Immunosuppressive and cytotoxic properties of Ochratoxin-A and protective role of Selenium in Rabbits

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Abstract:
Twenty New-Zealand white rabbits about 6-8 weeks of age were housed individually in metal cages. All animals were house-caged in a temperature controlled and artificially illuminated room (12 hrs./light/dark cycles). The rabbits were maintained on a standard basal diet and tested for aflatoxin B1, ochratoxin- A (OTA), CIT and fumonisin B1by using HPTLC before the experiment. Rabbits were randomly distributed into four groups of each five rabbits treated as follows: Group I was given a diet containing 0.75 mg OTA/kg feed, group II was given selenium preparation in the sodium selenite/kg body weight, group III as given both selenium and OTA and group IV was fed a standard mycotoxins free basal diet.

Blood samples were collected from each rabbit via ear vein after one week from experiment. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by using Histopaque. Serum samples were collected from each rabbit at 20^{th} day at the end of experiment. The Comate assay was done for observation of DNA fragment migration patterns using fluorescent microscope. Also applied Lymphocyte Transformation Test (LTT), Polymorph nuclear (PMN) cells, Interlukin-6, TNF-α and IL-12 by using ELISA test.

The present study induced significant elevation of IL12, IL6 and TNF-x of rabbit serum. Selenium treated group with OTA reduces the cytokines in compare with OTA treated group. Also gp.II didn't record any significant alteration in the level of cytokines in compare with control group. OTA treated group reduced phagocytosis%, killing% and LSI while selenium treated group significantly augment phagocytosis%, killing% and LSI compare with control group. OTA produce significant increase of DNA damage as estimated tailed cell%, Tail length, DNA% and Tail moment. On the other hand, selenium treated group with OTA shows diminish significantly DNA damage in compare with OTA treated group.

The present study concluded that the administration of selenium able to reduce the immunosuppressive and cytotoxic effects of ochratoxin-A.

Introduction:

Ochratoxin- a (OTA) is a mycotoxins produced by some species of fungi such as *Aspergillus* and *Penicillium*. OTA is found as a contaminant of a variety of animal and human foods, including cereal and grain products, coffee, beer, and wine ***(Nicoletta et al, 2005).*** OTA has been identified in blood, bile, and urine of humans and animals after consumption of contaminated food ***(Petkova et al , 1988)*** and has been implicated as one of the etiological agents in Balkan endemic nephropathy, a chronic renal disease involving progressive renal fibrosis and impaired renal function, where contamination with much OTA is described ***(Vrabcheva, 2004).*** OTA is also immunosuppressive, teratogenic, genotoxic, and carcinogenic and affects blood coagulation and carbohydrate metabolism ***(Pfohl, 2002).***

International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). Currently, the mode of carcinogenic action by OTA is unknown. OTA is genotoxic following oxidative metabolism. This activity is thought to play a central role in OTA-mediated carcinogenesis and may be divided into direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action ***(Pfohl , 2002).***

OTA-induced liver injury involves a reduction in the ability to counterbalance oxidative stress, maybe leading to altered gap junction intercellular communication and loss of cell adhesion and polarity. This suggests that mild oxidative damage might be a key factor, in combination with other cytotoxic effects, in triggering the promotion of liver tumors after exposure to OTA ***(Gagliano, 2000).***

Selenium (Se) supplementation markedly decreased the incidence and severity of neuroleptics-induced cardiac lesions and these findings may serve as a basis for further evaluation of the protective role of Se supplementation in neuroleptics-treated patients. However, Se supplementation in normal animals without Se deficiency was also shown to be cardio toxic *(Jacques , et al, **2009).***

The present investigation designed to elucidate the genotoxic and immunosuppressive roles of Ochratoxin A in rabbits. Furthermore, the current study clarifies the protective function of selenium.

Material and Methods

Experimental animals:

Twenty New Zealand White rabbits, 6-8 weeks of age, were housed individually in metalcages. All animals were house-caged in a temperature-controlled and artificiallyilluminated room (12 h light/dark cycles). The rabbits were maintained on a standard basal diet. Prior to commencing the research,

the basal experimental diet was tested in the laboratory for aflatoxin B1, OTA, CIT and fumonisin B1. No detectable levels of thesemycotoxins were observed in HP-TLC and spectrophotometry analysis (lowest detection limit 1 to 5 µg/kgfeed). Fresh green fodder was provided along with water ad libitum. UV-Vis-Spectrophotometer (CAMAG, Switzerland) at 333 nm against the standard toxin procured from Sigma Chemicals Limited, USA ***(Brown et al, 1967).*** After one week of acclimatization period, the animals were randomly distributed into four groups of five and treated as follows: Group I was given a diet containing 0.75 mg OTA (Sigma-Aldrich chemical company)/kg feed; Group II, was given selenium preparation in the form of oral dose 0.5 mg Sodium selenite (Sigma-Aldrich chemical company)/kg body weight; Group III: was given both selenium and OTA and finally, Group IV was fed a standard mycotoxins free basal diet.

Blood Samples:

Heparinized and non- heparinized blood samples were collected from each rabbit via ear vein, one week post experiment. Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were isolated as per method of ***(Boyum, 1968***), using Histopaque (Sigma-Aldrich).serum and heparinized blood samples were collected from each rat at the 20th day from the end of experiment.

Comet Assay:

Cell suspension (100 µl) was mixed with 600 µl of low-melting agarose (0.8% in PBS). 100 µl of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining with ethidium bromide 20µg/ml at 4°C. The observation was with the samples still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope (With excitation filter 420-490nm [issue 510nm]). The comets tails lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of EtBr-stained DNA using a 40X objective on a fluorescent microscope.

Although any image analysis system may be suitable for the quantitation of SCGE data, we use a Comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail

moment. Generally, 50 to 100 randomly selected cells are analyzed per sample ***(Singh et al, 1988).***

*Lymphocyte Transformation Test (*LTT*):* LTT was performed according to ***Uma et al (1999).*** Briefly, Peripheral Blood Mononuclear Cells (PBMCs) isolated from rabbit of each group were stimulated in triplicate with mitogen Con a (Sigma) and a set of PBMCs of the same rabbit was kept as unstimulated control. PBMCs were cultured in 96-well tissue culture plates at 37°C at 5%CO_2 for 72 hours. At the end of 120 hours MTT assay was performed and 25 μl of MTT [3-(4, 5- dimethyl trizol-2-yl)-2, 5-diphenyl tetrazolium bromide] dye (5mg/ml) was added to each well of the tissue culture plate. Plate was incubated at 37°C for 4 hours. After incubation 150 μl of DMSO(dimethyl sulfoxide) was added to each well and incubated overnight at 37°C and absorbance was read at 570 nm. Proliferation of lymphocytes was indicated by optical density value of the well with test samples and that of the unused wells. For analysis of data, a signal-to-noise ratio, that is, lymphocyte stimulation Index (LSI) was calculated for individual goats using the following formula: Average OD at 570nm in stimulated wells/Average OD at 570 nm in no stimulated control wells.

Polymorph nuclear (PMN) cells were isolated from blood by the method describe by ***Rouse et al. (1980).*** The mixture of PMN and bacteria (*Staph. aureus*) were incubated at 37°C for 2 hours with regular stirring and then the mixture was centrifuged at 20 Xg for 5 minutes at 4°C. The supernatants was used to estimate the percentage of bacteria phagocytized (Phagocytosis%). The mixture of bacteria and PMN were treated with one cycle of freezing and thawing and the percentage of bacteria killed (Killing %) was estimated according to the formula described by ***Woldehiwet and Rowan (1990).***

Measurement of *IL-6, TNF-α* and *IL-2* levels by enzyme linked immunosorbent assay(ELISA):Serum IL-6 , TNF-α and IL-2 levels were measured by using a polyclonal ELISA kits (Rapid Bio Lab., Calabasas, California, USA)following the manufacturer's instructions. Briefly,the anti-IL-6 capture polyclonal antibody was adsorbed on a polystyrene 96-well plate and the IL-6 present in the sample was bound to the antibody coated wells. The biotinylated anti-IL-6 detecting pAb was added to bind the IL-6 captured by the first antibody. After washing, avidin-peroxidase (Sigma,USA) was added to the wells to detect the biotinylated detecting antibody and finally 2,2′-azinobis(3- thylbenthiazoline-6-sulfonic acid) (ABTS; Sigma, USA) substrate was added and a coloredproduct was formed in proportion to the amount of IL-6 present in the sample, which was

measured at optical density 405 nm (*OD*405) with an ELISA microplate reader (model 450, Bio-Rad, Chicago,Illinois, USA). A standard curve was generated, and the IL-6 concentration (pg/ml) of the samples was calculated. The measurement of TNF-α and IL-2 are similar to that of IL-6. All determinations were performed by full-time technical personnel.

Data was analyzed using SPSS version 16. Descriptive statistics, independent t-tests and Pearson correlation coefficient were assessed.

RESULTS

The present investigation elucidate that Ochratoxin induced significant elevation of IL2, IL6 and TNF-α of rabbit's serum. Selenium treatment simultaneouslywith ochratoxin exposure (Group II) reduces the measured cytokines in compare with ochratoxin treated group (Group I). Selenium treated group (group II) didn't record any significant alteration in the level of cytokines in compare with control group (group IV) (Table, 1).

Table (2) shows the effect of ochratoxin on phagocytosis%, killing% and LST.Ochratoxin treatment significantly reduced phagocytosis %, killing% and LSI in compare with control group. Meanwhile, selenium treaded group significantly augment phagocytosis %, killing% and LSI in compare with control group. Concurrent ochratoxin exposure and selenium induced non-significant changes in phagocytosis %, killing% and LSI in compare with control group.

The effect of OTA and selenium on DNA damage of lymphocytes was determined using comet assay. The recorded results illustrate that OTA exposure produce significant increase of DNA damage as estimated Tailed cells %, Tail length Tail, DNA % and Tail moment. On the other hand, Selenium treatment simultaneouslywith OTA exposure diminishes significantly DNA damage in compare with ochratoxin treated group (Table,3 &Photo, 1).

Table (1): *Effect of Ochratoxin and selenium on IL2, IL6 and TNF-α of rabbits*

	Group I	Group II	Group III	Group VI
IL 2 (Pg/ml	132.69±2.64*	93.58±4.61	110.85±3.44*	95.15±2.58
IL6 (Pg/ml)	188.52±4.59*	145.85±5.08	161.52±2.89*	153.64±3.99
TNF-α (Pg/ml)	235.58±5.61*	196.41±5.61	214.59±3.64*	205.47±5.72

Table(2):*Effect of Ochratoxin and selenium on phagocytosis%, killing% and lymphocyte stimulation Index (LSI) of rabbits.*

	Group I	Group II	Group III	Group VI
Phagocytosis%	75.31±2.64*	93.16±3.41*	82.33±3.55	85.59±2.92
Killing%	72.64±2.05*	89.04±2.52*	77.42±2.18	80.31±2.66
LSI	1.13±0.16*	2.41±0.23*	1.49±0.12	1.55±0.05

Table(3): *Effect of OTA and selenium on DNA damage of rabbits using comet assay.*

	Group I	Group II	Group III	Group VI
Tailed cells %	20.52±1.64*	4.52±0.33	16.44±0.92*	5.51±0.22
Untailed cells %	79.58±2.11*	95.11±3.61	83.31±3.22*	94.05±4.58
Tail length (µm)	6.97±0.41*	2.05±0.12	4.88±0.29*	2.67±0.12
Tail DNA %	5.58±0.33*	1.52±0.11	4.19±0.36*	1.85±0.05
Tail moment (Units)	38.89±3.51*	3.12±0.49	20.45±1.05*	4.94±0.21

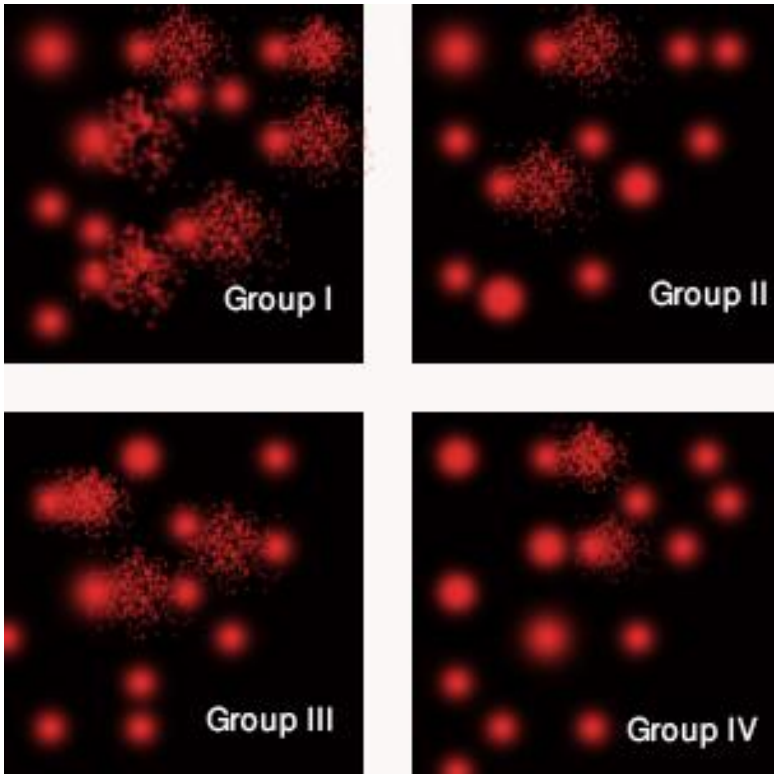

Photo (1): of OTA and selenium on DNA damage of rabbitsUsing comet assay

DISCUSSION:

OTA is an immunosuppressant fungal compound, produced by toxigenic species of Aspergillus and Penicillium fungi in a wide variety of climates and geographical regions. The contamination of food by these mycotoxins takes place primarily during preharvest periods. Almost all types of food can be contaminated. In addition, its chemical stability against heat and during industrial food processing makes OTA one of the most

abundant food contaminating mycotoxins.Oral exposure to OTA caused tumors at several different tissue sites in mice and rats. Dietary administration of ochratoxin A caused benign and/or malignant liver tumors (hepatocellular adenomaor carcinoma) in mice of both sexes and benign and malignant kidney tumors (renal-cell adenoma and carcinoma) in male mice ***(IARC, 1987).***

The present investigation recorded that ochratoxin induced a significant elevation of IL2, IL6 and TNF-α of rabbit's serum. Selenium treatment simultaneously with ochratoxin exposure (Group II) reduces the measured cytokines in compare with ochratoxin treated group (Group I). These findings were agreed with ***Petzinger & Weidenbach (2002).*** They found that OTA causes a significant release of TNF-α and IL-6 from blood-free perfused rat liver equivalent to the release induced by LPS in the same system. Arachidonic acid and its metabolites exerted both stimulatory and inhibitory effects on OTA-mediated TNF-α release from blood-free perfused rat liver ***(Al-Anati et al., 2005).***OTA caused a marked increase in IL-2 production ***(Marin et al, 1996).*** On the other hand, OTA inhibited IL-2 production from porcine lymphocytes which were stimulated by concanavalin a ***(Harvey et al, 1992).*** Others found that OTA did not interfere with IL-2 levels released from stimulated murine lymphocytes ***(Thuvander et al, 1995).*** Equally important as immune cell and organ decline are the effects of cytokine production, which could lead to activation of lymphocytes, macrophages by OTA. Anincrease of the pro-apoptotic cytokines TNF-α and IL-6 by OTAcould trigger further organ size reduction and cell depletion.Also, Selenium supplementation did suppress the enhancing effect of TNF-alpha on HIV-1 replication in vitro in acutely infected human monocytes, but not in T lymphocytes. Selenium supplementation also increased the activities of the selenoproteins, glutathione peroxidase (GPx) and thioredoxin reductase (TR), which serve as cellular antioxidants ***(Hori et al, 1997)***.Low plasma selenium concentrations always accompany with high interleukin-6 concentrations in adults presenting with pulmonary tuberculosis ***(Van Lettow and Semba,2005).***Selenium reduce interleukin- II production and interleukin-II receptor expression in patients with chronic hepatitis ***(Shui-Xiang et al, 2004).***

The current study elucidates that ochratoxin treatment significantly reduced phagocytosis %, killing% and LSI in compare with control group. Meanwhile, selenium treated group significantly augment phagocytosis %, killing% and LSI in compare with control group. Concurrent ochratoxin exposure and selenium induced non-significant

changes in phagocytosis %, killing% and LSI in compare with control group. These findings were on the same line of ***Singh et al (1990).***They recorded significant reduction in cell mediated immune response as well as depression in phagocytic activity of splenic macrophages in broiler chickens fed with diet intoxicated with OTA. Reductions in the cell-mediated immune responses, in the OTA treated rabbits, as assessed lymphocyte proliferation assay, were supported by earlier observations in OTA treated poultry ***(Ramadevi et al, 1996)*** and rats ***(Satheesh et al, 2005),*** where significant cellular immunosuppression was observed. The stimulation index of the lymphocyte transformation test in rabbits indicated a significantly lower cellular immune response in OTA and combination groups. This may be due to the effect of these toxins on the immunocompetent organs ***(Dwivedi and Burns, 2012).*** Moreover, ***Hassan et al (2001) and Ramadan et al (2001)*** found that selenium augment phagocytosis %, killing% and LSI in horses and buffaloes respectively.

The recorded data illustrate that Ochratoxin exposure produce significant increase of DNA damage as estimated Tailed cells %, Tail length Tail,DNA % and Tail moment. On the other hand, Selenium treatment simultaneously with ochratoxin exposure diminishes significantly DNA damage in compare with ochratoxin treated group. These data supported by the findings of ***Mally et al (2011).*** They found that OTA induced oxidative DNA lesions since they are not typically caused by chemical carcinogens which form covalent DNA adducts. Together, with the lack of evidence for formation of lipophilic DNA adducts as assessed by post labeling, these data suggest that OTA may cause genetic damage in both target and no target tissues independent of direct covalent binding to DNA. Moreover, OTA concentration in kidney tissue strongly correlated with tail intensity and tail moment values. These results confirm the genotoxic potential of OTA, and show that the severity of DNA lesions in kidney correlates with OTA concentration***(Domijan, 2006).***Results demonstrated by ***Jing et al (2012)*** revealed that oxidative pathway in OTA mediated cytotoxicity in human immune cells, which including the ROS accumulation–oxidative DNAdamage–G1 arrest and apoptosis. Our results provide new insights into the molecular mechanisms by which OTA might promote immunotoxicity. The present DNA protective role of selenium agree with the findings of ***Dziaman et al (2009),*** it is likely that the selenium supplementation of the patients is responsible for the increase of BER enzymes activities, which in turn may result in

reduction of oxidative DNA damage. In addition, ***Rafferty et al (2003)*** recorded that Selenite and selenomethionine protect keratinocytes from UVR-induced oxidative damage, but not from formation of UVR-induced excision repair sites. Recently, ***Gad (2012)*** reported that selenium protect sperm DNA damage due to methoxychlor exposure.

The present study concluded that the administration of selenium able to reduce the immunosuppressive and cytotoxic effects of ochratoxin.

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الخصائص السامه للأوكراتوكسين- أ و تأثيره المناعى المثبط و دور وقائى للسيلينيوم فى الأرانب

أحمد فتحى

وحدة المناعة الدوائية – معهد بحوث التناسليات – مركز البحوث الزراعيه – جيزة- ج.م.ع

تم ايواء عشرين من الارانب البيضاء النيوزيلندى تتراوح أعمارهمما بين ٦-٨ أسابيع فى أقفاص معدنية . كانت جميع الحيوانات فى قفص منفرد فى درجة حرارة محكمه و غرفة مضائة بشكل مصطنع (١٢ ساعة / ضوء / دورات ظلام). و أستمرت الارانب على نظام غذائى متوازن و مختبر للأفلاتوكسين B1 ، أوكراتوكسين-أ (OTA) ، و الفيومونيسين و CIT بأستخدام جهاز HPTLC قبل التجربة . و قد وزعت الارانب عشوائيا الى أربع مجموعات كل مجموعة خمس أرانب على النحو التالى : المجموعة الاولى أعطيت نظاما غذائيا يحتوى على ٠,٧٥ ملجرام أوكراتوكسين-أ / كجم علف ، أعطيت المجموعة الثانية السيلينيوم فى شكل صوديوم سيلينيت/ كجم من وزن الجسم ، و المجموعة الثالثة أعطيت كلا من السيلينيوم و الأوكراتوكسين-أ ، و المجموعة الرابعة أعطيت غذاء خالى من السموم الفطرية كمجموعة ضابطة .

تم جمع عينات الدم من كل أرنب عن طريق وريد الأذن بعد أسبوع واحد من التجربة . تم عزل خلايا الدم وحيدات النوى المحيطية (PBMCs) بأستخدام Histopaque . وتم جمع عينات مصل من كل أرنب فى يوم ٢٠ فى نهاية التجربة . و قد تم الفحص عن طريق أختبار الكوميت لمراقبة أنماط الهجرة فى جزئ الحمض النووى بأستخدام المجهر الفلورسنتى . و تطبيق أختبار الليمفوسيت أيضا (LTT) ، وتعدد خلايا الأشكال النووية (PMN) ، و الانترلوكين- ٦ ، و معامل تحول الخلايا – ألفا و الأنترلوكين- ١٢ بأستخدام أختبار الاليزا . وكانت نتائج هذه الدراسة أرتفاعا كبيرا فى نسبة كلا من الانترلوكين ١٢ و الانترلوكين ٦ و معامل تحول الخلايا – ألفا فى أرانب المجموعة الاولى ، اما المجموعة التى تلقت العلاج بالسيلينيوم مع الأوكراتوكسين فقد قللت من تأثيرالسيتوكينز بالمقارنه مع المجموعة الاولى التى أخذت الاوكراتوكسين فقط . فى حين أن المجموعة الثانية لم تسجل أى تغيير كبير فى مستوى السيتوكينز بمالمقارنة بالمجموعة الضابطة . فى المجموعة الاولى التى أخذت الاوكراتوكسين أخفضت نسبة الخلايا الأكولة ، و نسبة الخلايا المقاتلة ، و نسبة خلايا الليمفوسيت ، بينما المجموعة الثانية التى أخذت السيلينيوم فقط أظهرت بشكل ملحوظ زيادة فى نسبة الخلايا الأكولة ، و نسبة الخلايا المقاتلة ، و نسبة خلايا الليمفوسيت مقارنة بالمجموعة الضابطة . و فى المجموعة الأولى التى أخذت الأوكراتوكسين - أ أنتجت زيادة كبيرة فى تلف الحمض النووى و من ناحية أخرى كانت نتيجة المجموعة الثالثة التى أخذت السيلينيوم مع الأوكراتوكسين-أ قللت بشكل كبير التلف فى الحمض النووى بالمقارنة مع المجموعة الأولى التى أخذت الأوكراتوكسين- أ فقط .

و نستخلص من هذه الدراسة الى أن السيلينيوم قادر على الحد من تأثير خصائص الأوكراتوكسين- أ السام للخلايا و المثبط للمناعة .