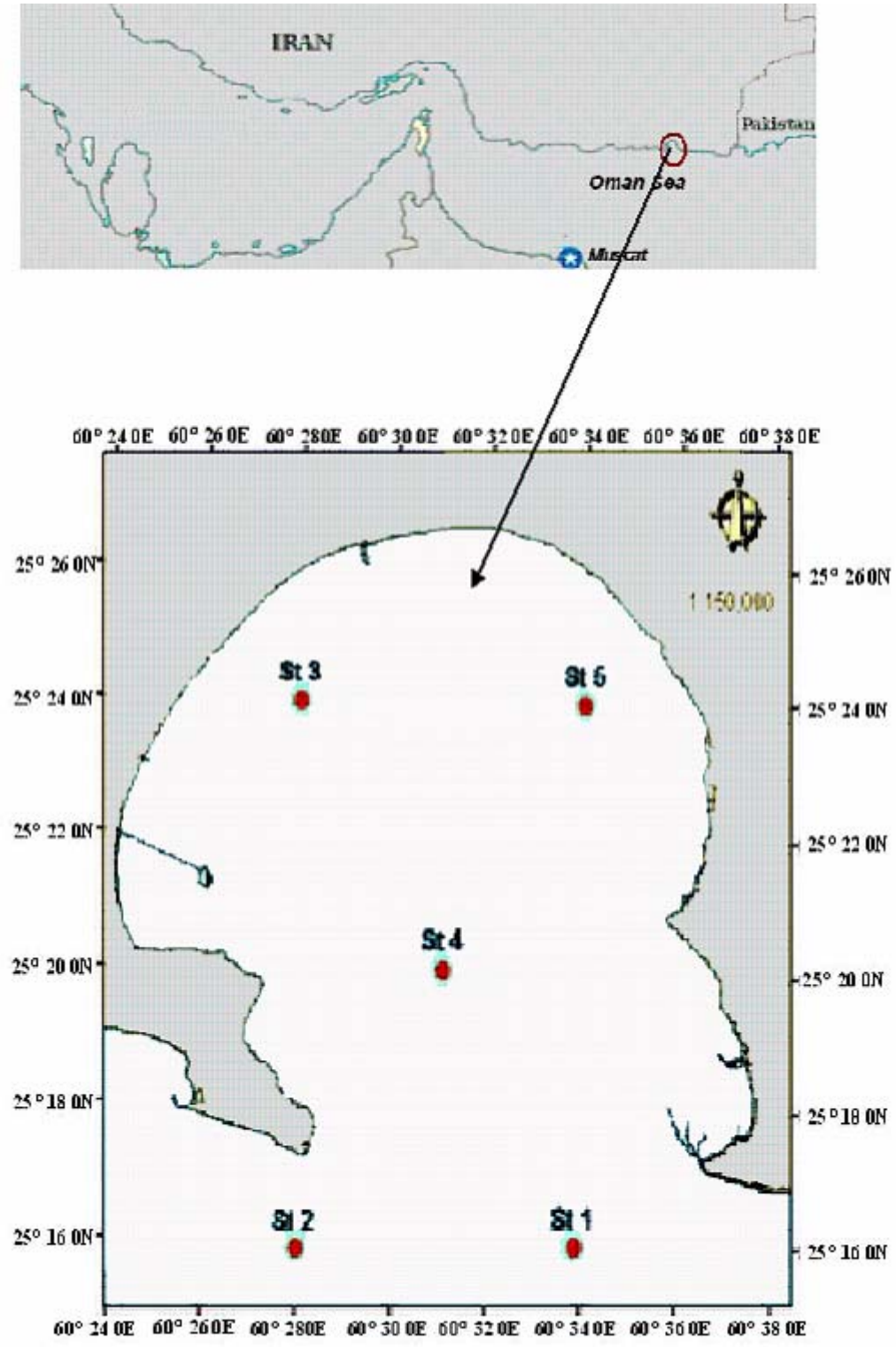

Figure 1. Sampling stations in the Chabahar Bay-Oman Sea

2.2. Sampling

Four sampling cruises were carried out in August 2007 (SW-monsoon), November 2007 (post-monsoon), February 2008 (NE-monsoon), and May 2008 (pre-monsoon). Five stations were investigated throughout the Chabahar Bay. Two stations (St. 1 and 2) were located far from shore waters with 22 m depth, another two stations were near the shore with 6m depth (St. 3 and 5) and the final station (St. 4) was located in the middle of the bay with 12m depth. Zooplankton was collected by using 100-µm mesh nets equipped with Hydrobios flow meter, from near the bottom to the surface at each station where physico-chemical variables were determined. The samples were preserved immediately in 4-5% formalin, bufferered to a pH of 8 with sodim tetraborate (borax). Organisms were identified to groups and copepods to species level (whenever possible) and counted. Density was expressed as individuals per m^{-3} (individual. m^{-3}).Only adult copepods were counted (Somoue *et al.,* 2005).

2.3. Data Analysis

Species diversity was calculated using Shannon–Weaver diversity index (Shannon–Weaver, 1963) and Species richness (Margalef, 1968). The data were further subjected to hierarchical cluster and multidimensional scaling (MDS) analyses to identify the similarity between stations based on composition of copepod species that were calculated as Braye Curtis similarity index using PRIMER (Clarke and Warwick, 1994).

3. Results

3.1. Environmental variables

The mean of water temperature varied from (20.53 ± 0.20°C) in NE-monsoon to (29.92 ± 0.05°C) in SW-monsoon. The mean of salinity ranged from (36.7 ± 0.06 Psu) in SW-monsoon to (36.91Psu) in pre-monsoon (Fig.2b). The minimum and maximum values of chlorophyll-*a* concentrations were noticed (0.77 ± 0.08 mg. m^{-3}) in SW-monsoon to (1.84 ± 0.92 mg. m^{-3}) in NE-monsoon (Fig.2c). The average of dissolved oxygen (DO) ranged from 5.66 ± 0.05 ml.l^{-1} in SW-monsoon to 8.80 ± 0.03 ml.l^{-1} in NE-monsoon (Fig.2d).

Pre-monsoon season showed the maximum Silicate (SiO_4) concentration (av. 0.031 ± 0.006 mg. m^{-3}) whereas it was minimum during the NE-monsoon (av.0.017 ± 0.05 mg. m^{-3}) (Fig. 2e). The variation of nitrate (NO_3) was (av. 0.026 ± 0.004 mg. m^{-3}) in pre-monsoon (av; 0.002 ± 0.0002 mg. m^{-3}) in post-monsoon (Fig. 2f). Minimum and maximum values of phosphate (PO_4) were from 0.015 ± 0.006 mg. m^{-3} during the NE-monsoon season to 0.008 ± 0.002 mg. m^{-3} during the SW-monsoon season (Fig. 2g).

3.2. Copepoda abundance and composition

Copepods were the dominant group during four seasons, reaching 69.73% (1253.57 ± 302.65 ind. m^{-3}) during pre-monsoon, 67.02% (613.30 ± 326.35 ind.m^{-3}) during SW-monsoon, 62.58% (594.12 ± 54.11 ind. m^{-3}) during post-monsoon and 47.38% (904.17 ± 161.7 ind. m^{-3}) in NE-monsoon (Fig 3; Fig 4). Copepod abundance increased significantly ($p<0.05$) during pre-monsoon than other seasons. There were significant spatial differences during SW-monsoon, post-monsoon and NE-monsoon ($p<0.05$).

3.3. Community structure

Totally, 75 copepod species including 5 orders, 19 families, and 21 genera were identified during four cruises in Chabahar Bay. Some species were observed only in one season with lowest abundance (less than 25 ind. m^{-3}) (Table1) such as '*Saphirina gastric aphirina nigromoculata* (at station 2) *Lucicutia flavicormis*, *Lucicutia gaussae* (at stations 1 and 3) and one species from Monstrilloida (at station 1) that only occurred during post-monsoon. *Oithona fallax, Paracanadica truncate,Euchatea marina* were observed at station 1(only during NE-monsoon). *Bestiolina similis*, *Delius nudus* just appeared during pre-monsoon (at station 2). *Euterpina acotifrons*, *Macrosetella gracilis* and *Microsetella rosea* were observed highest (44.87% from total copepoda) in pre-monsoon. *Temora turbinate* were greater during pre-monsoon (7.94%) and NE-monsoon (12.06%). Moreover, Psododiaptomidae species increased remarkably during NE-monsoon (7.57%) and were rare in other seasons. Results of cluster analyses based on copepod abundance revealed the presence of 2 main groups (groups *I* and *II*) during each season (Fig 5).

4. Discussion:

Zooplankton abundance in Chabahar Bay follows a cycle related to the monsoonal winds. In Chabahar bay, climatic changes and other environmental variables due to monsoonal winds play a key role in zooplankton community (Fazeli, 2008).

Nutrient enrichment is a key factor regulating temporal variations in zooplankton (mostly copepods) in coastal environments (Garcia and Lopez, 1989). Further, phytoplankton is a source of nourishment of herbivorous copepods (Tan *et al.,* 2004), but in present study there was not positive correlation between chlorophyll-a concentration and copepods abundance. According to (Tranter, 1973) there was a time lag between phytoplankton bloom and the increase of zooplankton. Our results showed chlorophyll-a concentration increased with phosphate concentration ($p<0.01$) (Table 2). Domestic sewage and industrial effluents noticed around the bay may also be responsible for increase of nutrients (mostly phosphate) and chlorophyll-a concentration consequently. This fact suggests that an earlier increase of phytoplankton must have favored such an increase in copepod and that the early bloom of phytoplankton may have used up the phosphate in this area.

Baars (1998) noticed during the SW- monsoon, that upwelling occurs in almost the entire western Arabian Sea, giving high primary productivity and vast diatom blooms, especially off Oman. This contrast with our result is because these areas are greatly affected by monsoonal winds and zooplankton densities were highest in the upwelling seasons, but upwelling was not reported in Chabahar Bay (Wilson, 2000).

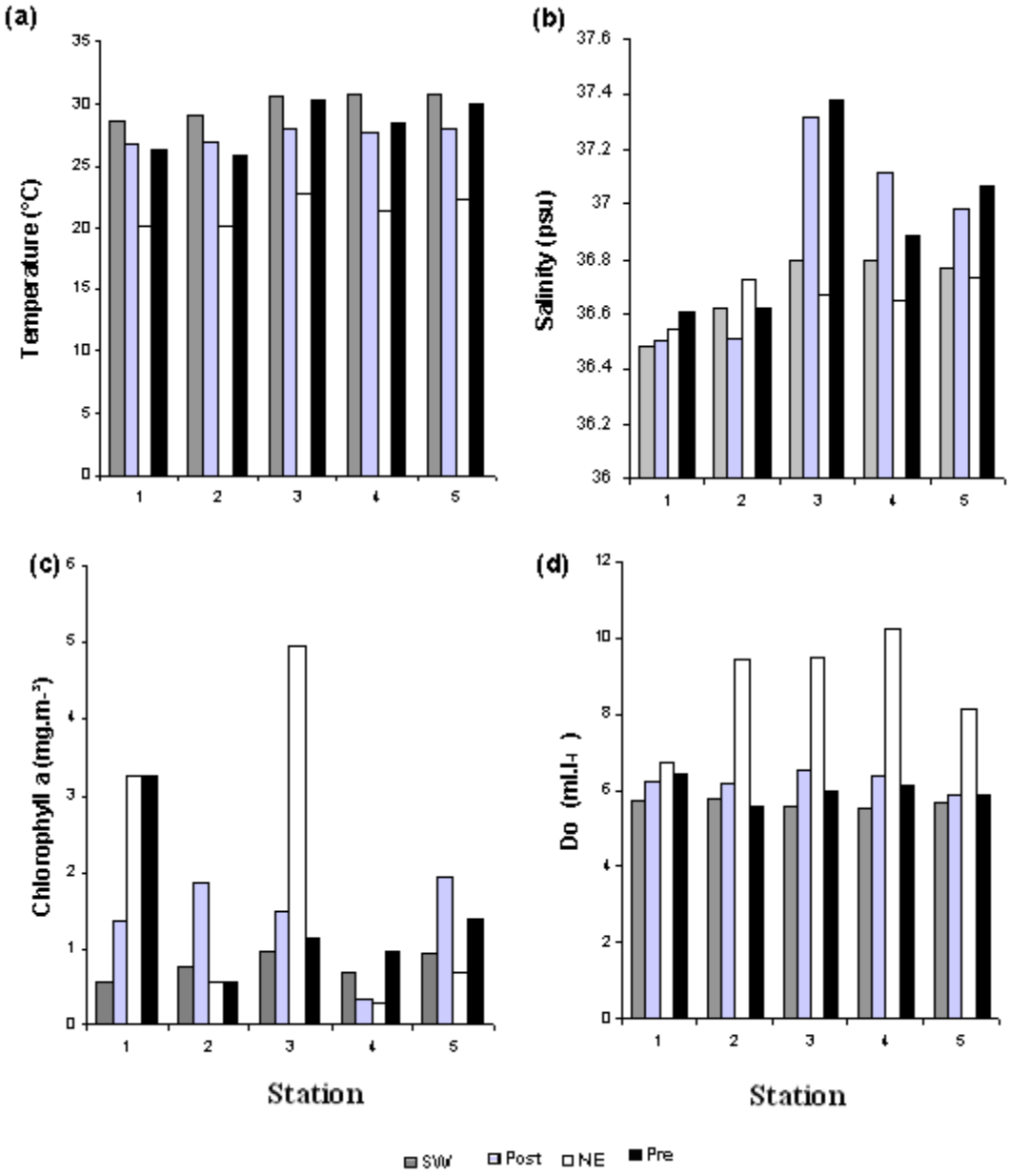

Figure 2. a, b, c, d, Distribution of major physico-chemical variables during monsoonal seasons (x axis as stations and Y axis as physico-chemical variables).

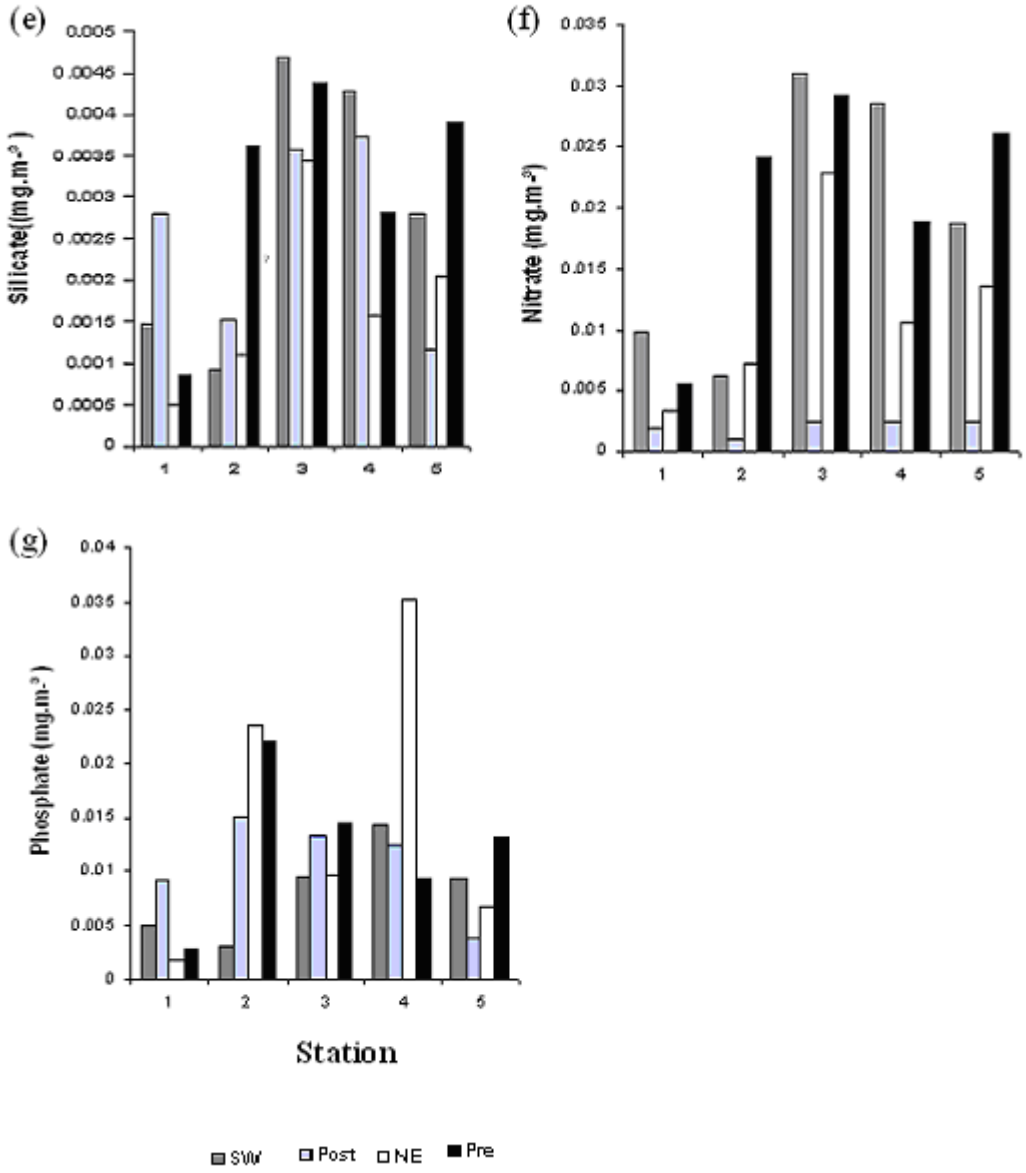

Figure 2. e, f, g, Distribution of major physico-chemical variables during monsoonal seasons (x axis as stations and Y axis as physico-chemical variables).

Since, there was no correlation between copepoda abundance and temperature-salinity, we conclude that the abundance of copepoda does not have any relationship with these parameters in Chabahar bay, while in some tropical embayments and estuaries in India, accelerate of zooplankton production during the periods of high salinity was documented by (Baidya and Choudhury, 1984; Tiwari and Nair 1993) and high temperature by (Li *et al.,* 2008).

Copepod diversity and richness were related to copepod abundance inversely. Species richness was enhanced towards the far from the shore and middle stations of the bay (Table 3). This is because organisms living in near shore waters seem to be adapted to the qualification and unable to thrive in offshore area (Faure, 1951). Increase of diversity and richness indices in far from shore communities is common in Indian Ocean (Madhupratap, 1986). This trend was observed in waters of Africa by (Okemwa, 1990) in Tudor Bay, (Mwaluma, 1997) in Kenya, (Osore, 1992; Osore, 1994) in Gazi Bay and (James *et al.,* 2003) in Media creek. In India, in the Bay of Bengal and Cochin backwaters, the similar trend were reported by (Pillai *et al.,* 1973; Nair *et al.* 1981; Tiwari and Vijayyalakshmi, 1993), who attributed this high diversity to the calmer, more stable oceanic waters.

In this study *Oithona nana* had much abundance through the year. This species is a euryhaline and euryterm species in tropical water (Nishida, 1985). Most abundance of copepod species during the pre-monsoon belongs to Harpacticoida in particular *Macrosetlla gracilis* ، *Microsetella rosea* ،*Euterpina acutifrons* and Corycaeus *andrewsi.*

In conclusion, chlorophyll-*a* concentration appear to be the important factor leading to increase of copepod

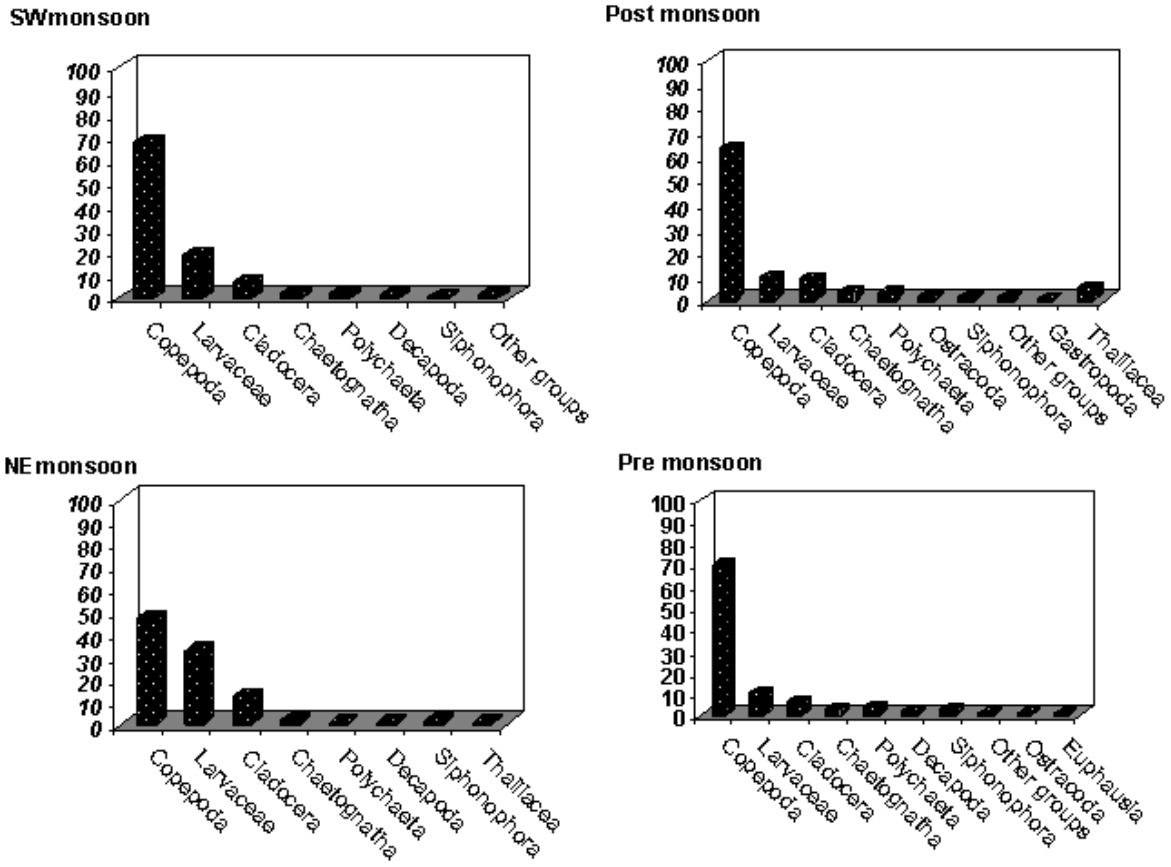

Figure 3. Percentage of zooplankton groups (%) during monsoonal seasons in Chabahar Bay.

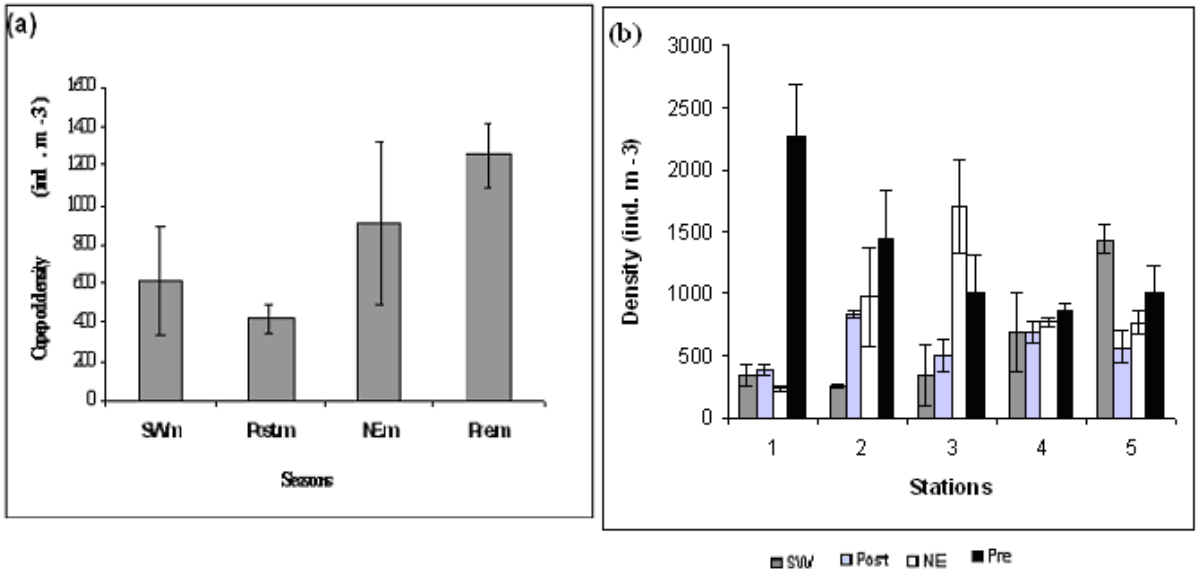

Figure 4. Seasonal (a) and spatial (b) distribution of copepoda during monsoonal seasons in Chabahar Bay.

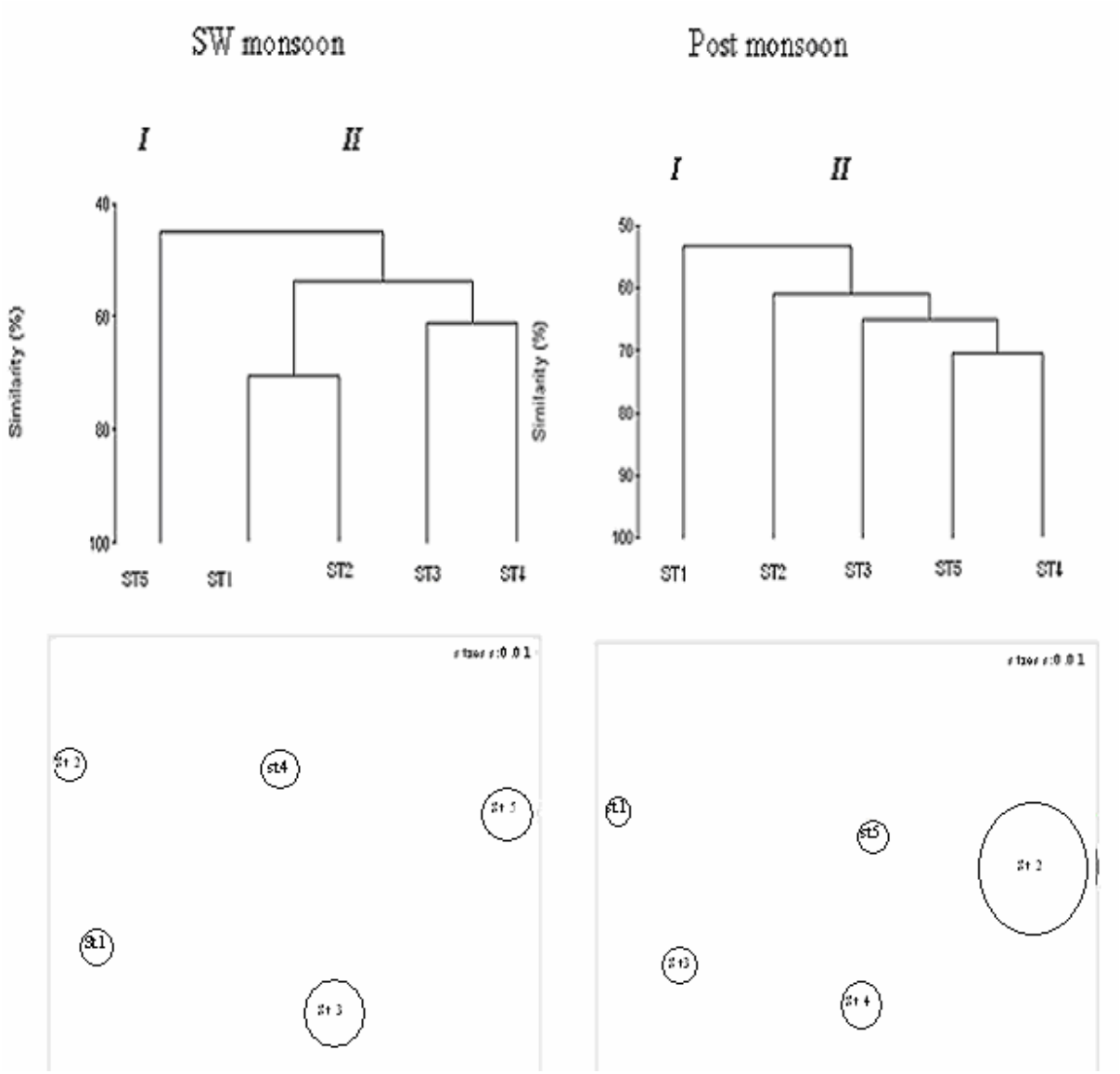

Figure 5. Cluster and MDS analyses showing similarity of stations during monsoonal seasons based on copepod composition in Chabahar Bay.

*Figure 5 continues in the next page.

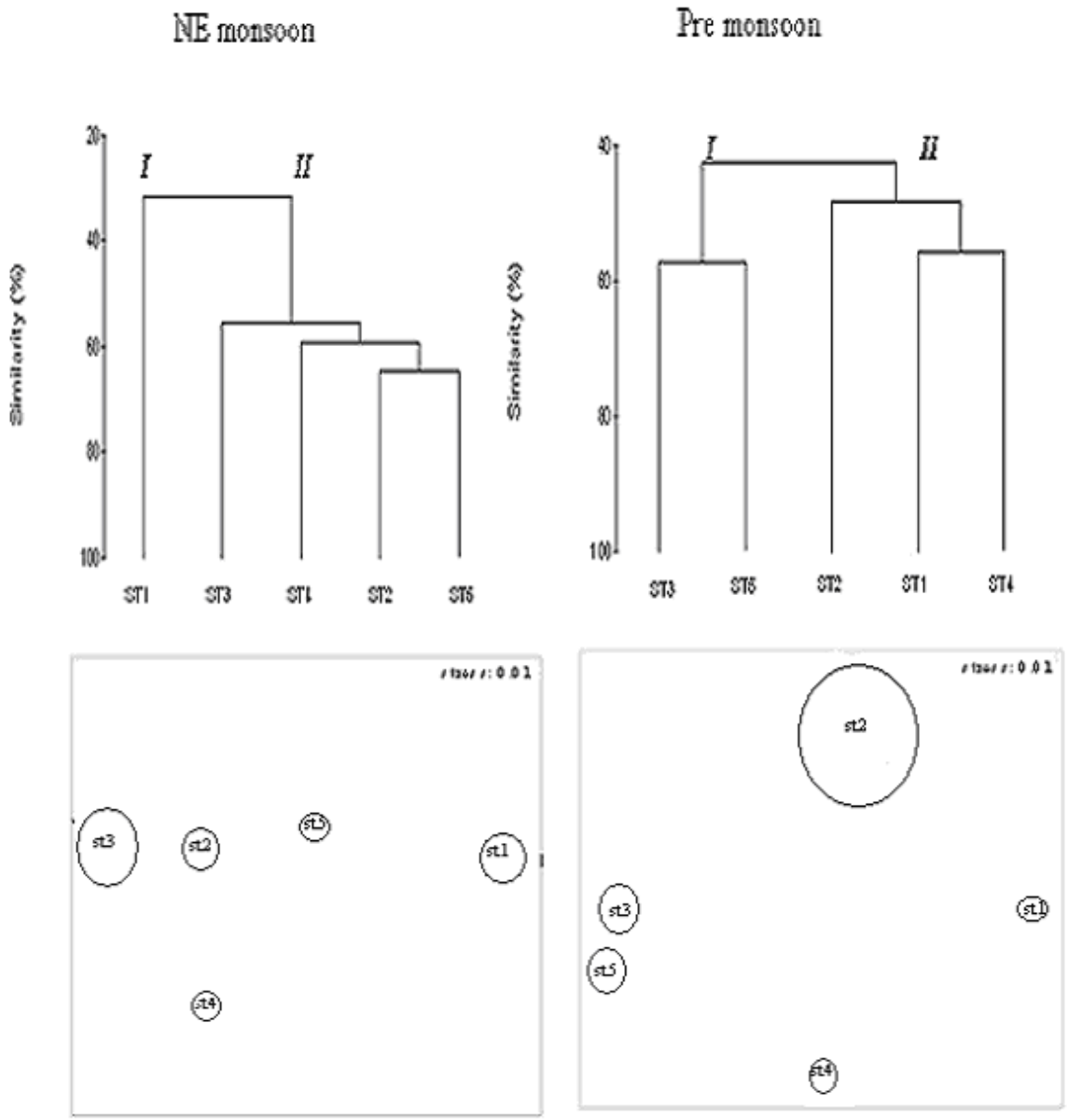

Figure 5. Cluster and MDS analyses showing similarity of stations during monsoonal seasons based on copepod composition in Chabahar Bay.

Table 1. Abundance of copepod species (ind. m^{-3}) in monsoonal seasons in Chabahar Bay.

Species	SW .m	post. m	NE. m	pre.m
Paracalanus crassirostris	1125.62	2830. 62	255 .24	548.34
P.elegans	2782.11	4162.53	1558.07	516.59
P.aculatus	56.1676	1701.56	160	47.418
P.denudatus	579.69	540.17	165	-
P.parvus	189.02	140.55	125	87.129
*P.*sp	600.52	1350.36	-	288.60
Acrocalanus longicornis	1230.81	339.7	222.22	-
A.gracilis	2152.94	61.54	630.48	-
A.gibber	2479.67	187.59	246.96	-
A.monochus	1374.52	87.90	316.75	259.74
*A.*sp	1219.27	541.49	525.71	412.67
T. desicaudata	89.43	1584.75	40	-
T.turbinata	677.3	5698.92	9291.32	5717.78
T.stylifera	-	299.07	-	-
Eucalanus subcrassus	221.32	626.23	1004.45	-
E. morcantus	-	210.12	-	-
E.sp	87	-	31.75	-
E. crassus	231.25	133.25	31.75	-
E. attenuatus	41.03	-	40	-
E.monochus	85.10	49.84	-	-
E. pileatus	44.07	-	-	-
E. halinus	44.07	-	-	-
Acartia pasifica	644.97	-	-	-
A.erythraea	47.60	-	-	-
A.sp	187.56	277.50	111.11	225.11
A. longirenis	205.66	389.75	236.11	-
Clausocalanus furcatus	-	-	430.58	-
C. gracilis	-	110.42		-
C. minor	-	-	766.96	-
C.sp	600.52	-	285	-
Calocalanus styliremis	45.30	42.60	-	-
C. plumulosus	-	63.06	31.75	-
C.pavo	47.60	55		
Pseudodiptomus sp	136.03	-	4994.04	299.32
P.marinus	-	-	589.21	-
Centropages furcatus	-	-	713.22	-
C. tenuremis	690.54	384.41	9441.95	839
Labidocera sp	27518.82	84.49	375.24	-
Pontellina	-	90. 115	-	-
Paracanadica truncata	-	-	191/11	-
Lucicutia sp	-	-	31/75	-
L. *flavicormis*	-	20.51	-	-
L. *gaussae*	-	22.04	-	-
Bestiulina similis	-	-	-	130
Delius nudus	-	-	-	127
Euchata marina	-	-	31.75	-

Oithona oculata	1952.91	1742.31	1558.78	1619.05
O. attenuata	3600.99	2762.99	1891.85	965.99
O.nana	3551.84	5254.90	10203.44	6323.23
O. bremicornis	336.41	2496.91	3761.27	1238.10
O.simplex	1343.38	5624.04	750.95	761.90
O. rigida	3883.92	1913.12	1284.20	1111.11
O.plumifera	45.35	1058.42	591.11	894.66
O. sp	5270.54	5911.14	5735.79	7163.06
O.fallax	-	-	31.75	-
Corycaus pacificus	194.72	311.33	166.98	-
C. andrewsi	1005.18	659.05	1528.07	5125.54
C. asiaticus	321.52	279.52	236.11	1910.13
C. erythraeus	90.52	2013	267.86	-
C. sp	725.13	390.30	166.96	285.71
C. affinis	-	243.68	125	331.07
C. dahli	-	384.77	361.11	-
C. speciosus	-	65.86	220.24	-
Oncea media	608.28	3626.43	2907.78	-
O. venusta	703	1860.83	634.20	259.74
O. clevei	417.74	1174.20	160	-
O. minuta	44.07	670.01	-	387.72
Sapphirina sp	-	-	331.35	-
S. gastrica	-	44.35	-	-
S. nigromoculata	-	45.35	-	-
Clytemnestra scutellata	-	123.08	1227.88	704.81
Macrosetlla gracilis	302.14	2375.38	753.49	14286.96
Microsetella rosea	172.92	280.86	366.96	8116.86
Euterpina acutifrons	4058.41	8791.82	4831.53	10440.73
Monstrilloida	-	105.17	-	-

Table 2. Pearson correlation of major environmental parameters and copepoda density

Variables	Chl-*a*	Temperature	Salinity	DO	SiO3	NO3	PO4
Copepod density	0.05	-0.26	-0.08	0.20	-	-	-
Chl- *a*	1.00	-0.38	0.06	0.33	0.05	0.05	0.93**

('*' significant at 0.05 level; '**' significant at 0.01 level).

Table 3. Diversity index (H') and species richness (D) of Copepoda in monsoonal seasons.

Stations	SW .m	post .m	NE .m	pre. m
1	3.09 (4.10)	3.03 (4.59)	2.89 (4.39)	2.52 (1.65)
2	3.41 (4.11)	3.34 (4.23)	2.67 (2.40)	2.47 (1.90)
3	2.40 (1.35)	2.62 (2.99)	2.19 (1.44)	1.96 (1.57)
4	2.73 (3.13)	2.85 (3.58)	2.88 (2.97)	2.14 (1.84)
5	1.15 (1.71)	2.33 (1.79)	2.54 (2.55)	1.93 (1.04)
Mean	2.55 (2.88)	2.83 (3.43)	2.63 (2.75)	2.20 (1.60)
Number species	50	58	57	33

abundance (after a time lag) and associated phosphate concentrations during pre-monsoon.

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Nandrolone Decanoate Administration to Male Rats Induces Oxidative Stress, Seminiferous Tubules Abnormalities, and Sperm DNA Fragmentation

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Abstract

The present study was conducted to evaluate the effects of Nandrolone Decanoate (an anabolic steroid) on the level of oxidative stress markers, sperm chromatin integrity, seminiferous tubules structure, and spermatogonia/Sertoli cell ratio in adult rat. Rats were divided into three groups: control, low (3mg/Kg) and high-dose (10mg/Kg) Nandrolone Decanoate-receiving groups. Seminal fluid analysis was performed, and the serum was used to evaluate testosterone level. Testicular oxidative stress markers were measured and routine histological preparation and androgen receptor immunochemistry was used to evaluate the effects of Nandrolone Decanoate injection on seminiferous tubules. Injection of Nandrolone Decanoate caused an increase in the production of thiobarbituric acid-reactive substances in the testes of treated rats. The level of sperm DNA fragmentation and the percentage of seminiferous tubules showing maturation arrest were also increased in treated animals. The absolute numbers of spermatogonia and Sertoli cells in the rats receiving Nandrolone Decanoate decreased significantly; however, the ratio of spermatogonia/Sertoli cells did not. Administration of anabolic steroids at supraphysiological doses leads to multiple pathological changes in the reproductive system of treated rats. Testosterone or its derivatives such as Nandrolone Decanoate are being abused commonly. Athletes, coaches, and physicians should be aware of their harmful side effects.

الملخص

تهدف الدراسة الحالية إلى تقييم آثار حقن Nandrolone Decanoate (واحد من الستيرويدات البنائية) على مستوى الجهد الاوكسيجيني، وسلامة المادة الوراثية للحيوانات المنوية، و شكل ومكونات الأنابيب المنوية، ونسبة الخلايا المنوية الأم للخلايا المساعدة (خلايا سرتولي) في الجرذان التي تم حقنها. تم تقسيم الجرذان إلى ثلاثة مجموعات: مجموعة الضبط و مجموعة حقنت بتركيز متدني من Decanoate Nandrolone (3مج/كغم) و مجموعة حقنت بتركيز عالي (10مج/كغم). توصلت الدراسة إلى أن الإستخدام المفرط لمركب Nandrolone Decanoate أدى إلى إفساد الحمض النووي للحيوانات المنوية و إلى إيقاف عملية تكوين الحيوانات المنوية في مراحل مختلفة. ولم يحدث أي تغيير في نسبة الخلايا المنوية الأم إلى خلايا سرتولي، ولكن العدد المطلق لهذه الخلايا قلَ بشكل كبير. وبالإضافة إلى ذلك، حقن الستيرويدات البنائية تسبب في زيادة إنتاج Acid Thiobarbituric دون حدوث أي تغيير في مستوى GSH في خصية الجراذين التي حقنت ب Nandrolone Decanoate. إن إساءة استعمال الستيرويدات البنائية بدأت تصبح مشكلة أساسية في مجال الصحة العامة وهو ما يعني ضرورة تنفيذ البرامج التعليمية لتوعية وتحذير المراهقين والمرشدين على حد سواء عن الآثار الجانبية والسلبية لهذه العقارات على صحة مستخدميها.

Keywords: Germinal Epithelium, TBARS, Glutathione, Anabolic Steroids, Sertoli Cells, Androgen Receptor.

1. Introduction

Anabolic-androgenic steroids (AAS) are synthetic compounds which are based on the structure of testosterone, and are used to treat various conditions such as reproductive system dysfunction, breast cancer and anemia (Thiblin and Petersson, 2005). Three basic modifications are made to the structure of testosterone to enhance deliverability and potency and slow down rate of degradation. The first of these modifications (class I) involves esterification of testosterone at the 17-β-hydroxy location (Hall and Hall, 2005). This modification, which is made to injectable AAS, slows down degradation but enhances androgenic properties (Hall and Hall, 2005).

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Nandrolone Decanoate (ND) is a class I AAS. The second modification (class II) results from alkylation at the 17-α-hydroxy position, which depresses hepatic degradation (Wilson, 1988). The third modification (class III) has been used to generate together oral and injectable formulations. One pattern of class III modifications results in an AAS with analogous properties to that of Class II AAS, but with a decreased or missing hepatic effect (Wilson, 1988; Hall and Hall, 2005).

AAS are taken by abusing athletes at supraphysiological doses; such doses are usually 10 to 100 fold the recommended therapeutic dose (Clark *et al.,* 1997). Traditionally, AAS have been abused in drug-use cycles of 6-14 weeks followed by a drug-free period to prevent building up tolerance to AAS (Karila *et al.,* 2004).

Defining the exact adverse effects of AAS abuse is very hard due to the lack of clinical trials which mimics AAS abuse by athletes (Hall and Hall, 2005). In addition, almost all major tissues in the body have androgen receptors, and thus AAS abuse affects almost all body systems (Karila *et al.,* 2004). Side effects of AAS abuse include liver failure, acne, a decrease in high-density lipoprotein (HDL) levels and hepatic adenomas (Boyadjiev et al, 2000; Hall and Hall, 2005), mood fluctuation, aggressive behavior, violence and suicide attempts (Clark *et al.,* 1997).

One of the most pronounced effects of AAS abuse is the negative impact on the hypothalamus-pituitary-gonadal axis (*Takahashi et al.,* 2004). In men abusing AAS, there is a noticeable reduction of serum testosterone level as well as FSH and LH (Jarow and Lipshultz, 1990). In addition, AAS abuse has been linked to reduced quality and quantity of semen which could be related to infertility (Torres-Calleja *et al.*, 2001). Long-term AAS administration results in reduction in testicular weight, testicular atrophy and abnormal morphology (Jarow and Lipshultz, 1990).

Although many studies have investigated the effects of testosterone suppression on spermatogenesis through sperm output evaluation (Torres-Calleja *et al.*, 2001), there are no reports on the effects of AAS abuse and thus testosterone suppression on Sertoli cells number and function, structural changes of the testis or sperm chromatin integrity. Hence, this work was conducted to evaluate the effects of administration of ND at supraphysiological doses on oxidative stress biomarkers and male reproductive system.

2. Experimental Procedure

2.1. Animals and treatments

Animal care, handling, and all of the experiments performed were approved by the Hashemite University Institutional Animal Care and Use Committee.

Forty-five adult Wister male rats (*Rattus norvegicus*), weighing between 150–300g (three to four months old), were randomly selected from the laboratory animal center at The Hashemite University (Zarqa-Jordan). The animals were housed individually in an air-conditioned room (12 hr dark/12 hr light) at 23 ± 2 ºC, and had free access to tap water and standard food pellet. After one week of acclimatization, the 45 animals were randomly divided into three groups of 15 each: Group 1 (control), receiving 10 mg/kg/wk of the vehicle peanut oil, Group 2 (low dose), receiving 3 mg/kg/wk of ND (N.V. Organon Oss Holland), and Group 3 (high dose), receiving 10 mg/kg /wk of ND Treatments were administered weekly via intramuscular injection (0.1ml) for 14 weeks. The doses of ND administered to rats and period of administration mimics one cycle of AAS abuse by athletes (Hall and Hall, 2005; Mirkhani *et al.,* 2005; Trenton and Currier, 2005).

2.2. Sample collection and processing

One week after the final drug administration, body weight was measured and the rats were killed rapidly under ether anesthesia followed by cervical dislocation. The following samples were collected and processed:

2.2.1. Serum

Blood was collected via heart puncture, centrifuged at 2,000 rpm for 10 min and the supernatant (serum) was stored at -20ºC. The serum was used to measure total serum testosterone which was determined using AxSYM ® Testosterone test (Abbott, USA). The test is based on Micropar t ical Enzyme Immunoassay (MEIA) technology for the quant itat ive determinat ion of total testosterone in serum (Gonzalez-Sagrado *et al.,* 2000) .

2.2.2. Sperm suspension

The left vas deferens of the rats from the three different groups was exposed and 1 cm of its distal portion was removed. The diffusion method was used to collect semen (Seed *et al.,* 1996). Sperm suspension was used to calculate sperm count and motility. Sperm samples were collected from distal region (cauda) of the left epididymis (Syntin and Robaire, 2001) and were used for evaluation of sperm morphology and chromatin integrity by acridine orange staining. For sperm morphology analysis, 20 µl of sperm suspension were smeared, air dried and stained with hematoxylin and eosin (H&E). 200 sperm per sample were evaluated for normal and abnormal sperm forms [such as angulated (bent) sperm, broken sperm (i.e., headless, tailless), short sperm, and coiled-tailed sperm]. Sperm DNA fragmentation was evaluated by acridine orange staining which was performed according to the method of Tejada *et al.* (1984) and Chohan *et al.* (2004). An average of 200 sperm cells was counted on each slide by the same examiner. Upon excitation, the monomeric acridine orange bound to double-stranded DNA fluoresces green. The aggregated acridine orange on single-stranded DNA fluoresce a spectrum of yellow-orange to red (Chohan *et al.,* 2004).

2.2.3. Right testis

The right testis was removed and cut in half. The first half was used to measure oxidative stress markers. Lipid peroxidation products were quant ified by the thiobarbituric acid (TBA) method (Ohkawa et al., 1979) and expressed as Thiobarbituric Acid-Reactive Substances (TBARS) Levels (nmoles/g protein). Total glutathione content (GSH) and oxidized glutathione (GSSG) were determined by the method of Anderson (1985) and the results were expressed as nmoles of GSH or GSSG/mg protein.

The second half of the right testis was used to analyse the effects of ND on the expression of androgen receptor

by immunoblotting. Briefly, the tissues were washed with cold PBS and then immersed in lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaF, 2% SDS, 5 mM DTT, 2 mM EGTA). Equivalent amounts of protein were resolved by SDS-PAGE on 7.5% gel, transferred to nitrocellulose membrane and immunoblotted with rabbit polyclonal anti-androgen receptor (AR) primary antibody (1:2,000) (Sigma, USA). Blots are representative of at least two independent experiments. Left testis

The left testis was removed and weighed. The volume of testis prior to fixation was estimated using the immersion method (Plethysmometer, Ugo Basile, Italy). The tissue was fixed in 10 % neutral formalin fixative and embedded in paraffin. The tissue was then used either for routine H&E staining or for androgen receptor immunochemistry.

To study the process of spermatogenesis and on the basis of standard qualitative interpretation of H&E staining, testis sections were classified as follows: (i) Normal histology: almost all tubules showing elongating spermatids in each cross section, (ii) Early maturation arrest of spermatogenesis (EMA): most tubules showing spermatogenesis arrest at the level of primary of spermatocyte, with spermatids never observed, and (iii) Late maturation arrest (LMA) of spermatogenesis: most tubules showing spermatogenesis with only round spermatids being observed.

In order to determine the spermatogenic capacity of the male rat, the number of spermatogonia supported by one Sertoli cell was counted. Sertoli cells were identified by positive staining with rabbit polyclonal anti-androgen receptor (AR) primary antibody (1:200) (Sigma, USA). The primary/secondary antibody reaction was detected using high sensitivity streptavidin conjugated to horseradish peroxidase (HSS-HRP) and revealed by 3, 3 diaminobenzidine (DAB) chromogen. The sections were counterstained with Mayer's hematoxylin. A total of five seminiferous tubules per animal were randomly selected, and the number of spermatogonia and Sertoli cells were counted (Watanabe, 2005). All histological observations were carried out using bright field microscope (Nikon E800, Japan).

2.3. Statistical analysis

All samples were processed individually and measured in triplicate in the same assay. Data are stated as mean ± Standard Error of the Mean (SEM).When appropriate, data were analyzed by a one-way ANOVA test. A significant level of 0.05 was considered appropriate.

3. Results

The effects of ND administration on several parameters of serum, testis, and semen in male rats were investigated.

3.1. Effect of ND administration on body weight

The body weight for all animals in the three groups was measured before and after treatments (Fig. 1). After 14 weeks, the final body weight of animals in the three groups was not significantly different as compared to their weight at the beginning of the study, but the rate of weight gain was more in the control group; although not significant (p=0.582).

3.2. Effects of ND administration on testosterone level

Measuring total testosterone level in the serum of the control and treated groups indicated that injection of ND caused a significant decrease in testosterone level in both the low (5.3 ± 3.7 pg/ml) and the high-dose (60.1 ± 19.3 pg/ml) ND receiving groups (p=0.001) as compared to the control group (633.3 ± 210.1 pg/ml).

3.3. Effects of ND administration on sperm characteristics

The average sperm concentration of both groups receiving low (31.4 ± 5.9x10^6/ml) and high (44.7 ± 5.9x10^6/ml) doses of ND was significantly decreased as compared to that of the control group (116.0 ± 1.1 x10^6/ml) (p=0.001). 78.1 ± 2.7% of sperm extracted from the vas deferens of rats in the control group showed progressive forward motility. In contrast, treatment of rats with the low and high doses of ND caused a significant decrease (p=0.001) in the percentage of progressively motile sperm (39.2 ± 4.1% and 19.4 ± 3.1%, respectively).

The percentage of sperm with normal morphology was statistically decreased (p=0.001) in the low-and high-dose receiving groups (61.8 ± 4.7% and 59.4 ± 5.0%, respectively) as compared to the control group (92.9 ± 1.1%).

The effect of ND administration on sperm chromatin integrity was evaluated by acridine orange staining. 200 sperm per slide were counted, and the percentage of sperm with single-strand DNA (Red, yellow-orange/total x 100) was calculated (Fig. 2A). The percentage of sperm with defected DNA was significantly increased in the treated rats receiving low and high doses of ND (72.3% and 53.3%, respectively) as compared to the control group (19.7%) (p=0.001) (Fig. 2B).

3.4. Effects of ND administration on testes

The volume of the testes decreased significantly in animals which received low (0.81 ± 0.14 ml) and high doses (0.93 ± 0.02 ml) (p=0.001) of ND in comparison with the animals in the control group (1.78 ± 0.04 ml).

Injection of ND caused a significant increase in TBARS production in the testis of both the low and the high-dose receiving groups as compared to the control group (p=0.001) (Fig. 3). On the other hand, the testicular levels of both total glutathione (GSH) and oxidized glutathione (GSSG) were not significantly different (p=0.519 and 0.551, respectively) between treated and untreated groups (Table 1).

Table 1. Effect of Nandrolone Decanoate administration on testicular glutathione levels. Values are expressed as means ± SEM (n in each group=3-6)

Group	Total GSH (nmoles/mg protein)	GSSG (nmoles/mg protein)
Control	18.9 ± 1.2	2.4 ± 0.15
Low Dose	21.1 ± 2.3	2.1 ± 0.09
High Dose	19.3 ± 2.1	2.9 ± 0.35

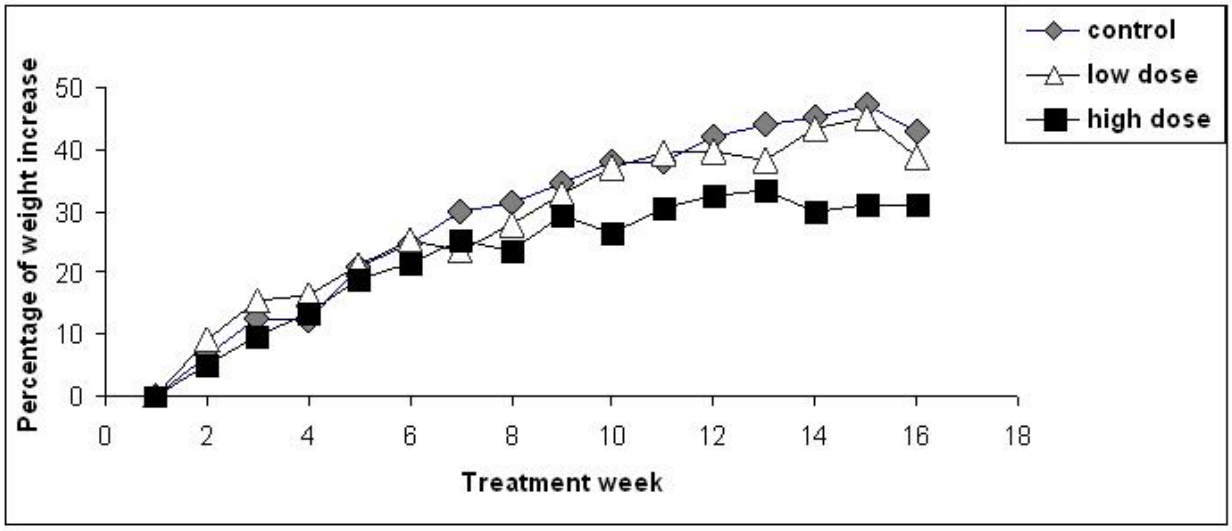

Figure 1. Effect of Nandrolone Decanoate administration on body weight. Body weight is expressed as (final body weight – initial body weight /initial body weight ×100). n=15 in each group.

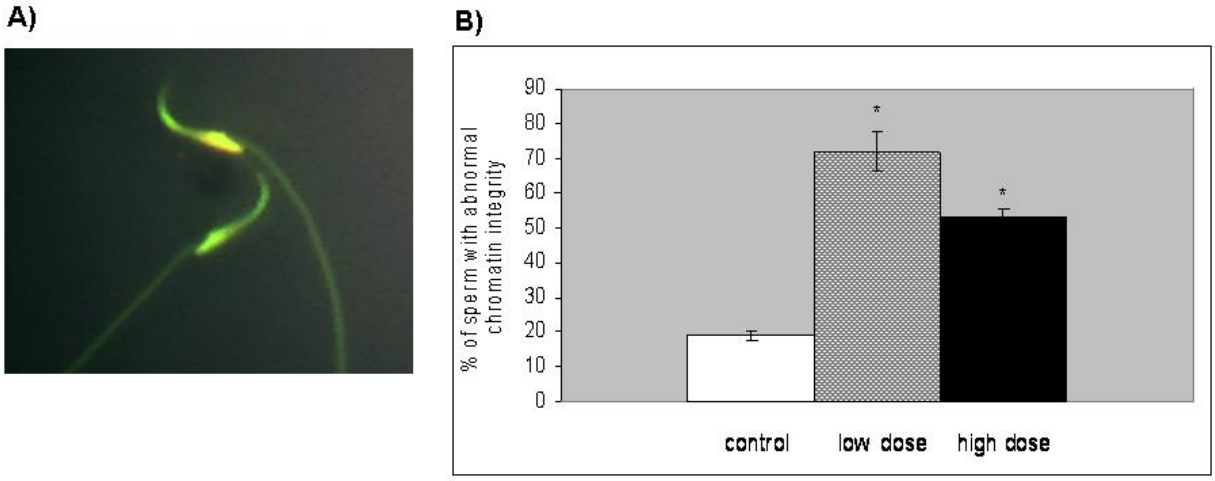

Figure 2. Effect of Nandrolone Decanoate administration on sperm chromatin integrity. A) Acridine orange test applied to rat sperm showing a sperm with intact chromatin (green) and a sperm with damaged chromatin (yellow-orange), B) The percentage of sperm with abnormal chromatin integrity was higher in treated groups as compared to the control group. (Values are expressed as mean ± SEM, n =15 in each group, *p=0.001).

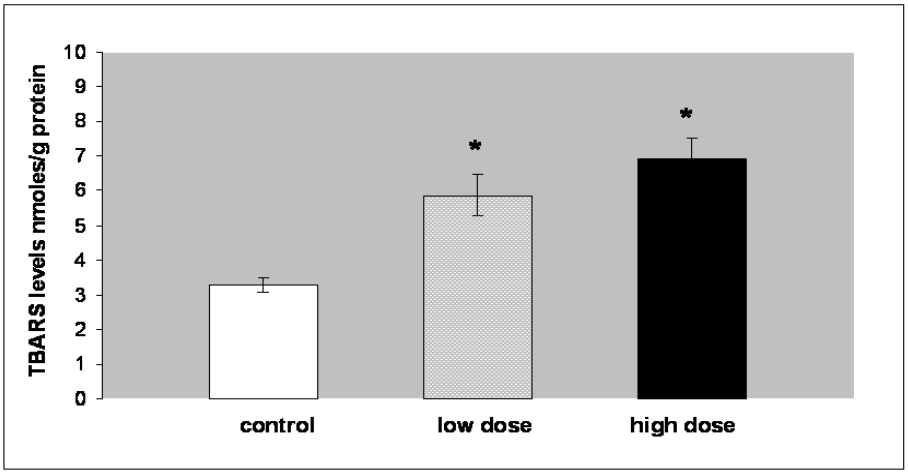

Figure 3. Effect of Nandrolone Decanoate administration on testicular TBARS levels. Both low and high-dose receiving groups had a higher level of TBARS as compared to the control group. (Values are expressed as mean ± SEM, n = 6-9 in each group, *p=0.001).

After analyzing different cross sections of testes of control and treated animals, obvious differences were noted. One of these differences was atrophy (low number of germ cells) in the seminiferous tubules (ST) of treated rats (receiving low or high doses of ND) with large focal areas, and a noticeable decreased accumulation of spermatozoa within the lumen (hypospermatogenesis) (Fig. 4B) as compared to ST of control rats (Fig. 4A). Also, some ST in the testes of treated rats showed abnormal architecture of the seminiferous epithelium (Fig. 4C), and maturation arrest (early or late) (Fig. 4D and E, respectively). In addition to the abnormal patterns of spermatogenesis mentioned, normal seminiferous tubules were also found in cross sections of testes of treated rats. A summary of spermatogenesis patterns in control and treated rats is shown in Table (2).

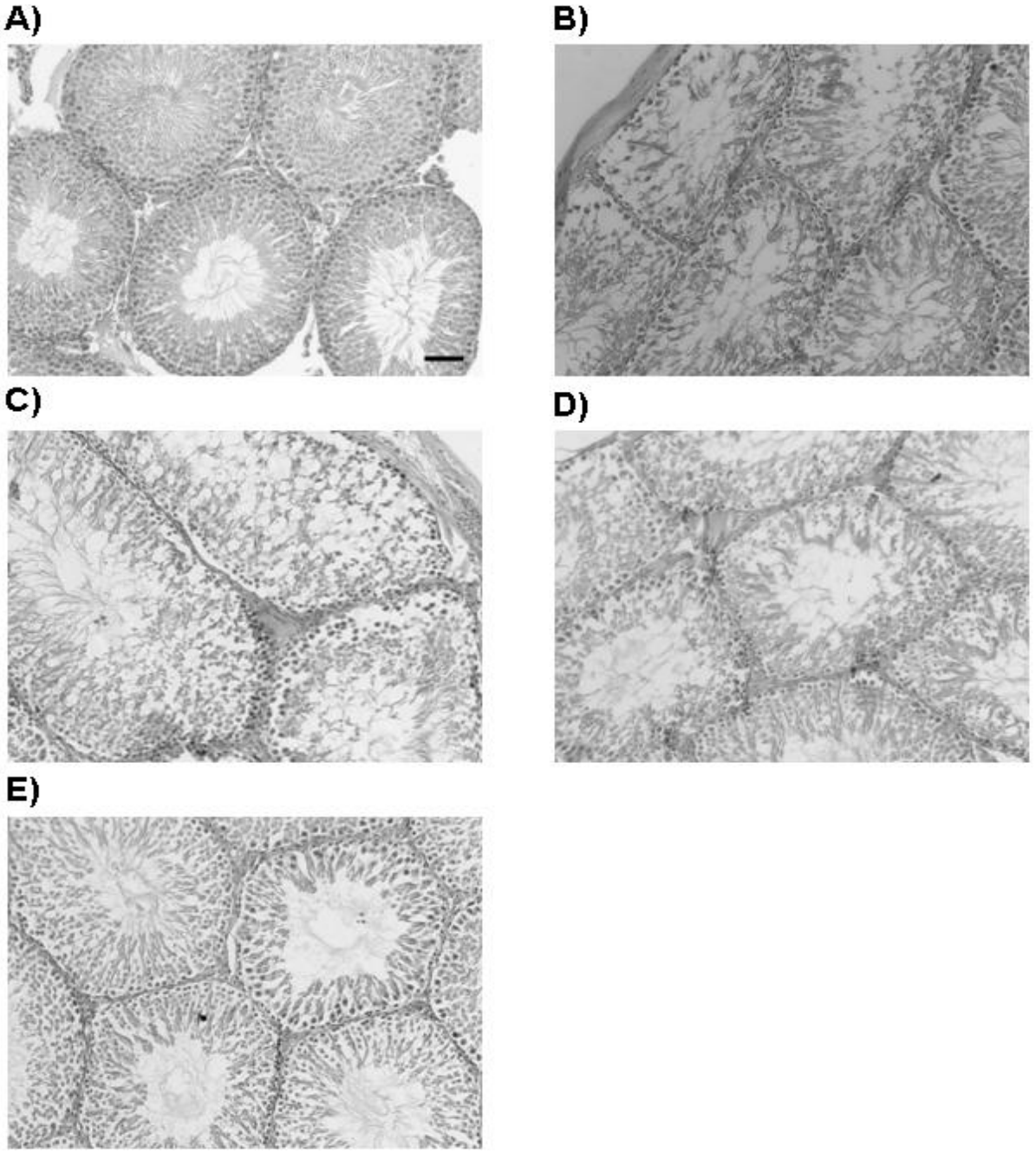

Figure 4. Histological appearance of representative examples of seminiferous tubules (ST) of control and treated rats stained with hematoxylin and eosin. A) A cross section of ST from control rat showing normal architecture of germinal epithelium, i.e., multiple layers of germ cells ranging from spermatogonia, 1^o spermatocytes, 2^o spermatocytes, round and elongated spermatids and sperm in the lumen, B) A cross section of ST from a low-dose treated rat displaying atrophic germinal epithelium (2-3 layers of germ cells) and almost lacking sperm in the lumen, C) A cross section of ST from a low-dose treated rat showing abnormal organization of the germinal epithelium, D) A cross section of ST from a low-dose treated rat showing early maturation arrest in which germ cells differentiation stops at 1^o spermatocytes, E) A cross section of ST from a high-dose treated rat displaying round spermatids as the last stage of germ cell differentiation (late maturation arrest). Scale bar = 50 μm.

Table 2. Different patterns of spermatogenesis observed in control and Nandrolone Decanoate -receiving rats. Values represents mean of percentages ± SEM, n in each group=7, *: p=0.001

Group	% of ST showing normal spermatogenesis	% of ST showing maturation Arrest		% of ST showing hypo-spermatogenesis
		Early	Late	
Control	94.1 ± 0.26	4.3 ± 0.3	1.2 ± 0.1	0.4 ± 0.3
Low Dose	21.1 ±1.2*	44.3 ± 1.2*	20.7 ± 1.8*	13.9 ± 1.5*
High Dose	20.7 ± 2.1*	30.0 ± 1.1*	33.7 ± 1.8*	15.6 ± 1.2*

Finally, we examined the pathological changes that might have occurred to spermatogonia and Sertoli cells in the testes of treated rats. Immunohistochemical localization of AR expression within seminiferous tubules was restricted to nuclei of Sertoli cells (Fig. 5A). Spermatogonia in seminiferous tubules of control rats were closely packed and the nuclei were darkly-stained, and the nuclei of Sertoli cells were located regularly in the periphery of the tubules (Fig. 5A). Seminiferous tubules of treated rats (receiving either low or high doses of ND) showed abnormal organization of spermatogenic cells (Fig. 5B), and the number of layers of germ cells was reduced. In addition, the morphology, nuclear size and position of Sertoli cells were changed in the STs of treated rats (Figure 5B). The number of spermatogonia in the testes of rats in both low and high-dose receiving groups was significantly decreased as compared to the control (p=0.001) (Fig. 5C). In addition, there were fewer Sertoli cells in the seminiferous tubules of ND-treated animals than in those of the control rats (p=0.001) (Fig. 5C), which was confirmed by analyzing the level of AR expression by immunoblotting of extracted testicular proteins (Fig. 5D). Quantification of normalized AR levels in treated animals relative to that of controls (lane 1,2) revealed that extracts from both low- (lane 3) and high-dose receiving groups (lane 4) showed a reduction (32% and 47%, respectively) in AR expression as compared to extracts of control animals (Fig. 5D). However, the ratio of spermatogonia to Sertoli cells in the treated rats was not significantly different from that of the control group (p=0.287).

4. Discussion

One of the most prominent effects of AAS is the negative impact on the pituitary-gonadal axis (Aubert *et al.,* 1985; Takahashi *et al.,* 2004).AAS stimulates hypogonadotrophic hypogonadism coupled with decreased serum testosterone concentrations (Harkness *et al.*, 1975; Schurmeyer *et al.*, 1984; Jarow and Lipshultz, 19908). In the current study, total serum testosterone level in treated rats was lower than in control animals, which is in accord with what was previously reported.

The dysfunction accounting for these abnormalities is supposed to be steroid-induced suppression of gonadotrophin production; it was reported that there is a noticeable depression of serum FSH and LH in men abusing AAS (Harkness *et al.*, 1975; Clerico *et al.*, 1981). This in turn results in a condition of impaired spermatogenesis including oligozoospermia to azoospermia, abnormalities of sperm motility and morphology (Ramaswamy *et al.*, 2000; Torres-Calleja *et al.*, 2001; Takahashi *et al.*, 2004). Suppression of sperm output is attributed to the degree of inhibition of germ cell development which is related to the degree of FSH, LH and testosterone suppression (O'Donnell *et al.*, 2001). This could explain decreased sperm concentration, motility and normal morphology induced by long-term ND administration to rats. Also, it could explain the maturation arrest noted in the ST of treated rats (Table 2).

Since little information is available on the effects of 17-β-alkylated steroid treatment on oxidative stress markers, we aimed to investigate whether prolonged treatment of rats with low or high doses of ND modified oxidative stress markers through studying TBARS production as a result of lipid peroxidation and redox status of glutathione (GSH). Figure 3 shows that prolonged administration of ND induced a significant increase (p=0.001) in TBARS levels which serves as an index of extended lipid peroxidation. However, this treatment did not modify testicular levels of GSH in treated rats (Table 1). To our knowledge, this is the first time that the effects of ND treatment on oxidative stress biomarker levels have been studied. AAS seek to maximize the anabolic effects and overcome the catabolic pathways thus increasing anabolic pathways, so the possibility of oxidative stress condition could increase (Saborido *et al.*, 1993; Molano *et al.*, 1997).

It was shown previously that prolonged administration of Stanazolol (17-α-alkylated AAS) provoked dysfunction of the mitochondria respiratory chain complexes and mono-oxygenase systems; it would be possible that these alterations were accompanied by an increased reactive oxygen species (ROS) generation (Saborido *et al.*, 1993; Molano *et al.*, 1997; Pey *et al.*, 2003).

One more supposed source of free radicals generation is cytochrome P450 cholesterol side-chain cleavage enzyme isoforms of the steroidogenic pathway during their catalytic cycles, these isoforms have been shown to release ROS under basal conditions especially with uncoupled substrates (Chang *et al.*, 1995). Consequently, metabolism of high doses of ND by cytochrome P450 mono-oxygenases would have increased greatly the production of ROS resulting in a state of oxidative stress and up-regulation of the activity of the antioxidant enzymes such as SOD and glutathione peroxidase (GPx), gluthatione reductase (GR) and catalase (Georgiou *et al.*, 1987; Diemer *et al.*, 2003; Pey *et al.*, 2003; Chen *et al.*, 2005). However, the mechanism through which the intramuscular injection of ND could be associated with free radical production is unidentified at present.

On the other hand, there was no change on GSH level (Table 1), which might be due to the body using other antioxidant defense mechanisms such as the activities of

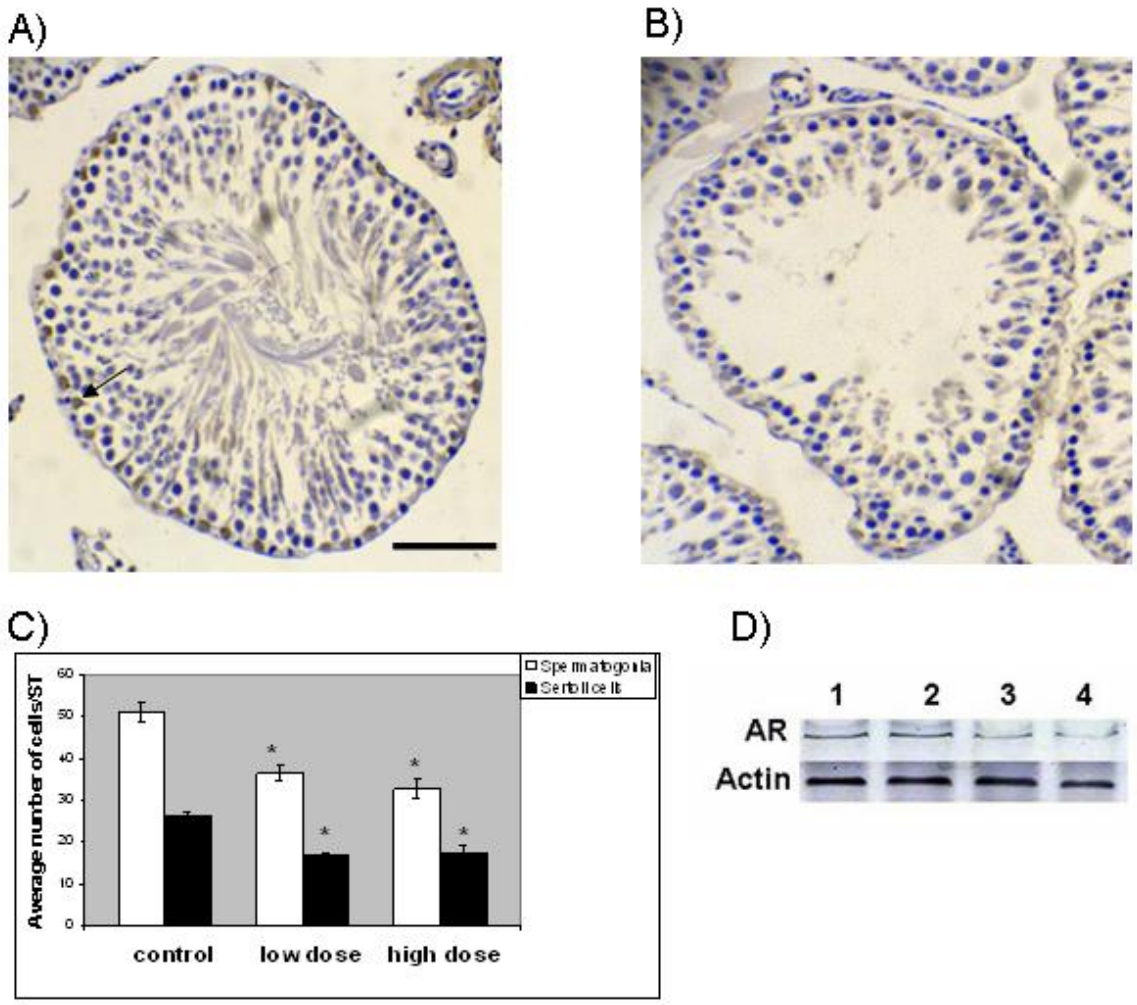

Figure 5. Effect of Nandrolone Decanoate administration on spermatogonia and Sertoli cells. A) A representative example of a cross section of a ST from control rat immunostained for androgen receptor, the outermost layer shows darkly stained spermatogonia and evenly placed Sertoli cells (arrow), B) A representative example of a cross section in ST of a low-dose treated rat immunostained for androgen receptor, showing reduced number of germ cells, and misplaced Sertoli cells. Scale bar = 50 µm. C) The number of spermatogonia and Sertoli cells in the ST of treated rats was significantly reduced as compared to the control group, values are expressed as mean ± SEM, n=5, **p*=0.001, D) Immunoblot analysis for AR levels. Testis lysates from control (lanes1, 2), low (lane 3) and high-dose receiving rats (lane 4) where immunoblotted with anti-AR antibody.

several scavenging enzymes [SOD, GPx, GR, catalase and hemoxygenase-1 (HO-1)] (Georgiou *et al.*, 1987; Diemer *et al.*, 2003; Chen *et al.*, 2005).

To our knowledge there is no available data that evaluates the effect of ND on sperm chromatin integrity. Sperm DNA fragmentation could be due to several reasons such as deficiency in recombination during spermatogenesis (Bannister and Schimenti, 2004). Exposure of mature spermatozoa to excessive levels of ROS produced by immature sperm during migration from the seminiferous tubules to the epididymis could also lead to fragmented DNA (Ollero *et al.*, 2001). In addition, abnormal spermatid maturation could result in DNA-fragmented sperm.

Around 80-90% of the weight of each testis consists of tightly packed ST (Greenspan and Gardner, 1994). The major supporters of spermatogenesis process are the Sertoli cells which take up a volume of around 17–19% of the seminiferous tubules of adult rats. Sertoli cells are the only somatic cells in direct connection to germ cells (Mruk and Yan, 2004).

Androgen action in the testis, as in other tissues, is mediated through androgen receptor (AR) transcriptional activation (Bremner *et al.*, 1994). Inside Sertoli cells, testosterone is selectively bound to the androgen receptor and activation of the receptor will result in initiation and maintenance of the spermatogenic process and inhibition of germ cell apoptosis (Dohle *et al.*, 2003). In testes, ARs are expressed in the somatic Leydig, peritubular myoid and Sertoli cells as well as to rete testis, the epithelial cells of the epididymis, and prostate (Vornberger *et al.*, 1994; Bilinska *et al.*, 2005).

AR expression is maintained by endogenous testicular androgens; withdrawal of testosterone is known to lead to disruption of spermatogenesis (Kerr *et al.,* 1985). After long-term hypophysectomy and elimination of residual testosterone, spermatogenesis rarely proceeds beyond meiosis, with very few round spermatids observed and elongated spermatids nearly non-existing (Franca *et al.,* 1998). In the current work, injection of male rats with low or high doses of ND resulted in reduction of testosterone, which caused maturation arrest at the primary

spermatocyte level (Fig.4D), and at the spermatid level (Fig.4E).

In addition, it was found that loss of AR activity from Sertoli cells would lead to spermatogenic failure resulting in incomplete meiosis and collapse to transition of spermatocytes to haploid round spermatids (Birgner et al., 2008; Holdcraft and Braun, 2008).In the current study, the immunoexpression of AR was scored as the number of positive nuclei within the boundaries of STs (Fig. 5A) and by immunoblotting (5D), and it was found that ND-administration caused a reduction in the number of Sertoli cells expressing AR (Fig.5C), which in turn could explain the maturation arrest observed in treated animals (Table 2) and testicular atrophy (Fig.4B).

Sertoli cells play an important role in organizing the somatic cell lineages and in determining the structure of the testis (McLaren, 2000); they also support a finite number of germ cells, and thus their number determines the spermatogenic capacity of the adult (Orth *et al*., 1988; Sharpe, 1994; McLachlan *et al.,* 1996). Our results indicate an AAS-induced reduction in Sertoli cell number (Fig.5C) which might be due to a structural response of Sertoli cells to deprivation of testosterone (Watanabe, 2005). The reduction in Sertoli cell number in treated rats could have resulted in a subsequent reduction in the number of spermatogonia (Fig.5C) leading eventually to a decrease in sperm count.

There are no reports on the effects of testosterone suppression on spermatogonia cell differentiation. In addition, the quantitative structural changes of the testis caused by AAS abuse received little or no attention. The current study reports a number of abnormalities in the architecture of the seminiferous tubules of treated rats (Table 2) namely atrophy in the ST (Fig. 4B), abnormal organization of the germinal epithelium (Fig. 4C), and maturation arrest (Fig. 4D and E).

Injection of male rats with low or high doses of ND caused a reduction in testicular volume as compared to control rats. Estimation of testicular volume is a good indicator of testicular atrophy, as evident in Fig. 4B. The decrease in testis volume might be a consequence of reduction in seminiferous tubules length (Noorafshan *et al.,* 2005), or could have resulted from a negative feedback on the hypothalamic-pituitary axis with consequent testicular atrophy (Dohle *et al.,* 2003). On the other hand, and as reported by others (Takahashi *et al.*, 2004), body weight of the experimental animals did not differ from controls over the route of treatment (Fig.1).

One final observation from the current work is that in some of the situations studied (effects of ND administration on TBRAS levels, motility and morphology of sperm, number of Sertoli cells and degree of late maturation arrest in ST), the actions of ND were directly receptor and dose dependent; the more drug injected the more adverse the side effect. When ND acts in a receptor mediated mode, we can assume that the high concentration of ND injected to rats can overcome the fact that AR has low affinity to ND (Saartok *et al.,* 1984). In contrast, the effects of ND injection on level of sperm DNA fragmentation, serum testosterone concentration, sperm concentration, and the degree of ST early maturation arrest were receptor- and dose-independent. This observation about ND action could provide some evidence that ND has two distinct modes of actions, receptor-dependent and independent. However, both of ND modes of action in the testicular tissue may be closely linked (Rommerts, 1998).

Androgen deficiency may have serious consequences in men, and this condition requires diagnosis and appropriate treatment. When administered properly, androgens are safe; however, when testosterone or its derivatives (such as AAS) are abused at supraphysiological doses, they may cause considerable harm. AAS are commonly used in our society, and physicians should be aware of their physiological effects. The present work reports that intramuscular injection of male rats with commonly used anabolic androgenic steroid ND (3 or 10 mg\kg) for 14 weeks was deleterious to the structure of rat testes. These effects included testicular atrophy, maturation arrest in seminiferous tubules, severe depletion of the absolute number of spermatogenic and Sertoli cells, and marked suppression of sperm count. In addition, ND administrations caused testosterone depression, enhanced lipid peroxidation, as well as severe fragmentation in the DNA of sperm of treated rats. Although the concentration of ND which was administered to male rats is comparable to what is injected by AAS abusers, we are aware that caution should be taken when such results are extrapolated from animal to man.Acknowledgements

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Impact of Magnetic Application on the Parameters Related to Growth of Chickpea (*Cicer arietinum* L.)

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Abstract

The morphological data were used in this study for the evaluation of five chickpea (*Cicer arietinum* L.) varieties at the University of Sulaimanyah, College of Agriculture in 2010. Seeds of different varieties of chickpea were exposed in batches to static magnetic fields (1500 gauss of magnetic force) for 30, 50 and 70 min. Then, the magnetic seed were placed between two layers of moist germination paper in petri dish. They were placed in the germination incubator at 20°C in an upright position. After 8 days, different plant growth parameters were tested such as germination percentage, root length, shoot length, root fresh weight, shoot fresh weight, root dry weight and shoot dry weight, based on normal seedlings. The results showed that magnetic field application enhanced seed performance in terms of laboratory germination: seedling length and seedling fresh and dry weight compared to unexposed control. However, the response varied with duration of exposure. Among the various duration exposures, 50 and 70 min. exposures gave best results. A pot experiment was carried out in a greenhouse was aimed at finding the effect of a constant magnetic field on the root and shoot system, as well as on yield of spring chickpea. Seeds are grown in plastic cups for 4 months and irrigated with magnetized water which prepared by using static magnetic field. Six to fourteen plants were tagged for morphological data collection. The results showed that magnetized seeds irrigated with magnetized water have enhanced seed performance in terms of plant height, number of brancha, number of leaves, number of leaflets root and shoot fresh weight, root and shoot dry weight, the total photosynthetic pigments (chlorophyll a, b, and carotenoids) and yield in some varieties.

الملخص

اجري البحث فى قسم المحاصيل الحقلية-كلية الزراعة- جامعة السليمانية لعام 2010 على خمسة أصناف من الحمص (Cicer arietinum L) للمعرفة تاثير المعالجة المغناطيسية لماء الرى والبذور على بعض صفات النمو المتعلقة بالبادرة و النبات الحمص.

استخدمت الاجهزة المغناطيسية بقطر 1 بوصة. اجريت عملية مغنطة البذور والماء بقوة 1500 كاوس للفترات 30 و 50 و 70 دقيقة. ووضعت البذور الممغنطة بين طبقتين من ورق رطبة داخل اطباق بترى. ووضعت الاطباق في حاضنة الإنبات عند 20 درجة مئوية. بعد 8 أيام ، تم قياس البادرات الطبيعية على أساس: نسبة الإنبات، طول الجذور ، طول الساق ، الوزن الطرى للجذر ، الوزن الطرى للمجموع خضرى، الوزن الجاف للجذر والوزن الجاف للمجموع خضرى. و أوضحت النتائج أن تطبيق المجال المغناطيسي ادت الى تحسن البذور من حيث المعايير المختبرية: طول البادرات، وزن الطرى والجاف للبادرات بشكل ملحوظ مقارنة مع البذور التي لم تتعرض للمعالجة المغناطيسية فى بعض الاصناف. ومع ذلك ، فسجلت استجابات مختلفه باختلاف مدة التعرض للمعالجة المغناطيسية. وكانت افضل النتائج عن المعاملة معالجة البذور مغناطيسياً لمدة 50 و 70 دقيقة (كل على حدة).

وتم اثناء البحث، زراعة بذور الحمص في البيوت الزجاجية داخل التربة وذلك لايجاد تأثير المعالجة المغناطيسية على النمو الجذرى والخضرى وانتاج الحمص. حيث زرعت البذور (بذور الممغنطة و بذور غير الممغنطة) في أكواب بلاستيكية لمدة 4 أشهر وسقيت النباتات مع الماء الممغنط (للبذور الممغنطة) والماء الحنفية (للبذور غير الممغنطة). وأستخدمت 6-14 نباتاً لجمع البيانات المورفولوجية. وأظهرت النتائج حصول زيادة معنوية في معدل ارتفاع النبات ، عدد الفروع، عدد الوريقات، عدد الاوراق، الوزن الطرى و الجاف للجذر، الوزن الطري والجاف للمجموع خضرى، صبغات التمثيل الضوئي (الكلوروفيل أ ، ب والكاروتينات) ، والانتاج البذور في بعض الأصناف من الحمص عند المعالجة المغناطيسية المزدوجة لماء الرى والبذور مقارنة مع البذور والماء غير الممغنطة.

Keywords: Chickpea, Magnetic field, growth parameter.

1. Introduction

Chickpea (*Cicer arietinum* L.), is the third most important cool season food legume in the world after dry beans and peas (FAOSTAT, 2006). Chickpea is a diploid with 2n = 2x = 16 (Arumuganathan and Earle, 1991) having a genome size of approximately 931 Mbp. Chickpea is a self-pollinated crop. Cross-pollination is rare; only 0-1 % is reported (Singh, 1987).

The genus *Cicer* belongs to the family Leguminosae, subfamily Papilionoideae, tribe *Cicereae* Alef and comprises 43 species, nine of which being annual including chickpea (*Cicer arietinum* L.), while the rest are perennial (Van der Maesen, 1987). Chickpea is currently

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cultivated in over 40 countries worldwide and grown on 11 million hectares producing around 9 million ton in 2005 growing season (FAOSTAT, 2006). Two main types of chickpea cultivars are grown globally– *kabuli* and *desi,* representing two diverse gene pools. The *kabuli* types are generally grown in the Mediterranean region including Southern Europe, Western Asia and Northern Africa and the *desi* types are grown mainly in Ethiopia and Indian subcontinent (Pundir *et al.,* 1985). Chickpea are grown usually as a rained cool-weather crop or as a dry climate crop in semi-arid regions.

Pre-sowing seed treatment including chemical and physical treatments like electrical, microwave and irradiation are known to improve seed performance. Physical methods are not only cost effective; they also significantly improve the yield without adversely affecting the environment. They influence the physiological and biochemical process in the seeds, and thereby contribute to greater vigor and improved crop stand. Therefore, physical pre-sowing seed treatment for enhancing the seed performance, if standardized, can lead to commercial application. Alexander and Doijode (1995) noted that aged onion (Allium cepa) and rice (Oryza sativa) seeds exposed to a weak electromagnetic field for 12 h increased the germination shoot and root length of seedlings. Celestino et al. (2000) reported enhanced germination and growth of Cork oak (Quercus suber) seedlings when exposed to chronic magnetic field. Harichand et al. (2002) reported that exposure of magnetic field (10 mT; 40 h) increased plant height, seed weight per spike and yield of wheat. Aladjadjiyan (2002) observed that the magnetic field stimulated the shoot development of maize and led to an increase in germinating energy, fresh weight and shoot length. Growth of the germinated Vicia faba seedlings was enhanced by the application of power frequency magnetic fields (100 mT) that were supported by increased mitotic index and 3H-thymidine uptake (Rajendra et al., 2005). In broad bean (Phaseolus lunatus) and pea (Pisum sativum*)* cultivars the magnetic stimulation of seeds improved the sprouting and emergence of seed and resulted in higher pod number and seed yield (Podlesny et al., 2005). Harichand et al. (2002) reported that exposure of magnetic field (10 mT; 40 h) increased plant height, seed weight per spike and yield of wheat (Triticum aestivum).

Magnetic treatment of water irrigation is an acknowledged technique for achieving high water use efficiencies due to its effect on some physical and chemical properties of water and soil (Noran *et al.,* 1996). These changes result in an increased ability of soil to get rid of salt and consequently better assimilation of nutrients and fertilizers in plant during the vegetative growth period. Magnetizing methods among different physical and chemical methods of natural water treatment attract a special attention due to their ecological purity, safety, and simplicity. Magnetically treated water (MTW) is the water that is subjected to treatment by a magnetic field. The use of MTW is common in various branches of industry as a precaution against accumulation of scale in the water supply system, cooling tower, thermal and solar heating installation (Lin and Yotvat, 1989). The major objective of this study is to: evaluate the effect of magnetic application on some growth parameters of some chickpea varieties.

2. Materials and Methods

2.1. Plant material

The plant material comprised of five varieties of chickpea including Rania, Chamchamal, Sangaw, FLIP98-133C (screened for their very sensitivity to *Ascochyta rabiei*) and FLIP83-48C (screened for their resistance to *Ascochyta rabiei*). All of the above material was obtained from Sulaimanyah Agricultural Research Center, Sulaimanyah, Iraq. Healthy seeds with identical dimensions were selected by visual observation.

2.2. Magnetic treatment

2.2.1. Germination characteristic

This research was carried out in the 2009-2010 at College of Agriculture, University of Sulaimanyah to determine the impact of magnetic application on five chickpea varieties. A complete randomized design with three replications was used. Chickpea seeds were placed in magnetron (one inch of diameter and 1500 gauss of magnetic force as shown in Fig. 1) in a cylindrical shaped sample holder. One hundred visibly mature, healthy seeds were treated by magnetic field for various durations ranging from 30 min (T30), 50 min (T50) and 70 min (T70). Seed germination was achieved in three replications each with 15 seeds placed on two layers of moist filter paper in Petri dishes (imbibed with 12 ml of magnetized water). They were placed in the germination incubator at 20 °C in an upright position. After 8 days, germinated seeds were grouped as normal, abnormal seedling, fresh ungerminated and dead seeds. Germination percentage was calculated based on normal seedlings. The seedlings from each replicate were randomly taken for measuring shoot and root length (using a ruler), and shoot and root fresh weight. Subsequently, they were dried in an oven at 90 °C for 48h and the dry weight of these seedlings was measured.

2.2.2. Greenhouse experiment

This research was carried out in 2010 (15.01.2010-18.05.2010) at the College of Agriculture, University of Sulaimanyah so as to determine the impact of magnetic application on five chickpea varieties grown under greenhouse conditions. A complete randomized design with ten replications was used. Two groups of chickpea (*Cicer arietinum L.*) seeds (magnetized group and control group for each variety) are selected with twenty seeds for each variety. Two seeds were sown (in 15.01.2010) in a plastic cup (25 cm height, 7 cm diameter) in 3 cm depth of soil containing mix (2 soil: 1 peat moss). Group (1) contained ten plastic cups (2 seeds/plastic cup) containing magnetized seed and irrigated with magnetized water, while group (2) contained ten plastic cups (2 seeds/plastic cup) containing unmagnetized seeds and irrigated with tap water. Irrigation was provided as and when required. The plastic cups were maintained in greenhouse under natural light. After four days the seedlings started to emerge over the soil level.

2.3. Quantitative morphological traits

Growth and developmental characteristics, including, plant height, number of primary branches, leaves, leaflets,

roots, fresh and dry weight of root and shoot, total pigment (Carotene + Chlorophylls a and b) of 28 days old plants were measured:

1. Plant height: Height of plants (cm) from ground to the highest part of the plant by using a ruler.
2. Number of primary branches: Actual counts of primary branches on the main stem per plants.
3. Number of leaves and leaflets: Actual counts of number of leaves and leaflets per plant.
4. Root biomass: Weight (g) of fresh and dry root per plant.
5. Shoot biomass: Weight (g) of fresh and dry shoot per plant
6. Total pigments (Carotene + Chlorophylls a and b)
7. Grain yield: Dried weight (g) of seed per plant at 12% moisture content.

2.4. Chlorophyll and carotene assay

Photosynthetic pigments were extracted according to Mochizuki method (Mochizuki *et al.,* 2001). One gram of fresh leaves of 28 days old plants (mixture of small specimens picked up from all the 14 plants) were ground in liquid nitrogen, using a mortar and pestle. Ten milliliter of 80% acetone was added to a 15 ml Falcon tube, and mixed in dark for 15 min (note: chlorophylls degrade under light). The mixture was filtered through two Whatman filter papers. The absorbance (A) of chlorophyll and carotene content was measured, with three replications, at three-wave lengths 470, 646 and 663 using spectrophotometry. The chlorophyll and carotene concentrations are calculated as follows:

Chlorophyll a (mg/g) = [12.21× A663 - 2.81 × A646] ×V/1000×W

Chlorophyll b (mg/g) = [20.13 × A646 - 5.03 × A663] ×V/1000×W

Carotenoids (mg/g) = [1000 × A470 – 3.27 × (Chl a) – [104 × (Chl b)/227] ×V/1000×W

Total pigments = chlorophyll a + chlorophyll b + carotenoids

Where V = volume of the extract (ml); W = Weight of fresh leaves (g).

Figure 1: Showing Magnetron apparatus.

2.5. Statistical analysis

Statistical analyses were conducted using SPSS for windows (version 18). The variance analyses (ANOVA) was used to test the main effects of magnetic field. The Duncan's test was done to find the significant differences between each magnetic treatment and control at level 5% (Levesque, 2007).

3. Results and Discussion

3.1. Seedling growth (laboratory experiment)

Concerning the germination percentage, exposure of chickpea seeds to different time of magnetic field did not show significant difference between the control and others treatments for all varieties (Table 1). Other developmental growth parameters including: root length of 8 days seedlings, showed significant differences among the treatments: Control, T30, T50 and T70 for Chamchamal, Sangaw and FLIP98-133C, while Rania and FLIP83-48C revealed non-significant difference among the treatments: Control, T30, T50 and T70 (Table 1). From these results, it was found that the average length of root of Chamchamal, Sangaw and FLIP98-133C was more than the average length of control. The improvement root length over untreated seeds (control seeds) was 40-45% for the varieties Chamchamal and Sangaw.

The results revealed that shoot length was increased in treatment T50 for Rania, Chamchamal and Sangaw (Table 1). There was no significant difference among the treatments: Control, T30, T50 and T70 for FLIP83-48C. On the other hand, the treatment T70 shared the maximum shoot length for FLIP98-133C. The improvement over control (untreated seeds) was 73 and 26 % for the varieties: Chamchamal and Sangaw respectively (Table 1). For root fresh weight, significant positive value was observed among the treatments: Control, T30, T50 and T70 in all varieties except FLIP83-48C. The value of improvement, when compared with the control (untreated seeds), varied between 40 to 80% (Table 1).

The parameter, shoot fresh weight, showed the significant positive value for the varieties: Chamchamal and FLIP98-133C, when compared with the control (untreated seeds). The percentage of improvement was 50-90% (Table 1). Root dry weight was significantly higher than the control in most of the treatments for all varieties except Rania. The percentage of improvement was 40-50% when compared to the control (Table 2). The average seedling shoot dry weights are tending to increase in treatments: T30, T50 and T70 for Chamchamal, T50 for Sangaw and T70 for FLIP98-133C (Table 2).

Table 1: Effect of seed pretreatment by magnetic field and its duration on germination characteristic: germination percentage, root length, shoot length, root fresh weight and shoot fresh weight.

Germination percentage (%)					
Treatments	Rania	Chamchamal	Sangaw	FLIP98-133C	FLIP83-48C
Control	90.33a	93.33a	95.55a	73.33a	93.33a
T30	92.33a	93.33a	93.33a	73.33a	93.33a
T50	95.55a	93.33a	95.55a	86.66a	93.33a
T70	93.33a	95.55a	95.55a	82.22a	91.11a
Root length (cm)/plant					
Control	4.18a	4.45b	4.84b	3.59a	1.81a
T30	4.26a	5.54ab	4.98b	2.81b	1.57a
T50	4.13a	6.18a	5.58ab	2.88b	1.94a
T70	4.65a	6.48a	6.54a	3.64a	1.78a
Shoot length (cm)/plant					
Control	2.26ab	2.10b	2.61b	1.84b	1.22a
T30	2.04b	3.28a	2.63b	1.62b	1.11a
T50	2.64a	3.92a	3.41a	1.84b	1.18a
T70	2.37ab	3.65a	3.29a	2.75a	1.19a
Root fresh weight (mg)/plant					
Control	114.23b	70.12b	132.26b	92.56b	68.21a
T30	116.9b	85.53b	154.1ab	88.9b	61.23a
T50	131.26a	134a	176.76a	102.5b	63.13a
T70	136.26a	135.7a	187.8a	129.33a	56.9a
Shoot fresh weight (mg)/plant					
Control	62.96ab	46.43c	70.13b	41.53b	32.55a
T30	58.36b	77.3b	74.05b	36.26b	39.66a
T50	75.36a	98.03a	120.03a	45.16b	34.4a
T70	61.1ab	88.33ab	89.26b	60.06a	36.23a

*Means designated with the same letter (s) do not differ significantly from each other according to Duncan's multiple range tests, $p \leq 0.05$.

Table 2: Effect of seed pretreatment by magnetic treatment and its duration on germination characteristic: root dry weight and shoot dry weight.

Root dry weight (mg)/plant					
Treatments	Rania	Chamchamal	Sangaw	FLIP98-133C	FLIP83-48C
Control	14.45a	11.31b	11.88b	10.54b	5.78ab
T30	14.253a	14.14a	13.97ab	11.05b	4.95b
T50	14.98a	14.92a	15.44a	11.83ab	6.29a
T70	14.60a	14.92a	14.54a	14.13a	5.84ab
Shoot dry weight (mg)/plant					
Control	8.14ab	7.33b	8.41b	5.69b	4.24a
T30	8.19ab	10.35a	9.37b	5.73b	4.39a
T50	8.8a	11.79a	11.99a	5.93b	4.36a
T70	7.85b	10.02a	9.86b	7.54a	4.70a

*Means designated with the same letter (s) do not differ significantly from each other according to Duncan's multiple range tests, $p \leq 0.05$.

Researchers carried out an experiment study on water absorption by lettuce seeds previously treated in a stationary magnetic field of 1 to 10 mT. They reported an increase in water uptake rate due to the applied magnetic field, which may explain the increase in the germination seed of treated lettuce seeds (Calatayud *et al.,* 2003). Lentil seedlings from magnetically pretreated seeds grew better than the untreated, and also biomass and root growth were significantly increased (Ahmad *et al.,* 2009). Biomass increase needs metabolic changes particularly increasing protein synthesis (Lebedev and Litvinenko, 1977). The mechanism of stimulating effect of magnetic field-treatment on seed germination and seedling growth was unknown. Although most seemed to involve changes in intracellular levels of Ca^{+2} and in other ionic current density across cellular membrane (Florez *et al.,* 2004) which caused alteration in osmotic pressure and changes in capacity of cellular tissues to absorb water (Calatayud *et al.,* 2003). Magnetic fields can remove bounded of Ca^{+2} from cell membrane which is essential for the stability of membranes. Consequently, their loss will increase temporary pore formation under the mechanical stresses from pressure differences within cell and abrasion by its moving content. The magnetic field could increase an inner energy which is distributed among the atoms causing accelerated metabolism (Campbell, 1977).

In conclusion, the magnetic field pre-treatment for 50 or 70 min enhanced root length, shoot length, root fresh weight, shoot fresh weight, root dry weight and shoot dry weight compared with the control. Seed germination percentage did not show any significant differences between the treatments times for all varieties.

3.2. Greenhouse experiment

Cumulative plant growth data (plant height, number of branch, number of leaves, number of leaflets and total pigments) were measured. The most significant different growth was observed in plants grown from magnetized seeds and water for the treatments T50 and T70 (Tables 3 and 4). Growth data were measured onthe 28th days after seeding. The greatest plant height was observed in plants grown up from seeds magnetized for 50 (T50) and 70 min (T70) and irrigated with magnetized water for all varieties of chickpea except Rania (Table 3). This result was in agreement with that obtained by Algozari and Yao (2006) in the increasing of plant height as a result of magnetizing of seeds and water. They reported that the magnetic application lead to easy breakthrough of water for the cell membrane of plants. The easy breakthrough of water leads to better absorption of water and mineral by plant roots (Barefoot and Reich, 1992). Kronenberg *et al.* (2005) showed that the magnetic application lead to an increase in the availability of minerals in soil through the increasing of solubility of salts and minerals. The increasing of solubility of salts and minerals lead to the increasing of macro and micro elements from soil and division and elongation of cell during the plant growth. However, it was found that the pretreatment of seeds for 50 (T50) and 70 min (T70) by magnetic field and irrigation by magnetized water had more growth in branch compared with the control plants in all varieties except FLIP98-133C and FLIP83-48C (Table 3). Also these data allow us to find significant differences among the treatments: Control, T30, T50 and T70 for the number of leaves and leaflets of chickpea plants (Tables 3). Total pigments content (Chlorophylls a, b and Carotene) increased significantly (20-25%) for Chamchamal, FLIP98-133C and FLIP83-48C with respect to control groups at T30 and T50. On the other hand, Rania did not show significance difference between the control and the magnetic treatments: T30, T50 and T70 (Table 3). Total fresh weight (biomass) of plants was measured on the 28th days after planting (Table 4). Shoot fresh weight, root fresh weight, root dry weight and shoot dry weight were significantly affected by magnetic application compared with the control. Significant genotypic variability was detected in all genotypes except Rania for shoot fresh weight (Table 4). Comparatively higher shoot fresh weight was obtained at magnetic treatment T70. The greatest increase (improved value) in shoot fresh weight was recorded with Chamchamal at T70. Analysis of variance for root fresh weight traits computed from the chickpea, treated or non-treated by magnetic

Table 3: Effect of magnetized seed and water on plant height, number of branch, number of leaves, number of leaflets and total pigments (Chlorophyll a + Chlorophyll b + Carotene) of chickpea grown in soil under greenhouse condition.

Plant height (cm)/plant					
Treatments	Rania	Chamchamal	Sangaw	FLIP98-133C	FLIP83-48C
Control	17.35a	15.44b	14.93b	13.80b	13.40b
T30	16.52a	16.58ab	15.42b	15.26ab	14.97ab
T50	16.37a	17.52a	16.99ab	15.69a	16.58a
T70	17.85a	17.97a	18.13a	14.34ab	15.61ab
Number of branch/plant					
Control	3.13b	3.13b	4.1b	4.4a	3.83a
T30	3.7ab	3.73a	4.13b	4.26a	3.96a
T50	3.43ab	3.86a	4.33ab	4.36a	4.3a
T70	3.9a	3.76a	4.66a	4.56a	4.23a
Number of leaves/plant					
Control	12.5ab	11.7b	16.83b	15.76b	14.43b
T30	11.73b	11.6b	17.06b	16.2b	15.8ab
T50	14a	14.86a	19.73a	19.06a	17.7a
T70	13.93a	15.23a	20.73a	18.86a	16.33ab
Number of leaflets/plant					
Control	79.26b	88.26bc	102.93b	82.36b	75.9c
T30	79.7b	77.46c	101.46b	82.6b	88.96bc
T50	89.33ab	98.96ab	114.86ab	111.83a	105.96a
T70	96.1a	109.16a	122.8a	103.16a	96.96ab
Total pigments (Chlorophyll a, b + Carotene) (mg/g fresh weight)/plant					
Control	0.253a	0.199bc	0.220a	0.201d	0.165b
T30	0.258a	0.189c	0.218a	0.235b	0.202a
T50	0.241a	0.240a	0.211a	0.236a	0.167b
T70	0.251a	0.201b	0.201b	0.219c	0.161c

*Means designated with the same letter (s) do not differ significantly from each other according to Duncan's multiple range tests, $p \leq 0.05$.

Table 4: Effect of magnetized seed and water on shoot fresh weight, root fresh weight, shoot dry weight, root dry weight and seed yield/plant of chickpea grown in soil under greenhouse condition.

Treatments	Rania	Chamchamal	Sangaw	FLIP98-133C	FLIP83-48C
Shoot fresh weight (g)/plant					
Control	2.79a	1.63b	3.17b	2.07c	2.36c
T30	2.68a	1.5c	3.4a	2.19b	2.5b
T50	2.87a	2.75a	2.95c	2.23ab	3a
T70	2.67a	2.83a	3.03bc	2.29a	3.03a
Root fresh weight (g)/plant					
Control	4.83b	3.55c	5.11b	3.45d	4c
T30	4.66b	3.20c	5.33b	4.2c	5.23ab
T50	6.86a	4.2b	5.05b	4.48b	5.35a
T70	5.55b	4.83a	5.73a	5.46a	4.86b
Shoot dry weight (g)/plant					
Control	0.359c	0.18d	0.57b	0.43b	0.43a
T30	0.425b	0.31c	0.601b	0.44b	0.46a
T50	0.453a	0.44b	0.605b	0.47a	0.46a
T70	0.445ab	0.48a	0.79a	0.44b	0.47a
Root dry weight (g)/plant					
Control	0.51c	0.31b	0.46b	0.27d	0.259c
T30	0.515bc	0.25c	0.47b	0.32c	0.40a
T50	0.525b	0.32b	0.50b	0.44a	0.36b
T70	0.67a	0.42a	0.589a	0.42b	0.38b
Seed yield (g)/plant					
Control	3.05c	3.01c	3.41b	2.61b	2.28d
T30	3.27b	3.2b	3.28b	2.76b	2.93c
T50	4.00a	3.76a	4.18a	3.36a	3.1b
T70	3.98a	3.65a	4.15a	3.33a	3.22a

*Means designated with the same letter (s) do not differ significantly from each other according to Duncan's multiple range tests, $p \leq 0.05$.

field, showed significance differences. The highest increased in root fresh weight was recorded by FLIP98-133C at T70 compared with the control (Table 4). Significant genotypic variability was detected in all genotypes except FLIP83-48C for shoot dry weight. The greatest increase (improved value) in shoot dry weight was recorded by Chamchamal at T70 compared with the control (Table 4). Analysis of variance for root dry weight traits computed from the chickpea treated or non-treated by magnetic field, showed significance differences. The highest increase in root dry weight was recorded by FLIP98-133C at T70 compared with the control (Table 4). The current study showed that seeds yield per plant was significantly affected by the duration of exposure to magnetic field as indicated by the significant one-way based on ANOVA ($p< 0.05$) (Table 4). Rania (T50), Chamchamal (T50), Sangaw (T50), FLIP98-133C (T50) and FLIP83-48C (T70) showed an increase of 31, 25, 23, 29 and 41% respectively of seeds yield compared with the control (Table 4). The increasing of seed yield as a result of increased plant growth and the effectiveness of the shoots in the photosynthesis process (Kronenberg et al., 2005).

The results obtained in this growth test allow us to conclude that magnetic treatment improves the stages of growth in higher plants. In general the seedlings from seeds magnetically pretreated grew taller and heavier than untreated controls. These seedlings showed greatly improved root characteristics. The stimulatory effect of the application of different magnetic times on the growth data

reported in this study was in agreement with that obtained by other researchers. Florez *et al.* (2007) observed an increase in the initial growth stages and an early sprouting of maize and rice seeds exposed to 125 and 250mT stationary magnetic field. Martinez *et al.* (2002) observed similar effects on wheat and barley seeds magnetically treated. The mechanisms are not well known yet, but several theories have been proposed, including biochemical changes or altered enzyme activities by Phirke *et al.* (1996). Chickpea seedlings from magnetically pretreated seeds grew more than the untreated, and also biomass and root growth were significantly increased.

Magnetic field is known as an environmental factor which affects gene expression. Therefore, augmentation of biological reactions like protein synthesis, biomass would increase too. Moreover, transcriptional factors are under effect of magnetic field stimulation (Xi and Ling, 1994). The effects of magnetic fields are superimposed on endogenous rhythms in some situations leading to inhibition, and in others to stimulation, while sometimes no effects had been reported. Moreover, magnetic fields have the ability to change water properties, thus magnetized water increased rice chlorophyll content (Tian *et al.,* 1989). Similary, Racuciu *et al.* (2008) reported that long magnetic field exposure has the ability to increase assimilatory pigments. This fact was confirmed by several studies for different plants where magnetic field treatment increased the chlorophyll content in sugar beet (*Beta vulgaris* L.) leaves (Rochalska, 2005). Additionally, studies by Atak *et al.* (2007) involving magnetic field impact on soybean (*Glycine max* L.) confirmed that magnetic field significantly increased chlorophyll a, chlorophyll b and total chlorophyll contents. Whereas, magnetic field short exposure is accompanied with increases in chlorophyll a, chlorophyll b and total chlorophyll contents (Atak *et al*., 2000). The reduction of pigments explained by Commoner *et al.* (1956), that chemical with unpaired electrons possess a magnetic moment plays an important role in electron transfer and kinetics of chemical reactions. The electrons with magnetic moments can be oriented in the external magnetic field. As a result of the interaction between the external magnetic field and the magnetic moment of unpaired electrons, the energy is absorbed. Chloroplasts have magnetic moments and could be affected by the absorbed energy at a high dose of magnetic field which can disturb the pigments synthesis. Other possible explanations for the decline in pigments content are that carotenoids may be consumed in radical scavenging reactions (Strzalka *et al.,* 2003), or free radicals inhibited the pigments synthesis through affecting photosynthesis enzymes.

In conclusion, magnetic field could be used as a stimulator for growth related reactions. Photosynthetic pigments content have shown a significant increase in response to magnetic fields at low dose. Short exposure to alternating magnetic field had a positive impact, whereas long exposure had a negative effect on pigments content similar to magnetic field effect on proline (Dhawi Al-Khayri, 2008). Using magnetic field treatment could be a promising technique for agricultural improvements but extensive research is required, using different levels of magnetic field doses to determine the optimum dose. We can conclude that exposure of dry chickpea seeds to static magnetic field for different durations significantly increased laboratory germination characteristics. On the other hand, the exposure of seed to magnetic field and irrigation with magnetized water revealed the stimulatory influence on the following growth characters: significant enhancement of the fresh tissue mass, assimilated total pigments level, increase of the average plants height, number of branch, number of leaf and leaflet in some varieties of chickpea.

In conclusion, the exposure of seed to magnetic field and irrigation with magnetized water revealed the stimulatory influence on the plants: significant enhancement of the fresh tissue mass, assimilatory total pigments level, increase of the average plants height, number of branch, number of leaves and leaflets in some varieties of chickpea. We recommend the following:

1. Studying the effect of magnetic field on the flowering.
2. Evaluation of expression of genes, total proteins and metabolic compounds related to the growth.
3. Studying the effect of magnetic field on the resistance to biotic (*Ascochyta rabiei*) and abiotic stress (Drought).

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In vitro Study of Adult Bone Marrow Mesenchymal Stem Cells Derived from Albino Rats and their Cardiomyogenic Differentiation

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Abstract

Mesenchymal stem cells (MSCs) are pluripotent adult stem cells residing within the bone marrow (BM) capable of self renewal, production of large number of differentiated progeny and regeneration of tissues. This study aimed at isolating the adult rats BM-MSCs and stimulating their *in vitro* differentiation into cardiomocytes. The BM, isolated from young male white rats *(Rattus norvegicus albinos)* and maintained in culture at 37C˚ and 5%CO_2. The culture system which permits to direct differentiation of the MSCs into cardiomyocyte-like characteristics was treated with rat embryonic heart muscle extract (HME), and differentiated cells were detected with immunocytochemistry examination using anti - myosin and anti - cardiotin antibodies as markers. The MSCs had fibroblast like morphology before HME treatment, but their morphology began to change after treatment. Treated MSCs increased in size and had formed a ball and a stick like morphology and were connecting with adjoining cells after one week and began to form myotube like structures at the end of second week. After three weeks of treatment, the differentiated cells could be distinguished by the presence of a number of branches and began to interface with each other to form cardiac like cells. The differentiated cells were positive for myosin and cardiotin. The morphological changes were not seen in control cells. We can conclude that HME may contain certain growth factors that stimulate the differentiation of BM-MSCs to the muscular pathway.

الملخص

تتواجد الخلايا الجذعية اللحمية ضمن البيئة الدقيقة لنقي العظم وهي خلايا جذعيه بالغة متعددة القوى، لها القدرة على التكاثر والتجدد وتكوين أعداد كبيرة من الخلايا المتمايزة إضافة إلى قابليتها على تجديد الأنسجة التالفة. تهدف هذه الدراسة إلى عزل وإنماء الخلايا الجذعية اللحمية البالغة لنقي عظم الجرذان ومن ثم تحفيز نموها وتمايزها خارج الجسم الحي إلى الخلايا العضلية القلبية. عزلت الخلايا الجذعية اللحمية لنقي العظم من ذكور الجرذان البيضاء اليافعة *(Rattus norvegicus albinos)* وتم إدامتها في الوسط ألزرعي وبدرجة حرارة 37C˚ و 5% غاز ثاني اوكسيد الكربون.ولتوجيه تمايز هذه الخلايا إلى خلايا شبيهة بالخلايا العضلية القلبية تم معاملتها بمستخلص عضلات قلوب أجنة الجرذان. وبعدها تم الكشف على هذه الخلايا المتمايزة باستعمال الفحص الكيميائي الخلوي المناعي وذلك باستعمال معلمات (واسمات) متخصصة لذلك مثل anti-myosin and anti-cardiotin. تبدو الخلايا الجذعية اللحمية قبل المعاملة بمستخلص عضلات القلب بشكل شبيه بالأرومة الليفية ولكن يبدأ شكلها بالتغاير بعد المعاملة ، حيث تبدأ الخلايا بالزيادة في حجمها وتكون شكل شبيه بالكرة ومن ثم يتغير شكلها ويصبح شبيه بالقضيب وبعد أسبوع واحد من المعاملة تبدأ هذه الخلايا بالارتباط بالخلايا المجاورة لها ، وفي نهاية الأسبوع الثاني تبدأ بتكوين تراكيب شبيهة بالأنبوب العضلي، ولكن بعد ثلاثة أسابيع يصبح بالإمكان تمييز هذه الخلايا المتمايزة وذلك بوجود عدد من التفرعات المتصلة مكونة خلايا شبيهة بالخلايا القلبية. ولكن هذه التغيرات لم تلاحظ في مجموعة السيطرة. أظهرت الخلايا المتمايزة استجابة موجبة لكلا النوعين من الواسمات. من خلال نتائج هذه الدراسة ممكن أن نستنتج بان مستخلص عضلات القلب قد يحتوي على عوامل نمو معينة والتي بدورها تقوم بتحفيز تمايز الخلايا الجذعية اللحمية لنقي العظم إلى الاتجاه العضلي.

Keywords: Bone marrow, Cardiomyogenic differentiation, Mesenchymal stem cells, Cell culture..

1. Introduction

Bone marrow stem cells (BMSCs) have myogenic potential and are therefore promising candidates for multiple cell-based therapies for myocardial diseases (Orlic *et al*., 2001). Recent attention has focused on BM as a source of stem cells which can be collected from adults and used for transplantation without posing ethical questions or creating problems of tissue matching and rejection (Hassink *et al*., 2003).

Mesenchymal stem cells (MSCs) are pluripotent adult stem cells residing within the BM microenvironment (Makino *et al*., 1999). Mesenchymal stem cells have an adherent nature and are expandable in culture and can differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells (Prockop, 1997) and cardiomyocytes (Wang *et al*., 2000). Cardiomyogenic differentiation of stem cells has been vastly reported (Tomita *et al*., 1999; Kehat *et al*., 2001). The cells undergoing cardiomyogenic

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differentiation achieve the cardiomyocyte phenotype through the expression of specific genes encoding various transcriptional factors and structural and regulatory proteins (Peng *et al*., 2002).

The potential of MSCs to differentiate into myogenic cells was first reported by (Wakitani *et al*., 1995) and then by a number of other investigators (Tomita *et al*., 1999; 2002; Xu *et al*., 2004). Early *in vitro* studies of the primary culture of rat MSCs treated with 10µmol/L 5-azacytidinem (a DNA demethylating chemical compound used to induce cardiomyogenic differentiation), showed that after 2 weeks of treatment, murine BM-MSCs formed myotube like structure and expressed myocardial specific proteins, such as cardiac troponin I and cardiac myosin heavy chain (MHC). These data suggest that stromal stem cells including MSCs differentiate into cardiomyocytes under appropriate culture conditions (Tomita *et al*., 1999). The same results were also demonstrated by Makino *et al*., 1999; Fukuda, 2001 and Bittira *et al*., 2002. At a concentration of 3 µmol/L for 1 week, 5-azacytidine induced BMCs into cardiomyogenic cells. These cells stained positive for myosin, actin and desmin and showed spontaneous beating at 3 weeks after treatment. Electron microscopy revealed a cardiomyocyte like structure including typical sarcomeres, a centrally positioned nucleus and a trial granule.

An interesting study from Xu *et al*., 2004 showed that the MSCs of human BM when treated with 10µmol/L 5-azacytidine appeared spindle shaped with irregular processes and the myogenic cells differentiated from MSCs were positive for beta–MHC, desmin and alpha cardiac actin.

After-wards, various strategies have been adopted for directed differentiation of BM-MSCs into cardiomyocytes by culturing BM-MSCs in *vitro* using culture media supplemented with retinoic acid (RA), dimethyl sulphoxide (DMSO) and 5-azacytidine (Heng *et al*., 2004; Antonitsis *et al*.,2007). The study of AL-Jumely, (2006) showed that mice BM-Hematopoetic stem cells (HSCs) when treated with heart muscle extract (HME) of newborn mice differentiated into muscular pathway *in vitro*, and these cells are stained positive for myosin.

The current study aimed at isolating and cultivating the BM-MSCs from young rats and stimulating their growth and differentiation *in vitro* into cardiomyocytes using HME.

2. Materials and Methods

Young male white rats (50-55 day old) *(Rattus norvegicus albinos)* weighing 180-200gm were used for the isolation of MSCs from the BM. These animals were obtained from the animal breeding house of the Medical Research Unit - College of Medicine - Al-Nahrain University - Baghdad - Iraq.

2.1. Isolation of bone marrow derived mesenchymal cells

Bone marrow derived mesenchymal stem cells cultures were prepared according to the protocol of Wakitani *et al*., 1995. Briefly, under sterile conditions, the femur and tibiae of the rats were excised, with special attention given to remove all connective tissue attached to bones. Bone marrow was extruded from these bones by flushing the BM cavity using a syringe with 20-gauge needle filled with culture medium (Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS)). The harvested BMCs were gently pipetted to break up cell clumps in order to obtain cell suspension. After a homogenous cell suspension was achieved, the cells were centrifuged at 2000 rpm for 10 minutes and the cell pellet was resuspended in 3ml of culture medium.

The cell suspension was loaded carefully onto 5ml of 60% percoll in sterile conical tube, and then centrifuged for 20-25 minutes at 2000 rpm at 8Cº. The mononuclear cells (MNCs) were retrieved from the buffy coat layer by sterile Pasteur pipette and placed in 5ml sterile conical tube. The cells were washed two to three times with PBS to remove the percoll and centrifuged at 2000 rpm for 10 minutes at 8 Cº.

2.2. Culturing and expansion of MSCs

The cell suspension was seeded in 50cm plastic tissue culture flasks with 5 ml culture medium and maintained at 37C˚ in a humidified atmosphere with 5% CO_2 for two weeks. Cultures of MSCs were inspected and refeed every three days and passaged when the MSCs have reached approximately 80% confluence (Javazon *et al*., 2001). The mesenchymal population was isolated on the basis of its ability to adhere to the culture plate.

2.3. Cardiomyocytes differentiation of BM-MSCs in vitro:

To stimulate the differentiation of MSCs *in vitro* to the cardiomyocytes progeny, the second passage of rat BM-MSCs were resuspended after trypsin treatment and seeded into 4-well culture plates at a density of $1X10^6$ cell \ well. The second day after seeding, the tissue culture plates were divided into two groups as follows:

- Control group: which were treated with MEM +10% FCS only without HME.
- Treated group: which were treated with MEM +10% FCS and 0.1µl HME per 1ml medium. The cells of this group were cultured for three different periods: 1week, 2weeks and 3weeks.

The HME were prepared from the hearts of rat embryos of (18-19 days old) following the general principle of embryo extract preparation as described by New (1966) and modified by (Hammash and Waheed, 2004; and AL-Jumely, 2006).

The medium was changed twice a week until the experiment was terminated. After that, the changes in morphology of treated cultures were reported and compared with the untreated (control) cultures. The cells in both groups were fixed with 4% phosphate buffered formalin for 10 minutes. The cells were detected using immunocytochemistry examination which was performed with primary monoclonal antibodies against anti-cardiotin and anti-myosin (Pochampally *et al*., 2004; Xu *et al*., 2004).

3. Results

3.1. Effect of HME treatment on MSCs

The results of the current study showed that the MSCs before HME treatment appeared elongated and flattened with a fibroblast like morphology (spindle like uni-polar or bipolar shape) (Fig. 1A). But after HME treatment, the

cells began to proliferate and differentiate during the first week (Fig. 1B). Approximately, 50% of all adherent cells had enlarged or increased in size and had formed a ball like appearance (Fig. 1C) or lengthened in one direction and formed a stick like morphology, these cells began to connect with adjoining cells in one week (Fig. 1D).

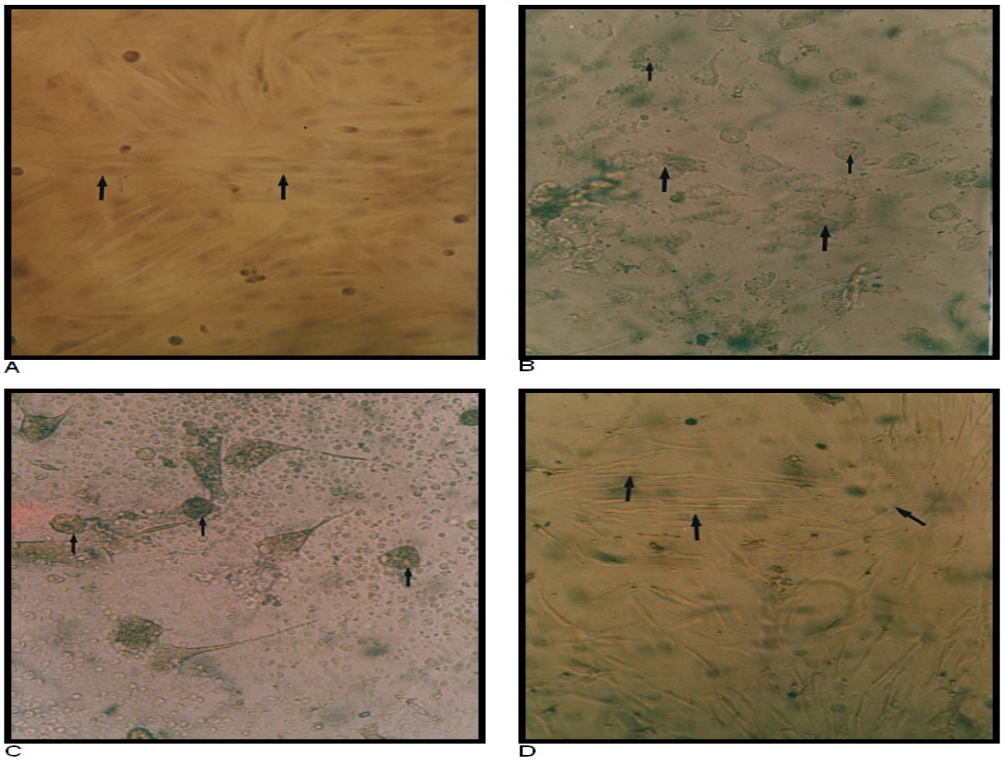

Figure1: The morphology of differentiated MSCs *in vitro* in the first week of the second passage (before and after treatment with HME). (A): MSCs had fibroblast-like morphology (arrows) before HME treatment (X100.8). (B): the cells treated with HME began to proliferate (arrows) (X160). (C): some of adherent cells treated with HME enlarged and formed a ball-like appearance (arrows) (X160). (D): most of adherent cells lengthened in one direction and had formed stick-like morphology (arrows) and began to connect with adjoining cells (X100.8).

At the end of second week, the cells began to connect to each others and then formed myotube-like structure (Fig. 2A). After three weeks, we noticed that most of the cells are mononuclear and some of them are binuclear but a few are extremely multinucleated (Fig. 2 B, C). The differentiated cells can be distinguished by the presence of a number of branches (Fig. 3 A, B), and these cells began to interface with each other to form cardiac-like cells (Fig. 3 C). These morphologies changes of BM-MSCs during exposure to HME in treated groups during different exposed periods were not seen in control groups.

3.2. Immunocytochemical Examination for Differentiation of Mesenchymal Stem Cells in vitro

To determine if HME treatment can induce MSCs to express cardiac muscle specific or related proteins, an immunocytochemistry examination was performed with monoclonal antibodies against myosin and cardiotin (Fig. 4, Table 1).

The immunostaining of the differentiated MSCs with anti-myosin and anti-cardiotin at two and three weeks after HME treatment showed that about 80% of the resulting differentiated cells expressed these proteins which was found in the longitudinal sarcoplasmic reticulum of mature cardiomyocytes (Fig. 4 A, B, C).

In contrast, the control groups expressed negative responses for these general (myosin) and specialized (cardiotin) (Fig 4 D).

4. Discussion

4.1. Effects of HME on MSC differentiation

Mesenchymal stem cells which have been isolated from BM can be expanded and induced either *in vitro* or *in vivo* to terminally differentiate into osteoblasts, chondrocytes, neural cells, myotubes and hematopoietic-supporting stroma (Dennis and Charbord, 2002). The BM-MSCs can also be differentiated into skeletal and cardiac muscles using appropriate environmental conditions plus several growth factors (Tomita *et al*., 2002; Xu *et al*., 2004; Yoon *et al*., 2008). To direct the differentiation of MSCs into specialized population, the growth conditions of MSCs need to be changed in specific ways, such as adding growth factors to the culture medium or changing the chemical composition of the surface on which MSCs grow (Odorico *et al*., 2001). Consequently, we substituted these growth factors using rat embryonic HME as a crude source of stimulating factors for directing the differentiation of MSCs *in vitro*.

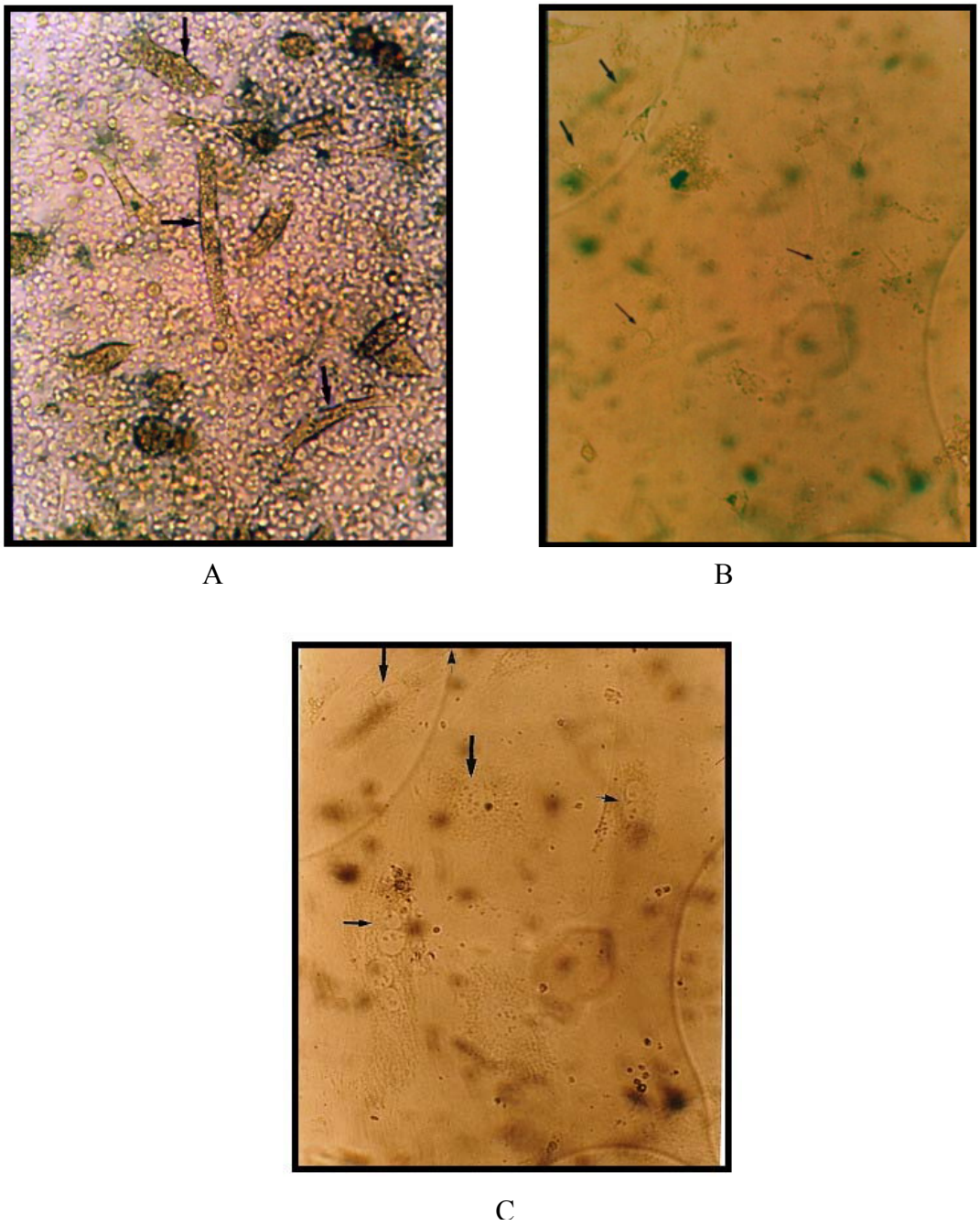

Figure 2: The morphology of differentiated MSCs *in vitro* at second passage after treatment with HME. (A): at the end of second week of culturing, the MSC cells connected with adjoining cells and began to form myotube-like structure (arrows) (X160). (B): at the third week of culturing, most of the cells are mononuclear (thick arrows) and some are binuclear (head arrow) but few are multinuclear (thin arrow) (X100.8). (C): the cells at third week, some of cells are mononuclear (thick arrows) and the other are binuclear (thin arrows) (X100.8).

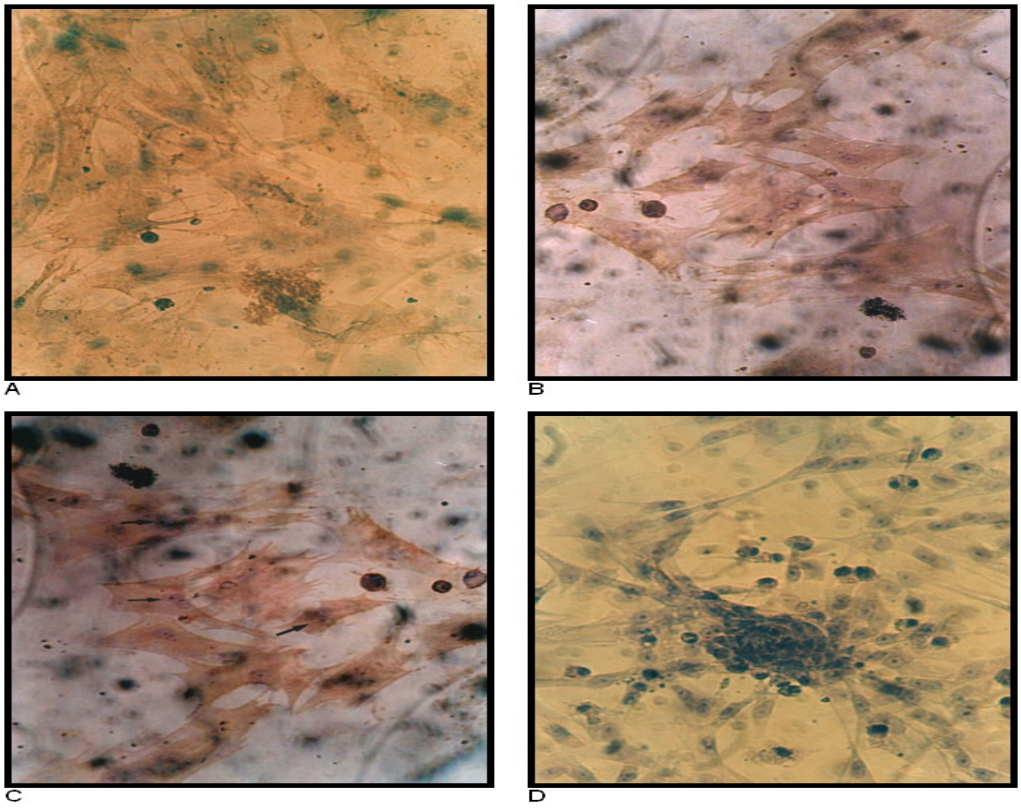

Figure4: Immunocytochemical analysis for differentiation of MSCs in vitro in treated groups with HME at second passage. (A): The most of differentiated cells were positive for anti-myosin marker (brown color) (X100.8). (B): the most of differentiated cells were positive for anti-cardiotin marker (X100.8). (C): the differentiated cells at three weeks after HME treatment were positive for anti-cardiotin marker and some of these cells appeared binuclear (arrows) (X160). D: the MSCs in control groups were negative for anti-cardiotin marker (X100.8).

Table1: Markers commonly used in the present study and characterized differentiated cell types.

Markers	Dilution	Cellular distribution	Properties
Anti-myosin	1:10	Myoblasts, Myocytes	A component of structural and contractile protein found in myocyte
Anti-cardiotin	1:50	Neonatal and mature cardiomyocyte	A component of structural and contractile protein found in cardiomyocyte

The direction of differentiation of MSCs has been performed for the first time after treatment with HME at a concentration of 0.1µl/1mL media, which is considered as the optimal concentration for differentiation into myogenic cells with cardiomyocyte-like characteristics for different exposure periods. From the results of the present study, we observed the differentiation of BM-MSCs into cardiac-like cells in treated groups compared with control groups. This is confirmed with immunocytochemical analysis and the results of the present study are consistent with many of prior reports (Wakitani *et al*., 1995; Makino *et al*., 1999) that suggested that using 5-azacytidine induced BM-MSCs to differentiate into myogenic cells. These adherent and differentiated cells increased in size and formed a ball like appearance or formed a stick like morphology, and then formed myotube like structure which is a feature that was not present or detected in the control groups. The effects of HME treatment in stimulation and differentiation of MSCs in culture was similar to the effect of several materials such as Amphotericin-B (Wakitani *et al*., 1995) or the drug 5-azacytidine (Makino *et al*., 1999).

The precise mechanism of how to induce BM-MCs to differentiate into muscle cells is unknown. In studies on myogenic differentiation of the mouse embryonic cell line with 5-azacytidine, Konieczy *et al*., (1984) proposed that these cells contain a myogenic determination locus in a methylated state with a transcriptionally inactive phase, which becomes demethylated and transcriptionally active with 5-azacytidine causing the cells to differentiate into myogenic cells .

The use of HME to stimulate the differentiation of MSCs took three weeks of treatment which is longer than the period which caused the differentiation of MSc using the 5-azacytidine; the latter was for 24 h only (Makino *et al*., 1999; Xu *et al*., 2004).

The role of these embryonic extract and as mentioned by Leor *et al*., (1996) is that most embryonic tissues are regarded as an important source of extracting factors that stimulate the growth and differentiation of stem cells into

special direction. The newly differentiated cells can then be used for therapeutic angiogenesis.

The critical role of extract also was observed by Waheed (Un-published data) when the induction of muscle differentiation from embryonic stem cells (ESCs) was carried in media containing New Born Bovine Serum (NBBS) and embryonic muscle extract. Additionally, the study made by (AL-Jumely, 2006) demonstrated that the differentiation of HSCs into muscle like cells *in vitro* could be done treating the colony of HSCs with HME of new born mice.

4.2. Immunocytochemical Examination for Differentiation of Mesenchymal Stem Cells in vitro

The results of the immunocytochemistry examination showed that the differentiated cells were positive when detected by anti-myosin antibody. This result is similar to different studies such as (Makino *et al*., 1999; AL-Jumely, 2006) who suggested that the expression may be associated with activation of the myosin gene.

Myosin is known to be an early marker of myogenic differentiation and myosin filaments are very important structures of muscle tissues that play an important role in contraction (Grigoriadis *et al.,* 1988; Yablonka-Reuent, 2005). The immunostaining analysis using anti-cardiotin marker demonstrated that most of the differentiated cells expressed this protein. These findings are similar to that described by Pochampally *et al*., (2004).

Cardiotin is a high molecular weight protein complex (300KDa) located in the longitudinal sarcoplasmic reticulum (SR) of cardiac muscle. The cardiotin structure consists of subunits of 60KDa and 100KDa. During cardiac contractile dysfunction, a decreased in SR activity is detected (Schaart *et al*., 1993).

According to the results of (Yoon *et al*., 2002) study, it was concluded that the cells from BM-MSCs co-cultured with rat neonatal cardiomyocytes expressed cardiac troponin I and other cardiac-specific proteins. When the rat cardiomyocytes were removed from the culture medium, the differentiation did not occur. These results confirm the important results obtained from the current study. We observed that MSCs when exposed to the culture medium without adding HME, the cells did not differentiate and were negative for anti-cardiotin marker.

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Mycobiota Associated with Sugarcane (*Saccharum officinarum* L.) Cultivars in Iraq.

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Abstract

During the study of the mycobiota associated with four sugarcane (*Saccharum officinarum* L.) cultivars (CO331, Co976, CP5-68, and Missan 1) currently cultivated for sugar production at sugarcane factory at Missan governorate, Southern Iraq, ten teleomorphic ascomycetes have been reported. These include *Arxiomyces zubairiensis*, *Chaetomium atrobrnneum, C.convolutum, C.elatum, C.globosum*, *C.murorum, C.spiralotrichum, Coniochaeta saccardoi, Kerinia nitida* and *Leptosphaeria sacchari*. All the identified species are reported for the first time on sugarcane plant in Iraq. A brief description along with photographs is provided for the reported species.

الملخص

خلال دراسة المجموعة الفطرية المصاحبة لاربعة أصناف لنبات سكر القصب المزروعة حاليا في حقول الشركة العامة لصناعة السكر في ميسان – جنوب العراق، تم تشخيص عشرة أنواع من الفطريات الكيسية وهي:
Arxiomyces zubairiensis, *Chaetomium atrobrnneum, C.convolutum, C.elatum, C.globosum*, *C.murorum, C.spiralotrichum, Coniochaeta saccardoi, Kerinia nitida* and *Leptosphaeria .sacchari*.
جميع الأنواع المشخصة تسجل لأول مرة على نبات سكر القصب في العراق. وصفت الأنواع المسجلة باختصار ومعززة بالصور الفوتوغرافية.

Keywords: Sugarcane, Mycobiota, Ascomycetes, Iraq.

1. Introduction

Sugarcane (*Saccharum officinarum* L) is grown in tropical and subtropical regions of the world. The crop is introduced by the Arabs in the eight century A.D to the Mediterranean, Mesopotamia, Egypt, North Africa and Andalusia. By the tenth century, sugarcane cultivation was well established and some sources indicated that there was no village in Mesopotamia that did not grow sugarcane crop (Watson,1983). However, in recent Iraq, the production of the crop is restricted to Missan governorate, Southern Iraq (31 40 N-47 40 E). The first commercial production of the crop in Iraq was in 1965 after the building of a sugarcane factory at Al-Majar Al-Kabir town to the south of Missan governorate. The area cultivated with the crop is about 6000 hectar with average cane yield of 43.21 t/he which is far below the existing potential (Anonymous, 2002).

Several pathogenic and saprophytic fungi have been reported as a mycobiota associated with sugarcane plant in several parts of the world (Watanabe, 1974, 1975 a,b,c; Zummo,1986, Sivanesan and Walter,1986; Dosayla *et al*.,1993; Magarey,1986,1995; Mena Partalos *et al*.,1995; Fernandez *et al*,.1995; Egan *et al*.,1997; Lopez Mena *et al*.,1999; Aoki, 2000).

In Iraq, however, studies on the mycobiota associated with sugarcane crop were restricted to internal reports made by the staff of the Directorate of General State for Sugarcane Production at Missan on the incidence of sugarcane smut (*Ustilago scitaminea*) on the cultivar NCO310 at Missan fields (Karam, 1983,1987) and on the microbiota responsible for deterioration of sugarcane juice (Mansour *et al*.1979). More recently, Abdullah and Saleh (2010) reported 16 mitosporic fungi assigned to the genera *Alternaria* (5 species), *Bipolaris* (4 species), *Curvularia* , *Exserohilum* (3 species each) and *Drechslera* (1 species). This paper reports the identification of ten ascomycetous fungi.

2. Materials and Methods

A total of 60 pieces of living and dead leaves and stems (2 cm long) from four sugarcane cultivars or hybrids (C0331, C0976, CP5-68 and Missan1) were washed for several times with tap water and then rinsed three times with sterile distilled water. Washed pieces were plated on moist blotter and in plates containing PDA medium (200g potato, 20g dextrose, 20g agar and 1L distilled water) with chloramphenicol (250 mg/l) and incubated under 12h of darkness alternating with 12h of cool white fluorescent light. Plates were examined every 3 d for 3 wk. To achieve pure cultures, ascospores were transferred from the natural substrates or from the PDA plates to new PDA plates.

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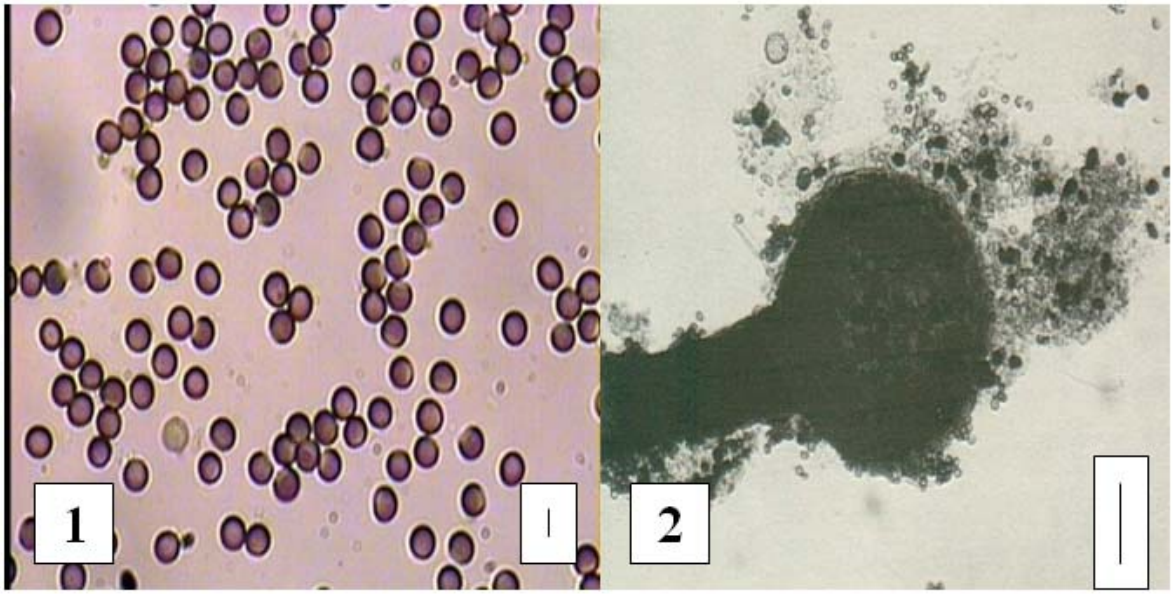
Identification of isolates was made according to Malloch and Cain (1971), Arx *et al.* , (1986), Sivanesan and Waller(1986), Checa *et al*., (1988) and Abdullah and Al-Saadoon (1994). Specimens (dried cultures) of the reported species have been deposited at Basrah University herbarium (BSRA).

3. Results and Discussion

Arxiomyces zubairiensis Abdullah and Al-Saadoon. Marina Mesopotamica 9:246 (1994). Figs.1 and 2.



Figures (1 and 2). *Arxiomyces zubairiensis*, 1-ascospores, 2-ascomata.

Bar 1= 5µm Bar 2= 50µm.

Ascomata superficial to semi-immersed, were dark brown to black due to spore mas, globose, 170-250 µm, glabrous or slightly hairy, neck-pale , yellow brown , cylindric, 40-80 X 35-40 µm. Asci 4-spored, broadly clavate, 9-11.5 X 13-16 µm, and evanescent. Ascospores in turn were globose to subglobose with a truncate base and rounded apex ,4-5 µm diameter, and at first hyaline becomes dark brown to black in mass, and smooth walled, with a single large basal germ pore, 3.5-4 µm diameter.

Specimen examined: BSRA 11155. On dead stem of cultivar C0331, November, 2001. This is the first record of the species on sugarcane plant. The type species was originally described from Iraq (Abdullah and Al-Saadoon,1994) parasitizing *Stachybotrys* sp.on *Phragmitis australis* dead stem collected from tidal zone of Khawr Al-Zubair canal, Southern Iraq. *A.zubairiensis* differes from two other known species in the genus (*A. vitis* (Fuckel) P.F.Cannon and D.Hawksworth and *A. campanulatus* Horie, Udagawa and P.F.Cannon) by its globose to subglobose ascospores, whereas ,the former two species are characterized by having ovoid to ellipsoidal ascospores.

Chaetomium atrobrunneum L.M.Ames. Mycologia 41:641 (1949).Figs.3 and 4. Ascomata superficial, ostiolate, 60 – 150 µm. Terminal hairs arising around the ostiole , straight, septate, smooth brown in colour, tapering at end, 3-4 µm broad at base. Lateral hairs are similar but slightly shorter. Asci 8-spored, clavate, vanescent. Ascospores fusiform or elongate pyriform, grey brown at maturity, 9-15 X 4-7.5 µm, with subapical germ pore. Specimen examined: BSRA 11160.Isolated from stem of P52-68 cultivar, March, 2002.

This is the first record of the fungus on sugarcane plant in Iraq. However, the fungus has been repeatedly isolated from Iraq from soil at date palm plantation (Abdullah and Zora,1993), from corn grains (Abdullah and Al-Mousawi, 2006), from medicinal plants (Abdullah *et al*., 2002;2008), from sediment of Shatt Al-Arab River and Southern marshes (Abdullah and Abbas ,2008; Abdullah *et al*., 2010).

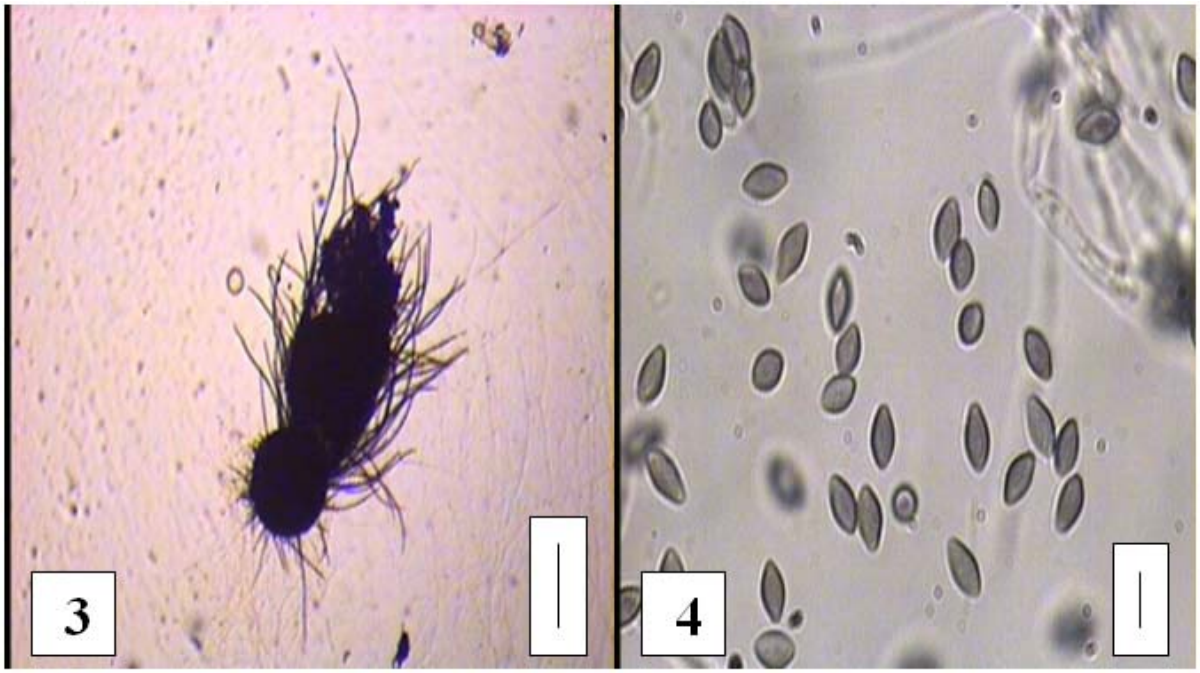
Chaetomium convolutum Chivers. Proc.Amer.Acad. 48:85 (1912). Figs.5 and 6.

Ascomata obovate to ovate, dark brown, superficial 130-200 µm. Terminl hairs are spirally coiled, brown, septate, thick-walled , verrucose, or warty, and 4-5 µm thick. Lateral hairs are seta-like, olive to brown, shorter than terminal hairs. Asci 8-spored, clavate, evanescent. Ascospores limoniform, slightly apiculate at both ends, pale brown at maturity, bilaterally flattened, 6-9 X 4-7 µm.

Specimen examined: BSRA 11161. Isolated from dead stem of C0371 cultivar, May, 2001. This is the first record for the species from Iraq.

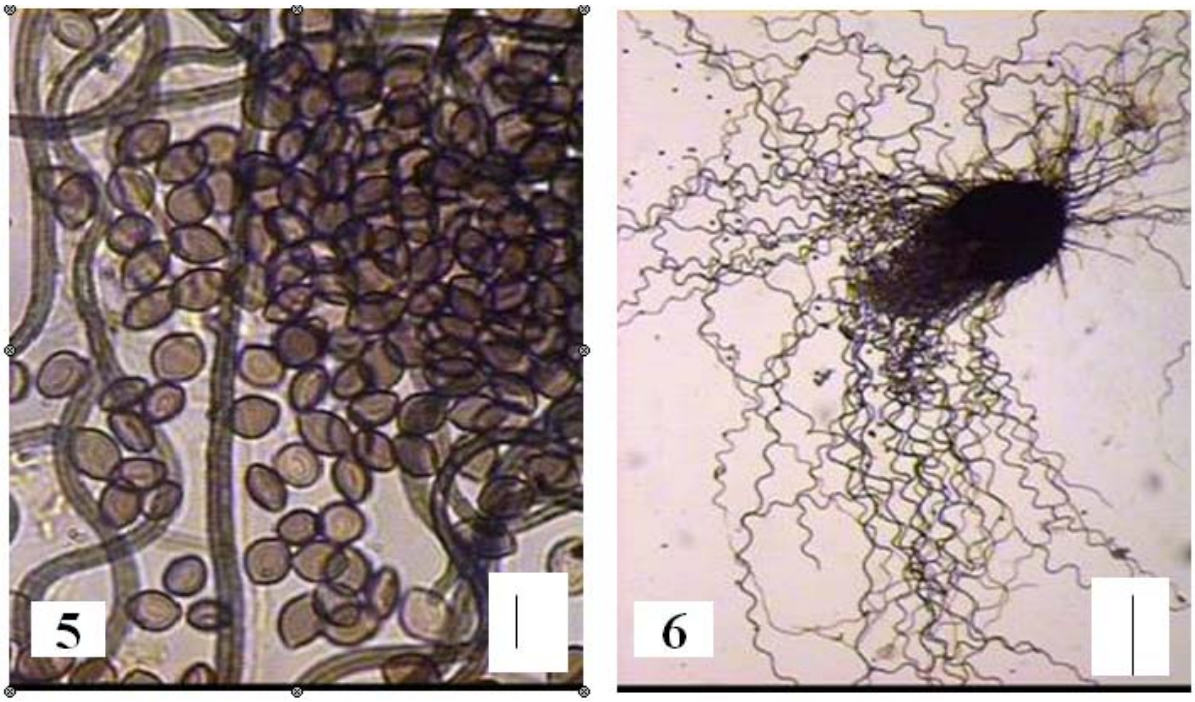
Chaetomium elatum Kunze, Mycol. Hefte 1:16 (1817). Figs.7 and 8.

Ascomata are spherical or ovate, superficial, 170-350 µm. Hairs are long dichotomously branched, verrucose or warty, septate, 4-5 µm thick at base. Asci are 8-spored, clavate, evanescent, 30-40 X 12-18 µm. Ascospores are liminiform, thick-walled, 7-11 X 6.5-8 µm, bilaterally flattened , and brown at maturity, with an apical germ pore. Specimen examined: BSRA 11162. Isolated from leaves of C0331 cultivar, September 2001.



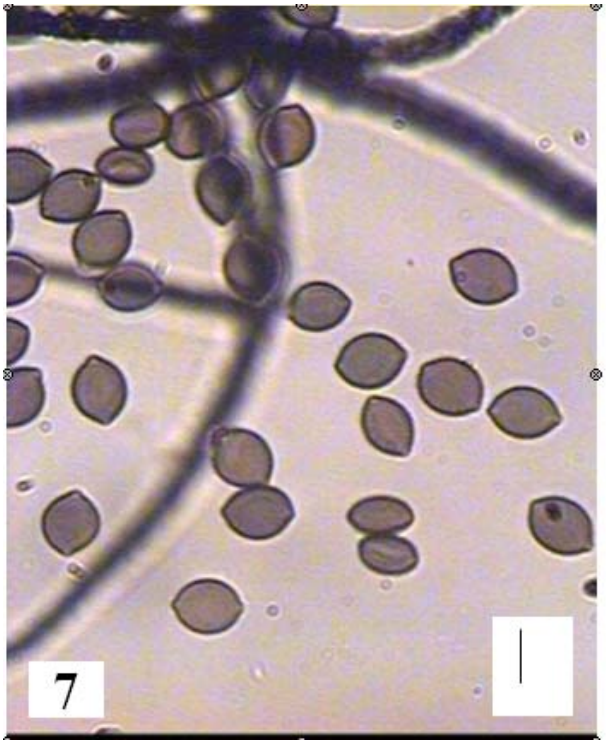
Figures (3 and 4). *Chaetomium atrobrunneum*, 3-ascomata, 4-ascospores.

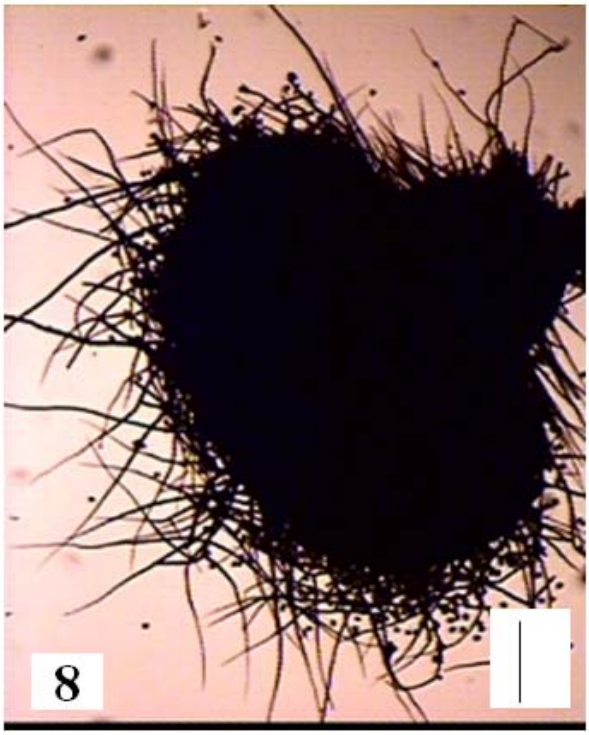
Bar 3= 100 µm. Bar 4= 10µm.



Figures (5 and 6). *Chaetomium convolutum*. 5-ascospores, 6-ascomata.

Bar 5=10µm. Bar 6= 100µm.





Figures (7 and 8). *Chaetomium elatum*. 7-ascospores, 8-ascomata.

Bar 7=10µm. Bar 8=100µm.

This is the first report for the fungus on sugarcane plant in Iraq. However, the fungus has been previously isolated from different habitats in Iraq (Abdullah and Zora,1993), Abdullah and Al-Mousawi, 2006; Abdullah and Abbas ,2008).

Chaetomium globosum Kunze. Mykol.Hefte 1:16(1817).

Figs.9 and 10. Ascomata olive brown, ovate or obovate, ostiolate,superficial, 160-270 µm in size.

Ascomatal hairs flexuous, undulate or coiled, usually unbranched,septate, brownish, 3-5 µm wide. Asci 8-spored, clavate, 30-36 X 11-15 µm, evanescent. Ascospores liminiform usually basiapiculate, brownish at maturity, 9-12 X 8-10 µm, with an apiculate germ pore.

Specimen examined: BSRA 11163. Isolated from stem of Missan 1 cultivar, September,2001. The species is common to all cultivars. This is the first report for the species on sugarcane plant in Iraq. However the fungus has been reported from different habitats in Iraq, including desert soil (Abdullah *et al*., 1986), corn grains (Abdullah and Al-Mousawi,2006), surface sediments of rivers and marshes (Abdullah and Abbas, 2008; Abdullah *et al.,* 2010) and herbal drugs (Abdullah *et al.,* 2002). The species has been reported on sugarcane in India and Pakistan (Sivanesan and Waller,1968) and from sugarcane in Cuba (Hernandez *et al*., 1995).

Chaetomium murorum Corda. Icon.Fung. 1:24 (1837). Figs.11 and 12.

Ascomata superficial, spherical, dark olive, 145-230 µm. Ascomatal hairs long, flexuous or undulate, thick-walled, septate, brown, 4-6 µm thick. Asci 8-spored, clavate 30-45 X 10-20 µm, evanescent. Ascospores ellipsoidal, 10-15 X 7-9 µm, with a distinct apical germ pore. Specimen examined: BSRA 11164. Isolated from leaves of cultivar C0331, January, 2001.

This is the first report for the species on sugarcane plant in Iraq. However, it has been isolated from other sources (Abdullah and Zora,1993; Abdullah and Abbas, 2008).

Chaetomium spiralotrichum Lodha. J.Indian Bot.Soc.43:134 (1964). Figs,13 and 14. Ascomata spherical to ovate, ostiolate, dark brown, 110-180 µm. Ascomatal hairs flexuous, or spirally coiled, often forming long coils, indistinctly septate. Asci evanescent, 30-38 X 13-16 µm. Ascospores ellipsoidal, olivaceous brown, 9-11 X 5-7 µm with a distinct apical germ pore. Specimen examined: BSRA 11165. Isolated from dead leaves of Missan 1cultivar, November, 2001. This is the first report for the fungus in Iraq.

Coniochaeta saccardoi (Marchal) Cain. Univ. Toronto Stud. Bot. Ser.38:65 (1934).Figs.15-18. Ascomata superficial,globose to pyriform, ostiolate, dark brown to black, 105 -280 µm, covered with setae, 20-80 X5-7 µm, swollen at base. Asci 8-spored, cylindrical, without distinct apical ring, 70-80 X5-8 µm. Ascospores uniseriate , one-celled, dark brown to black, narrowly ellipsoid, 8-10 X4-6 µm with longitudinal germ slit.

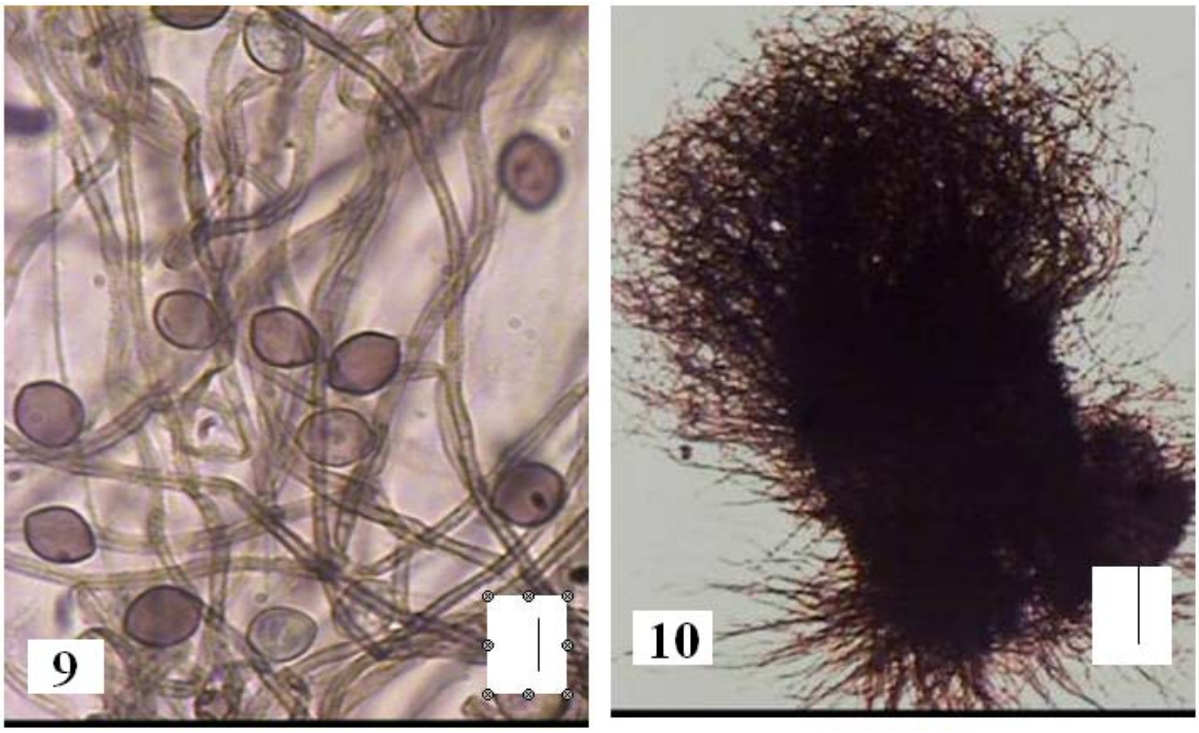
Specimen examined: BSRA 11166. Isolated from dead leaves and stems of Missan 1 cultivar. March, 2001.

This is the first report for the fungus on sugarcane plant in Iraq. However, the species was previously reported on submerged dead palm leaves (Al-Saadoon and Abdullah,2001).

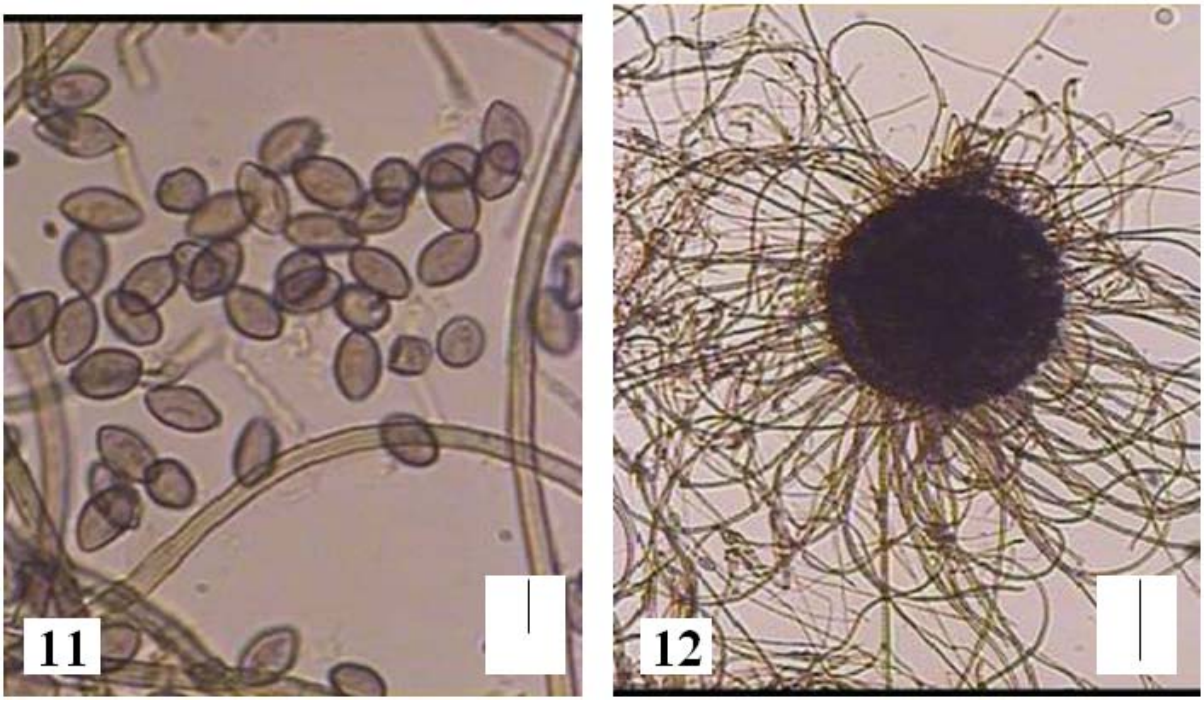
Kernia nitida (Saccado)Nieuwland. Amer. Midland Natur. 4:379 (1916). Figs,19 and 20.

Ascomata varying in shape, irregular in outline, black opaque, non-ostiolate, 120-220 µm. Ascomatal appendages

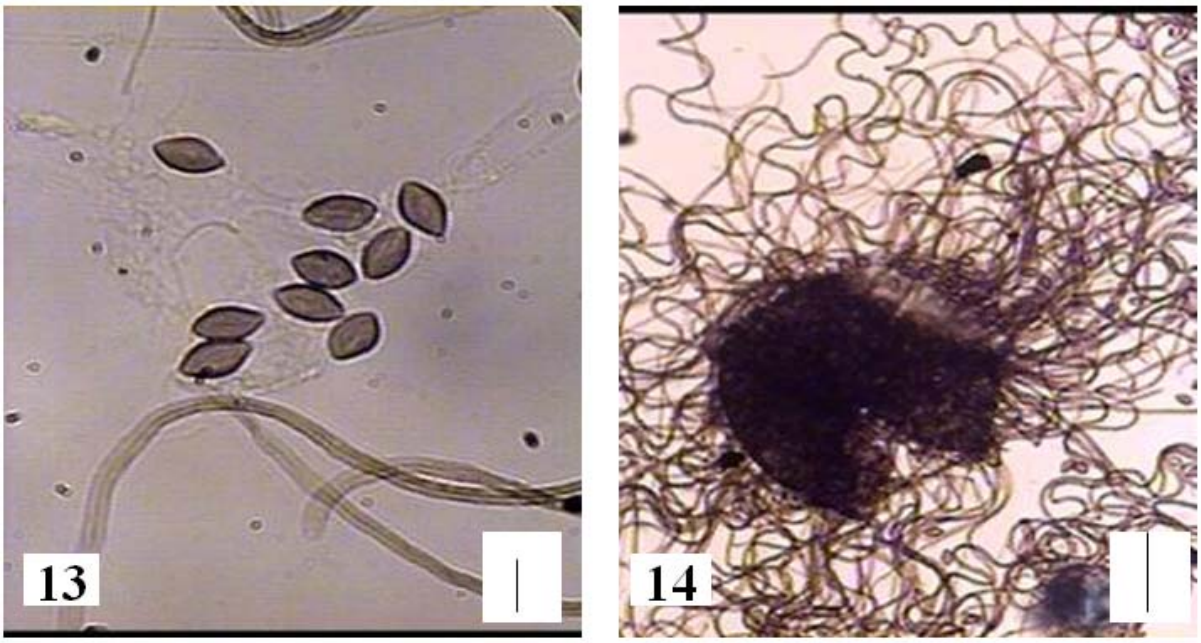
arising in fascicles from two points, black, thick-walled, unbranched up to 1000 µm long and 5-7 µm wide. Asci 8-spored, ovoid to globose, evanescent. Ascospores 4-6 X3-4 µm, ellipsoidal, orange brown to copper-coloured in wet mass, thin-walled, smooth with a prominent de Bary bubble, with germ pore at each end. Specimen examined: BSRA 11177. Isolated from dead stem of C0331 cultivar. March,2002. This is the first report for the fungus on sugarcane plant in Iraq.



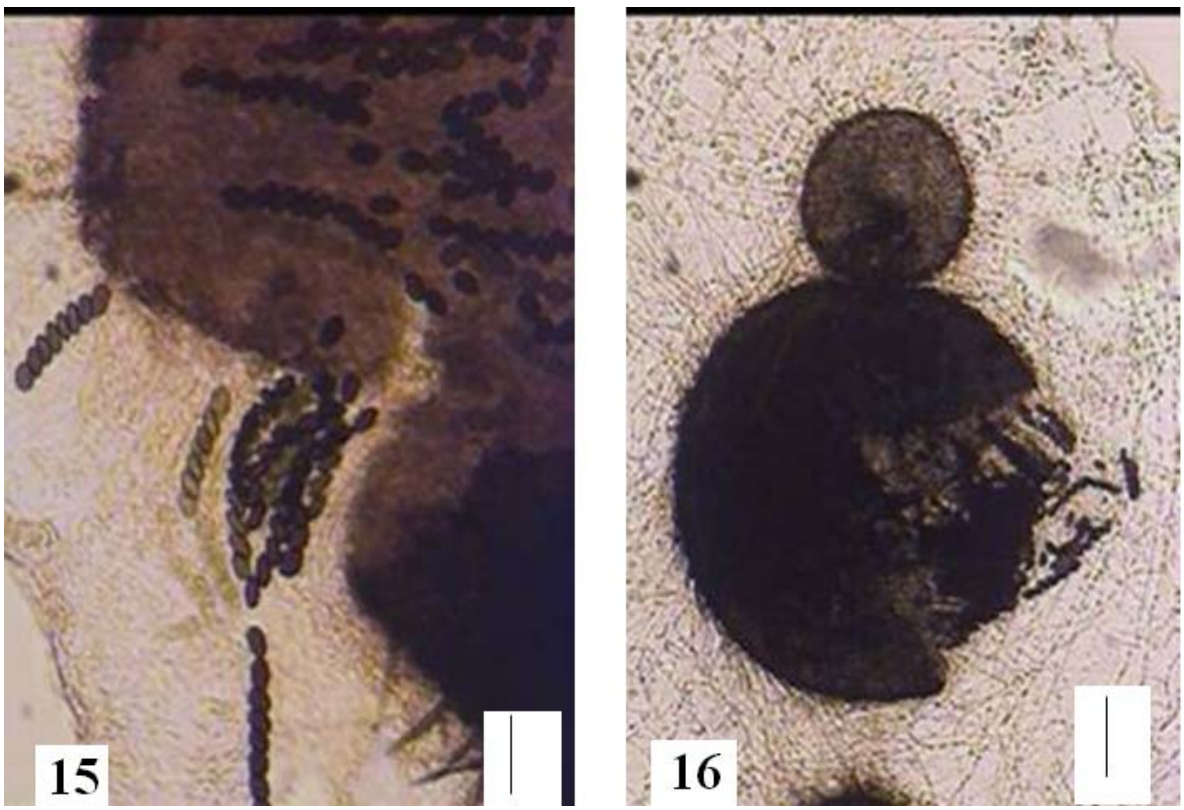
Figures (9 and 10). *Chaetomium globosum*. 9-ascospores, 10-ascomata.
Bar 9= 10µm. Bar 10=100µm.



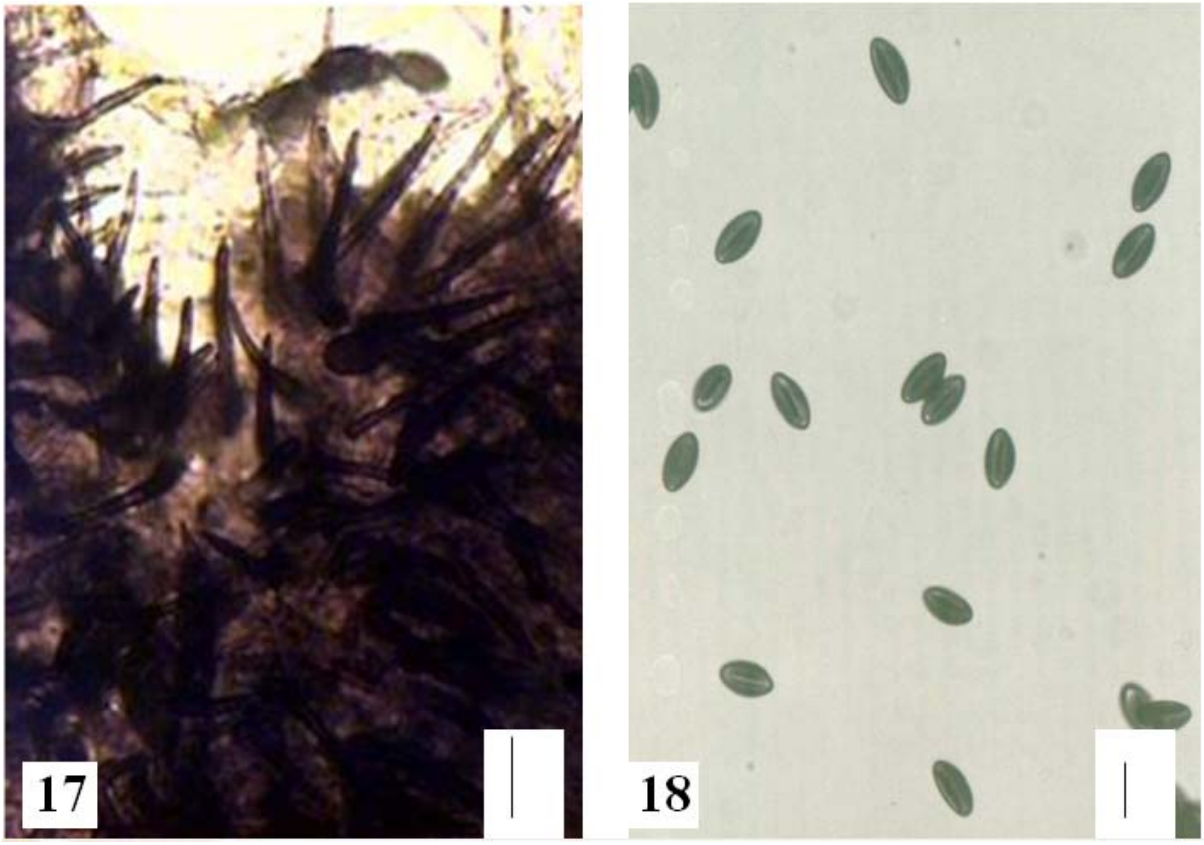
Figures (11 and 12). *Chaetomium murorum*. 11-ascospores, 12-immature ascomata.
Bar 11=10µm. Bar 12=100µm.



Figures (13 and 14). *Chaetomium spiralotrichum*. 13-ascospores, 14-ascomata.
Bar 13= 5µm. Bar14=100µm.



Figures (15 - 16). *Coniochaeta saccardoi.* 15-asci, 16- ascomata, 17-setae. 18-ascospores.
Bar 15=50µm, Bar 16=100µm, Bar 17= 50µm, Bar 18= 10µm.



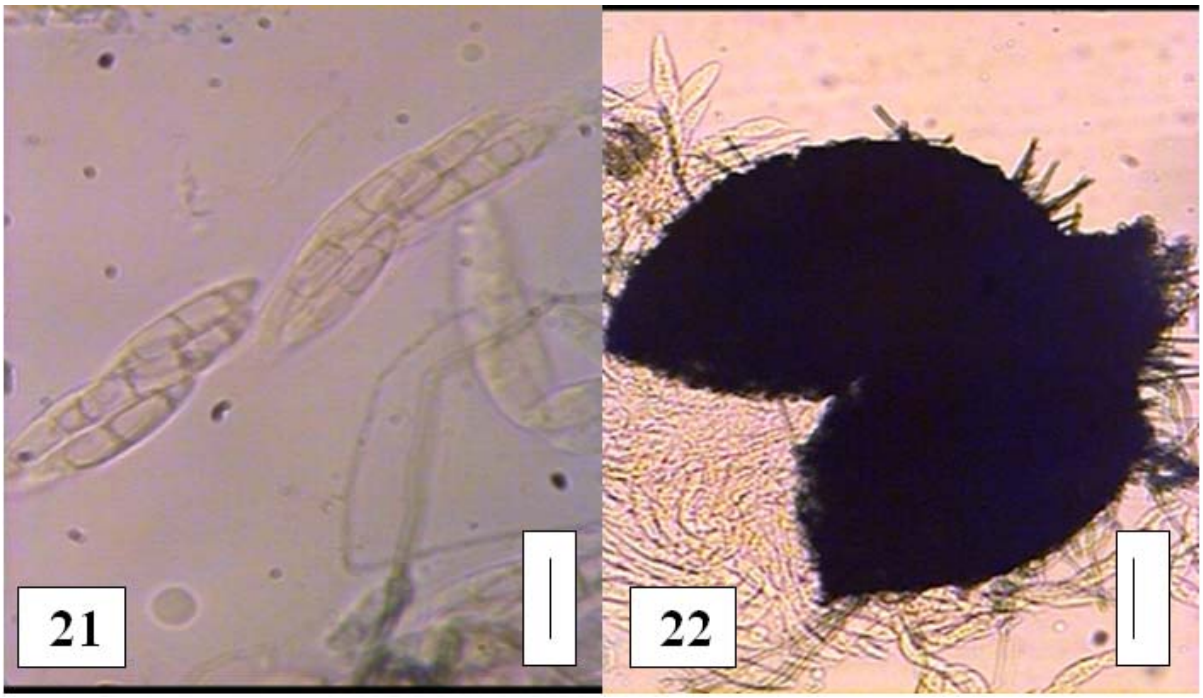
Figures (17 - 18). *Coniochaeta saccardoi.* 15-asci, 16- ascomata, 17-setae. 18-ascospores.

Bar 15=50µm, Bar 16=100µm, Bar 17= 50µm, Bar 18= 10µm.

Figures (19 and 20). *Kernia nitida* 19-ascospores. 20-ascomata.

Bar 19= 5 µm. Bar 20= 100µm.

The species is commonly found on dung of various herbivore animals as well as frequently isolated from soil and decaying plant materials (Malloch and Cain,1971). In Iraq, however, the fungus was isolated from various dung types (Abdullah,1982), and from soil in several occasions (Abdullah *et al.,* 1986,2007). *Leptosphaeria sacchari* Van Breda de Haan. Meded. Proefstat suilcerr.W.Jeva 3:25 (1892).Figs.21 and 22.



Figures (21 and 22). *Leptosphaeria sacchari*. 21- asci and ascospores. 22- ascomata.

Bar 21= 10µm. Bar 22= 50 µm.

Ascomata globose to subglobose up to 200 µm size. Asci oblong-cylindric, 8-spored 40-60 X 8-12 µm. Ascospores oblong, fusoid, straight to somewhat curved, biseriate, subhyaline to light yellow brown, 3-septate, 18-23 X3-5.5 µm. Specimen examind: BSRA11178. Isolated from living leaves of CO331, July ,2001.

This is the first record for the species in Iraq. The fungus was identified among the fungi causing ring spot of sugarcane leaf disease with a worldwide distribution (Hudson, 1962; Sivanesan and Waller,1986).

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