Journal of Cell and Animal Biology

Volume 8 Number 8, August 2014
ISSN 1996-0867

Academic Journals

ABOUT JCAB

The **Journal of Cell and Animal Biology (JCAB)** (ISSN 1996-0867) is published Monthly (one volume per year) by Academic Journals.

Journal of Cell and Animal Biology (JCAB) provides rapid publication (monthly) of articles in all areas of cell and animal biology such as Cellular metabolism, Cellular differentiation, Alcoholic fermentation etc. All articles published in JCAB are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email jcab@academicjournals.org.

With questions or concerns, please contact the Editorial Office at jcab@academicjournals.org.

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the Journal of Cell and Animal Biology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Journal of Cell and Animal Biology

Table of Contents: Volume 8 Number 8 August, 2014

ARTICLES

academicJournals

Vol. 8(8), pp. 136-155, August 2014
DOI: 10.5897/JCAB2014.0420
Article Number: 272370947316
ISSN 1996-0867

http://www.academicjournals.org/JCAB

Journal of Cell and Animal Biology

Full Length Research Paper

Effects of Aflatoxin B1 on the skeletal system of rabbit (*Oryctolagus cuniculus*) fetuses

S. M. El-Nahla, H. M. Imam, E. A. Moussa and A. R. Ghanam*

Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

Received 5 August, 2014; Accepted 27 August, 2014

The present study was carried out to determine the effects of aflatoxinB1 (AFB1) on the skeletal system of the fetuses of balady rabbits. The female animals were divided into three groups, one control and two treated, the control group contained four dams, the intoxicated group with 0.1 mg/kg contained six dams while the intoxicated with 0.05 mg/kg contained three dams. A dose of 0.1 and 0.05 mg/kg/day AFB1 was administered by gastric intubation to pregnant rabbits on the 6th-21st day of pregnancy. The fetuses were obtained through the uterine incisionat different stages of gestation according to the group. The lengths and weights of the fetuses as well as absolute organs weights were measured, revealing the statistically significant differences between the control and intoxicated with 0.05 mg/kg group at 29th day of gestation ($p<0.001$) while there were non-significant decrease of control and intoxicated with 0.1 mg/kg at 22nd day of gestation. The observed gross anomalies included wrinkled skin, enlarged eye socket and microphthalmic eyes in both groups. The heart of treated group showed reduction in size with wide ventricular lumen and shallow inter ventricular groove in intoxicated group with a dose of 0.05 mg/kg AFB1. Regarding the skeletal anomalies, there were incomplete ossification in some of the skull bones, the laminae of the vertebral arches throughout the vertebral column remain cartilaginous. The sternum was incompletely ossified. Most of the appendicular skeleton bones were grossly shorter and remained in cartilaginous state.

Key words: Aflatoxin B1 (AFB1), rabbits, teratogenicity, ossification.

INTRODUCTION

Aflatoxins, metabolites of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, are frequent contaminants of a number of staple foods, particularly maize and ground nuts, in subsistence farming communities in tropical and sub-tropical climates in sub-Saharan Africa, Eastern Asia and parts of South America. Increased expression or deregulation of the c-myc and c-Ha-ras genes may play an important role in the development of hepatomas induced by AFB1 (Tashiro et al., 1986). 7,8-Benzoflavone stimulates the metabolic activation of aflatoxin B1 to mutagens by human liver (Buening et al., 1978). A mouse hepatoma cell line, Hepa-1, is highly sensitive to the toxic effects of Aflatoxin B1 (AFB1) (Kärenlampi, 1987). Consumption of a CMRN-containing diet provides substantial protection against the initiation of AFB1 hepatocarcinogenesis in the rat (Kelly et al., 2000).

The metabolism of AFM1 and AFB1 has been studied *in vitro* using human liver microsomes. Formation of primary

*Corresponding author. E-mail: dr_amrelbialy@yahoo.com or amrelbialy@vet.suez.edu.eg.

metabolites associated with metabolic activation to the respect-tive epoxides reflected the differences between the carcinogenic potentials of the two toxins and, similar to AFB1, the conjugation of AFM1 epoxide with reduced GSH was catalyzed by mouse, but not human liver cytosol (Decad et al., 1977). The nuclei and microsomes are capable of metabolizing aflatoxin B1 into aflatoxin M1, aflatoxin Q1, and two unidentified fluorescent compounds in the presence of fortified NADPH generating system (Yoshizawa et al.. 1981). The levels of expression of many of the forms of cytochrome P-450 involved in AFB1 metabolism are known to be highly sensitive to environmental factors. This indicates that such factors will be an important determinant in individual susceptibility to the tumorigenic action of AFB1 (Forrester et al., 1990).

Among commonly occurring mycotoxins, aflatoxin B1 (AFB1) gained immense importance due to its biological effects and widespread toxicity (Trail et al., 1995). AFB1 is one of stable mycotoxins commonly produced by toxigenic strains of *Aspergillus*: *Aspergillus* flavus, *Aspergillus parasiticus* and *Aspergillus fumigatus*, that are ubiquitous in hot/humid conditions and natural contaminates of food and feed stuff (Hussein and Brasel, 2001; Diekman and Green, 1992). In 1993, WHO-international agency for research stated that rabbits are exclusively recommended by regulatory bodies for developmental toxicity studies also classified AFB1 as class 1 carcinogen and regulate its level to a very low concentration in traded commodities (20 ppb in grains, 05 ppb in milk and 4 ppb in feeds).

Congenital malformations are structural anomalies which take place during embryogenesis. Several medicines and certain chemicals may cause malformations or permanent defects which may lead to death passing through fetal circulation, which is called (teratogenesis) (Brendel et al., 1989).

Development of embryo is affected by teratogens mostly during the process of organogenesis, which is recognized as the time period from the occurrence of the neural plaque to closure of the plate (Stanley and Bower, 1986; Vickers and Brackley, 2002). It begins usually on the 6^{th}–18^{th} day in rabbit (Petrere et al., 1993; Wangikar et al. 2005) during this period, teratogenic agents can lead to significant congenital anomalies.

Rabbits and ducks are the most sensitive species to aflatoxin B1 (AFB1) with the oral LD50 0.3 and 0.36 mg/kg bodyweight, horses and sheep are moderately susceptible to oral LD50 1.0 and 2.0 mg/kg body weight respectively, while chickens are relatively resistant to oral LD50 6.5 mg/kg body weight (Marquardt and Fronhlich, 1992; Pier, 1992). Also, Clark et al. (1980) determined the acute oral LD50 of AFB1 for rabbits as 0.3 mg/kg body weight, while Wangikar et al. (2005) mentioned that 0.1 mg/kg body weight AFB1 is the minimum teratogenic dose in rabbits which interfere with intrauterine development during gestation days 6-18.

However, the literatures about the teratogenic study in rabbits are meager and the rabbit is the most sensitive species to mycotoxins and greatly similar to humans in early developmental patterns (Beaudion et al., 2003) and the extra-embryonic membranes of rabbits are closely related to that of human (Foote and Carney, 2000). This study was planned to elucidate the effects of AFB1on the skeletal system of rabbit fetuses by administering different doses during gestation period of 6-18 day. Literature on this is lacking

MATERIALS AND METHODS

Experimental animals and mating procedures

Sexually mature (2.5±0.5 kg), virgin apparently healthy balady rabbits of both sexes (2 males &13 females) from 3-4 months old were used. The animals were kept for two weeks for acclimatization with the housing conditions. The female rabbits in estrous were mated with males and considered as day zero of conception. The pregnant animals were divided into three groups and each individual was kept in a separate cage. All animals were kept under a constant day/night cycle (12 h L/12 h D) for 29 days (pregnancy period). All animals received routinely *ad libitum* standard rabbit diet (18% crude protein containing granule pellet) free of any mycotoxins at Central Laboratory of Residue Analysis of Pesticides and Heavy metals in Food, Agricultural Research Center, Ministry of Agricultural, Egypt. Tap water was supplied *ad libitum* on a daily basis. The fetuses were taken by uterine incision after slaughter of the dams.

Method of treatment

The control group (4 animals): two animals were dosed by gastric intubation with a dose of 0.2 ml corn oil/kg/day from 6^{th}-18^{th} day of the pregnancy, slaughtered at 29^{th} day of gestation. One animal dosed from 6^{th}-13^{th} was slaughtered at 14^{th} day of gestation and the last one dosed from 14^{th}-21^{st} with the same dose, was slaughtered at 22^{nd} day of gestation

The intoxicated group (3 animals): received AFB1 with a dose of 0.05 mg/kg dissolved in corn oil 0.2 ml/kg/day from 6^{th}-18^{th} day of the pregnancy, was slaughtered at 29^{th} day of gestation. The intoxicated group (6 animals): three received AFB1 with a dose of 0.1 mg/kg dissolved in corn oil 0.2 ml/kg/day from 6^{th}-13^{th} day, died on 14^{th} day of gestation and three received the same dose from 14^{th}-21^{st} day of the pregnancy, died at 22^{nd} day of gestation.

Fetus preparation

All the collected fetuses were weighed separately and their crown rump length (CRL) was measured. The fetuses were carefully examined for gross morphology, visceral anomalies (liver, heart, kidney, brain and eye) as well as the skeleton.

Fetus staining for skeleton

Ten fetuses from the control group, 10 fetuses from the intoxicated group with a dose of 0.05 mg and 10 from the intoxicated group with a dose of 0.1 mg at 22^{nd} day gestation were randomly selected and fixed in absolute ethyl alcohol and stained with Alizarin Red-S-AlcianBlue to define the mineralized areas and the cartilage, respectively. The amount of mineralization was calculated as the length of stained portion of the bone by Alizarin Red-S.

The fetuses were firstly fixed in 95% ethyl alcohol for 7 days and subsequently put in pure acetone for degreasing for 3 days. Then,

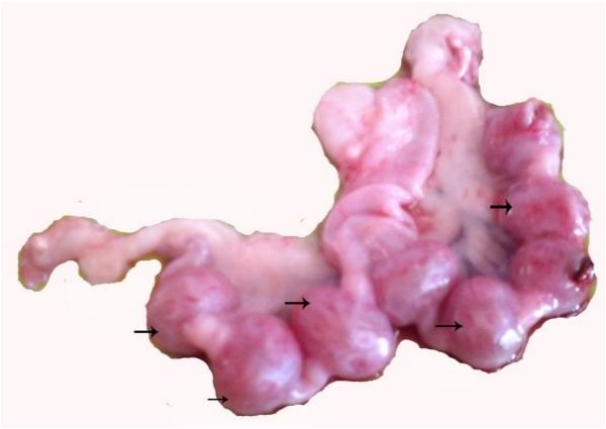

Figure 1. A photograph showing live embryos (arrows) inside the uteri of control rabbit at 14th day of gestation.

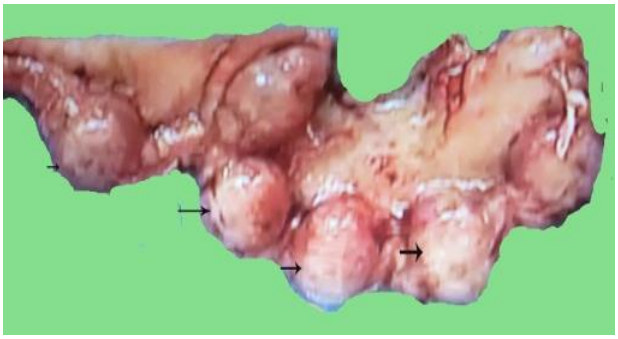

Figure 2. A photograph showing dead embryos inside uteri of treated dam with 0.1mg/kg AFB1at 14th day of gestation.

skin and internal organs were totally removed to achieve better staining results.

1. They were stained according to the method described by Inouye (1976) and Young et al. (2000).
2. Then the specimen were transferred to transparency process using ascending series of glycerol and 1% aqueous solution of KOH after which they were preserved in 100% glycerin.
3. The stained preparations were carefully examined using Stereo-microscope (OPTIKA) to illustrate the different parts of the bones of both axial and appendicular skeleton.

Statistical analysis

The individual data on relative organ weight, CRL and fetal weight were subjected to descriptive statistics (mean and standard deviation), followed by analysis of variance (ANOVA) and T-test according to Argyrous (2005) using Statistical Package for Social Science (SPSS) software program version 16.0.and Excel Microsoft office program (2003). Statistical significance was accepted at $P < 0.05$.

Nomenclature used in this study was adapted according to Nomina Anatomica Veterinaria (2005) and Nomina Embryologica Veterinaria (2003) whenever possible.

RESULTS

Macromorphometric measurements and gross anatomical findings

During the period of the experiment, there were no mortalities of any control dams as well as dams of intoxicated group with a dose of 0.05 mg/kg AFB1 while the treated group with high dose of 0.1 mg/kg did not complete gestation and all dams died even when they were administered from 14th -21st day of gestation.

The fetuses of control group and intoxicated with a dose of 0.05 mg were alive at 29th day of gestation after slaughtering of the dams. While the dams intoxicated with 0.1 mg/kg AFB1 from 6th day died at 14th day; three fetuses were of olive seed in size and suffered from total hemorrhage and necrosis. Neither the limb buds nor the brain ventricles formed as compared to the control fetuses. The dams intoxicated with the same dose from 14th day of gestation aborted at 22nd day of dead fetuses with sever vaginal bleeding and died (Figures 1 and 2).

The mean fetal weight of intoxicated group with 0.05 mg/kg and control fetuses was 28±1.29 and 54.28±8.26, respectively while the mean crown rump length was 9.84±0.21 and 11.57±0.47 respectively (Table 1 and Figure 3). They were significantly reduced when compared with the control. While the fetuses of the treated group with 0.1 mg/kg at 22nd day of gestation, showed comparable fetal weights of intoxicated and control groups as 16.005±3.28 and 16.34±0.56 respectively and slight decrease in crown rump (CRL), 7.17± 0.47 and 7.18± 0.37 respectively (Table 2 and Figures 4 and 5).

The gross anomalies of treated fetuses with 0.05 mg/kg at 29th day and 0.1mg/kg at 22nd day were severe subcutaneous congestion at different regions including abdomen and head mainly in the eye region. In addition, enlarged eye socket with micro-ophthalmic eye and wrinkled skin was seen (Figures 3 and 5). The treated fetuses with a dose of 0.05 mg/kg AFB1 showed small sized liver (Figure 8) as well as small sized heart with shallow interventricular grooves and wider ventricular
lumen as compared to control group at 29th day of gestation (Figures 6 and 7).

The mean absolute weight of different fetal organs (liver and gall bladder, stomach and intestine, heart and lungs and kidneys) of treated dams with a dose of 0.05 mg/kg was significantly decreased as compared to control fetuses at 29th days as follow (2.52 ± 0.296 and3.23 ± 0.23, 1.84 ± 0.11 and 2.47 ± 0.11, 1.69±0.11 and 1.89±0.09, 0.247± 0.011 and 0.33±0.036, respectively) (Table 1 and Figure 9). While fetuses of treated dams with dose of 0.1 mg/kg AFB1 which were slaughtered at 22nd day of gestation showed non-significant decrease in absolute organs weight (liver and gall bladder, stomach and intestine and kidneys) as compared to control fetuses at the same age as follows (1.31±0.09 and 1.4±0.13, 1.01±0.029 and 1.08±0.13, 0.21±0.028 and 0.23±0.022, respectively), while heart and lungs of treated fetuses were significantly

Table 1. Mean ±SD for fetuses at 29th day of gestation of both control and treated with 0.05 mg/kg AFB1.

Measurements	Control fetuses	Treated Fetuses	T	P-value	Df
Body weight (g)	54.28^{a}±8.26	28.00^{b}± 1.29	8	0.001	12
CRL (cm)	11.57^{a}±0.47	9.84^{b}±0.21	9	0.001	12
Liver and gallbladder weight (g)	3.23^{a}±0.23	2.52^{b}±0.296	5	0.001	12
Stomach and intestine weight (g)	2.47^{a}±0.11	1.84^{b}±0.11	10	0.001	12
Heart and lungs Weight (g)	1.89^{a}±0.092	1.69^{b}±0.11	4	0.002	12
Kidneys Weight (g)	0.33^{a}±0.0365	0.247^{b}±0.011	6	0.001	12

P- value = 0.0001. Within the same raw means with different superscripts considered as highly significant (p ≤ 0.01).

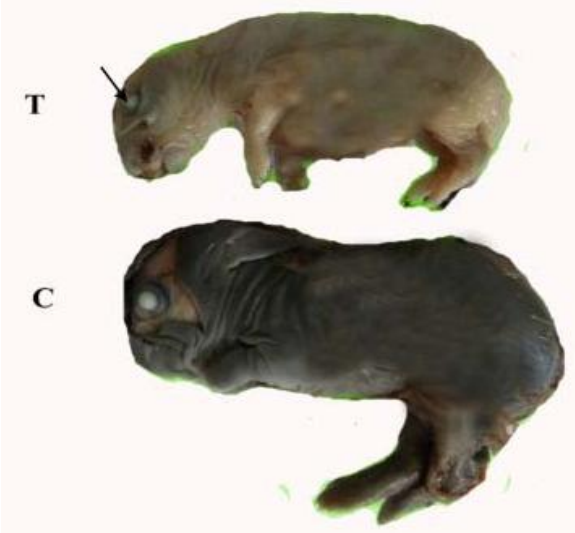

Figure 3. A photograph showing significant decrease of CRL of control (C) and fetuses of treated dams (T) with a dose of 0.05 mg/kg AFB1 at 29th day of gestation with micro-ophthalmic eye (arrow) and a dose of 0.05 mg/kg at 29th day of gestation.

Table 2. Mean ±SD for fetuses at 22nd day of gestation of both control and treated with 0.1mg/kg AFB1.

Measurements	Control fetuses	Treated Fetuses	T	Df	p-value
Body weight (g)	16.34^{a}±.56	16.005^{a}± 3.28	0.268	12	0.794
CRL (cm)	7.18^{a}± 0.372	7.17^{a}± 0.47	-0.063	12	0.951
Liver and gallbladder weight (g)	1.40^{a}± 0.13	1.31^{a}± 0.09	1.677	12	0.119
Stomach and intestine weight (g)	1.08^{a}± 0.13	1.01^{a}±0.029	1.58	12	0.139
Heart and lungs weight (g)	0.797^{a}±0.035	0.746^{b}±0.051	2.20	12	0.048
Kidneys weight (g)	0.23^{a}±0.022	0.21^{a}±0.028	1.378	12	0.193

Within the same raw, means with different superscripts are considered significant (p≤ 0.05).

decreased as compared to the control (0.746± 0.051 and 0.797± 0.035, respectively) (Table 2 and Figure 10).

Skeletal anomalies

The bony and cartilage elements of the fetal skeletons were examined using double staining technique. The bony components were stained red with alizarin Red- S while the cartilages component was stained with alcian Blue. The length and rate of ossification of the long bone can be calculated by the amount of mineralization that stained red by alizarin red-S stain.

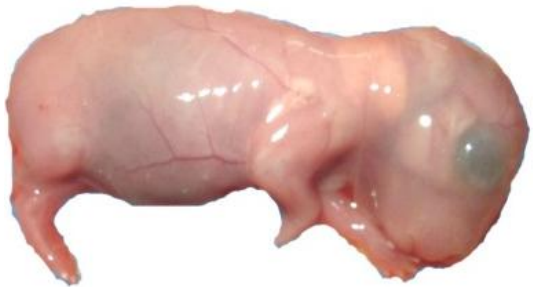

Figure 4. A photograph showing control fetus at 22nd day of gestation with intact and normal skin.

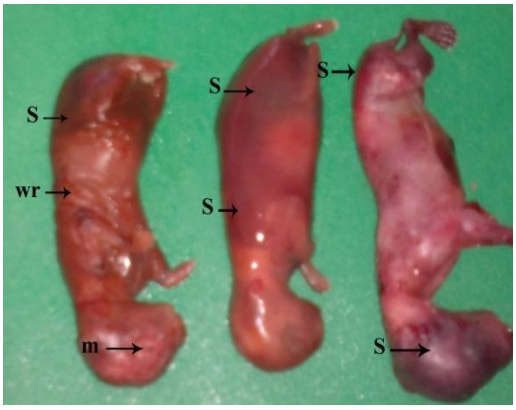

Figure 5. A photograph showing fetuses of treated dams with 0.1 mg/kg AFB1 at 22nd day of gestation with subcutaneous congestion at different regions including abdomen and head mainly in the eye region (S), sever wrinkled skin (WR) and microphthalmic eye (m).

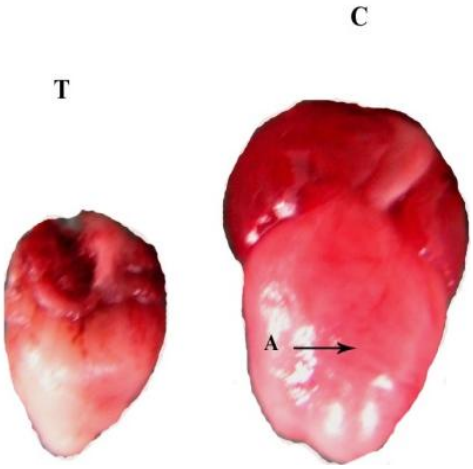

Figure 6. A photograph showing heart of fetues of treated dams (T) with 0.05 mg/kg AFB1 at 29th day of gestation with small sized heart and shallow inter-ventricular groove as compared to the control fetus (C). Inter ventricular groove (A)

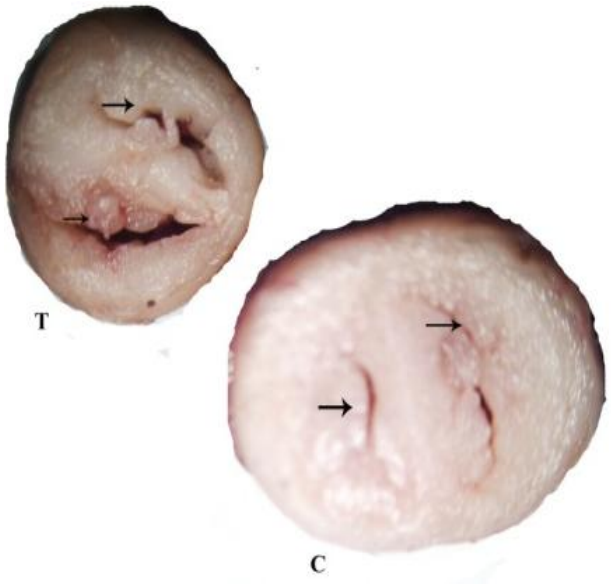

Figure 7. A photograph showing cross section of ventricular mass of fetuses of treated dams (T) with 0.05 mg/kg having wider ventricular lumen than that of control fetus (C) at 29th day of gestation.

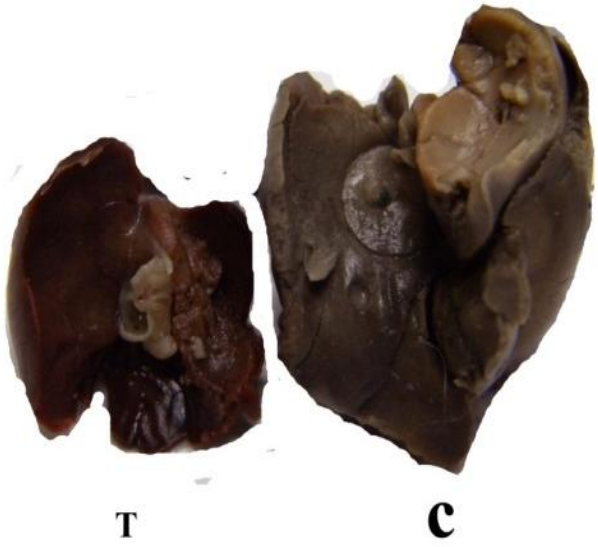

Figure 8. A photograph showing small sized liver of fetuses of treated dams (T) with 0.05 mg/kg at 29th day of gestation as compared to control one (C).

Skeleton axiale (axial skeleton)

Many of the skull bones developed through the process of intramembranous ossification, which does not involve a cartilaginous template. While bones at the base of the skull including the occipital and basisphenoid bones developed through endochondral ossification.

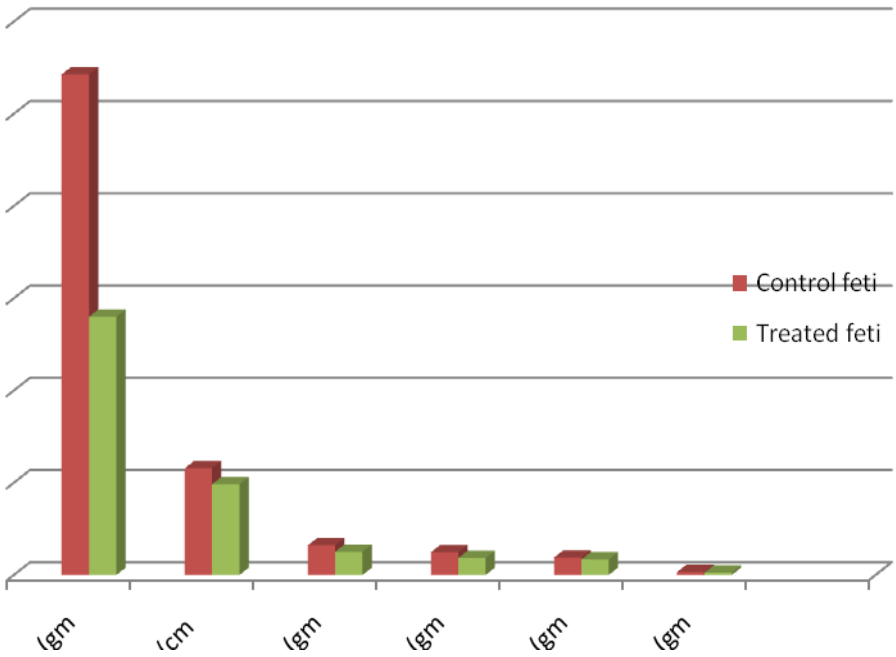

Figure 9. A histogram showing gross parameters for both control and fetuses of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation.

Figure 10. A histogram showing gross parameters for both control and fetuses of treated dam with 0.1 mg/kg AFB1 at 22nd day of gestation.

Control fetuses and fetuses of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation

The skull of control fetuses showed incomplete ossification between parietal and frontal bones where the rostral fontanelle was small and still open and the closure of the sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones,

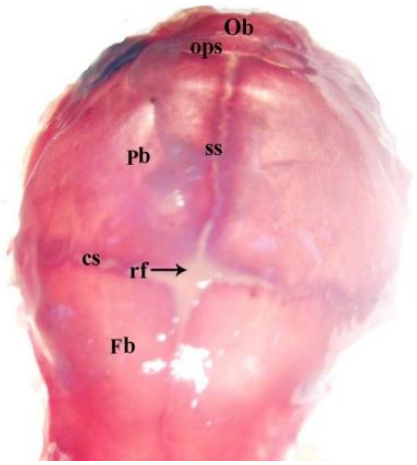

Figure 11. A photograph of dorsal view of skeleton craniale of control fetus at 29th day of gestation showing open small rostral fontanelle (rf) and well established sutures (occipito-parietal (OPS), sagittal suture (SS) and coronal suture (CS). Parietal bone (Pb) and frontal bone (Fb)

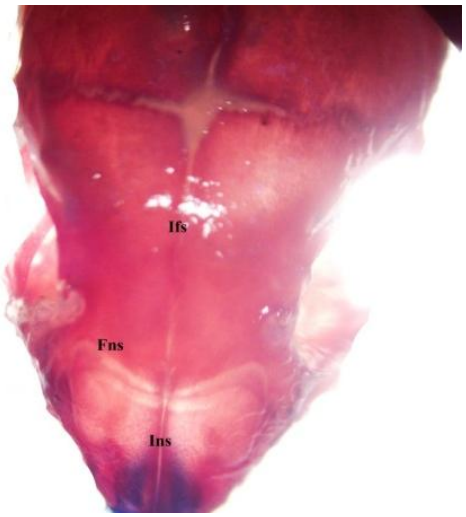

Figure 12. A photograph of dorsal view of skeleton craniale of control fetus at 29th day of gestation showing well established closed interfrontal (Ifs), frontonasl (Fn) and internasal (Ins) sutures.

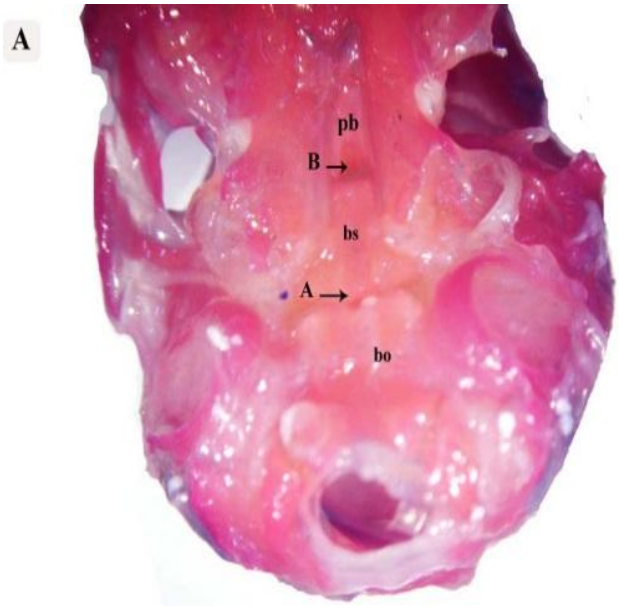

Figure 13A. A photograph of ventral view of skeleton craniale of control fetus at 29th day of gestation showing nearly complete ossification between basioccipital (bo) and basisphenoid (bs) bones (A) and presence of cartilaginous plate (B) between the basisphenoid and pre sphenoid (pb) bones.

the interfrontal suture between the two frontal bones as well as the occipito-parietal suture between the parietal and occipital bones) were established but still not closed, while the interfrontal, frontonasal and internasal sutures were well established and closed (Figures 11 and 12). There was nearly complete ossification between basioccipital and basisphenoid bones as well as between the presphenoid and vomer bones while the basisphenoid and presphenoid bones were still connected by a plate of cartilage (Figure 13A and B). Meanwhile, the treated fetuses with dose of 0.05 mg/kg AFB1 showed wide membranous rostral fontanelle. All the developing sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones, the interfrontal suture between the two frontal bones, the occipito-parietal suture between the parietal and occipital bones, the frontonasal sutures between the frontal and nasal bones as well as the internasal suture between the two nasal bones) had smooth adjacent margins and separated from each other by membranes (Figure 14). The ventral surface of the skull of this group of fetuses showed cartilaginous plates between basioccipital and basisphenoid bones, between the pre sphenoid and vomer bones as well as between the basisphenoid and pre sphenoid bones (Figure 15).

Regarding the vertebral column (columnavertebralis), the bodies and arches of all vertebrae of the control fetuses were markedly stained red due to their ossification (Figure 16) while in the one treated with a dose of 0.05 mg/kg AFB1, the lamina of the vertebrae is the only part stained blue because they remain cartilaginous and other parts of the vertebrae (bodies and pedicles) stained red (Figure 17). The transverse processes of the lumbar vertebrae were reduced in the treated fetuses as compared to the control (Figures 18 and 19).

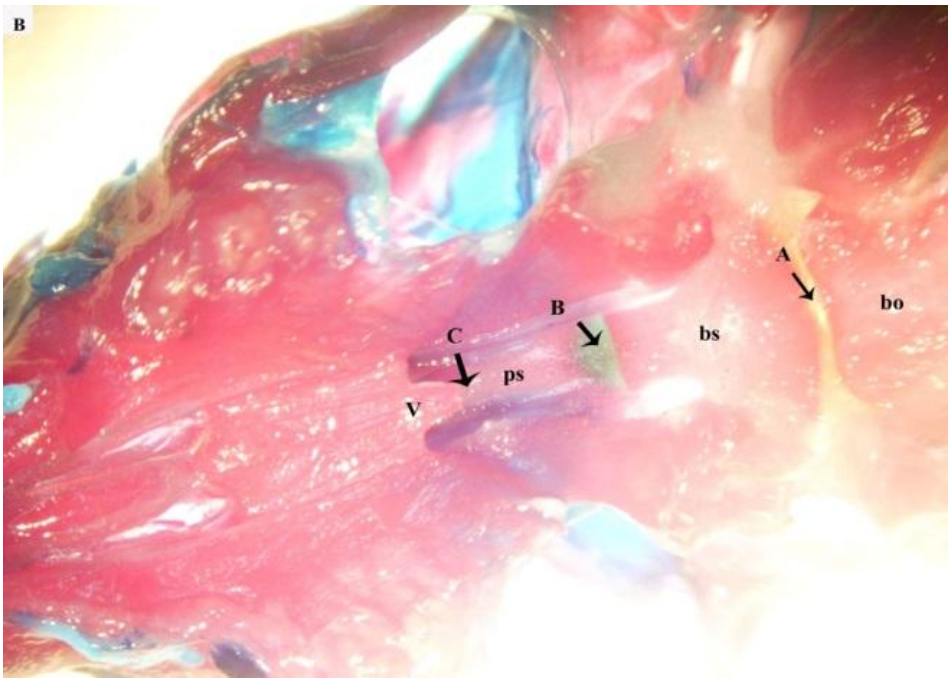

Figure 13B. A photograph of ventral view of skeleton craniale of control fetus at 29th day of gestation showing nearly complete ossification (A) between basioccipital (bo) and basisphenoid (bs) bones as well as between the presphenoid (ps) and vomer (V) bones (C) while the basisphenoid and presphenoid bones were still connected by a plate of cartilage (B).

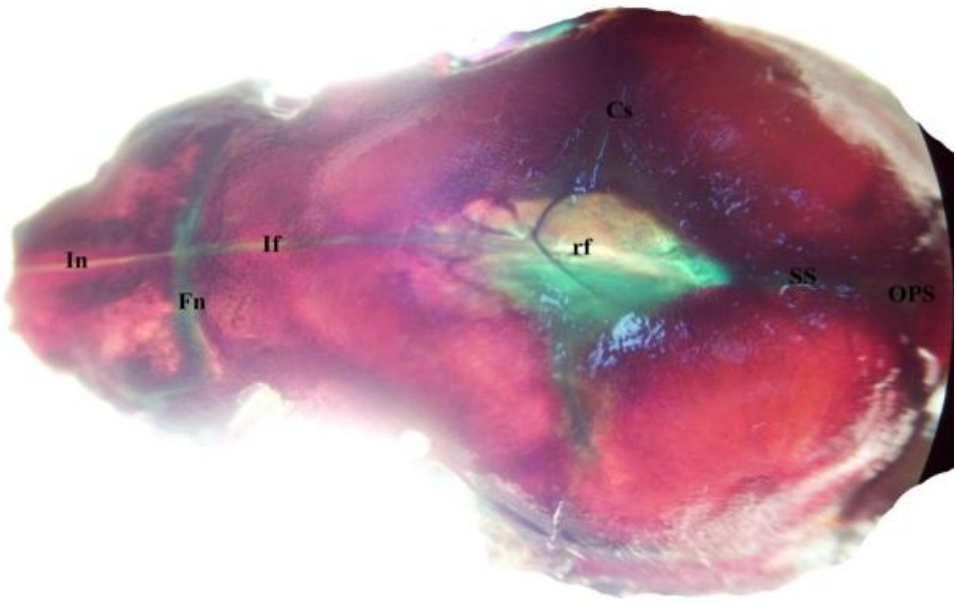

Figure 14. A photograph of dorsal view of skeleton craniale of fetus of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation showing wide rostral fontanelle (rf), well established smooth edged sutures (sagittal suture (SS), coronal suture (Cs), occipito-parietal suture (Ops), internasal suture (In), frontonasal suture (Fn) and interfrontal suture (If).

The sternebrae of the control fetuses formed clearly six bony segments completely ossified and stained red while that of treated fetuses with a dose of 0.05 mg/kg AFB1 had five segments with faint red coloration due to delay in their ossification (Figures 20 and 21).

The ribs of the control fetuses were markedly stained

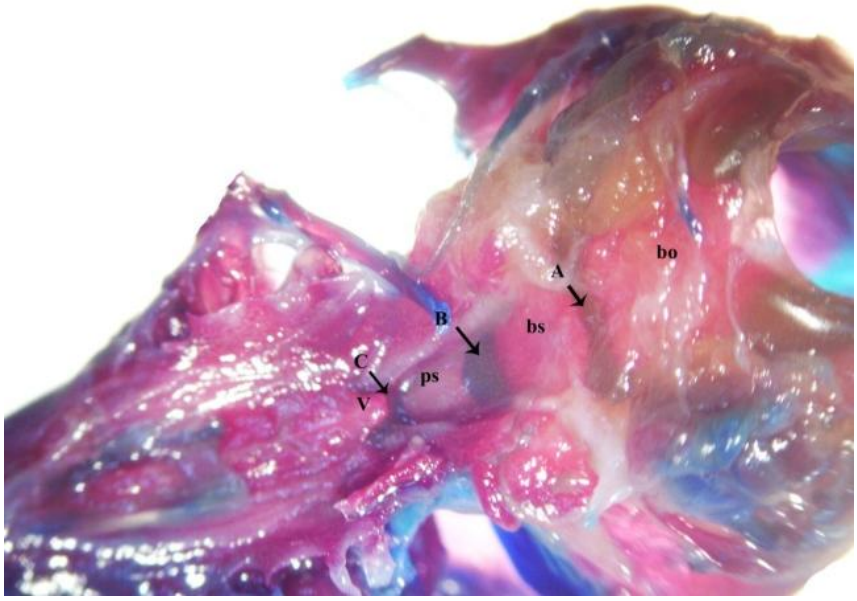

Figure 15. A photograph of ventral view of skeleton craniale of fetus of treated dams with a dose of 0.05 mg/kg AFB1 showing cartilaginous plates (A) between pars basilariesoccipitale (bo) and pars basisphenoidale bones (bs), (C) between the pre sphenoidale (ps) and vomer (V), as well as (B) between the osbasisphenoidale and ospre sphenoidd bones.

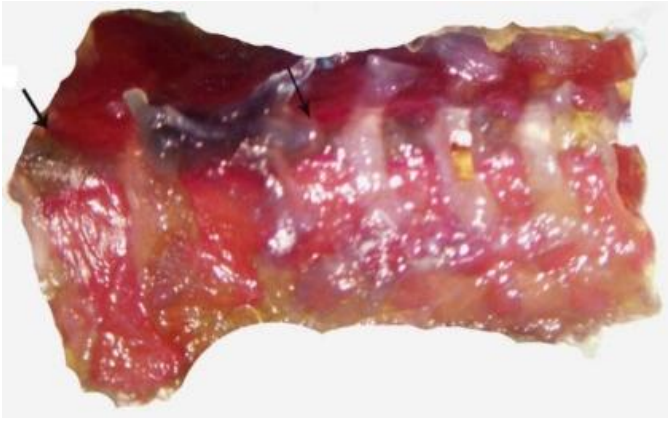

Figure 16. A photograph showing ossified cervical vertebrae of control fetus at 29th day of gestation (arrow).

red due to their complete ossification (Figure 22C) while the treated fetus with a dose of 0.05 mg/kg AFB1 show dark violet due to incomplete ossification of ribs (Figure 22T).

Control fetuses and fetuses of treated dams with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation

The skull of control fetuses showed wide rostral fontanelle and all the sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones, the interfrontal suture between the two frontal bones, the occipito-parietal suture between the parietal and occipital bones, frontonasal suture between the frontal and nasal bones and inter nasal suture between the two nasal bones) were well established but still not closely connected by membranes (Figures 23 and 24). While, in the treated group, the rostral part of the parietal bones and the caudal part of the frontal bones was still membranous (unossified), and there was very wide rostral fontanelle. Also, all the developing sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones, the interfrontal suture between the two frontal bones, the occipito-parietal suture between the parietal and occipital bones, frontonasal suture between the frontal and nasal bones and inter nasal suture between the two nasal bones) could be defined by a membranes between their smooth adjacent margins and the caudal fontanelle represented by a wide membrane (Figure 25).

The ventral surface of skull control fetuses showed incomplete ossification between pars basilarisos occipital and osbasisphenoidale and between osbasisphenoidale and ospresphenoidale (Figure 26) while the treated fetuses with a dose of 0.1 mg /kg AFB1, showed complete separation between the developing pars basilarisosoccipital and osbasisphenoidale, between osbasisphenoidale and ospresphenoidale as well as between ospresphenoidale and the vomer. There were

Figure 18. A photograph of dorsal view of thoracic and lumbar vertebrae of control fetus showing the ossification of their bodies and arches and well formed processustransversus of vertebrae lumbales at 29th day of gestation (arrows).

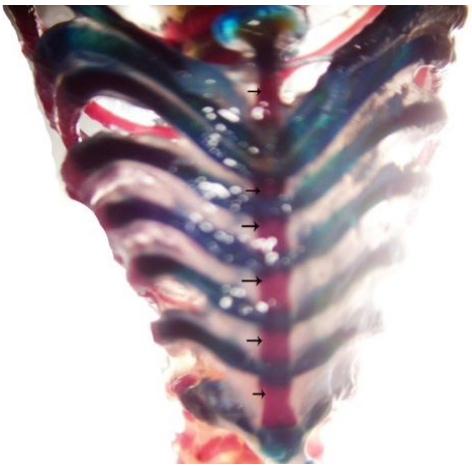

Figure 20. A photograph of control fetus sternum at 29th day of gestation showing complete ossification of six sternebral segments (arrows).

Figure 19. A photograph of dorsal view of thoracic and lumbar vertebrae of fetus of treated dam with 0.05 mg/kg AFB1 showing blue stained vertebral laminae and reduced processustransversus of vertebrae lumbales at 29th day of gestation (arrows).

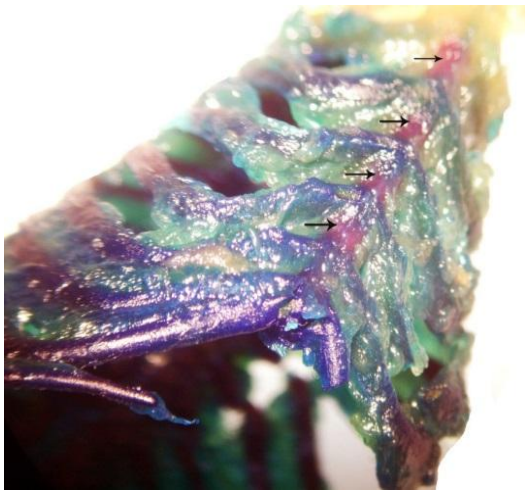

Figure 21. A photograph of sternum of fetus of treated dam with 0.05 mg/kg AFB1 at 29th day of gestation showing incomplete ossification of five segments (arrows).

no cartilaginous templates between the previous mentioned bones in this group of fetuses. The vomer seemed to be a thin delicate plate of bone (Figure 27).

Regarding the vertebral column in the control group, there were incomplete ossification of the vertebral arches of the cervical vertebrae, thoracic vertebrae and lumbar vertebrae and ossification of their bodies (Figure 28A and B) while the caudal vertebrae showed ossification in their bodies (Figure 30). The treated fetuses with a dose of 0.1 mg/kg AFB1 showed all the vertebrae throughout the vertebral column were smaller in size than that of the control ones. Also, there was delay in the development of the vertebral arches, there were wide separation between the pedicles in the cervical vertebrae and this space became narrow in the thoracic region containing cartilaginous plate. Also, this separation between the pedicles became wide again in the lumbar and sacral regions contained a faint blue cartilaginous plates (Figure 29A and B) while the caudal vertebrae showed the commencement of ossification in the bodies of the 1st two caudal vertebrae and the rest of the vertebrae remained cartilaginous (Figure 31).

The sternum of the control fetuses showed ossified five

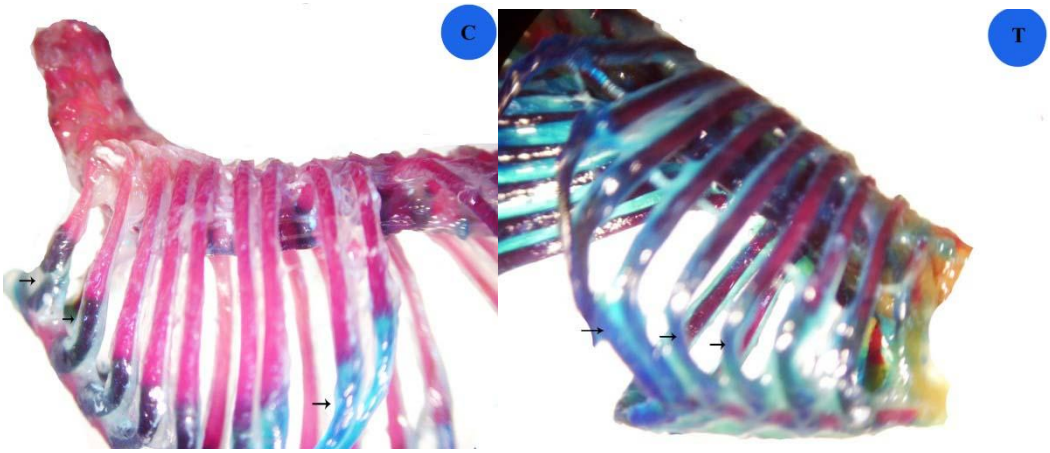

Figure 22. A photograph of control fetus (C) showing complete ossification of ribs and fetus of treated dam (T) with a dose of 0.05 mg/kg AFB1 showing incomplete ossification of ribs at 29th day of gestation (arrows).

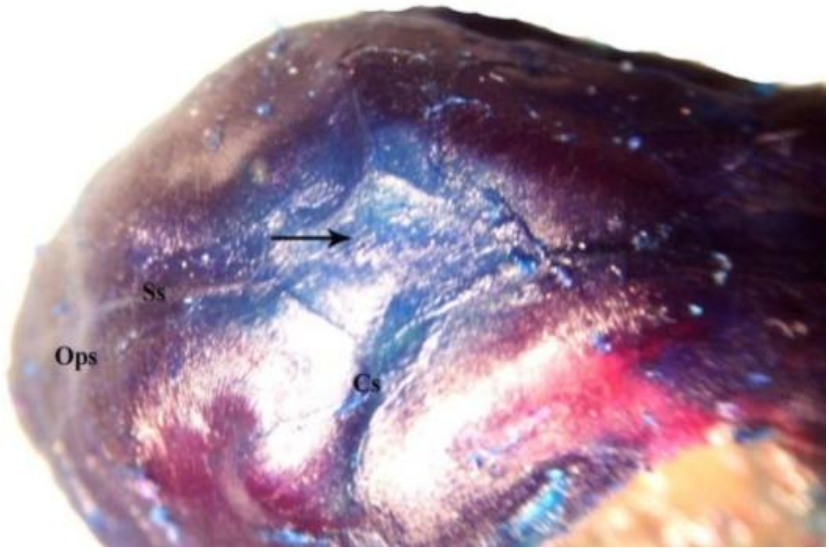

Figure 23. A photograph of dorsal view of skull of control fetus at 22nd day of gestation showing wide rostral fontanelle (arrow) and smooth edged sutures (occipito-parietal (Ops), sagittal (Ss) and coronal (Cs) connected by membranes.

sternebrae and one sternebra still cartilaginous (Figure 32). While, the treated fetuses sternum had three ossified sternebrae and the rest sternebrae were still cartilaginous (Figure 33).

Appendicular skeleton

Control fetuses and fetuses of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation

The bones of the fore limb (scapula, humerus and radius and ulna) of the control fetuses were grossly longer than those of the treated fetuses with a dose of 0.05 mg/kg AFB1 (Figures 34, 35 and 36). In the control group, the proximal and distal extremities of the radius and ulna and metacarpi as well as the carpus were still cartilaginous while, the treated group showed blue coloration for both extremities of radius and ulna and their shafts showed violet color due to incomplete ossification of the diaphysis. The carpus, the proximal and distal extremities of the metacarpi and the second phalanges of all digits had no cartilaginous templates in this treated group (Figure 36).

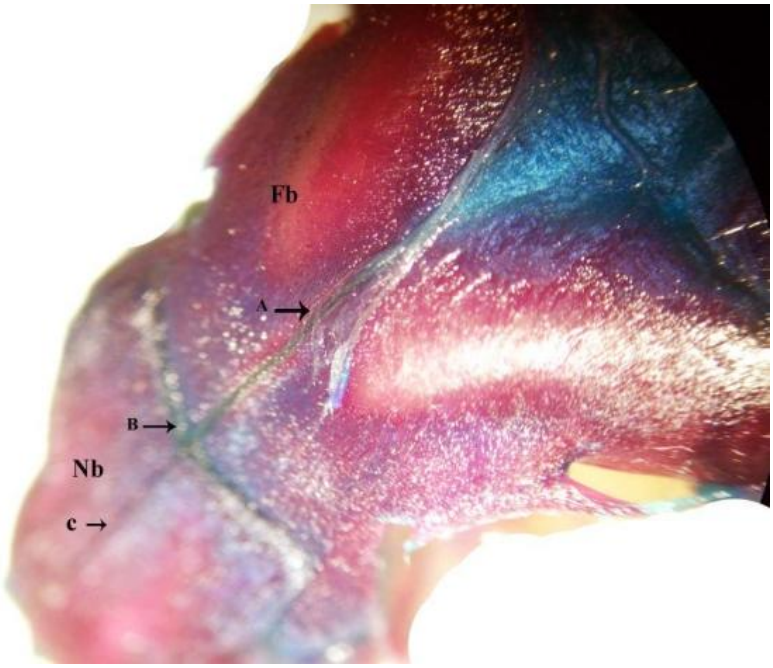

Figure 24. A photograph of dorsal view of control fetus skull at 22nd day of gestation showing well established interfrontal suture (A), frontonasal suture (B) and internasal sutures (C). Frontal bone (Fb) and nasal bone (Nb)

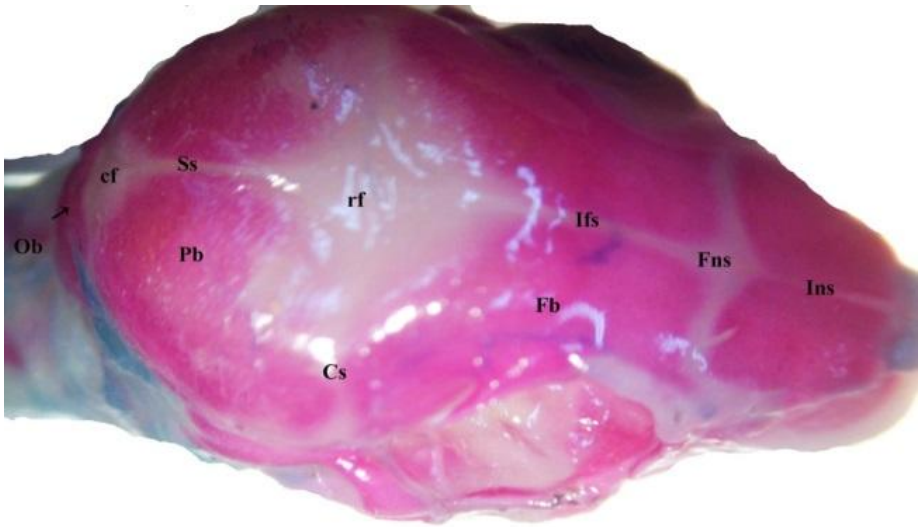

Figure 25. A photograph of dorsal view of the skull of fetus of treated dam with a dose of 0.1 mg/kg AFB1 showing wide caudal fontanelle (cf) and very wide rostral fontanelle (rf) in addition to membranous connection between the developing sutures: sagittal suture (Ss), coronal suture (Cs), interfrontal suture (Ifs), frontonasal suture (Fns) and internasal sutures (Ins).

The bones of the hind limb (oscoaxe, femur and tibia and fibula) of the control fetuses were grossly longer than those of the treated fetuses with a dose of 0.05 mg/kg AFB1 (Figures 37 and 38). The oscoaxe of the treated fetuses had a very small ossified pubis than that of the control (Figure 37). The distal extremity of tibia as well as

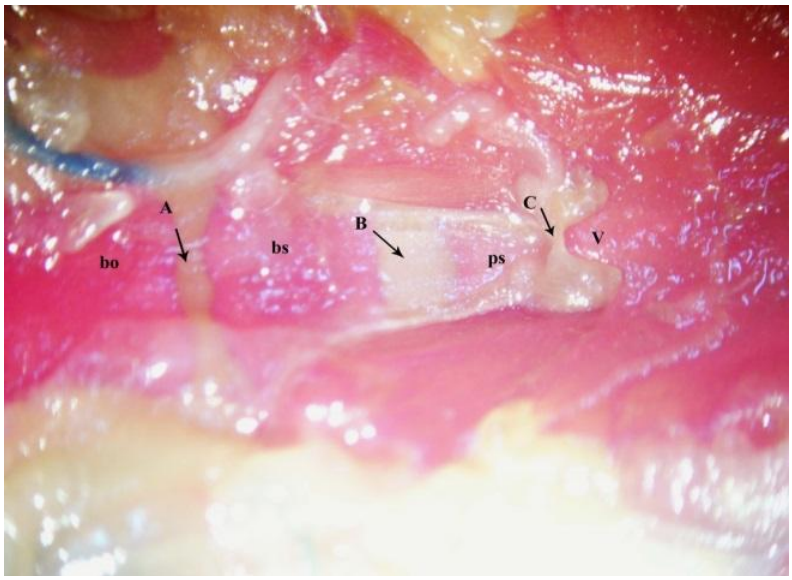

Figure 26. A photograph of ventral view skull of control fetus at 22nd day of gestation showing incomplete ossification (A) between pars basilarisosoccipitale (bo) and osbasisphenoidale (bs), (B) between osbasisphenoidale (bs) and ospresphenoidale (ps) and (C) between ospresphenoidale (ps) and vomer bone (V).

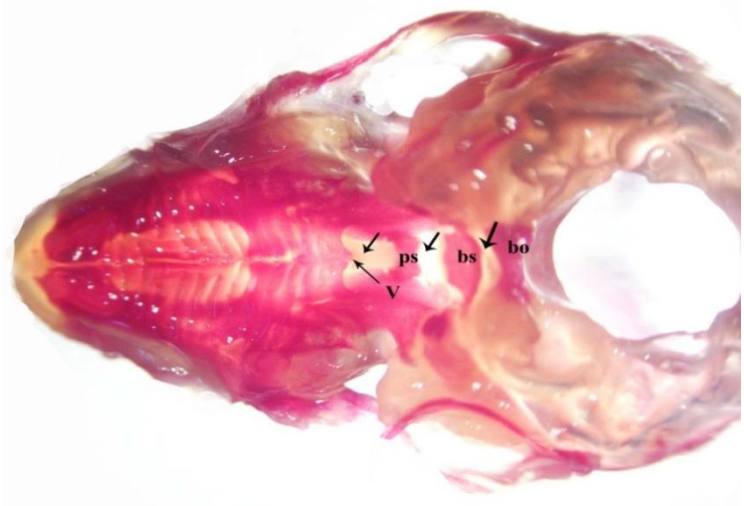

Figure 27. A photograph of ventral surface of skull of fetus of treated dam with 0.1 mg/kg AFB1, showing separation between pars basilarisosoccipitale (bo), osbasisphenoidale(bs), ospresphenoidale (ps) and vomer (V) (arrows) at 22nd day of gestation.

the distal raw of tarsus in the control group had cartilaginous templates and the talus and calcaneus showed incomplete ossification while the treated group showed no cartilaginous templates for the distal extremity of tibia, central and distal raws of tarsaus and showed commencement of ossification of talus and calcaneus (Figures 39, 40 and 41).

Control fetuses and fetuses of treated dams with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation

The bones of the fore limb (humerus, radius and ulna) of the control fetuses were grossly longer in length than that of the treated fetuses with a dose of 0.1 mg/kg AFB1 (Figures 43 and 44). The scapulae of both groups were

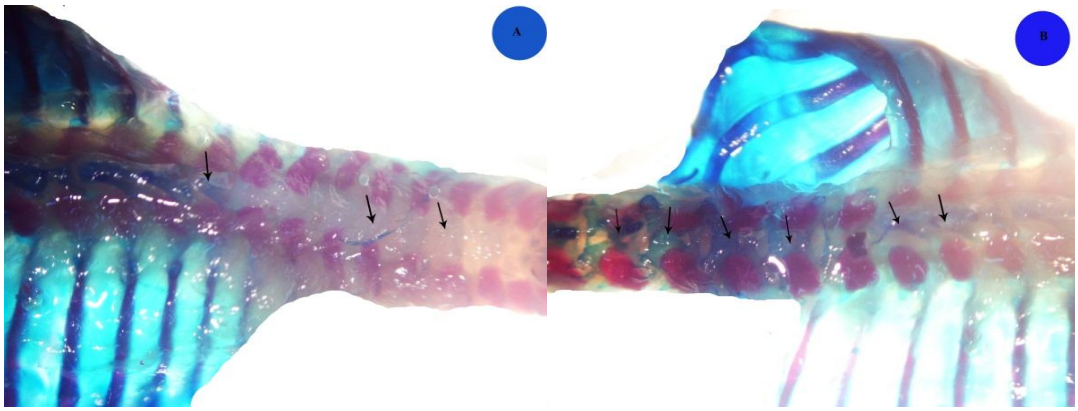

Figure 28. A: A photograph showing incomplete ossification of the vertebral arches of the cervical vertebrae. B: Incomplete ossification of the vertebral arches of thoracic and lumbar vertebra of the control fetuses at 22nd day of gestation (arrows).

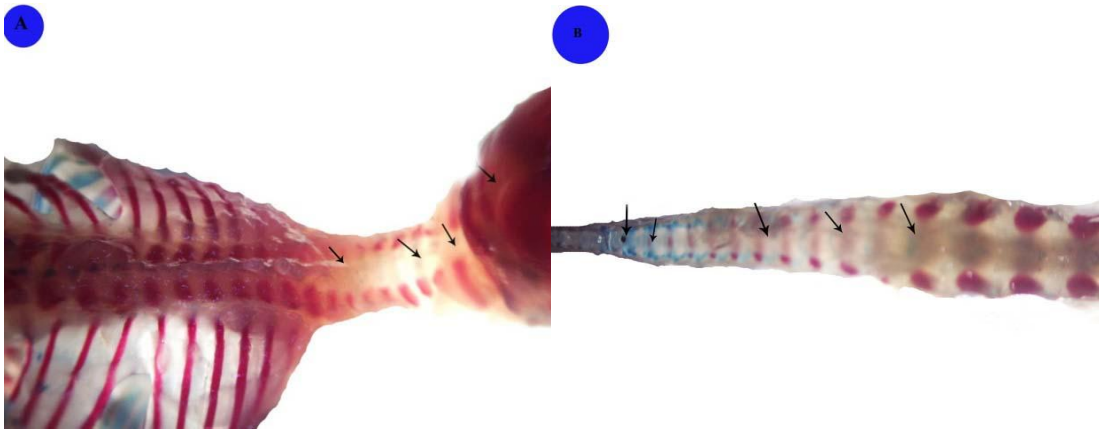

Figure 29A. A photograph showing reduced vertebral size as well as a wide separation of the thoracic vertebral arches. 29B: wide separation of pedicles of sacral vertebra with faint cartilaginous templates of fetus of treated dam with 0.1 mg at 22nd of gestation (arrows).

Figure 30. A photograph showing complete ossification of the bodies of the caudal vertebrae of control fetuses at 22nd day of gestation (arrows).

Figure 31. A photograph of the caudal vertebrae of fetuses of treated dam with a dose of 0.1 mg/kg AFB1 showing ossification of the bodies of the 1st and 2nd caudal vertebrae (A) and the rest remain cartilaginous at 22nd day of gestation (arrows).

somewhat similar in length (Figure 42). The control group showed incomplete ossification of distal extremities of radius and ulna. There was beginning of ossification in the carpus proceeding from its medial toward the lateral aspect where the lateral bones (ulnar carpal and 4th carpal bones) had cartilaginous templates. The shafts of the metacarpi were ossified while their extremities still cartilaginous and the phalanges of all digits were ossified.

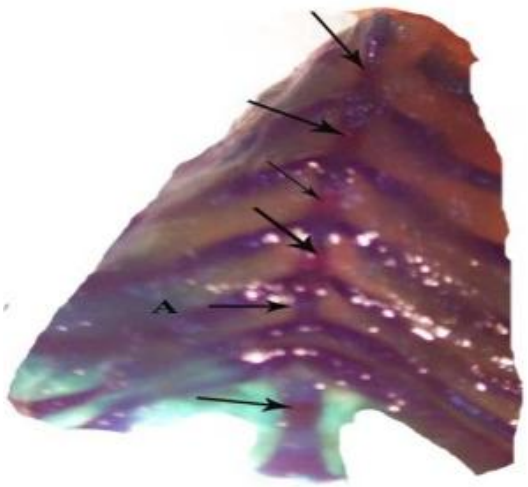

Figure 32. A photograph of control fetus sternum at 22nd day of gestation showing ossified 5 bony sternebrae (arrows) and one sternebra still cartilaginous (A).

Figure 33. A photograph of fetuses of treated dam, with 0.1 mg/kg AFB1 at 22nd day of gestation showing ossified three sternebrae (**arrows**) while the rest remained in cartilaginous template.

In the treated group, the proximal and distal extremities of radius and ulna, the carpus, the metacarpus of the 1st and 5th digit as well as the 1st and 2nd phalanges of all digits had cartilaginous templates (Figure 44).

The bones of the hind limb (femur and tibia and fibula) of the control fetuses were grossly longer in length than that of the treated fetuses with a dose of 0.1 mg/kg AFB1(Figures 46 and 47). The oscoaxe had ossified ilium and ischium while the pubis was still small cartilaginous part in both control and treated group and also appeared approximately equal to each other in length (Figure 45). The control fetuses showed complete fusion between the distal extremities of tibia and fibula, the tarsus had a

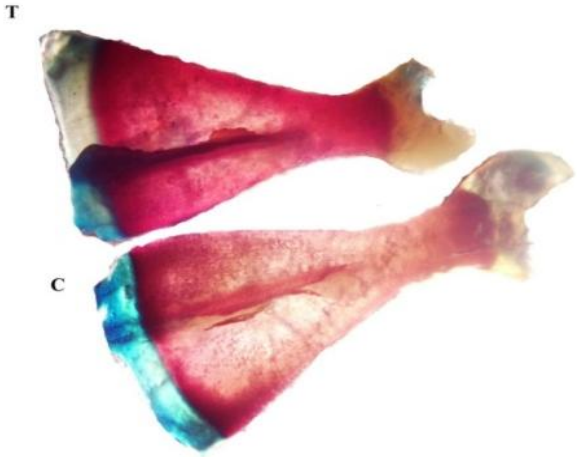

Figure 34. A photograph-showing scapula of control fetuses (**C**) was grossly longer than that of the fetuses of treated dams (T) with a dose of 0.05 mg/kg AFB1 at 29th days of gestation.

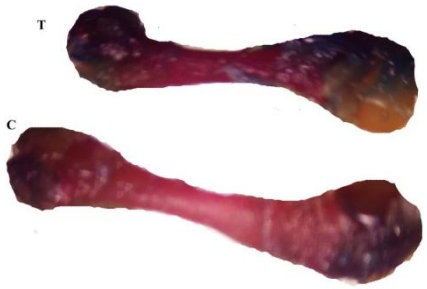

Figure 35. A photograph showing humerus of control fetuses (C) was grossly longer than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation.

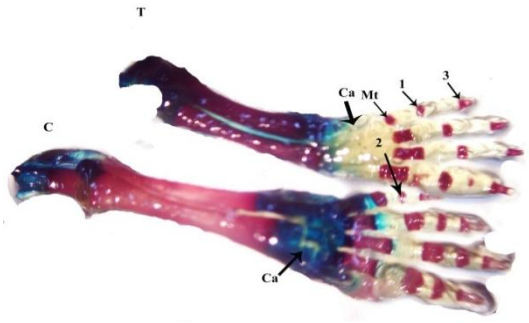

Figure 36. A photograph showing grossly longer radius and ulna of control fetuses (C) than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation. also there were no cartilaginous templates of carpus (Ca), proximal and distal extremity of metacarpi (Mt) and 2nd phalanx of the treated fetuses. 1st phalanx (1), 2nd phalanx (2) and 3rd phalanx (3).

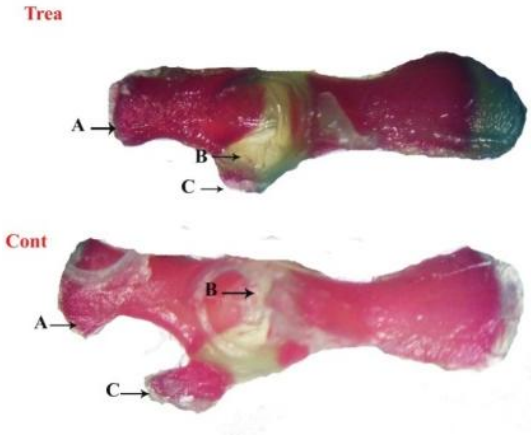

Figure 37. A photograph showing oscoaxe of control fetuses (Cont) was grossly longer than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation, also the os pubis of treated fetuses represented a small ossified bone (C).

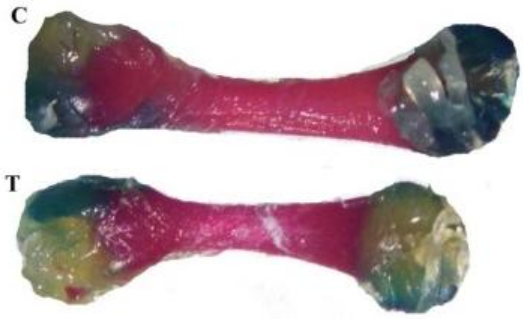

Figure 38. A photograph showing os femur of control fetuses (C) was grossly longer than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation.

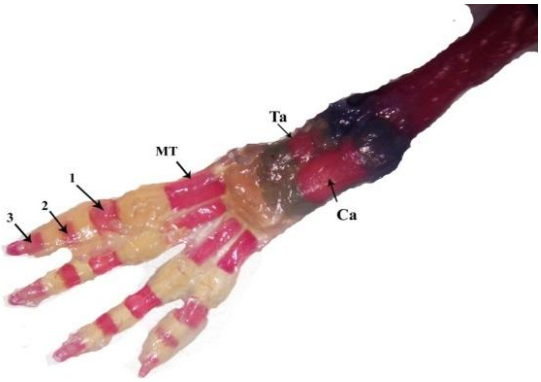

Figure 39. A photograph showing completely ossified metatarsus (MT) and digital phalanges (1, 2 and 3) of the control fetuses at 29th days of gestation as well as tarsal bones, the talus (Ta) and calcaneus (Ca) were the only ossified and the remaining bones of the tarsus had a cartilaginous templates.

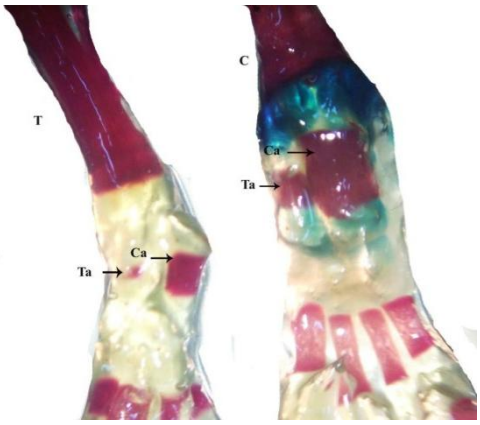

Figure 41. A photograph of planter aspect of pes region of control fetuses (C) and fetuses of treated dams (T) showing tarsus with beginning of ossification of talus (Ta) and calcaneus (Ca) while the rest had no cartilaginous templates in the treated one.

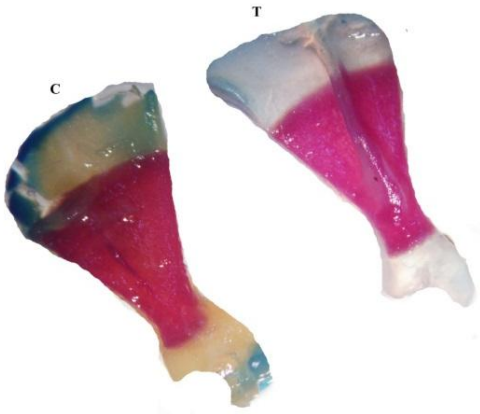

Figure 42. A photograph showing scapula of control fetuses (C) was grossly similar to that of the treated one (T) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation.

Figure 43. A photograph showing humerus of control fetuses (C) was grossly longer than that of treated one (T) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation. Also, the extremities of the treated one were stained faint blue.

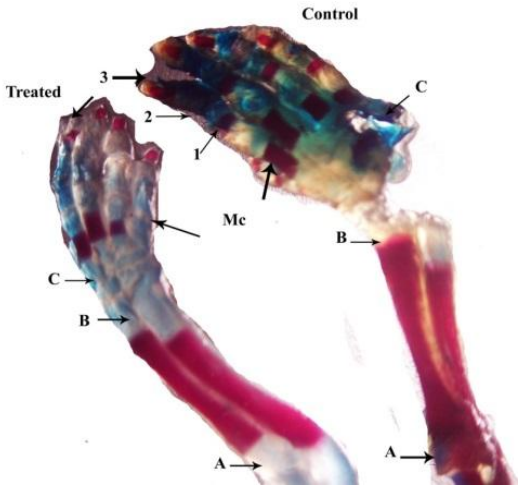

Figure 44. A photograph showing radius and ulna of control fetuses (control) was grossly longer than that of the treated one (treated) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation. Also, the proximal (A) and distal (B) extremity of radius and ulna and carpus (C) of treated fetuses had cartilaginous template. In addition, 1st and 5th metacarpi of the treated fetuses and 1st and 2nd phalanx of all digits had cartilaginous templates. 1st phalanx (1), 2nd phalanx (2) and 3rd phalanx (3).

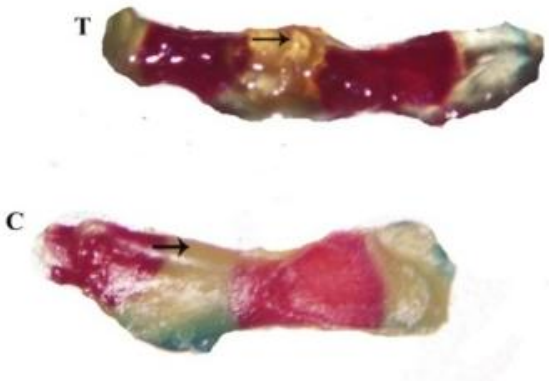

Figure 45. A photograph showing oscoaxe of control feuses (**C**) was grossly similar to the treated one (**T**) with 0.1 mg/kg AFB1 at 22nd day of gestation.

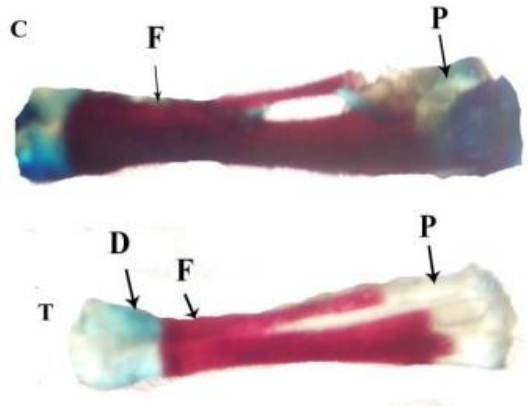

Figure 47. A photograph showing tibia and fibula of control fetuses (C) was grossly longer than that of treated one (T) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation, the fibula of the treated fetus was still long bone with incomplete fusion (F) and the proximal ext. of tibia did not form cartilaginous template (P).

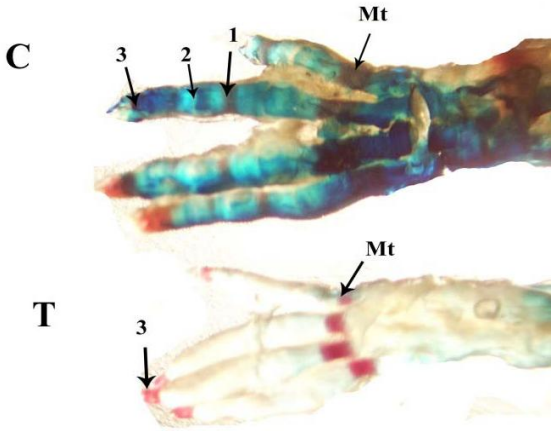

Figure 48. A photograph of pes region showing ossified shaft of metatarsi (Mt) and 3rd phalanx, the proximal and distal ext. of metatarsi as well as the 1st phalanx had cartilaginous templates while the 2nd phalanx had no cartilaenous template in treated fetus (T) with a dose of 0.1 mg AFB1 at 22nd day of gestation and control fetus (C) show ossified shaft of metatarsi (Mt), 1st, 2nd and 3rd phalanges (1, 2 and 3).

cartilaginous template except the calcaneus bone as well as the phalanges of the digits became ossified. While, in the treated group, the tibia and fibulawere still completely separated long bones. The distal extremities of the previous bones as well as tarsus except calcaneus bone were still cartilaginous. Also, the shafts of metatarsi were the only ossified parts as the distal and proximal extremities of these bones in addition to the 1st phalanges of all digits had cartilaginous templates. The 2nd phalanges of the digits had no cartilaginous templates and the 3rd phalanges were ossified (Figure 48).

DISCUSSION

Animal experiment is the first scientific preference to learn whether a substance does have a teratogenic effect or not. It is the most acceptable way for drug producers to determine potential teratogenic effect of any given drug on human health. In relation to that, this study documented the teratogenic effect of aflatoxin B1 on the skeletal development of the rabbit fetuses which have

similarity to human in the early developmental pattern (Beaudion et al., 2003) and the extra-embryonic membranes were more closely related to that of human (Foote and Carney, 2000). It was worthy to mention that the selection of the rabbit in this study might be attributed to its economic importance and is the most sensitive species to AFB1 as mentioned by Marquardt and Fronhlich (1992) and Pier (1992).

In the present study, the oral route of administration of AFB1 to pregnant rabbits was selected to ensure the administration of exact amount of mycotoxins to the animals and was considered to be the most accurate way to give a fixed dose. This method of administration was confirmed by Arora (1982). Also, aflatoxins are absorbed mainly from GIT by passive diffusion transferred to hepatic portal blood and very little amount appeared to be transferred into the lymphatic system as reported by Hsieh and Wong (1994).

In pregnant rabbits, the teratogenic doses of AFB1 mentioned by Reddy and Rao (2001) were 1-3 ppm during day zero of conception to one weak post partum. While, Wangikar et al. (2005) used different doses of 0.025, 0.05 and 0.1 mg/kg by gastric intubation during the whole length of organogenesis period and inferred that 0.1 mg/kg body weight was the minimum teratogenic dose. In our study, we administrated the doses of 0.05 and 0.1 mg/kg dissolved in 0.2 ml corn oil by gastric intubation. In this respect, the dose of 0.05mg/kg in rabbits under investigation was the minimum teratogenic dose while 0.1 mg /kg was lethal for both dams and fetuses.

In the current work, the dosing administered during period of organogenesis from 6^{th}-18^{th} day of gestation simulate that recorded by Petrere et al. (1993) and Wangikar et al. (2005) in rabbits. The chosen time of dosing was during the process of organogenesis in the present study due to the teratogenic agents which could lead to significant congenital anomalies as documented by Stanely and Bower (1986) and Vickers and Brackley (2002).

Fetal observations

Our results revealed that the fetuses of dead dams at 14^{th} day of gestation intoxicated orally with a dose of 0.1 mg/kgAFB1 were necrosed with total hemorrhage as well as no closure of the neural tube, while Le Breton et al. (1964) showed that the dosing of pregnant rats intoxicated AFB1 with a dose of 0.3 mg/ kg intraperitoneal causes fetal deaths and hemorrhage at utero placental junction. The death of fetuses of the treated dams with a dose of 0.1 mg/kg AFB1 at 22^{nd} day of gestation did not correlate with that given by Wangikar et al. (2005) where there was no fetal mortalities for the same dose in pregnant rabbits.

It was also found that the fetal body weights and crown rump lengths in treated fetuses with a dose of 0.1 mg/ kg at 22^{nd} day of gestation were comparable to the control fetuses; the results were not in accordance with that of Wangikar et al. (2005) for treated rabbit fetuses with the same dose at 29^{th} day of gestation and El-Tahan (2013) in rats with the same dose at 21^{st} day of gestation.

In agreement with Wangikar et al. (2005) in rabbits and Wangikar et al. (2004a, b) in rat the present study revealed no fetal mortalities for the dose of 0.05 mg/kg body weight throughout the period of gestation.

In the rabbit fetuses of the dams treated with a dose of 0.05 mg/kg AFB1, there were significant decrease in both fetal body weight and CRL as compared to control fetuses at 29^{th} day of gestation, the results disagreed with that of Wangikar et al. (2005) in fetal weights and agreed with CRL for the same dose and animal.

Fetuses gross anomalies found in our work, including wrinkled skin and enlarged eye socket by both doses were also observed by Wangikar et al. (2005) in rabbits with a dose of 0.1 mg/kg and in rat by Sharma and Sahai (1987) by different doses (7, 3.5, 1.4 and 0.7 mg/kg) body weight and El-Tahan (2013) by a dose of 0.1 mg/kg.

The current study revealed other fetal gross anomalies for both treated doses as sever subcutaneous congestion at different regions including abdomen and head mainly in the eye region; such observations have not been recorded before.

The visceral anomalies as microphthalmiceyes and cardiac defects (small sized heart and wide ventricular lumen) in the fetuses in the study of Wangikar, et al. (2005) showed microphthalmiceyes and fusion of auriculo-ventricular valves.

The absolute fetal organs: liver, kidneys, stomach and intestine, heart and lung weights was significantly deceased in treated fetuses with 0.05 mg/kg at 29^{th} day as compared to control fetuses, similar observations were found in liver and kidneys in male rabbits intoxicated with AFB1 (Orsi et al., 2007).

Fetal skeletal anomalies

As a result of the rarity and unavailability of the literature on the skeletal anomalies induced by AFB1 among the laboratory animals, it was not possible to make a detailed discussion for our results.

Calcium transfer from the mother to the fetus and neonate during pregnancy and lactation plays an extremely important role in the bone health of the mother and infant. Calcium aids in bone health through all ages but is especially crucial during pregnancy and lactation. Development of a novel class of BMP antagonists could lead to new treatments for traumatically and genetically induced heterotopic ossification (Weber et al., 2001). The skeletal elements of the axial and appendicular skeleton are preformed as cartilage templates by a mechanism called endochondral ossification. During this process, a cartilage

template is formed in which chondrocytes proliferate and differentiate into hypertrophic chondrocytes and are gradually replaced by bone (Wuelling and Vortkamp, 2011). Osx regulates chondrocyte differentiation and bone growth in growth plate chondrocytes, suggesting an autonomous function of Osx in chondrocytes during endochondral ossification (Oh et al., 2012). Molecules regulates chondrocyte formation, chondrocyte maturation, and osteoblast differentiation, all key processes of endochondral bone development. These include the roles of the secreted proteins IHH, PTHrP, BMPs, WNTs and FGFs, their receptors and transcription factors such as SOX9, RUNX2 and OSX, in regulating chondrocyte and osteoblast biology (Long and Ornitz, 2013).

The available literature recognized only the teratogenesis of the skeleton from the aspects of incomplete or delay ossification of the bones of axial and appendicular skeleton or reduction or absence of some bones. So, the present thesis focused on tracing the effects of AFB1 by two doses (0.1 and 0.05 mg/kg) body weight on craniofacial intramembranous bones which grow normally by ossification at the sutures. Also, the endochondral ossification of the skull base progressed from caudal to rostral direction, from occipital bone, then proceeded to sphenoid, presphenoid and finally to the vomer. Also, the thesis focused on the endochondral ossification of the appendicular skeleton.

The fetuses of treated dams in this work by a dose of 0.1 mg/kg AFB1 showed the commencement of the membranous ossification of the bones of the dorsal surface of skull and no cartilaginous template were formed between the bones of the ventral aspect of the skull while Wangikar et al. (2004b, 2005) mentioned incomplete ossification of the skull bones in the same animal with same dose of AFB1 as well as El-Tahan (2013) in rat fetuses with the same dose.

Regarding the vertebral column, Wangikar et al. (2004a and 2005) observed agenesis of the caudal vertebrae in the rabbit fetuses treated by a dose of 0.1 mg/kg OTA and AFB1 respectively, while the treated group of fetuses with the same dose of AFB1 in the present study showed the commencement of ossification in the bodies of the 1st two caudal vertebrae while the rest of the vertebrae remained cartilaginous.

In this work, all the vertebrae were reduced in size in treated group with a dose of 0.1 mg/kg AFB than the control one, the result which was in a line with that of El-Tahan (2013) in intoxicated rat fetuses with the same dose and toxin.

The sternum of the control rabbit fetuses showed five ossified sternebrae and one sternebra still cartilaginous. While in treated fetuses with a dose of 0.1 mg/kg, sternum had three ossified sternebrae only and the rest still cartilaginous, which was conflicting with El-Tahan (2013) in the rat fetuses with the same dose with failure of sternebrae ossification.

Our results revealed detailed information on each bone in both limbs about length and state of ossification for the treated groups by both doses. In the treated fetuses with a dose of 0.1 mg/kg AFB1, the distal extremities of radius and ulna, the carpus, the metacarpi of the 1st and 5th digit as well as the 1st and 2nd phalanges of all digits had cartilaginous drafts. Also, the distal extremities of the tibia and fibula as well as tarsus except calcaneus bone were still cartilaginous and the shafts of metatarsi were the only ossified parts in addition to the distal and proximal extremities of these bones and the 1st phalanges of all digits had cartilaginous templates while the 2nd phalanges of the digits had no cartilaginous templates. El-Tahan (2013) observed failure or incomplete ossification of long and flat bones of both fore and hind limb in rat fetuses of intoxicated dams with a dose of 0.1 mg/kg AFB1

3D culture system reveals numerous biological features and widely accepted to be useful for the assessment of toxic compound, but animal experiments are essential considering the effect of microenvironment. For instance, Yoshida et al. (2013) performed the 3D-culture of sebaceous gland cells and was successful in inducing the polarity as epithelial cells and basal membrane formation, but it remains to be known, the interaction between sebaceous glands and hair follicle niche cells. Further, recent cancer researchers emphasized the importance of spheroids rich in cancer stem cells (Yoshida et al., 2013). Invasive behavior of tumor cells in a 3D environment mimick the tumor microenvironment (Brekhman and Neufeld, 2009). Bone morphogenetic proteins (BMPs) regulate cell proliferation, differentiation and motility, and have also been reported to be involved in cancer pathogenesis. BMP4 reduces breast cancer cell proliferation. BMP4 partly recapitulates in 3D culture growth suppressive abilities previously seen in 2D culture (Ampuja et al., 2013).

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Ampuja M1, Jokimäki R, Juuti-Uusitalo K, Rodriguez-Martinez A, Alarmo EL, Kallioniemi A (2013). BMP4 inhibits the proliferation of breast cancer cells and induces an MMP-dependent migratory phenotype in MDA-MB-231 cells in 3D environment. BMC Cancer 13:429.

Argyrous G (2005). Statistics for Research with a guide to SPSS, 2, Sage Publications, London

Arora RG (1982). Mycotoxin induced effects on the prenatal devel-opment in mice:a teratopathologic and autoradiographic study ofaflatoxin B1, ochratoxin Aand zearalenone. Ph.D. Thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Beaudion S, Barbet P, Bargy F (2003). Developmental Stages in the Rabbit Embryo: Guidelines to Choose an Appropriate Experimental Model. Fetal. Diagn. Ther.18:422-427.

Brekhman V1, Neufeld G (2009). A novel asymmetric 3D in-vitro assay for the study of tumor cell invasion. BMC Cancer 9:415.

Brendel R, Duhamel KC, Shepard TH (1989):Embryotoxic drugs. Biol.

Res. Preg. 6(1):1-54.
Buening MK, Fortner JG, Kappas A, Corney AH (1978). 7,8-Benzoflavone stimulates the metabolic activation of aflatoxin B1 to mutagens by human liver. Biochem. Biophys. Res. Commun. 82(1):348-55.
Clark JD, Jain AV, Hatch RC, Mahaffey A (1980). Experimentally induced chronic aflatoxicosis in rabbits. Am. J. Vet. Res. 41:184-1845.
Decad GM, Hsieh DP, Byard JL (1977). Maintenance of cytochrome P-450 and metabolism of aflatoxin B1 in primary hepatocyte cultures. Biochem. Biophys. Res. Commun. 78(1):279-87.
Diekman MA, Green ML (1992). Mycotoxins and reproduction in domestic livestock. Anim. Sci. 70:1615-1627.
El-Tahan AS (2013). A study on the effects of mycotoxins on pregnant rats. Master thesis, Faculty of Veterinary Medicine, Suez Canal University, Pathology department.
Foote RH, Carney EW(2000). The rabbit as a model for reproductive and developmental toxicity studies. Reprod. Toxicol.14: 477–493.
Forrester LM, Neal GE, Judah DJ, Glancey MJ, Wolf CR (1990). Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B1 metabolism in human liver. Proc. Natl. Acad. Sci. USA 87(21):8306-10.
Hsieh PH, Wong JJ (1994). Pharmacokineticsand excretion of aflatoxins. In: DL Eaton and JD Groopman (ed.), The toxicology ofaflatoxins: human health, veterinary,And agricultural significance. Academic Press, San Diego, California. pp. 73-88.
Hussein HS, Brasel JM (2001). Toxicity, metabolism and impact of mycotoxins on human and animals. Toxicology 167:101-134.
Inouye M (1976). Differential staining of cartilage and bone in fetal mouse skeleton byalcian-blue and alizarin red. S. Cong. Anom. 16:171-3.
Kärenlampi SO(1987). Mechanism of cytotoxicity of aflatoxin B1: role of cytochrome P1-450. Biochem Biophys Res Commun. 145(2):854-60.
Kelly VP, Ellis EM, Manson MM, Chanas SA, Moffat GJ, McLeod R, Judah DJ, Neal GE, Hayes JD (2000). Chemoprevention of aflatoxin B1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B1-aldehyde reductase, the glutathione S-transferase A5 and P1 subunits, and NAD(P)H:quinone oxidoreductase in rat liver. Cancer Res. 60(4):957-69.
Le Breton E, Fragssinet C, Lafarg C, De Recondo AM (1964). Aflatoxin. Mechanisme de faction. Food Cosmet. Toxicol. 2: 675–677.
Long F, Ornitz DM (2013). Development of the endochondral skeleton. Cold Spring Harb. Perspect. Biol. 5(1):a008334.
Marquardt RR, Frohlich AA (1992). A review of recent advances in understanding ochratoxicosis. J. Anim. Sci. 70:3968-3988.
Nomina Anatomica Veterinaria (2005). 5th Ed. Prepared by International Committee on Veterinary Gross Anatomical Nomenclature (I. C.V.G.A.N) and authorized by the General Assembly of the World Association of Veterinary Anatomists(W. A. V.A),Konxiville, TN, USA.
Nomina Embryologica Veterinaria (2003). 2nd Edition. International Committee on Veterinary Gross Embryological Nomenclature (I. C.V.E.N) and authorized by general assembly of the world association of Veterinary Anatomists (W.A.V.A) Konxiville, TN, USA.
Oh JH, Park SY, de Crombrugghe B, Kim JE (2012). Chondrocyte-specific ablation of Osterix leads to impaired endochondral ossification. Biochem. Biophys. Res. Commun. 418(4):634-40.
Orsi RB, Oliveira CAF, Dilkin P, Yavie JG, Direitio GM, Correa B (2007). Effects of oral administration of aflatoxin B1 and fumonisin B1 in rabbits. Chem. Biol. Interact. 170:201-208
Petrere JA, Rohn WR, Grantham LE, Anderson JA (1993). Food restriction during organogenesis in rabbits: effects on reproduction andthe offspring. Fundam. Appl. Toxicol. 21:517-522.
Pier AC (1992). Major biological consequences of aflatoxicosis in animal production. Anim. Sci. 70:3964-3967.
Reddy G, Rao DGK (2001). Teratological effects of aflatoxin in rabbits. Ind. Vet. 78:900-902.
Sharma A, Sahai R (1987). Teratological effects of aflatoxin on rats (Rattusnovegocus). Ind. J. Anim. Res. 21 (1):35-40.
Stanley FC, Bower C (1986). Teratogenic drugs in pregnancy. Med. J. Aust. 11–12(145):596–9.
Tashiro F, Morimura S, Hayashi K, Makino R, Kawamura H, Horikoshi N, Nemoto K, Ohtsubo K, Sugimura T, Ueno Y (1986). Expression of the c-Ha-ras and c-myc genes in aflatoxin B1-induced hepatocellular carcinomas. Biochem. Biophys. Res. Commun. 138(2):858-64.
Trail F, Mahanthi N, Linz J (1995). Molecular biology of aflatoxin biosynthesis. Microbiology 141 (4):755-765.
Vickers M, Brackley K (2002). Drugs in pregnancy. Curr. Obstet. Gynaecol. 12:131–7.
Wangikar PB, Dwivedi P, Sinha N (2004b). Effects in rats of simultaneous prenatal exposure to ochratoxin A andaflatoxin B1.I. Maternal toxicity and fetal anomalies. Birth Defects Res. B Dev. Reprod. Toxicol. 71 (6):343-351.614.
Wangikar PB, Dwivedi P, Sharma AK, Sinha N (2005). Effects of aflatoxin B1on embryo fetal development in rabbits. Food Chem. Toxicol. 43:607–615.
Wangikar PB, Dwivedi P, Sharma AK, Telang AG, Bisht GS (2004a). Haemato-biochemical changes in experimental ochratoxicosis andaflatoxicosis alone and in combination in pregnant rabbits. Ind. J. Vet. Pathol. 28 (1): 64-67.
Weber FE, Eyrich G, Grätz KW, Thomas RM, Maly FE, Sailer HF (2001). Disulfide bridge conformers of mature BMP are inhibitors for heterotopic ossification. Biochem. Biophys. Res. Commun. 286(3):554-8.
World Health Organization, International Agency for Research on Cancer (IARC) (1993). Monographs on evaluation of carcinogenic risks to humans
Wuelling M, Vortkamp A (2011). Chondrocyte proliferation and differentiation. Endocr. Dev. 21:1.
Yoshida GJ, Saya H, Zouboulis CC (2013). Three-dimensional culture of sebaceous gland cells revealing the role of prostaglandin E2-induced activation of canonical Wnt signaling. Biochem. Biophys. Res. Commun. 438(4):640-6.
Yoshizawa H, Uchimaru R, Ueno Y (1981). Metabolism of aflatoxin B1 in the isolated nuclei of rat liver. J. Biochem. 89(2):443-52.
Young AD, Phipps DE, Astroff AB (2000). Large-scale double staining of rat fetal skeletons using alizarin red S and alcian blue.Teratology 61:273-6.

academicJournals

Vol. 8(8), pp. 156-160, August 2014
DOI: 10.5897/JCAB2014.0418
Article Number: 495737F47318
ISSN 1996-0867

http://www.academicjournals.org/JCAB

Journal of Cell and Animal Biology

Full Length Research Paper

Cd44 gene expression in mature and immature oocytes and fetus of Kermani and Baluchi sheep, and Rayeni and Tali goats

M. Aminafshar[1], V. Bahrampour[1]*, A. Baghizadeh[2], N. EmamJomeh Kashan[1] and M. R. Mohamad Abadi[3]

[1]Department of Animal Science, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman-Iran.
[3]Departments of Animal Science, ShahidBahonar University, Kerman, Iran.

Received 16 July, 2014; Accepted 29 July, 2014

CD44 family belongs to a large group of proteins that bind to hyaluronic acid. It has important role in oocyte maturation, fertilization and embryo development. We analyzed CD44 in oocytes and embryos of goat and different breeds of sheep. We used both Kermani and baluchi breeds of sheep, two Rayeni and Tali goats. Domestic animals kept after feeding for 3 months exude embryos and oocyte. After this, the animals were cured by keeping full progesterone for 14 days in the vagina. Gonadotropin-releasing hormone (GnRH) hormone was injected from the animals' vagina and made ready for sampling after 1 day. After that, mature oocyte was exuded from the animals' uterus and they were taken for slaughter. To get the embryos, male animals were kept beside them. After 6 days these animals were taken for slaughter. In another way, ovary collected from the slaughter house exuded immature oocyte. In the laboratory, immature and mature oocyte was produced. So by keeping sperm under environmental condition embryo culture is produced. The samples exude RNA using kit; after that, cDNA was produced by special protocol. The products produced by conventional PCR and Real time PCR were studied. Result shows that gene expression does not exist in immature oocyte of sheep. In Tali goat, expression of this gene was more than in Rayeni goat.

Key words: CD44 gene, gene expression, real time PCR, goat, sheep.

INTRODUCTION

Glycosaminoglycans (GAGs) play a main role in the proliferation and differentiation of a variety of cell types (Luz et al., 2012). The communication between the granulosa cells and the adjoining is vital for the attainment of oocyte aptitude (Assidi et al., 2008). The growth of cumulus cells might be absolutely connected to the ovulation, fertilization, and subsequent zygote growth (Chen et al., 1993).

Among the GAGs, hyaluronic acid (HA) is a lofty molecular weight polysaccharide found in the extracellular matrix of most animal tissues and is one of the most

*Corresponding author. E-mail: v_bahrampur@yahoo.com. Tel: +98 913 597 7707.

Table 1. Primer sequence to Real time PCR used to notice the presence of CD44 receptor in sheep and goat immature and mature oocytes and embryos.

Gene	Nucleotide sequence
CD44	5-CAACACCTCCCASTATGACAC-3
	5-TTCTTCTGCCCACACCTTCT-3
b-actin	5-CAACTGGGACGACATGGA-3
	5-TGGTGGTGAAGCTGTAGC-3

abundant GAGs in the uterine, oviductal andfollicular fluids (Archibong et al., 1987). Through the progression of ovulation, cumulus cells exude HA (Salustri et al., 1990). Moreover, HA adds to the average development of 1 and 2-cell porcine embryos (Miyano at al., 1994) as well as *in vitro* bovine embryo growth at the blastocyst stage (Furnus et al., 1998). HA mostly binds to CD44, which is a glycolprotein extensively expressed on the outside of many mammalian cells. CD44 exists as manifold isoforms expressed in an exact way for different cell types. These isoforms affect splicing and post-translational modifications, where they can be glycosylated in a different way (Opela et al., 2012). Cell exterior glycoprotein CD44 is present in mature oocytes and embryos in small number of classes of mammals such as bovine (Furnus et al., 2003). CD44 was not detected in immature oocytes in porcine (Yokoo et al., 2007). This information shows that CD44 is expressed throughout the maturation procedure, which suggests its significance in this phase. It is reasonable to assume that HA profile is directly proportional to the amount of CD44 in somatic cells surrounding the rising oocyte (Luz et al., 2012). There is a whole connection loss of cumulus growth in cumulus-oocyte complexes (COCs) and oocyte meiotic series (Allworth and Albertini, 1993). The growth of cumulus cells might be absolutely connected to the ovulation, fertilization, and subsequent zygote growth (opelia et al., 2012).

The purpose of this study was to investigate whether this gene is expressed in immature oocytes and embryos of goats.

MATERIALS AND METHODS

Natural ways for collecting mature oocytes and embryo

This procedure was performed as follows: First, estrous cycle synchronized was created in the vagina with a sponge containing 60 mg medroxyprogesterone acetate for 14 days. After 24 h, sponge was used to remove estrus from rams. After 90 h when the sheep was slaughtered, the estrus extracted was placed in the vagina. A syringe was then placed in a liquid at the lab; the fetus was confirmed under a microscope with a magnification of 10 to 50X; for only the morula stage embryos. In goats, estrus synchronization was done using CIDR and injections for 14 h; CIDR removal was performed in 5/2 ml of GnRH for 48-24 h; after CIDR was used to remove heat from the goats. For every 10 female goats, a male goat was used for mating them just like the other procedures used for sheep.

In vivo embryo production

Five hair ewes and goat (2 to 6 years old) were submitted for similar hormonal action as described above. Females were mated at the start of estrus and 24 h later, rams and male goat were used to form fertility. Recovery of embryo was performed by laparotomy for six and seven days during the first mating. Soon after genital area contact, every uterine horn was washed with 25-30 ml DMPBS. Embryo excellence and growth phase were evaluated under a microscope at 10 to 50X magnification. Embryos at the morula phase were then frozen (-80°C) using Real time PCR.

RNA

RNA was extracted using RNA Purification Kit, after which CDNA synthesis was carried out. In this protocol, the materials and reactions added to a system of numbered and color-coded labels are shown. Replication done using PARSGENOME MiR-Amp kit includes a three-step protocol.

Real-time PCR method

Replication done using PARSGENOME MiR-Amp kit includes a three-step protocol; the cDNA amplification was conducted with Real time using PCR primers to increase the specificity and yield of the PCR product (Table 1). Sum of RNA was remote as described above (Opiela et al., 2012). The comparative expressions levels of glyceraldehyde-3-phosphate dehydrogenize were used for normal marker gene expression in all samples. SYBR Green PCR Master Mix Kit (PARSGENOME, Iran) was used to do relative quantification of gene expression. Every reaction (total volume of 20 µL) consisted of total RNA (2 ng/µL), 1× of SYBR Green PCR master mix containing an optimized RT-PCR buffer, 2.5 nM of MgCl2, nucleotides, Taq DNA polymerase, SYBR Green and stabilisers, 200 nM each of the forward and reverse primer (Luz et al.2012), and 1× of RT/RNase block enzyme mixture. Thermal cycling conditions are as follows: 35 min at 50°C (for the first-strand synthesis); 12 min at 95°C; 40 cycles of 30 s at 95°C for denaturing; 60 s at 60°C for annealing; and 30s at 72°C for extension. Experiments were carried out using Master cycler apparatus (Eppendorf, UK Limited, Cambridge). GAPDH was used as an endogenous normal. The results for individual target genes were consistent with the relative endogenous standard. Each reaction PCR was sprinted in triplicate and the obtained results were averaged. Ct method was used for calculating the comparative quantification. Statistical analysis differences in transcripts level were assessed using ANOVA test.

RESULTS AND DISCUSSION

Quality and quantity of DNA and RNA were extracted from high contamination and bands were observed on agarose gel, using spectrophotometer. The Quantity and quality of extracted DNA were calculated and recorded (Figure 1). In the original Eppendorf tube, mix temperature gradient was attempted using Real time PCR. The mature oocytes of Kermani and Baluchi sheep were lower than that of Rhine goats; CD44 genes wereencoded at a temperature range of 55 to 60°C. CT criteria for selecting optimal binding temperature were high, and few ΔRn at 59.5 and 60.7°C for CD44 gene was selected.

The graphs show Ct at different temperatures and ct is the cycle that begins the Sigma growth chart. The melting

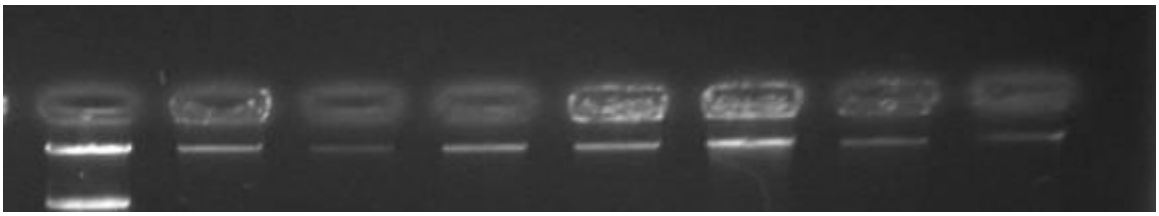

Figure 1. RNA was extracted from the agarose gel shows

Table 2. Results of thermal gradients CT internal control and CD44 gene.

Temperature gradient	Internal control gene CT	CD44 gene CT
57	23.2	29.1
58.2	22.6	28.62
61.9	21.8	24.31

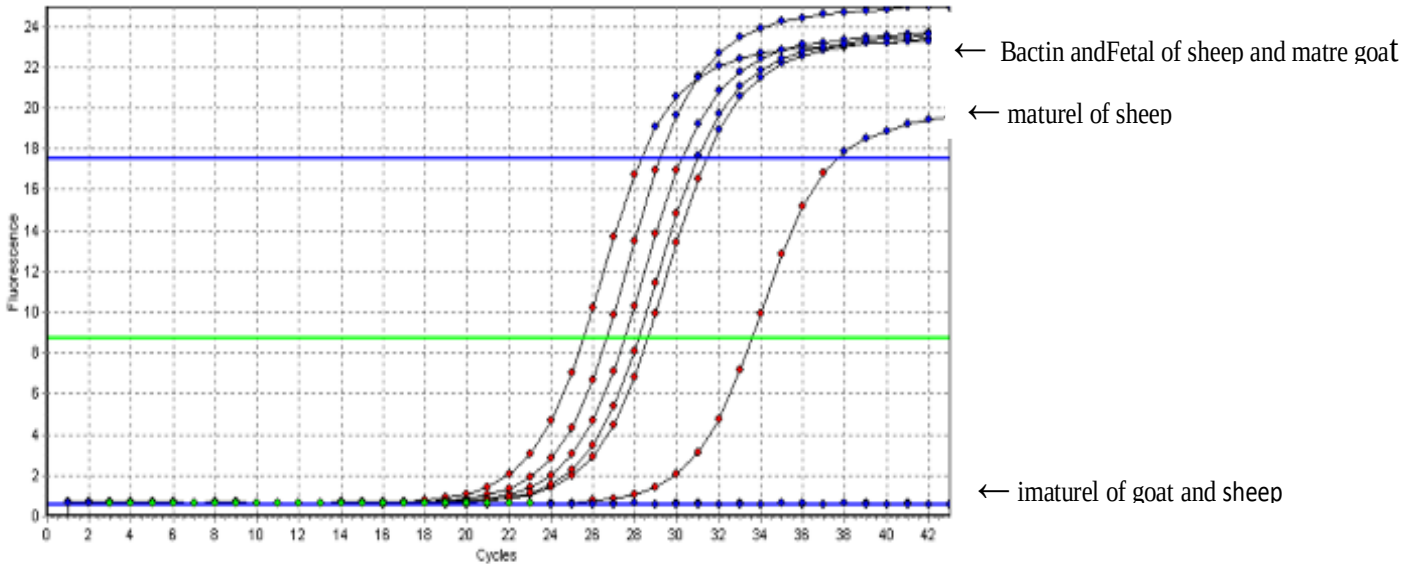

Figure 2. Curve of CD44 gene replication in immature oocytes, mature and fetal.

Table 3. Variance Analysis of CD44 gene expression in Baluchi, Tali sheep and Rayeni, Tali goat.

Sources changes	Mean-square	sum of squares	DF	F
Treatment	84.141	84.141	1	
Error	11.25	112.509	10	7.47
Total		196.65	11	

DF= degrees of freedom; Minimum significance level = P <0.05.

point of the CD44 gene in all tissues of the animals was 62°C. Table 2 shows the thermal gradients of threshold cycler. By using Software line Reg PCR, the cycle curve shows the fluorescence light and CD44 expression in all tissues (Figure 2). CD44 gene in all tissues of sheep and goats were expressed in immature oocytes, but this expression in other tissues was indistinguishable. Rates of PCR efficiency were different in tissues of different animals. Expression levels in sheep and goat were different due to the significant difference in sheep and goat breeds, as shown in Table 3.

So we can conclude that CD44 expression is asignificant

Table 4.sheep, goat CD44 gene expression data analysis

Sources changes	Mean-square	sum of squares	DF	F
Treatment	65.4	196.4	3	2616
Error	0.025	111.26	8	
Total		196.65	11	

DF= degrees of freedom; Minimum significance level = P <0.05.

Table 5. Comparison of Average.

Sample	Goat Embryo	Sheep Embryo	Mature oocyte Tali goat	Mature oocyte Baluchi sheep	mature oocyte Rayeni goat
Sheep embryo	2.97 *	-	-	-	-
Mature oocyte Tali goat	2.34 *	0.63	-	-	-
Mature oocyte Baluchi sheep	8.43 ***	5.45 **	6.99	-	-
Mature oocyte Rayeni goat	10 ***	6.03 **	6.66**	0.561	
Mature oocyte Kermani sheep	14.4***	11.5***	12.06***	5.971***	5.98**

test for comparison, which was done by examining the sample. In addition to the effect of race on expression levels of CD44, gene expression comparison between sheep and goats was done (Table 4). F that was obtained from Table 3 is higher than F in base table (P <0.05). So this test is more significant in the expression of these genes and also the obtained result was different between sheep and goat (Table 5) in the CD44 gene in comparison to the races at P <0.05 according to Duncan's method was studied.

Based on the table, it is clear that the greatest differences were found between goats' fetus and mature oocytes of sheep but minimum differences were found between Baluchi sheep mature oocytes and immature Rayeni goats' oocyte. Also, the greatest differences were found between mature oocytes of goats and Kermani sheep. From this table, we can conclude that CD44 gene expression in fetus of sheep and goats was higher than that of the other samples.

In this study, it is established for the first time that CD44 is expressed on mature oocytes and embryos of goat. Real time PCR was used to detect the expression of transcript. Similar result is reported in mature oocytes of additional mammalian class, like human (Toyokawa et al., 2005). Also we found that CD44 mRNA was not detected in immature oocytes. This is logical since immature oocytes need to get in touch with nearby granulose cells to allow nutrient passage and to get good development. This agrees with previous data on other mammalian class such as porcine (Campbell et al., 1995) and bovine (Luz et al., 2012), in which CD44 in immature oocytes was not detected.

It is now recognized that CD44 influences the growth of the cumulus cells throughout the oocyte maturation (oeplia et al., 2012), leading to fertility and excellence of oocytes (Luz et al., 2012). As the hyaluronan-CD44 communication is concerned in the introduction of meiotic recommence-ment, it was supposed that this receptor was expressed in the oocytes. However, it was established that this receptor is present only in cumulus cells, and not in the oocyte. New studies have shown that the meiotic maturation of oocytes is also a topic on regulation by the somatic section of the ovarian follicle (oplea et al., 2012). MPF that starts at the onset of meiotic recommencement is inhibited by intra-oocyte cAMP, which is transferred from cumulus cells via gap junctional communication inside COCs. Break of gap junctions in the COCs, which occurs in reply to the pre-ovulatory rush of gonadotropins (Assidi et al., 2008), leads to a drop in the intra-oocyte concentration of cAMP, followed by MPF activation and meiotic resumptions. The decrease of the intra-oocyte cAMP attentiveness was concealed by the inhibition of the interaction between hyaluronan and CD44. This result supports the concept that hyaluronan-CD44 interaction is concerned with the regulation of gap junctional communication and the termination of the cAMP flux from cumulus cells to oocytes (Yokoo et al., 2010).

No studies have established the role of the HA-CD44 system in oocyte maturation. However, one study (Luz et al., 2012) demonstrated that the squalor production of HA induced the phosphorylation of the CD44 receptor, most important for the start of kinase proteins, which are then translocated to nucleus. This flow is significant for mitogenic signal transduction and enough for the induction of cell propagation from the proto-oncogenic transcription factors (Daum et al., 1994). Since the mainconstituent in the extended cumulus is HA (Borg and Holland, 2008), this almost certainly explains the availability of the CD44 receptor in mature oocytes.

CD44 as well plays a role in embryo development up to

blastocyst phase (Kimura et al., 2007). In one trial (Oeplia et al., 2012), l mg/ml of HA was supplemented with the civilization medium; and the bovine embryos were established, which then developed to the blastocyst stage higher than when in a medium alone. These authors reported that the amalgamation of HA in a chemically defined medium obviously established the result of HA in the development of blastocyst configuration. This is in accord with the study of Miyano et al. (1994), who stated that the amount of degenerated porcine embryos were more inferior in the presence of HA than in its absence. It has been suggested that HA helps the development of embryos by regulating the action of factors that synthesize the embryo, in an autocrine way (Wheatley et al., 1993). Li et al. (2008) reported that at the beginning, production of HA occurs at about 18 h after the beginning of maturation. This is enthused by the growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), which trigger hyaluron synthase enzyme expression, responsible for synthesis of HA. The best growth of refined COCs requires the presence of substrates of HA synthesis and a prolonged cumulus mass that might absolutely influence oocyte feasibility (Chen et al., 1993). HA shaped obviously by granulosa cells also stop fragmentation or segmentation of oocytes *in vitro* (Sato et al., 1994).

The presence of CD44 in mature oocytes and embryos suggests the expression of HA throughout maturation and development. The result of this study could be helpful in the description investigation and understanding of the physiological role of CD44 in the reproductive processes of the ovine class. Additional studies are necessary to elucidate the proceedings in which CD44 and HA are involved during maturation and embryo growth in goats and sheep.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Allworth AE, Albertini DF(1993). Meiotic maturation in cultured bovine oocytes is accompanied by remodeling of the cumulus cell cytoskeleton. Dev. Biol. 158:101-112.

Archibong AE, Petters RM, Johnson BH (1989). Development of porcine embryos from one- and two-cell stages to blastocysts in culture medium supplemented with porcine oviductal fluid. Biol. Reprod. 41:1076-1083.

Assidi M, Dufort I, Ali A, Hamel M(2008). Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbolmyristate acetate in vitro. Biol. Reprod. 79:209-222.

Borg N, Holland M (2008).The effect of glycosaminoglycans on rat gametes in vitro and the associated signal pathway. Reproduction 135:311-319.

Campbell S, Swann HR, Aplin JD, Seif MW (1995). CD44 is expressed throughout pre-implantation human embryo development. Hum. Reprod. 10:425-430.

Chen L, Russell PT, Larsen WJ (1993). Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol. Reprod. Dev. 34: 87-93.

Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J (1994). The ins and outs of Raf kinases. Trends Biochem. Sci. 19:474-480.

Furnus CC, de Matos DG, Martinez AG (1998). Effect of hyaluronic acid on development of in vitro produced bovine embryos. Theriogenology 49:1489-1499.

Furnus CC, Valcarcel A, Dulout FN, Errecalde AL (2003). The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos. Theriogenology 60:1633-1644.

Kimura N, Hoshino Y, Totsukawa K, Sato E (2007). Cellular and molecular events during oocyte maturation in mammals: molecules of cumulus-oocyte complex matrix and signalling pathways regulating meiotic progression. Soc.Reprod.Fertil. Suppl. 63:327-342.

Li HK, Kuo TY, Yang HS, Chen LR (2008). Differential gene expression of bone morphogenetic protein 15 and growth differentiation factor 9 during in vitro maturation of porcine oocytes and early embryos. Anim.Reprod. Sci. 103:312-322.

Luz JV, Batista A, Souza SJA (2012).Expression of CD44 in sheep oocytes and preimplantation embryos.Genet. Mol. Res. 11: 799-809.

Miyano T, Hiro-Oka RE, Kano K, Miyake M (1994).Effects of hyaluronic acid on the development of 1- and 2-cell porcine embryos to the blastocyst stage in vitro.Theriogenology 41:1299-1305.

Opiela J, Latasiewicz E, Smorag Z (2012). Optimal concentration of hyaluronan and plant protein in different culture systems for in vitro maturation of bovine oocytes. Indian J. Exp. Biol. 12:839–846.

Salustri A, Yanagishita M, Hascall VC (1990).Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. J. Biol. Chem. 264:13840-13847.

Sato E, Inoue M, Takahashi Y, Toyoda Y (1994).Glycosaminoglycans prevent induction of fragmentation of porcine oocytes stimulated by dibutyryl cyclic adenosine 3',5'-monophosphate in culture. Cell Struct.Funct. 19:29-36.

Toyokawa K, Harayama H, Miyake M (2005). Exogenous hyaluronic acid enhances porcine parthenogenetic embryo development in vitro possibly mediated by CD44. Theriogenology 64:378-392.

Wheatley SC, Isacke CM, Crossley PH (1993). Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning. Development 119: 295-306.

Yokoo M, Kimura N, Sato E (2010).Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs. J.Reprod. Dev. 56:15-19.

Yokoo M, Shimizu T, Kimura N, Tunjung WA (2007).Role of the hyaluronan receptor CD44 during porcine oocyte maturation. J.Reprod. Dev. 53:263-270.

Journal of Cell and Animal Biology

Related Journals Published by Academic Journals

- *International Journal of Genetics and Molecular Biology*
- *Journal of Microbiology and Antimicrobials*
- *International Journal of Biodiversity and Conservation*
- *Journal of Bacteriology Research*
- *Journal of Developmental Biology and Tissue Engineering*
- *Journal of Evolutionary Biology Research*

academicJournals