Niger. J. Physiol. Sci. 35 (December 2020): 155 - 159
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Research Article

Consumption of Calcium Carbide-Ripened Banana by Pregnant Rats May Programme for Infertility in Female Offspring

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Summary: One of the substances used in force ripening fruits is commercial grade calcium carbide (CaC2) which contains impurities such as arsine and this has been associated with low birth weight and fetal loss. There is thus a need to further investigate additional risks on offspring. This study was thus designed to evaluate the possible effects of maternal consumption of banana pulp, force ripened with CaC_2, on the offspring. Sixteen pregnant rats were randomly divided into two test groups and controls of four rats each. Two test groups were fed with pelletized feed mixed with banana pulp ripened by commercial grade CaC_2 at concentrations of 50g/5kg and 100g/5kg while the controls had a group fed with pelletized feed mixed with normal ripened banana and another had only pelletized feed. This feeding pattern was done morning and evening *ad libitum* throughout the gestation period of twenty-one days after which only pelletized feed and water was administered. At delivery, all male offspring were separated and each dam was allowed eight female pups to nurse. Upon weaning after twenty-one days, the mothers were removed leaving eight female offspring in each group. Development of their reproductive system was monitored and recorded using parameters such as vaginal opening day (VOD) and reproductive hormonal assay at the sixth week. A fertility test was also carried out by introducing viable male rats for mating at sixth week postpartum. Trace amount of arsenic was found in the banana pulp of 100g/5kg CaC_2 group (0.35ppb). CaC_2 exposure was related to delayed onset in puberty, decreased serum FSH and a decreased fertility rate in the 100g/5kg CaC_2 group ($p<0.05$). Consumption of contaminated CaC_2 ripened fruits exposes humans to arsenic acid which has harmful effects on reproductive development of offspring.

Keywords*: calcium carbide, arsenic, reproductive hormone, Vaginal opening day, postnatal reproductive development, fertility rate*

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Received - June 2020; Accepted- October 2020

INTRODUCTION

Environmental contaminants including endocrine disrupting chemicals are causing adverse reproductive health worldwide (Rashtian *et al*. 2019). The intrauterine and early childhood periods are the most vulnerable windows for chemicals that may impair growth and organ development (Grandjean *et al*., 2008). Even the least concentration of these common environmental pollutants that would appear harmless in the short term to an adult will be very harmful to the developing fetus and during early child development (WHO 2002). Consumption of good and ripe fruits is a healthy diet habit recommended for all and that place an increase demand on the supply in Nigeria. There is thus the propensity to initiate artificial ripening of these fruits. Chemicals are commonly employed as artificial ripening agents and the substance commonly used in Nigeria is calcium carbide because it is relatively cheap (Singal, Kumud, and Thakral, 2012). Calcium carbide (CaC_2) in its pure form is not toxic but commercial grade CaC_2 also known as *masala* is extremely hazardous to the human body as it contains traces of arsenic and phosphorus. It is banned in many countries of the world, but it is freely available in Nigeria, India, Pakistan and other countries (Dhua and Siddiqui, 2010). Several studies suggest association between arsenic exposure and adverse pregnancy outcomes such as spontaneous abortion, stillbirth, and infant death (Ahmad *et al*. 2001; Milton *et al*. 2005; von Ehrenstein 2006; Rahman *et al*. 2007). However, limited studies exist on the programming of intra-uterine exposure to impure commercial grade CaC_2 on fertility of the surviving offspring and the molecular mechanism underlying this deleterious effect.

MATERIALS AND METHODS

Experimental layout for ripening of banana: Unripe matured bananas (*Musa spp*) was purchased locally and authenticated in the department of Plant Science and Biotechnology, University of Nigeria, Nsukka and was assigned a voucher's number UNH No 813. The banana was ripened using commercial grade calcium carbide in the form of solution according to the method used by Chandel et al. (2018). For the control groups, control one (T0) had rats fed with pelletized feed and water only while control two (T1) had five-kilogram banana fruit placed in five-kilogram capacity carton and allowed to ripen naturally (without using CaC_2) at ambient temperature. For the test groups, five-kilogram banana fruit was dipped in five litres of water containing 50g of CaC_2. This served as T2. Another five-kilogram banana fruit was dipped in five litres of water containing 100g of CaC_2. This was T3. T2 and T3 were kept

for 30 minutes after which the fruits were removed from the solution and air dried to remove adhering moisture. The treated fruits were placed in five-kilogram capacity carton each and allowed to ripen at ambient temperature. After which, banana from each group was separately fed into a Qlink Blender model QBL-20L330 China, and then homogenized. The resulting puree (juice) was introduced into plastic bottles, properly labelled and preserved in a refrigerator at 15ºC which was later used in the animal experiment according to Gbakon *et al* (2018)**.** Although the doses of CaC_2 used in this study have been used by a previous study (Chandel *et al*., 2018), the rationale behind the adoption of the doses was to ascertain the possible health effect that can arise from a concentration this low.

Experimental design: Sixteen nulliparous (weighing between 170-220g) rats were used for this study. After successful mating and pregnancy was confirmed by the presence of vaginal plug, the rats were randomly assigned into four groups namely T0, T1, T2, and T3. Each group had four rats each. The rats were fed with pelletized mash. Pulp from the previously ripened banana fruits from each group was taken for estimation of arsenic residues in the banana from the peel and pulp separately for each treatment by using wet digestion according to the method of Chandel *et al*. (2018), using ICP-AES (Inductively Coupled Argon Plasma-Atomic Emission Spectrometry, Buck Scientific 210 Variable Giant Pulse model). The level of arsenic residues in the various treatments was compared with the maximum contamination level of arsenic residue in fruits which is 0.05ppb (USDA, 2006).

The banana pulp was mixed with the pelletized mash and the rats in each group fed *ad libitum* according to Gbakon *et al* (2018). The blended banana was mixed with the rat feed according to the method by Igbinaduwa *et al*. (2016). The allotment of diet is shown in the table below.

Table 1:
Allotment of diet across the groups

Groups	CaC_2 level (g/kg of fruit)	Treatment diet
T0	0g	Normal rat feed +water
T1	0g	Banana pulp (without CaC_2) + feed + water
T2	50g/5kg	Banana pulp (with CaC_2) + feed + water
T3	100g/5kg	Banana pulp (with CaC_2) + feed + water

After delivery, the male pups were removed leaving only the female pups. Each dam was allowed eight female pups to nurse throughout the lactation period to eliminate any form of over nutrition or mal nutrition. At postnatal day twenty-one, the pups were weaned off while the mothers were removed from the groups. The vaginal opening day (VOD) for each rat per group was recorded and taken as the onset of puberty (Ojeda and Skinner, 2006). At Postnatal day 35 (fifth week postpartum), the week the vagina of rats opened in the control group, blood was taken from the dorsal aorta of four rats in the control group and subsequent groups at their respective vaginal opening day (VOD) for assay of reproductive hormone namely follicle stimulating hormone (FSH), Luteinizing hormone (LH) and Estrogen (E_2). The hormones were estimated by radioimmunoassay (RIA). These rats were marked thereafter. The marked four pups in each group were later sacrificed after blood had been obtained from them by cervical dislocation and ovaries harvested and preserved in formalin for tissue processing while the remaining four were subjected to fertility test by introducing viable and mature males to the female pups. Confirmation of pregnancy was a sign of fertility. The fertility rate per group was calculated according to (Anjum and Reddy 2015) with slight modification as:

$$\frac{number\ of\ pregnant\ rats}{total\ number\ of\ mated\ rats} X\ 100$$

Statistical analysis: Findings were tabulated and analyzed with results expressed as mean ± SEM. Statistical analysis was done using one-way Analysis of Variance (ANOVA). The results were compared using Post-hoc (tukey) test. Results were considered significant at $p< 0.05$

Ethical consideration: All procedures used in this study adhere to the ARRIVE (Animals in Research: Reporting *in Vivo* Experiments) guidelines for reporting animal research (Kilkenny *et al*. 2010; Tilson and Schroeder, 2013) and the ethical standards of this experiment is in accordance with the guidelines provided by the CPCSEA and World Medical Association Declaration of Helsinki on Ethical Principles for Medical Research involving experimental animals. It was equally approved by College of Medicine Research and Ethics Committee, University of Nigeria.

RESULTS

Arsenic residue analysis of CaC_2 ripened banana by atomic absorption: Between the treatment groups, significant elevation in mean arsenic deposition was observed in the peel of 50g/5kg CaC_2 and 100g/5kg CaC_2 treatments group ($p<0.05$) compared to naturally ripened banana group. Non-significant increase was observed in the pulp of the treatment groups in dose-dependent manner as seen in the Table 2.

Table 2:
Arsenic residue analysis of CaC_2 ripened banana by atomic absorption

Ripening treatments (T)	Arsenic residue (ppb) in different fruits parts	
	Banana Peel	Banana Pulp
T1 (naturally ripened)	0.00 ± 0.00	0.00 ± 0.00
T2 (dipping in 50g/5litres of CaC_2)	0.61 ± 0.19	0.30 ± 0.12
T3 (dipping in 100g/5litres of CaC_2)	1.28 ± 0.00*	0.35 ± 0.17

Values are mean ± SE
**significantly different from T2 at p<0.05*
Arsenic residue in the calcium carbide granules was found to be 1.71ppb.

Vaginal opening day (VOD) of female offspring exposed to prenatal CaC_2 ripened banana: In the Table 3, a Significant increase (P<0.05) was observed in vaginal opening day in 100g/5kg CaC_2 group when compared to control. There was a non-significant increase in the VOD of the naturally ripened banana group (T1) when compared with the control. A significant dose-dependent increase was also observed within the CaC_2 treatment group (T2 and T3).

Hormonal analysis by radioimmunoassay (RIA) on reproductive hormone of female offspring exposed to prenatal CaC_2 ripened banana: Significant dose-dependent decrease (P<0.05) was observed in mean plasma levels of FSH with all doses of CaC_2 compared to control. Within the treatments groups, 50g/5kg CaC_2 group showed a non-significant (p<0.05) increase in plasma levels of FSH, LH and estrogen compared to 100g/5kg CaC_2 group. The naturally ripened banana group (T1) significantly increased the levels of FSH, LH and estrogen (p<0.05) when compared to the CaC_2 treatment groups as shown in Table 4.

Fertility rate of female offspring exposed to prenatal CaC_2 ripened banana: A significant dose-dependent decrease in fertility rate was observed in 100g/5kg CaC_2 group when compared with the control and naturally ripened banana groups. A non-significant decrease in fertility rate was observed within the treatment groups as seen in the Table 5.

Table 3:
Vagina opening day (VOD) of female offspring exposed to prenatal CaC_2 ripened banana (*mean ± SE, n= 8)*

GROUPS	Vagina opening day (days)
TO	35 ± 0.74
TI	33 ± 0.62†
T2	36 ± 0.16*
T3	44 ± 0.48‡

* *Significantly different from T3 at p < 0.05*
† *Significantly different from T3 at p < 0.05*
‡ *Significantly different from T0 at p < 0.05*

Table 4:
Hormonal analysis by radioimmunoassay (RIA) on reproductive hormone of female offspring exposed to prenatal CaC_2 ripened banana (*mean ± SE, n= 4)*

Groups	FSH (mIU/mL)	LH (mIU/mL)	Estrogen (pg/mL)
T0	47.1±1.1	12.8±4.0	57.7±11.8
T1	44.0±1.6	20.6±0.6	67.8±4.6
T2	32.8±2.2*†	9.4±1.2†	37.9±3.7*†
T3	34.2±1.4*	9.6±1.1†	28.5±1.0*†

**significantly different from the control (T0) at p<0.05*
† *Significantly different from T1 at p<0.05*

Table 5:
Fertility rate of female offspring exposed to prenatal CaC_2 ripened banana

Groups	Fertility Rate (%)
T0	100
T1	100
T2	75
T3	50*

Values are expressed as mean ± SE, n=4;
**=significant when compared with T0 and T1 at p<0.05*

Histological observation of ovaries of female offspring exposed to prenatal CaC_2 ripened banana: From Plate 1, the graafian follicles of the naturally ripened banana group were very large with increased amount of follicular fluid when compared to other groups (Plate 1B). Among the treated group, the 50g/5kg group had same number of developing follicles but smaller with little or no fluid within the follicles while the 100g/5kg group had markedly reduced number of follicles with scanty follicular fluid (Plate1C & D). The number of developing follicles was observed to be more in the control group (Plate 1A) and it appeared to decrease with increase in the dose of CaC_2.

In Plate 2, the space between the granulosa cells and the theca interna is observed to be well demarcated in both control and naturally ripened banana groups while it is indistinguishable in both treatment groups as there is no marked demarcation between them. In Plate 2, the follicles are seen to be closed to each other in both the control and naturally ripened banana groups while they are markedly spaced in the treatment group and this increase is dose dependent.

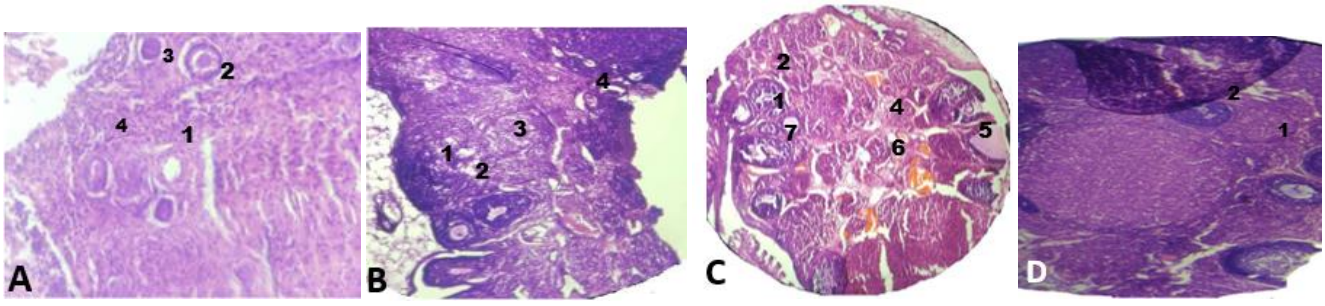

Plate 1:
Photomicrograph of ovary to offspring of control and CaC_2 treated mothers at puberty, showing number of developing follicles.(A) control (T0) showing normal proliferating developing ovarian tissue with the formation of primary follicles (B) Naturally ripened group (T1) showing normal proliferating developing ovarian tissue with the formation of primary follicles. (C) 50g/5kg CaC_2 group (T2) showing malformation of ovarian follicles with disappearance of the fluid in some follicles leading to solidification of some of the follicles. Hemorrhage can also be seen in the vascular channels. (D) 100g/5kg CaC_2 group (T3) showing reduction in number of developing ovarian follicles.

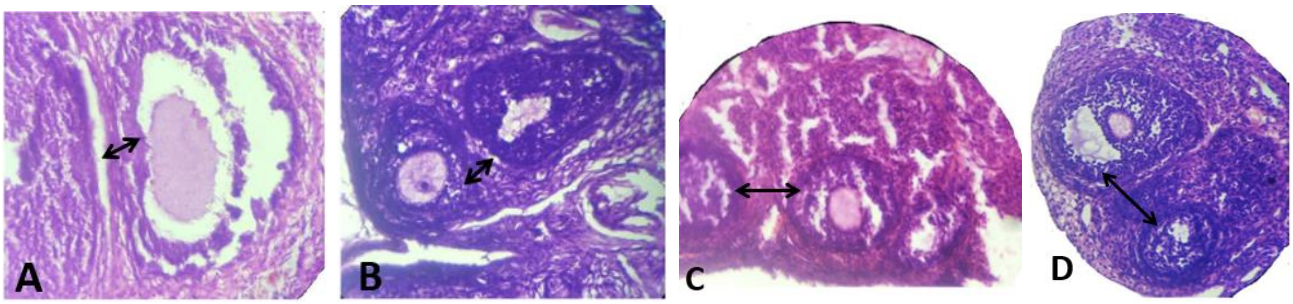

Plate 2:
Photomicrograph of ovaries to offspring of control and CaC_2 treated mothers at puberty, showing graafian follicle. The black arrows indicate the distance between the graafian follicles in each ovary.

The connective tissue around the follicles is moderately dense with clear vascular channels in the control and naturally ripened banana groups while it is relatively loose with congested vascular channels in the low dose group. However, the connective tissues in the high dose group are thicker and deeply hyalinized with fewer follicles Plate 2).

DISCUSSION

In this study we reported the effects of maternal exposure to CaC_2-ripened fruit on the reproductive development of the pups to unravel the mechanisms behind reproductive failures associated with arsenic exposure. It is evident from the result that maximum level of arsenic was found in the banana peel of 100g/5kg CaC_2 group but no significant difference in the concentration of arsenic was found in the banana pulp of both treatment groups. A possible explanation for this could be that the banana peel may act as a barrier preventing the influx of harmful chemicals to the pulp. A study has reported that some components of banana peel, such as Hydroxyl and Carboxyl of Pectin, have the ability to absorb heavy metals (Palacios, 2005). This could explain why the concentration of arsenic in the banana pulp, in both treatment groups, was fairly same despite the varied amount of CaC_2 used. All concentration of arsenic residue in the treatment groups exceeded the maximum contamination level of arsenic in fruits (0.05ppb) (USDA, 2006).

Age of vaginal opening and levels of circulating reproductive hormone have been used as predictors of puberty in mice (Kimberly *et al*., 1997). The observed delay in vaginal opening in group T3 (100g/5kg CaC_2 treated banana) could be linked to the aforementioned alterations in serum reproductive hormone. It has equally been reported that arsenic poisoning in trace amount can lead to one month's delay in the onset of puberty in female rats (Dávila-Esqueda *et al*., 2012). The mechanism explains that pre-pubertal exposure to arsine (III) acts peripherally to suppress circulating levels of IGF-1 resulting in delayed sexual maturation. It went further to identify a critical window of increased susceptibility to arsine (III) that may have a lasting impact on female reproductive function (Reilly *et al*., 2014). Contrary to the above, a study has reported that carbide accelerates puberty onset (Bafor *et al*., 2019).

Levels of circulating LH have been used as predictors of puberty in mice (Risma *et al*. 1997). Elevated serum LH, which is positively linked to precocious puberty (Risma *et al*. 1997), was observed only in the group that ate naturally ripened banana. A possible explanation for the observed increase in stimulation of LH and estrogen secretion could be banana-induced as certain meals have been reported to induce stimulation of hormone secretion (Schreihofer *et al*., 1996). Inorganic arsenic has been reported to suppress ovarian steroidogenesis, prolongs diestrus, and degenerates ovarian follicular cells (Navarro *et al*., 2004; Chattopadhyay *et al*., 2001; Zhang *et al*., 2000), which explains the observed decrease in serum concentration of LH, FSH and estrogen in this study. The mechanism behind the observed arsenic toxicity in the female reproductive system could be arsenic-induced changes in the levels of catecholamines in the brain. The elevation in serotonin and decrease in norepinephrine in the midbrain and diencephalon could lower gonadotrophin synthesis and secretion. Low gonadotrophin levels could in turn decrease activities of ovarian regulatory enzymes for steroidogenesis, a reasonable explanation for the reduction in all three hormones. The observed low FSH level may contribute to the observed decreased number of healthy follicles and increased number of malformed follicles seen in the treatment groups. Studies have equally shown that Arsenic also causes toxicity to estrogen production by interfering with its signaling pathways (Chatterjee and Chatterji, 2010; Bae-Jump *et al*., 2008; Watson and Yager, 2007). Watson and Yager (2007) showed that Arsenic disrupts the estrogen signalling pathways by suppressing the action of estradiol on the uterus and interaction of estrogen receptors with some transcription factors.

A significant decrease in fertility rate was seen in the 100g/5kg CaC_2 group when compared to the control ($p<0.05$). Bafor *et al*. (2019) reported that fruits ripened with calcium carbide negatively alter the female reproductive physiology. The notable difference in fertility between the control and treatment groups under experimental conditions, were due to the alterations in levels of reproductive hormones as well as changes in the architecture of the follicles.

A limitation to this study is the fact that attempt to get pure form of calcium carbide for this study proved abortive. Further work will be done with the pure form of calcium carbide once available to ascertain if the chemical in its pure form has any negative effect in the body.

In conclusion, consumption of commercial grade CaC_2 ripened fruits during pregnancy exposes humans to a significant deleterious effect on puberty onset, and fertility rate of female offspring.

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