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COMPARATIVE EFFECTS OF PRE-GESTATIONAL DOSES OF CLOMIPHENE CITRATE VERSUS LETROZOLE ON THE HEART OF DEVELOPING WISTAR RATS

Dare Isaac Olulana[1,2], Covenant Popoola[2]

1. Department of Surgery, College of Medicine, University of Ibadan, Ibadan, Nigeria

2. Department of Anatomy, College of Medicine, University of Ibadan, Ibadan, Nigeria

Correspondence to Dr Dare I. Olulana Department of Surgery, College of Medicine, University of Ibadan, Ibadan, Nigeria. Email:- isaacdare@yahoo.com. Phone:- +234-802-1196-701

ABSTRACT

Drug-induced ovulation is a therapy that allows women to ovulate and have the chance of conceiving. This therapy is used either in women with irregular ovulation or in patients undergoing In-vitro fertilization cycle to maximize egg production. The goal of drug-induced ovulation is to stimulate the ovaries to produce a single mature follicle and allow fertilization and pregnancy to occur. The drugs commonly used are clomiphene citrate and letrozole. There are indications that these drugs, when used prenatally, could interfere with the normal development of foetal hearts, though; there is a paucity of data on the lethal effects of drug-induced ovulation on the neonatal heart, particularly the left ventricle and ventricular septum. Forty-five adult female Wistar rats that weighed between 140-160g were used for the experiment. They were subdivided into three groups of 15 animals each. A vaginal smear was carried out to further divide the experimental animals based on their oestrous phases. A one-time dose of clomiphene citrate (clomid) was administered at 0.5mg/kg bodyweight, to one of the experimental groups, during the diestrous phase. The other experimental group received Letrozole at 0.025mg/kg bodyweight while the control group received normal feed and water liberally. Mating was done a day after administration of these drugs. Pups were sacrificed on postnatal day 21, the hearts of the pups were dissected out and processed for histological analysis. The result showed that the pups whose mothers received clomid had the lowest heart weights compared to the letrozole and control groups. The left ventricular wall thickness was greater in the Letrozole group and the level of necrosis was observed to be greater in the clomid group as compared to the other groups. These findings show that both clomid and letrozole have some effects on the hearts of the developing wistar rats.

Key Words: Clomiphene citrate, Letrozole, Heart, Wistar rats, Cardiac defects.

INTRODUCTION

Infertility is failure to achieve pregnancy by a couple despite regular unprotected sexual intercourse over a period of one year or the inability to carry pregnancy to term (Olooto, *et al.,* 2012). Drug-induced ovulation has proved to be one of the ways to manage anovulatory infertility and also to maximize egg production in women undergoing Assisted Reproductive Technology (ART) cycles (Katsikis *et al.,* 2006). Indications for the use of ovulation induction therapies include infrequent ovulation, anovulation, unexplained infertility despite regular ovulation, as well as in women undergoing In-vitro fertilization to maximize egg production (Wayne State University Physician Group, 2014).

The commonest drug used for induction of ovulation is Clomiphene citrate. It can be very effective, cheap and user-friendly. The dosage is 50-250 mg per day for 5 days to be commenced 2, 3, 4, or 5 days after spontaneous or induced menstrual bleeding. Starting with the lowest, the

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dose can be raised in increments of 50 mg per day per cycle until an ovulatory cycle is achieved. Though efficient in achieving ovulation, use of clomiphene is a risk factor for congenital anomalies (Sovino *et al.,* 2002). High serum levels of luteinizing hormone (LH) and especially the anti-oestrogenic effects of clomiphene on the endometrium and cervical mucus may make clomiphene citrate to interfere with fertility (Casper and Mitwally, 2006, Pavone and Bulun, 2013). The drug is rapidly absorbed in the intestine and remains in the body for about 5 days while the drug and its metabolite can be picked in the stool about 6 weeks after its administration. (Imani *et al.,* 2002). This is a probable explanation for its interference with cardiac development in utero.

Letrozole is cheap, has short half-life and insignificant side effects. Its use has been studied in women with polycystic ovary syndrome (PCOS), women who are resistant to clomiphene citrate, in intrauterine insemination and also in various protocols of mild stimulation for in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI). However, the efficacy and safety of the drug is not certain (Kar, 2013). At doses of 1–5 mg, letrozole inhibits estrogen levels by at least 97–99% (Polyzos *et al.,* 2011). In premenopausal women, letrozole stimulates the development of the follicles necessitating the use of aromatase inhibitors for ovulation induction. It does this by decreasing the serum levels of oestrogen which in turn stimulates the production of FSH by the pituitary gland. The resulting increase in FSH levels will stimulate the development of the follicles (Ferrero *et al.,* 2009). Investigation of a causal association between a drug therapy and congenital cardiac malformation must include knowledge of the timeline of normal cardiac development which begins in the third week of foetal development in humans.

Congenital anomalies are structural or functional defects that arise prenatally and may be identified during pregnancy, at birth, or later in infancy (WHO, 2016). They can have a serious, adverse effect on the health, development, or functional ability of the baby. The evidences that pregnancies following treatments by anti-estrogens (clomiphene citrate) and aromatase inhibitors (letrozole) have the potential of resulting into certain congenital anomalies have been inconclusive (Biljan *et al.,* 2005; Sharma *et al.,* 2014).

MATERIALS AND METHOD

Forty-five adult female Wistar rats weighing 140-160g were procured from the Central Animal House, University of Ibadan, Ibadan, Nigeria. The animals were acclimatized in well ventilated cages for 2 weeks with liberal access to feed and water. Vaginal smear cytology was done to determine their different phases of estrous cycle. They were randomly subdivided into three groups of fifteen animals each.

Group 1: Rats on normal feed and water

Group 2: Rats given 0.5mg of clomiphene citrate per kilogram bodyweight

Group 3: Rats given 0.025mg of letrozole per kilogram bodyweight

The required doses of clomiphene and letrozole were given to the rats according to the body weight of the rats using an oral cannula. Rats received a one-time administration during the diestrous phase specifically, because, this is the phase characterized by the development of primordial follicle. Clomiphene citrate tablets 50mg (Clomid – Sanofi Aventis, France) was purchased from the Pharmacy store and preserved appropriately. About 50mg of Clomid was dissolved in 50mls of distilled water to have 1mg of clomiphene citrate 1ml of water. Therefore, with an average weight of 150g, rats received a one-time single dose of 0.075mls with the use of an oral cannula. Letrozole tablets 2.5mg (Femara – Novartis, India) was also purchased from the Pharmacy store and

preserved appropriately. About 2.5mg of Letrozole was also dissolved in 50mls of water to have 0.05mg of Letrozole in 1ml of water. Therefore, with an average weight of 150g, rats received a one-time single dose of 0.075mls with the use of an oral cannula.

A day after administration of these drugs (pro-estrous phase), male rats were introduced prior to the oestrous phase to give an opportunity for fertilization during estrous phase. The rats were examined daily for vaginal plug and microscopically for sperm cells to confirm pregnancy. The resulting pups from these pregnancies were sacrificed 21 days after delivery. The hearts of the pup were dissected out, weighed and processed for histology using haematoxyline and eosin staining technique.

The average number of litters was noted and left ventricular wall thickness was also measured. The resulting values were expressed as Mean ± Standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the quantitative histological parameters among the groups. Post hoc Tukey's test was applied to show which groups' mean differs; p-values were calculated with 0.05 taken as being significant.

RESULTS

The average number of litters in Group 1 was (6.2±1.30), Group 2 (7.0±2.83) and (6.8±1.09) are comparably similar across the groups although, the average number of litters is slightly more in Group 2 (clomiphene citrate) than in Group 3 (Letrozole). The mean weight of the hearts of the pups was significantly lowest in Group 2 (0.205±0.004g) and highest in Group 1 (0.413±0.06g) animals (Table 1).

The average thickness of the wall of the left ventricles of the pups was significantly highest in Group 1 (207.2±110.9mm) and lowest in Group 2 (86.24±39.06mm). There was no hypertrophy of the left ventricular wall across the group but there were evidences of muscular degeneration among the experimental groups (Table 2).

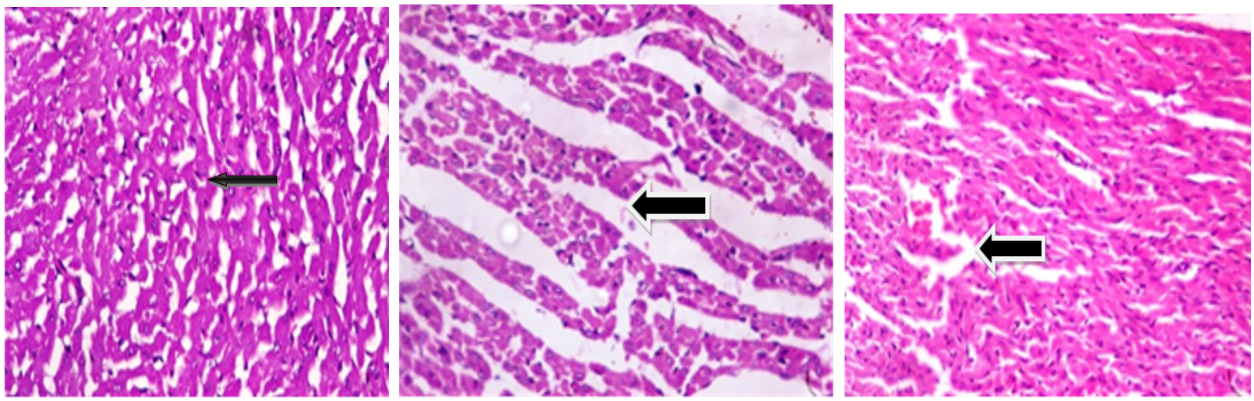
The microscopic examination of cardiac tissues of the left ventricles of the pups using H&E stains revealed that the cardiac tissue obtained from the day 21 pups of the control group were of normal myocardial histology (Fig. 1).

Table 1: Mean heart weights (in grammes)

Groups	Weights(g)
1 - Control	0.413 ± 0.06
11 - Clomiphene Citrate	0.205 ± 0.004
111 - Letrozol	0.237 ± 0.01

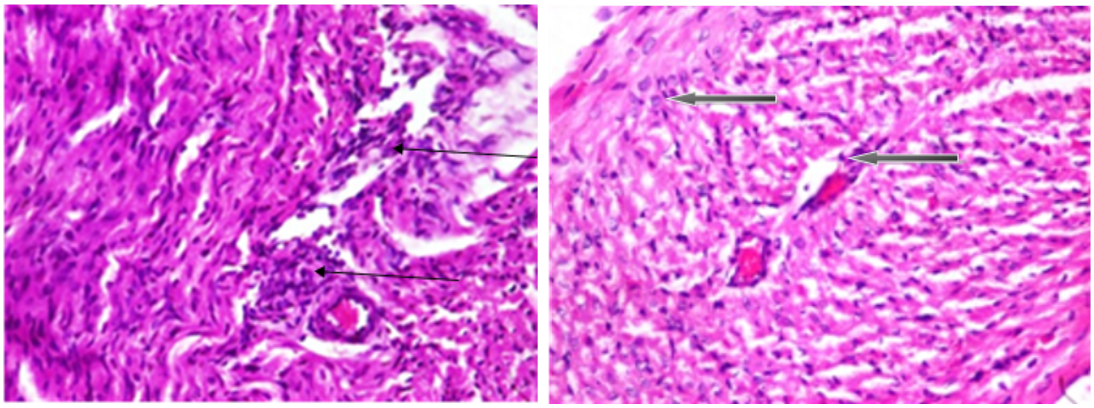
Table 2: Average left ventricular wall thickness (in millimeters)

Group	Left ventricular wall thickness (mm)
I – Control	86.24 ± 39.06
11 – Clomiphene Citrate	90.22 ± 55.38
111 – Letrozol	207.2 ± 110.9



A B C

Figure 1: Photomicrographs of the left ventricle of a heart section from the control (A), clomiphene citrate (B), letrozole (C) groups stained with Haematoxylin and Eosin X400. Figure A shows normal heart tissue with normal myocardial layer (black arrow). Figure B shows severe necrosis (black arrow) in the myocardial layer. Figure C shows mild necrosis in the myocardial layer (black arrow).



A B

Figure 2: Photomicrograph of the left ventricle of a heart section from the clomiphene citrate (A) and letrozole (B) groups stained by Haematoxylin and Eosin X400. Figure A shows focal area of infiltration of inflammatory cells (slender arrow), figure B shows few inflammatory cells and mild necrosis within the myocardium (thick arrow).

There were clear transverse striations and abundant cardiomyocytes in the myocardium. There was no inflammatory cell infiltration. About 70% of the cardiac tissues harvested from the clomiphene citrate group showed severe necrosis in the myocardial layer resulting in obvious separation of cardiac myofibrils. Also observed in this layer, was infiltration of inflammatory cells among necrotic cardiac myocytes. This occurrence was quite severe in the clomiphene citrate group. About 20% of the cardiac tissues obtained from the letrozole group showed mild necrosis and few inflammatory cells in the myocardium (Fig. 2). Apparently, the experimental groups presented with some level of myocardial infarction.

DISCUSSION

The incidence of multiple pregnancies is more common with the use of clomiphene citrate than letrozole (Sovino e*t al.,* 2002, Reefhuis *et al.,* 2011). More workers have reported above findings that letrozole use can be associated with a significantly lower multiple gestation rates than the use of clomiphene citrate (Mitwally *et al.,* 2005). This is also our observation in this study in which higher number of litters were born to the mothers who received clomiphene citrate compared to those in the letrozole group. Low birth weight might not necessarily amount to low heart weights and low heart weight has not been previously reported in humans or animals who received clomiphene citrate. However, in 1993, Dziadek, who carried out a study on a mouse model, reported that clomiphene citrate treatment during pregnancy was associated with decreased fetal growth, decreased implantation rates, and increased rates of fetal growth retardation among offspring of mice treated with clomiphene citrate just before ovulation in doses similar to those used in humans. In that study, it was concluded that preovulatory administration of clomiphene impairs uterine function, which subsequently reduces embryonic growth and development. These findings were corroborated by some other studies that reported low birth weight of babies born to women who conceived following treatment with clomiphene citrate (Sharma *et al.,* 2014, Forman *et al.,* 2007).

An observation of the highest and lowest means coupled with the standard deviation values of the different heart weights showed that there was a significant difference between the control and clomiphene citrate groups and between the control and letrozole groups with a p-value <0.05. However, there was no significant difference between the clomiphene citrate and letrozole groups. This implies that, these drugs have likely initiated some muscle cell degeneration resulting in loss of weight of the organs, compared to the control group which possessed the highest weight. Apparently, clomiphene citrate and letrozole function in a similar fashion; however, there is no evidence to suggest that clomiphene citrate has more deleterious effects since there was no significant difference between the two groups. Furthermore, the highest and lowest means with standard deviation values of the average number of litters born across the three groups showed no statistical significance. Hence, there was no significant difference between the control and experimental groups in regard to this outcome. Myocardial infarction (MI) in neonates is rare but has been described in cases of congenital heart disease, abnormal coronary arteries (anomalous origin and course of coronary arteries), thromboembolic events, and perinatal asphyxia (Leanne de Vetten *et al.,* 2011). One of the most common and clinically significant forms of acute cardiac injury is MI, and it results in ischemic death of cardiomyocytes (Ibanez *et al.,* 2015). MI triggers a complex inflammatory reaction accompanied by cytokine release and inflammatory leukocyte infiltration into the endangered myocardial region (Yellon and Hausenloy, 2007; Swirski and Nahrendorf, 2013; Eltzschig and Eckle, 2011). The inflammatory response and the expression of cytokines following MI are integral to the healing process and may be protective in the early stage of the MI through stimulation of myocyte autophagy and left ventricular remodelling; however, excessive inflammatory responses after MI are detrimental for cell survival and extracellular matrix integrity (Nahrendorf *et al.,* 2010; Nian *et al.,* 2004; Frangogiannis *et al.,* 2008). This may consequently impair left ventricular contractile function.

The degree of infarction that was observed amongst the experimental groups in this study, is indicative of some necrotic processes taking place in the cardiac tissue. Necrosis of cardiomyocytes presents with a mixed inflammatory response that becomes more mononuclear in character with macrophages phagocytizing cell debris (Clements *et al.* 2010). The presence of inflammation in these tissues was established by the accumulation of inflammatory cells in the myocardium of the left

ventricle. This observation was predominant in the cardiac tissues of pups belonging to the group that received clomiphene citrate and, to a lesser degree, in the cardiac tissues of pups in the letrozole group. The presence of inflammatory cells suggests a cardiac injury sequel to MI in the cardiac tissue. This group also presented with the lowest heart weights which is suggestive of muscle cells degeneration. The observed lack of a significant difference in the mean thickness of the wall of the left ventricles of the pups between the two experimental groups suggests that none of the drugs is superior to the other in terms of safety and damage to the ventricular wall.

In conclusion, Clomiphene citrate and letrozole have been considered to be safe for ovulation induction until recently. However, this study has been able to show the deleterious effects that these drugs could have on pups of Wistar rats. It remains to be seen whether this finding will be observed in same manner in similar study in humans.

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