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# THE EFFECT OF OCHRATOXIN A ON LIPID PEROXIDATION FROM OCHRATOXIGENIC FUNGI OBTAINED FROM DRIED CASSAVA POWDER GARRI USING AN ANIMAL MODEL.

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## ABSTRACT
Ochratoxin A (OTA) is a very dangerous Mycotoxins, and hence this study aimed at determining the role of Ochratoxin in the induction of lipid peroxidation. Ochratoxigenic *Aspergillus* species were isolated from garri sample and re-inoculated into fresh garri to produce Ochratoxin. The extracted pure Ochratoxin A was injected into the rat model at varied concentration of 0.75, 0.81 and 0.90ml/kg weight except for the control group that received no Ochratoxin A. The lipid peroxidation was measured using an assay kit from bio-division incorporation. The result of the study revealed *Aspergillus niger* has the most prevalent of the isolated Ochratoxigenic fungi 36(50%) followed by *Aspergillus carbonarius* 36(33.3%) and *Aspergillus ochraceus* 36(16.7%). The Ochratoxin A was found to significantly affect the weight gained ratio ($F= 185.19$, $9<0.05$) and the feed weight gained ratio ($F=185.19$, $P<0.05$). Also, Ochratoxin was found to have an increase the Melondialdehyde (MDA) in the treatment group relative of the control group ($F=135.93$, $P<0.05$). This study therefore clearly demonstrated a very strong link between Ochratoxin A and lipid peroxidation with this stated general objective of this study which is to investigate the role of Ochratoxin toxicity in lipid peroxidation and to investigate the effect Ochratoxin has on lipid peroxidation.

**KEYWORDS:** Ochratoxin A, lipid peroxidation, *Aspergillus*, rat model, melondialdehyde.

## INTRODUCTION
Mycotoxins are secondary metabolites of molds that exert toxic effect on animals and humans. The toxic effect of Mycotoxins are referred to as Mycotoxicosis, the severity of which depends on the toxicity of the toxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemicals to which the individual is exposed (Peraica *et al*., 1999). The Mycotoxins "Ochratoxin" is produced by certain *Penicillium* and *Aspergillus species* of storage fungi and it is found in variety of substrates including foods and feeds, (Hoehler *et al*., 1996). These fungi are known as saprophytes and can be present in different environments, field and warehouses, (Battilani and pietri, 2000). Among the *Aspergilli*, it is the section circumdati (the yellow *Aspergilli*), Which are well known as Ochratoxin producers, (Krogh, 1987). They have been studied especially on cereals, where *Aspergillus ochraceus* formerly known as *A. alutaceus* and *Penicillium verrucosum* were believed to be the relevant fungi for OTA presence in grapes,(Ospital *et al*., 1998) but Ochratoxin producing *Aspergillus carbonarius* and *A. niger* were identified in dried vine fruits in1999 (Codex Alimentarius commission, 1999).

The presence of Ochratoxin in several foods, (Dongo *et al*., 2008; Jayeola and Oluwadun, 2010; Ogunledun, 2007) and its accumulated effect such as Immunotoxicity, Neurotoxicity, Genotoxicity and possibly carcinogenicity has been documented, (Paraica *et al*.,1999).As a result, the international agency for research has classified it in group 2B as a human carcinogen, (IARC, 1993). The principal effect of this toxin is Nephrotoxicity, (Aish *et al*., 2000). It has been implicated in a human kidney disorder known as Balkan Endemic Nephropathy (BEN), (Aish *et al*., 1979). Ochratoxin has been suspected to be a risk factor for testicular cancer, (Jonsyn-ellin, 2000). It disturbs cellular physiology in multiple ways but it seems that the primary effects are associated with the enzymes involved in the

synthesis of the phenylalanine tRNA complex, (Marquardt and Frohlich, 1992). In addition, it inhibits mitochondrial ATP production, (Meisner *et al*., 1981) and stimulates lipid peroxidation, (Rahimtula *et al*., 1988). According to them, when Ochratoxin is added to rat liver chromosomes, it enhanced the rate of Nicotinamide Adenine dinucleotide Phosphate or Ascorbate dependent lipid peroxide as measured by MDA formation.

Peroxide or auto-oxidation of lipids exposed to oxygen is responsible not only for deterioration of foods (rancidity) but also for damage to tissues in vivo, where it may be a cause of Cancer, Inflammatory diseases, Atheleroscleroses, ageing among other factors, (Peraica *et al*., 1999). Taken together the above, there is thus the need to investigate the role of lipid peroxidation in Ochratoxin toxicity's.

## STATEMENT OF THE PROBLEM

In recent years, an outbreak of various diseases with link to tissue damage has been reported with no specific origin. This work therefore is directed at investigating if the ingestion of Ochratoxin in food commodities causes lipid peroxidation which has been shown to affect tissues.

## SIGNIFICANCE OF THE STUDY

The occurrence of Ochratoxin in food stuffs in global phenomenon and the role of peroxidation of lipids in Ochratoxin toxicity in an animal model remain relevant source of damages to tissues where it may cause various medical conditions. This research is therefore of medical importance as it will help to clarify if peroxidation is actually a result of Ochratoxin toxicity.

## MATERIALS AND METHOD

### Study site.

The study work was carried out at Olabisi Onabanjo University, Microbiology Laboratory, Ago-Iwoye, Ogun State, Nigeria and Federal College of Animal Health& Production Technology, Moor plantation Ibadan. A cross sectional study was conducted from July 2015 to September 2015 with an overall aim of determining the role of lipid peroxidation on Ochratoxin toxicity using an animal model.

### Preparation of the initial suspension

This was done using the method described by ISO 68877-1 (1999) with slight modification. 10g of each sample was added to 90ml of 0.1% (w/v) peptone water and homogenized by rolling between the palms at medium speed. The homogenate was passed through sterile Whatman filter paper to obtain the filtrates which was used to prepare serial 10 fold dilution by transfer of one millimeter of the homogenate ($10^{-1}$) into a tube containing 9ml of sterile 0.1% (w/v) peptone water. The mixture was then homogenized to make $10^{-2}$ dilution. To prepare further decimal dilutions, one millimeter of $10^{-2}$ dilutions was transferred into a tune containing 9ml of sterile 0.1% w/v peptone to make $10^{-3}$ dilution. These operations were repeated by using a new sterile pipette to obtain $10^{-4}$ through $10^{-10}$ dilutions.

### Isolation and identification of Ochratoxigenic fungi from garri

A portion (0.02ml) of the suspension prepared above was aseptically inoculated into Sabouraud Dextrose Agar plates using Pasteur pipette. This was allowed to be absorbed by the media. The Petri dishes were inoculated at 28$^{o}$C for 72-84 hours. They were then identified using both macroscopic and microscopic morphology.

### Microscopic examinations of the fungal isolates

After sub-culturing and subsequent incubation for a week at 28$^{o}$C, wet mount preparation of the isolated fungi in lacto phenol cotton blue dye were done and microscopic examination were carried out under a high power objective with dim light. The fungi of interest were then identified using standard microbiological methods already described, (Thomas *et al*., 2012).

### Speciation of potential ochratoxigenic *Aspergilli*

Spores harvested from the conidia of the *Aspergilli* on Sabouraud Dextrose Agar was suspended in Sabouraud Dextrose Broth, using a sterile inoculating wire loop, a spore suspension of the test *Aspergilli* was inoculated at three points equidistant from the center of Malt Extract Agar (MEA) plate and inoculated at 26-30$^{o}$C in the dark for 7 days. The distinguishing features of ochratoxigenic *Aspergilli* were used to identify based on the microscopic and macroscopic features of the isolate.

### Production and Analysis of Ochratoxin

Pure culture of *Aspergillus niger*, *Aspergillus ochraceus* and *Aspergillus carbonarius* obtained from dried cassava powders were employed for OTA production as production as described by (Trenk *et al*., 1971). OTA was produced through the fermentation of sterilized garri at 25±2$^{o}$C using the inoculated fungal culture for 2-3 weeks. OTA was extracted using chloroform and 10g of silica gel to 50g of the culture powder and then filtered. The filtrate was passed through a chromatography column containing activated silica gel and aqueous sodium bicarbonate. Initial elution was carried out with n-hexane, followed by benzene-acetic acid (98:2). Finally, OTA was estimated using Thin Layer Chromatography (TLC) and Ultra Violet-via Spectrometer at 333nm against standard toxin procured from sigma Chemical Limited, USA. The purity of the toxin was found to vary between 89 to 94% among the different species of ochratoxigenic fungi used.

### Induction of ochratoxicosis in a rat model

Male Sprague Dawley rats with an average initial weight of 250g were divided into four groups of six animals per group. Rats were housed in wire motion stainless steel cage in a room with controlled temperature, humidity and 12h light cycle. Feed and water were provided for 3 weeks and their feeds were the same throughout the

period. In addition to the above, rats in group 2,3 and 4 were injected with 0.75,0.81 and 0.90ml/kg weight of rat of the different Ochratoxin produced by the different *Aspergilli* while animals in group 1 serve as control. The experiment was done in triplicates to represent Ochratoxin from different *Aspergilli*.

**Determination of malondialdehyde as a bio-marker for lipid peroxidation**

**Sample preparation and extraction**

A sample of the liver, blood and plasma (2.5 g) was collected, the liver and 12 ml of 5 % Phosphotungistic acid solution and 1ml of Butylated Hydroxytoluene (BHT, $0.5l^{-1}$ in methanol as an antioxidant) was blended while the blood and plasma were mixed with 12 ml of 5 % Phosphotungisit acid solution acid and 1ml of Butylated hydroxytoluene (BHT, $0.5^{-1}$ in methanol as an antioxidant) were added, homogenized with a blender (Nakai, Japan) and centrifuged at 10,000rpm for 15 minutes to obtain clear supernatants designated as SL, SB and SP respectively to represent supernatants for liver, blood and plasma.

**Test procedure**

The assay reagents comprising Malondialdehyde (MDA) lysis buffer, Phospthotungistic acid solution, Butylated Hydroxytoluenes Tribarbituric acid and MDA (Malondialdehyde) standard from Bio-division incorporation (2012) were removed from the refrigerator and equilibrated to ambient temperature. To determine the total MDA level produced by the decomposition of lipid peroxides following TBA reaction in the liver, blood and plasma, the clear supernatants obtained above was mixed appropriately with 0.9mi of 0.36% TBA and 0.1M of $FeSO_4.7H_2O$ as a catalyst for decomposing hydro peroxides. These mixtures were further placed in a water bath for 30minutes in covered tubes. The MDA standard was also prepared according to manufacturer's instructions. Finally, the MDA in the supernatant mixture prepared above and the standard MDA were read colorimetrically at 553nm.

**Statistical analysis**

The data were analyzed using SPSS version 16 (SPSS institute, Inc. 2009). The mean of the Malondialdehyde, feed intake, initial weight gain of the rat, weight gain and feed too weight gain ratio were determined using Analysis of variance (ANOVA) while the weight gain and feed to weight gain ratio were determined as follows;

Weight gain in gram = final weight gain-original weight

$$\text{Weight gain in \%} = \frac{\text{final weight gain} - \text{original weight}}{\text{Original weight}}$$

$$\text{Feed to weight gain ratio} = \frac{\text{feed intake}}{\text{Weight gain in grams}}$$

**RESULTS**

The occurrence of a total of three *Aspergillus* species in 36 out of the 80 white garri powders collected from Sagamu and Ago-Iwoye, Ogun State, Nigeria was depicted in the table. These species include *A. ochraceus*, *A. niger* and *A. carbonarius* with different colony diameters and colors. All the isolates except A. niger produced yellow color on the reverse side of Malt Extract Agar (MEA) incubated at 25°C. The diameter of 50-70mm, 44-55mm was recorded for *A. niger*, *A. ochraceus* and *A. carbonarius* respectively. As shown in table 1 below:

**Table 1: Distribution of Ochratoxigenic fungi in white garri powders**

| Serial no | Fungal isolates | Number of isolates (%) | | |
|---|---|---|---|---|
| | | N | N | (%) |
| 1 | *Aspergillus niger* | 36 | 18 | 50 |
| 2 | *Aspergillus ochraceus* | 36 | 6 | 16.7 |
| 3 | *Aspergillus carbonarius* | 36 | 12 | 33.3 |

N= total number of *Aspergillus* species isolated from a sample size of 80 white garri powders
N= number of specific fungal isolated

Percentage $= (\%) = \frac{n}{N} x \frac{100}{1}$

The effect of Ochratoxin A on the performance of rat, an equal amount of feed was fed to the four groups of animal (rat) under study. The initial weight of the rat as shown were also the same, in order to effectively determine if the groups of animals injected with 0.75, 0.81 and 0.9mg/kg weight (group 2, 3 and 4 respectively) of Ochratoxin A would add weight with significant difference the control group of animals that were not injected with Ochratoxin. The weight gain varies from 20g for animals in group 1 to 4.8g for animal in group 2 and 4g for animals in group 3 and 4. The feed weight to weight gain ratio was therefore 8, 33.3, 40, and 40 for the animals in group 1, 2, 3 and 4 respectively. As shown in the table below.

**Table 2: Effect of Ochratoxin on the performance of rat**

| Parameters | Animal groups | | | |
|---|---|---|---|---|
| | Group 1 | Group 2 | Group 3 | Group 4 |
| Feed intake(g) | 400 | 400 | 400 | 400 |
| Initial weight of rat(g) | 250 | 250 | 250 | 250 |
| Final weight of rat(g) | 300 | 262 | 260 | 260 |
| Weight gain(g) | 50 | 12 | 10 | 10 |
| Weight gain (%) | 20 | 4.8 | 4 | 4 |
| Feed to weight gain ratio | 8 | 33.3 | 40 | 40 |

Table 3 below, depicts the comparative analysis of the effect of Ochratoxin on the performance of rats in the different groups. The weight gain in grams was found to be significantly different among the groups of animals investigated under a constant initial weight and feed intake ($F = 6259.7$, $P < 0.05$) while the feed to weight gain ratio was apparently higher in Ochratoxicosed groups of animals than in Ochratoxin free animals (F value = 185.19, $P < 0.05$).

**Table 3: Effect of Ochratoxin A on the performance of Rat, a comparative study of animals in different groups**

| Parameters | Animal groups | | | | | | |
|---|---|---|---|---|---|---|---|
| | n | Group 1 | Group 2 | Group 3 | Group 4 | F value | P value |
| Feed intake | 6 | 400±0.00 | 400±0.00 | 400±0.00 | 400±0.00 | 0.00 | >0.05 |
| Initial weight of rat(g) | 6 | 250±0.00 | 250±0.00 | 250±0.00 | 250±0.00 | 0.00 | >0.05 |
| Weight gain (g) | 6 | 50±0.00 | 12±0.00 | 10±0.00 | 10±0.00 | 6259.7 | <0.05 |
| Feed to weight gain ratio | 6 | 8.0±0.00 | 33.3±0.00 | 40±1.00 | 40±0.00 | 185.19 | <0.05 |

*n= number of animal in each group

The influence of Ochratoxin on the concentration of Malondialdohyde in blood, plasma and liver were represented in table below. A very high value of MDA was observed in the liver of the investigated rat relative to other tissues parts. Though the concentrations of MDA varies from one tissue to the other, the relative increase in the MDA concentrations were 18.8%, 100% and 131.8% for blood, 18.6%, 23.7% and 67.8% for plasma and 18.2%, 19.3% and 29.3% for liver. These data suggest that MDA values increases with increase in Ochratoxin injection in the experiment.

**Table 4: Effect of Ochratoxin on the concentration of Malondialdehyde in different tissues**

| Parameters | Animal groups | | | |
|---|---|---|---|---|
| | Group 1 | Group 2 | Group 3 | Group 4 |
| MDA, $nng^{-1}$ blood | 44 | 80 | 88 | 102 |
| MDA, $nng^{-1}$ plasma | 59 | 70 | 73 | 99 |
| MDA, $nng^{-1}$ liver | 352 | 416 | 420 | 455 |

**Appendix**

From table 4: Mathematically, If increase in Ochratoxin injection = IOA and increase in MDA concentration = IMDA

→ IOA □ IMDA, $\frac{\square\square\square}{\square\square\square\square} = \frac{\square\square\square\square\square}{\square\square\square\square}$

$K = \frac{\square\square\square}{\square\square\square\square}$ Where K = constant, which is derived using the equation above.

The relative measurement of the effect of Ochratoxin on MDA concentration in different tissues was statistically evaluated using Analysis of variance (ANOVA), (table 5). In all the tissues, increase in Ochratoxin injection was found to significantly lead to increase in MDA concentration. It therefore imply that; increase in Ochratoxin injection directly proportional to increase in MDA concentrations (F-value = 135.93, $P < 0.05$ for blood, F-value = 45.6 and 9784.3 for plasma and liver respectively).

**Table 5: Relative measurement of the effect of Ochratoxin on MDA concentration in different tissues**

| Parameters | Animal groups | | | | | |
|---|---|---|---|---|---|---|
| | Group 1 | Group 2 | Group 3 | Group 4 | F value | P value |
| MDA, $nng^{-1}$ blood | 44±0.00 | 80±1.00 | 88±4.00 | 102±1.00 | 135.93 | <0.05 |
| MDA, $nng^{-1}$ plasma | 59±0.00 | 70±5.00 | 73±3.00 | 99±0.00 | 45.6 | <0.05 |
| MDA, $nng^{-1}$ liver | 352±0.50 | 416±2.00 | 420±0.00 | 455±0.50 | 9784.3 | <0.05 |

## DISCUSSION

Garri circulating in Ogun state, Nigeria provides an excellent micro environment condition that favours the growth and survival of spoilage fungi (Thomas *et al*., 2012). The predominance of *Aspergillus niger* in the investigated garri samples is not surprising as this Xerophillic mold has also been reported in other stored foods (Essono *et al*. 2007). The presence of all the isolated *Aspergillus* species in Table 1 above represents a significant risk of Ochratoxin A contamination (Centre for Food Safety, 2006). Ochratoxin A has been referred to as the most toxic of all types of Ochratoxins known till date (Peraica *et al*., 1999). This toxic is Nephrotoxic, Immune-suppressive, Carcinogenic and Tetralogenic in all experimental animals tested so far (Peraica *et al*., 1999).

The injection of Ochratoxin A has different concentration to the groups of an animal model in this study revealed an apparent different in both control group and the treatment group of animal model in terms of weight gained in gram (F value = 6259.7, $P < 0.05$) and feed to weight gained in ratio at constant condition of feed intake and initial weight. This observation corroborates that of (Rahimtula *et al*., 1988) who also recorded similar findings. Even though this observation was glaring between the control and treatment group, low significant difference was however found between the animals that received 0.81 mg ml/lkg weight and 0.90 ml/kg of Ochratoxin A.

In addition to reducing the performance of all the investigated animals, Ochratoxin also induces lipid peroxidation (Rahimtula *et al*., 1988) as revealed by the increasing level of MDA relative to the control group [non-intoxicated group], MDA is known as an important biomarker of lipid peroxidation (Devasagayam *et al*., 2003, Wood *et al*., 2003). Lipid peroxidation has been defined as the oxidative deterioration of lipids containing a number of carbon-carbon double bonds (Devasagayam *et al*., 2003).

This reaction is well known to decrease activities o0f enzymes associated with membranes. Such lipid peroxidation results in inactivation of membrane pumps responsible for maintaining autoxidation of lipid known as lipid peroxidation causes rancidity in vitro and damages to tissues in view. Such damages may include, Cancer, Inflammatory diseases, Atheleroscleroses, aging among other factors (Devasagayam *et al*., 1999).

A very high value of MDA was observed in the liver of the study animal in relation to other tissues parts, this may due to the fact the liver is the target site for Ochratoxin (European Mycotoxins awareness network 2012). The relative high percentage of MDA in the liver than other tissues is an indication that specimen from the liver is the most appropriate when determining the effect of toxic compounds and lipid peroxidation (Knight *et al*., 2003).

## CONCLUSION

The result of this study clearly shows that Ochratoxin A induces lipid peroxidation. Further observation in this study revealed an elevated increase in MDA level with increase in Ochratoxin concentration this clearly demonstrates that Ochratoxin causes oxidative stress in the animal model (Schilter *et al*., 2005).

## RECOMMENDATION

Since it has been shown that there is a correlation between Ochratoxin A and lipid peroxidation, it is therefore recommended that strict control of quality in both industrialized and developing countries is necessary to avoid Ochratoxicosis. It can thereby be recommended that Ochratoxin A level should be monitored so that it does not go beyond the acceptable limit to avoid problems mentioned above.

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