JOURNAL OF CRITICAL REVIEWS

ISSN- 2394-5125 VOL 7, ISSUE 16, 2020

TERATOGENIC ACTIVITY OF CORN FUNGUS IN BIOASSAY VIA DUCK EMBRYOS AS TEST MEDIA

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Received: 16 March 2020 Revised and Accepted: 16 June 2020

Abstract— Teratogenesis is a prenatal toxicity characterized by structural or functional defects in the developing embryo or fetus. It also includes mutations, growth retardation and death of the embryo. Of all the birth defects, teratogens constitute to about 10% and other factors include genetic defects, infection and toxins. The teratogenicity of corn fungus may possibly contribute to rapid development of different defects on embryos which is caused by teratogens. In this study, the researcher used ethanol, agar, corn cobs, scalpel, petri dishes, syringes, pressure cooker, parafilm, and an incubator. Duck eggs were used for assaying. Treatments were prepared: T_0 for untreated eggs, T_1 to T_3 involved the use of reagents which were ethanol, fungi, and ethanol-fungi mixture respectively. Each setup was performed in 5 trials. The duck eggs were incubated for 7 days and were injected with 500 μL of reagents. The treated eggs were again incubated for 14 days. After incubation, ducklings and embryos were obtained. The samples were isolated for screening of occurring malformations and growth retardation. The reagent that yielded the least survival rate, hatching rate, and weight mean indicates potential of teratogenicity. Among all treatments, the reagent of fungi yielded the best results in the decrease of the given parameters. It was observed that the strains of A. flavus can cause the lowest survival rate of 0%, second lowest in hatching rate with a rate of 20%, and the lowest weight mean of 29.7 grams. Indicating the greatest teratogenicity among all treatments.

KEYWORDS— Growth Retardation, Hatching Rate, Survival Rate, Teratogenesis, Bioassay

I. INTRODUCTION

Corn or maize production is a major source of livelihood among the agricultural businesses in the Philippines. Corn, commonly known as the “second rice” is an important crop. In rural areas, in periods of rice shortage, white maize is their substitute. Another type of corn which can be fed to livestock is the yellow maize. Most of the time, this type of corn is fed to ducks.

Duck farming is common here in the country. Farmers feed their ducks with cracked yellow maize, corn kernels to provide the nutrients, and minerals and vitamins which the ducks need for healthy growth and development specifically its meat. They produce efficient animal protein –which makes this bird one of the high-rankers in the poultry industry. Another is their eggs, which is a high source of protein, too. And sometimes, some of these eggs are left to fertilize until they hatch to produce more of its species.

But corn is not that best for ducks or to their eggs. Corn is susceptible to all sorts of diseases, especially Ear Rot diseases caused by *Aspergillus flavus* fungus. This fungus grows favorably on hot and dry weather. It can be seen at the tip of the corn husk. It is said that this fungus produces toxins and carcinogens that can potentially kill live stocks when it is fed to them (Hedayati, M.T. 2007). In addition, it may cause congenital malfunctions on embryos, especially on ducks (*Anas platyrhynchos*) because these birds are more often fed with

corn.

Since corn mold has the presence of toxins that can cause damage to the organism as well as to its embryos specifically affecting their development. This includes growth retardation, death of the embryo or fetus, and carcinogenesis.

There were 510,000 deaths reported in 2010 due to congenital defects. Of all the birth defects, teratogens constitute to about 10% and other factors include genetic defects, poor maternal nutrition, infection and environmental toxins (Gaffield & Keeler, 2015).

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Corn plants thrive in countries with tropical climate. At the same time, during this season the fungus can grow and produce toxins on corn kernels. Fungus-infested corn in turn may be fed to ducks. Depending on the teratogenicity of the fungus, it may either affect the duck's health or its offspring. Hence, this study aimed specifically to determine the effects of this fungus to the embryos of ducks. Specifically, the study aimed to identify what specific mycotoxins that is present in corn fungus. Moreover, the teratogenicity of the corn fungus was measured in terms of the duck embryos' survival rate, hatching rate, mass of the embryos and yolk sac stability. These variables were compared.

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OBJECTIVES OF THE STUDY

This study aimed to determine the teratogenicity of corn fungus *Aspergillus flavus* in bioassay and as tested in duck embryos. Specifically, it aimed to answer the following questions: [1] What is the specific mycotoxin that is/are present in corn fungus that can cause teratogenesis on the duck embryos?; [2] What are the characteristics of the treated duck embryos compared to the untreated specimens in terms of survival rate, hatching rate, mass of the embryos, and yolk sac stability?; [3] Is there a significant difference among all treatments in terms of survival rate, hatching rate, and weight of the embryos?

II. MATERIALS AND METHODS

This study used experimental method of investigation wherein the teratogenic effects of corn fungus were determined using fungi identification by Morphology, fungal ethanolic extractions, In vivo injections, and Teratogenicity Assay. Specifically, this research study employed Random Block Design where in some extraneous variables were controlled by providing repetitions for each treatment (Kothari, 2004).

Table 1. Fungi Identification by Morphological Characteristics ((Muneera Al-Kahtani , 2014)

Colony Morphology	Mycelium	Spores	Conidiophores/sterigmata	Probable Organism
Velvety, yellow to green to brown	Blue mycelia woolly at first, white to yellow, then turn dark brown to black	Blue spores	Conidiophores variable in length, rough, spiny; sterigmata single and double, pointed in all directions	Flavus
Woolly at first, white, to yellow then turn dark brown to black	Blue/ brown mycelium	Blue spores	Sterigmata double, cover entire vesicle. Form radiate head	Niger

The materials used were 20 medium-sized fertile duck eggs, 20 moldy corn cobs, ethanol (95%), GRMO026-

Usually fast growing, pale or brightly colored	White/ pink mycelium	Black spores	Conidiophores may be single or branched with conidia	Fusarium
Grow quickly and colony may be gray, brown or black in color	Usually starts white before changing to a darker color	Dark brown to black spores	Conidiophores pale brown to olive brown straight or flexous	Alternaria

500G agar, and 100 mL distilled water. The equipment used were a scalpel, 3 petri dishes, 3 syringes, an alcohol burner, pressure cooker, hot plate stirrer, Erlenmeyer flask, beaker, graduated cylinder, gas stove, parafilm, and an egg incubator,

Twenty medium-size fertile duck eggs were obtained from a local balut producer. These were used for assaying the fungi-ethanol isolates and the other reagents. Before analysis, each egg was carefully scraped until they are smooth and thin using sandpaper and cleaned with ethanol. Egg candling process was employed using a flashlight and a tissue roll as an alternative to determine the position of the embryo. This procedure allowed the researchers to assess whether the eggs are fertilized or not. The eggs were incubated for 7 days. After 7 days, a small hole was made in the middle of the shell surface above the air chamber using a needle. Injections were performed through this hole, above the air chamber, with the syringe in horizontal position, without injecting the embryo itself. When the substances were injected in the air chambers, the eggs were held vertically with the large end pointing upward. The different substances were introduced with the dosage of the same amounts. The hole that was made was sealed using laboratory parafilm. They were incubated at 30℃ and 70% humidity for 14 days in an egg incubator.

A 5.2 gram GRM026-500G agar and 100 mL of distilled water were stirred for 2 minutes in a 250 mL glass Erlenmeyer flask. Once stirred, the mixture was autoclaved for 17-20 minutes in a pressure cooker. The agar mixture was poured into 3 petri dishes and was left to settle. They were sealed with parafilm and were set aside.

Moldy corn cobs were obtained from a local corn stand. The cobs were put in a Styrofoam cooler and were sprayed with water for further fungal growth. The cooler was sealed with packing tape and was left for 14 days to incubate and grow. At the end of 14 days, the fungi were scraped out of the cobs using a flame-sterilized scalpel and were transferred to petri dishes with agar. The fungal cultures were left to grow for 7 days.

The fungi that were obtained were identified using identification by morphology (Muneera Al-Kahtani , 2014) which is shown in Table 1. Meanwhile, after 7 days of culturing, 6 grams out of 10 grams of the fungi was obtained from the agar slants, they were scraped and transferred to a 600 mL beaker with 20 mL of 20% ethanol. The mixture was covered with parafilm and was shaken for 1 hour. The extract was then filtered using filter paper. The filtrates were transferred to petri dishes.

There were 4 treatments used in this study. Untreated duck eggs as Treatment 0 (T_0), pure fungal strains as Treatment 1 (T_1), pure ethanol as Treatment 2 (T_2), and fungi-ethanol mixture as Treatment 3 (T_3). For T_1, 4 g of pure *A. flavus* was scraped out of the agar slants, measured, and was transferred to a petri dish with 5 mL of water. For T_2, 5 mL of 95% ethanol was measured and transferred to a petri dish. For T_3, 20 mL of the fungi-ethanol mixture was measured and transferred to a petri dish. For every treatment, 500 µL (microliters) of each reagent was measured and injected to the duck eggs.

Hatched ducklings were obtained at the end of 21 days of incubation under strict aseptic conditions. The hatched ducklings were isolated for observation and screening of occurring malformations and growth retardation. Unhatched eggs were later on removed out of the shells and were individually weighed using a triple-beam balance and were observed for occurring malformations and growth retardation. The survival rate, hatching rate and yolk sac stability rate of the samples were computed using the data. To determine the teratogenicity of corn fungus, mean and standard deviation (s.d.) utilized. One-way Analysis of Variance (ANOVA) was employed to determine the p-value among the four treatments and was utilized to test the null hypothesis.

III. RESULTS AND DISCUSSIONS

Morphological Characteristics

Based from observations, the fungus was described as "velvety" with colors ranging from white to yellow to green or brown to black while the color of the spores was moss green.. Using the morphological

characteristics presented by Muneera Al-Kahtani (2014) it was later on identified as a strain of *Aspergillus flavus.*

Like other Aspergillus species, *A. flavus* has a worldwide distribution. This probably results from the production of numerous airborne conidia which easily disperse by air movement and possibly by insects. Atmosphere composition has a great impact on mold growth, with humidity being the most important variable (Gibson et al., 1994). *A. flavus* grows better with water activity (aw) between 0.86 and 0.96 (Vujanovic et al., 2001). The optimum temperature for A. flavus to grow is 37°C, but fungal growth can be observed at temperatures ranging from 12 to 48°C. Such a high optimum temperature contributes to its pathogenicity in humans (Hedayati et al., 2007).

Table 2. Mycotoxins and other Toxic Compounds Present in *Aspergillus flavus*

Toxic Compounds	Mycotoxins
sterigmatocystin	Aflatoxin B1
cyclopiazonic acid	Aflatoxin B2
kojic acid	Aflatoxin G1
bnitropropionic acid	Aflatoxin G2
aspertoxin	
aflatrem	
gliotoxin	
aspergillic acid	
dihydroxyaflavinine	
indole	
paspalinine	
versicolorin A	

Aspergillus flavus is an opportunistic pathogen of crops. It is important because it produces a teratogen such as aflatoxins as a secondary metabolite in the seeds of a number of crops both before and after harvest. Aflatoxin is a potent carcinogen that is highly regulated in most countries. In the field, aflatoxin is associated with drought-stressed oil seed crops including maize, peanut, cotton seed and tree nuts. Under the right conditions, the fungus will grow and produce aflatoxin in almost any stored crop seed. In storage, aflatoxin can be controlled by maintaining available moisture at levels below that which will support growth of *A. flavus* (Klich, 2007).

Mycotoxins are fungal secondary metabolites that are potentially harmful to animals or humans. The word 'aflatoxin' came from '*Aspergillus flavus* toxin', since *A. flavus* and *A. parasiticus* are the predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage (Yu et al., 2004). The aflatoxins B1, B2, G1 and G2 are the major four toxins amongst at least 16 structurally related toxins (Goldblatt, 1969). Aflatoxin B1 is particularly important, since it is the most toxic and potent hepatocarcinogenic natural compound ever characterized (Bennett & Klich, 2003). Different *A. flavus* strains may or may not produce either aflatoxin B1 and/or B2. Other toxic compounds produced by A. flavus are sterigmatocystin, cyclopiazonic acid, kojic acid, bnitropropionic acid, aspertoxin, aflatrem, gliotoxin and aspergillic acid. In addition A. flavus may produce some other secondary metabolites such as dihydroxyaflavinine, indole, paspalinine and versicolorin A.

Teratogenic Properties

Table 3. Survival Rate of the Duck Embryos

Treatment	X	Y	Survival Rate (%)
T_0	2	3	40%±0.54772*

T_1	0	5	0% ± 0.00000*
T_2	0	5	0% ± 0.00000*
T_3	2	3	40% ±0.54772*

*Note: X= Number of Live Ducklings; Y= Number of Dead Ducklings; T_0= untreated; T_1= ethanol; T_2= fungi; T_3= fungi-ethanol mixture; Survival Rate expressed with s.d.; *not significant*

Table 3 shows the survival rate of the embryos per treatment. Survival rate was the percentage of duck embryos that hatched, observed and described as alive after hatching. Among all treatments, T_0 and T_3 yielded the highest survival rate while T_1 and T_2 the lowest survival rate. This implies that ethanol and the pure fungi caused or contributed in the underdevelopment of embryos. Ethanol usually contains 95% alcohol which is known to be lethal to microorganisms and can disturb an embryo's development. A number of studies have revealed teratological effects of alcohol in man, and investigations have also been performed to demonstrate such effects in experimental animals (Hollstedt, Olsson & Rydberg, 1977). On the other hand, *Aspergillus flavus* fungus usually produce aflatoxins and other toxic compounds which are considered teratogenic and carcinogenic (Klich et al, 2007) thus supporting the observed effect. Meanwhile T_3 resulted to 40% survival rate which was actually the mixture of ethanol and fungi which caused 0% survival rate individually. This actually implies that while two reagents were in isolation, they caused teratogenicity, when these were mixed ethanol negated the potency of the fungi. Ethanol contains 95% alcohol which is an antiseptic.

A similar study did not use parameters like survival rate and hatching rate but researchers stated that growth retardation, underdevelopment and premature birth were signs of teratogenesis (e.g. Nationwide Cerebral Palsy Resource Network). Survival rate and hatching rate among duck eggs are signs of growth retardation and underdevelopment. Hence, low survival rate and hatching rate are considered teratogenic effects.

Table 4. Hatching Rate

Treatment	**Hatching Rate (%)**
T_0	40%±0.54772[a]
T_1	0% ±0.00000[b]
T_2	20%± 0.44721[c]
T_3	100 %± 0.00000**

*Note: X= Number of Hatched Eggs; Y= Number of Unhatched Eggs; T_0= untreated; T_1= ethanol; T_2= fungi; T_3= fungi-ethanol mixture; hatching rate expressed with ± s.d.; **significant with b and c*

Table 4 shows the hatching rate of the embryos per treatment. These were the eggs that were observed as hatched and showing chances of hatching including the dead ones but with observable characteristics of being hatched such as cracks on the egg shell surfaces. Among all treatments, T_3 yielded the highest hatching rate while T_1 (ethanol) yielded the lowest hatching rate. Results clearly showed that ethanol and the pure fungi contributed in the underdevelopment of embryos. But the mixture of 20% ethanol and pure fungi proved that it did not cause underdevelopment. This shows that while pure fungi caused underdevelopment and pure ethanol also caused underdevelopment, the ethanol-fungi mixture resulted otherwise to a positive effect. Mixing the two reagents somehow reacted with each other negating the toxicity of each test sample.

With a p-value of 0.002, it is noted that there was a significant difference among the treatments in the hatching rate of duck embryos. The hatching rates of the test subjects as affected by the reagents injected were significantly different among the treatments. Further analysis showed that the reagents had different degrees of influence on the duck embryos and these effects. T_3 showed the highest percentage of hatching which means that the reagent causes the least sign of underdevelopment. T_1 showed the lowest percentage of hatching, which proves that the reagent causes the most effect of underdevelopment.

The chance for an egg to hatch is affected by the condition of the embryo during the developmental stage. If along the process, the embryo became unstable, then a low chance of hatching is possible. Teratogenic substances when introduced to embryos during the developmental stage can cause unacceptable hatching rate among duck eggs. The fungi which propagate among corn cubs can cause teratogenic effect among egg embryos when introduced to these.

Table 5 shows the weights of the embryos after treatment and 14 days of incubation. The weight of the embryo can be used to denote any possible growth retardation. The embryo which had the least weight means the greater growth retardation. Based from the table, among all treatments, T_0 yielded the highest weight mean while T_2 yielded the lowest weight mean. Therefore, the embryos treated with fungi (A. flavus) had the greatest growth retardation. This further proved that the fungi caused teratogenic effect among the embryos as indicated by their growth retardation.

Table 5. Comparison of Weight Means of Embryos Treated

Treatment	Weight Mean (grams)
T_0	**42.2**±5.02[a]
T_1	**30.4**±5.37[b]
T_2	**29.7**±5.74[c]
T_3	**37.46**±10.03**

*Note: T_0= untreated; T_1= ethanol; T_2= fungi; T_3= fungi-ethanol mixture; weight mean± s.d given as mean of five values expressed; **significant with b and c*

Furthermore, an ANOVA test identified that there was a significant difference among the treatments in terms of growth retardation as indicated by the weights of the embryo. Further analysis revealed that the reagents differently affected the weight of the embryos. T_0 yielded the highest mean weight of the embryo and T_2 caused the lowest mean weight which proved that this reagent caused the greatest growth retardation.

Table 6.Yolk Sac Stability Rate

Treatment	Yolk Sac Stability Rate (%)
T_0	100%
T_1	100%
T_2	0%
T_3	100%

Note: T_0= untreated; T_1= ethanol; T_2= fungi; T_3= fungi-ethanol mixture; X = Number of Embryos with Complete Yolk Sac; Y= Number of Embryos with Damaged Yolk Sac

Another indicator for teratogenic effect is the stability of egg yolk sac. The greater the possible teratogenic effect of a reagent is, the lesser the yolk sac is stable Table 6 shows the stability rate of the yolk sac of the embryos per treatment. The embryos that have stable yolk sacs after treatments were accounted. T_0, $T_1$and T_3 resulted to the greatest number of embryos with complete yolk sacs (100%). Meanwhile, T_2 resulted to 5 out of 5 embryos with unstable yolk sacs. This reagent yielded 100% instability rate among duck embryos. The yolk sacs were observed and described as "damaged." This observation coincides with the earlier findings that fungi caused underdevelopment of embryos. Specifically, the eggs treated with pure fungi did not hatch because the yolk sacs were unstable thus hindering growth and development.

IV. CONCLUSIONS AND RECOMMENDATIONS

After all the observations and analysis of data it was found that the pure fungal culture yielded the most disturbances and malformations among duck egg embryos. Specifically, this study signifies that pure fungal cultures contributed to underdevelopment with very low survival rate and hatching rate. It also contributed to growth retardation resulting to minimal weights among duck egg embryos. Thus, among the reagents tested, it was the pure fungi that caused teratogenic effects among duck embryos.

. The study was only limited among duck embryos subjected to teratogenicity assay. Future researchers are advised to introduce the fungi tested for teratogenic effects on embryos among hens which are about to lay eggs.

V. ACKNOWLEDGMENTS

Heartfelt gratitude is given to parents, family members, and friends for their never-ending financial, physical and moral support. Special thanks to the following are likewise given: Dr. Leonilyn M. Coroña, Mrs. Febe Juan, Ms. Jeremiah Taan, Ms. Sean Micu, Mr. Briant Baraquio, Ms. Darlene Calica, Ms. Cristine Suaso, Ms. Clarisse Sajonia, Ms, Dianne Bautista, Ms Sheela Cabida and Ms. Jade Lutrania for extending their help in performing and providing materials for this study. Gratitude is also extended to the Philippine Association of Research Practitioners, Educators and Statistical Software Users, Inc for the publication grant of this paper.

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