

**A descriptive study to provide evidence of the teratogenic and cellular effects of
Sibutramine and Ephedrine on cardiac- and liver-tissue of chick embryos**

Hester Magdalena Oberholzer, PhD^a, Ciska van der Schoor, MSc^a, Helena Taute, MSc^a,

Megan Jean Bester, PhD^a

^aDepartment of Anatomy, Faculty of Health Sciences, University of Pretoria, Arcadia, South Africa

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Corresponding author:

HM Oberholzer

Department of Anatomy

Faculty of Health Sciences

University of Pretoria

Private Bag x323

ARCADIA

0007

SOUTH AFRICA.

Email: nanette.oberholzer@up.ac.za

Tel: (+27)12 319 2533 / Fax: (+27)12 319 2240

Abstract

Exposure to drugs during pregnancy is a major concern, as some teratogenic compounds can influence normal foetal development. Although the use of drugs during pregnancy should generally be avoided, exposure of the developing foetus to teratogens may occur unknowingly since these compounds may be hidden in products that are being marketed as “all natural”. The aim of the current study was to investigate the possible teratogenic and cellular effects of sibutramine - a serotonin-norepinephrine reuptake inhibitor used in the treatment of obesity - on the heart and liver tissue of chick embryos. Ephedrine was used as a positive control. The chick embryo model was chosen because it has been used in studying developmental and experimental biology and teratology with great success. The

embryos were exposed to three different concentrations of sibutramine and ephedrine respectively. The results obtained revealed that both compounds exhibited embryotoxicity when compared to the control groups. Liver and heart tissue of the exposed embryos was severely affected by these compounds in a dose-related manner. Morphology similar to that of muscle dystrophy was observed in the heart, where the muscle tissue was infiltrated by adipose and connective tissue. Severe liver steatosis was also noted. A more in-depth investigation into the molecular pathways involved might provide more information on the exact mechanism of toxicity of these products influencing embryonic development.

Key words: sibutramine, ephedrine, teratogenic effects, heart tissue, liver tissue

INTRODUCTION

Several substances, including the serotonin-norepinephrine reuptake inhibitor sibutramine, are indicated in the treatment of obesity. Pregnant women might also be exposed since these products are marketed as being ‘all natural’ products, exposure might occur unknowingly. [Francia-Farje et al., 2010]. With the treatment of any disease in pregnant women, there is the potential that it will affect the developing foetus. This teratogenic potential of compounds has received much attention in research, especially after the thalidomide disaster of the 1950’s and 60’s [Froster and Baird, 1993; Dally, 1998]. Although patients are generally advised to avoid the use of drugs during pregnancy [Doering et al., 2002], exposure of the developing foetus to various compounds could occur long before a patient realizes she is pregnant. The evaluation of the teratogenic potential of compounds and strict control thereof is therefore essential.

In this study, the *in ovo* model was used to evaluate the possible teratogenic and cellular effects of sibutramine. The teratogenicity of sibutramine has not been well studied and very little published data based on human studies are available and animal studies have been found to be inconclusive [Einarson et al., 2004]. A major concern is the unregulated availability and use of this compound despite its withdrawal from most global markets. The unintended use is a major problem, especially in products being marketed as “all natural”

products. This complication has led to further uninformed use of this compound as physicians are not consulted in obtaining a prescription. The lack of published data on the use of this compound during gestation is a key limiting factor in making conclusions on its safe use. Both serotonin (5-HT) and norepinephrine (NE) have been described as teratogens and other serotonin–norepinephrine reuptake inhibitors (SNRIs) have also been implicated [Reddy et al., 1963; Hirsch and Fritz, 1981], therefore further investigations are essential as these compounds share similar mechanisms of action.

In the current study the effect of sibutramine on the development of the chick embryo was evaluated. Like sibutramine, ephedrine has also been marketed as a weight loss agent as well as athletic performance enhancer due to its stimulatory effect within the central nervous system (CNS). Ephedrine's structure is closely related to that of other amphetamines and catecholamines such as norepinephrine (NE) and possess strong alpha (α)- and beta (β)-adrenergic stimulatory effects [Andrews et al., 2008; Brown, et al., 2012]. Ephedrine has been well studied for teratogenic effects and was therefore used as positive control in the current study as it has been shown to induce cardiovascular malformations in chicken embryos using the *in ovo* method [Nishikawa et al., 1985a]. Although major congenital abnormalities have been reported on [Nishikawa et al., 1985a; 1985b; Hansen, et al., 2011; Brender, et al., 2012], limited information is available on the effect on the cellular structure and function.

The question that arises is whether embryonic exposure to sibutramine could lead to ultrastructural changes that might prove significant later in development or in adulthood, resulting in eventual disease. Therefore, the effect of sibutramine and positive control, ephedrine, on the tissue and cellular structure of the heart and liver of embryos was evaluated. The liver tissue was selected since sibutramine is metabolised in the liver, after absorption in the gastrointestinal tract, to its active metabolites M1 (desmethylsibutramine) and M2 (didesmethylsibutramine) [Bae et al., 2008]. Therefore, the liver would be the immediate site of toxicity. The heart was chosen since sibutramine use has been associated

with some adverse cardiovascular events including tachycardia, hypertension, and arrhythmias (James et al., 2010; Scheen, 2011).

The chick embryo model is a well-known and widely studied model that has been used with great success in research related to developmental biology, teratology and experimental biology and was therefore judged to be a relevant model of choice in the current study [Drake et al., 2006; Venter, 2014]. Cell lines usually represent a single cell type and the majority of commercial cell lines are cancer cell lines and therefore do not represent normal cells. In a developing embryo a cell can undergo a wide range of changes, from being pluripotent stem cells to committed stem cells and finally mature differentiated cells. Certain cell models and primary cell cultures can be used to investigate the effect of drugs on this cellular pathway of differentiation. The limitations of these models are that they focus usually on a single cell or tissue type. A further limitation of using primary cultures containing all cell types is that cell culture conditions often promote the growth of a single cell type (e.g. epithelium) while inhibiting the growth of another cell type (e.g. fibroblasts). In an embryo the tight biochemical control, via autocrine and paracrine factors derived from cells, tissue and other organs is maintained. In addition the effect of matrix factors required for the migration and differentiation are also present. Organogenesis is a tightly controlled sequence of events and any disruption of this process can result in congenital defects. Cell lines and primary cultures can be used to investigate the effect of a drug on cell viability, gene transcription and translation but provides no information on the long term consequence of these effects.

The chick embryo develops independently in an isolated environment, whereas rodent models are more complex and the effect of maternal absorption, distribution, metabolism and excretion must be taken into account. Therefore the chick embryo model is ideal for initial studies as this model is free of these confounding factors.

MATERIALS AND METHODS

Implementing the in ovo model

Fertilized Broiler Hatching eggs were obtained from Swanepoel Boerdery (Bronkhorstspuit, Gauteng, South Africa). Upon delivery the fertilized eggs were incubated at 37.5°C in humidified air for the duration of 13 days. The day of delivery was recorded as day 0. Ethical clearance was obtained from the animal ethics committee, University of Pretoria (ethical clearance number h005-13) for the use of embryonated eggs.

Inoculation of the eggs

On embryonic day 3 (Hamburger-Hamilton stage 19/20) the eggs were removed from the incubator and swabbed with 70% ethanol. Once removed from the incubator holes were drilled into the blunt end of the eggs using a sterile needle during which extreme care was taken not to puncture the underlying chorioallantoic membrane (CAM). Sibutramine and ephedrine, at three different concentrations (indicated in Table 1 below) were then administered through the drilled holes at various concentrations in such a way that it is placed through the air sac onto the CAM. Two control groups were used to assure that the methods employed do not interfere with the results obtained. Table 1 summarizes the experimental groups as well as the treatment regime of each group.

Table 1: *In ovo* experimental design

Experimental group	Exposure and dosages	Sample size (n)*
Control group (C:Hole)	Drilled hole, no exposure	13
Control group (C:H ₂ O)	100µl sterile water	13
Sibutramine (Sib0.5)	0.5µmol	16
Sibutramine (Sib5.0)	5.0µmol	16
Sibutramine (Sib10)	10µmol	16
Ephedrine (Eph0.5)	0.5µmol	16
Ephedrine (Eph5.0)	5.0µmol	16
Ephedrine (Eph10)	10µmol	16

Ephedrine has shown teratogenic potential in previous studies at concentrations of 0.5 and 5 μ mol. Results showed that the frequency of malformed embryos exposed to these concentrations of ephedrine was 8 and 26% respectively [Nishikawa et al., 1991]. As Ephedrine was used as the positive control in this study, a serial concentration range was chosen which incorporated both these as well as intermediate concentrations of the two different compounds so that valid reference points and comparisons could be established.

Following exposure, the injection site was swabbed with 70% ethanol and carefully sealed with paraffin wax (Thermo Scientific) and returned to the incubator. All manipulations were performed under aseptic conditions within a laminar flow hood.

Termination

On E13 the eggs were removed from the incubator and the embryos were removed from the eggs by carefully opening the blunt end of the egg and peeling away the CAM with forceps. The embryos were then carefully lifted out of the eggs with a spatula and placed in a petri dish containing PBS to prevent unnecessary dehydration. Each embryo was examined for any gross malformations or abnormalities and these, if noted, were documented (Table 2) before sample collection commenced.

Sample collection

Following termination heart and liver samples were dissected from the embryos and were processed for light (LM)- and transmission electron microscopy (TEM).

Tissue preparation for light microscopy (LM)

Samples were fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution. The tissue was removed from the fixative, and serially dehydrated in 30%, 50%, 70% and 90% ethanol, followed by three changes of absolute ethanol for 15 min/change. Tissue samples were then embedded in paraffin wax by implementing the Leica TP1020 Automatic Tissue Processor (Leica Microsystems, Wetzlar, Germany). Following dehydration the samples were placed in a 1:1 xylene:ethanol solution for 1 hour followed by one change of 100% xylene for 2 hours.

Finally, the samples were placed in 100% paraffin wax for 2 hours followed by a final change of 100% paraffin wax for 4 hours. Sections were then made using a microtome which were then mounted on glass slides and stained with H&E.

Tissue preparation for transmission electron microscopy (TEM)

Tissue samples were fixed in 2.5% GA/FA in 0.075M phosphate buffer for 1 hour and rinsed three times in 0.075M sodium potassium phosphate buffer (pH 7.4) for 15 minutes before being placed in secondary fixative, 1% osmium tetroxide, for 1 hour. Following fixation, the samples were rinsed again as described above. The samples were dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol and were then embedded in resin. Ultra-thin sections (70-100nm) were cut with an ultramicrotome, and contrasted with uranyl acetate for 15 minutes followed by 10 minutes of contrasting with lead citrate. Finally the samples were allowed to dry for a few minutes before examination with the JEM 2100F TEM (JEOL, Tokyo, Japan). All chemicals and reagents were obtained from Merck Pty (Ltd) (Modderfontein, South Africa) and were of analytical grade.

RESULTS

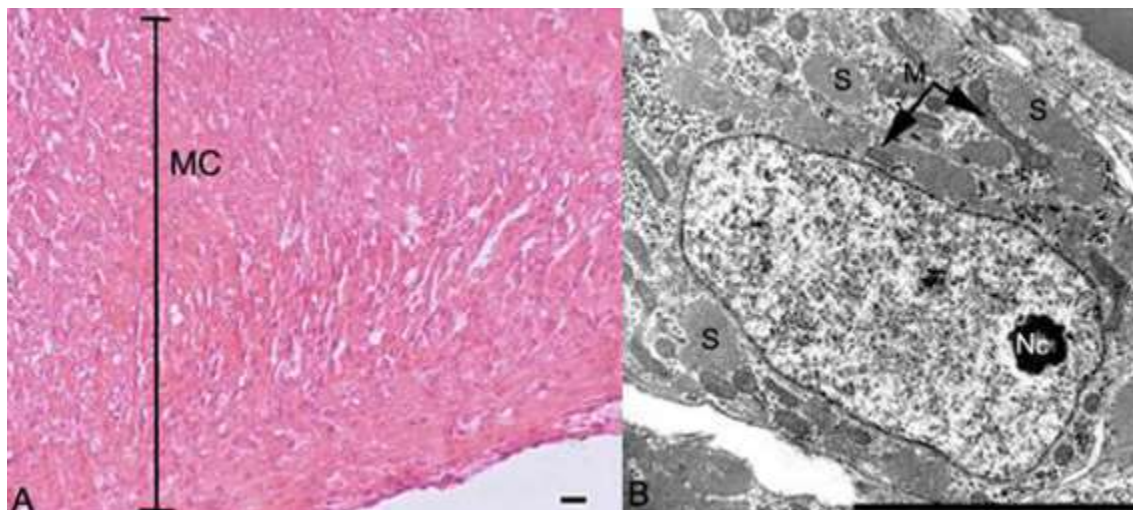


Figure 1: Heart samples collected from the control groups. **A & B:** Control LM and TEM, respectively. LM scale bar = 20 μ m, TEM = 5 μ m. (MC = myocardium; M = mitochondria; N_C = nucleus of cardiac muscle cell; S = sarcomere)

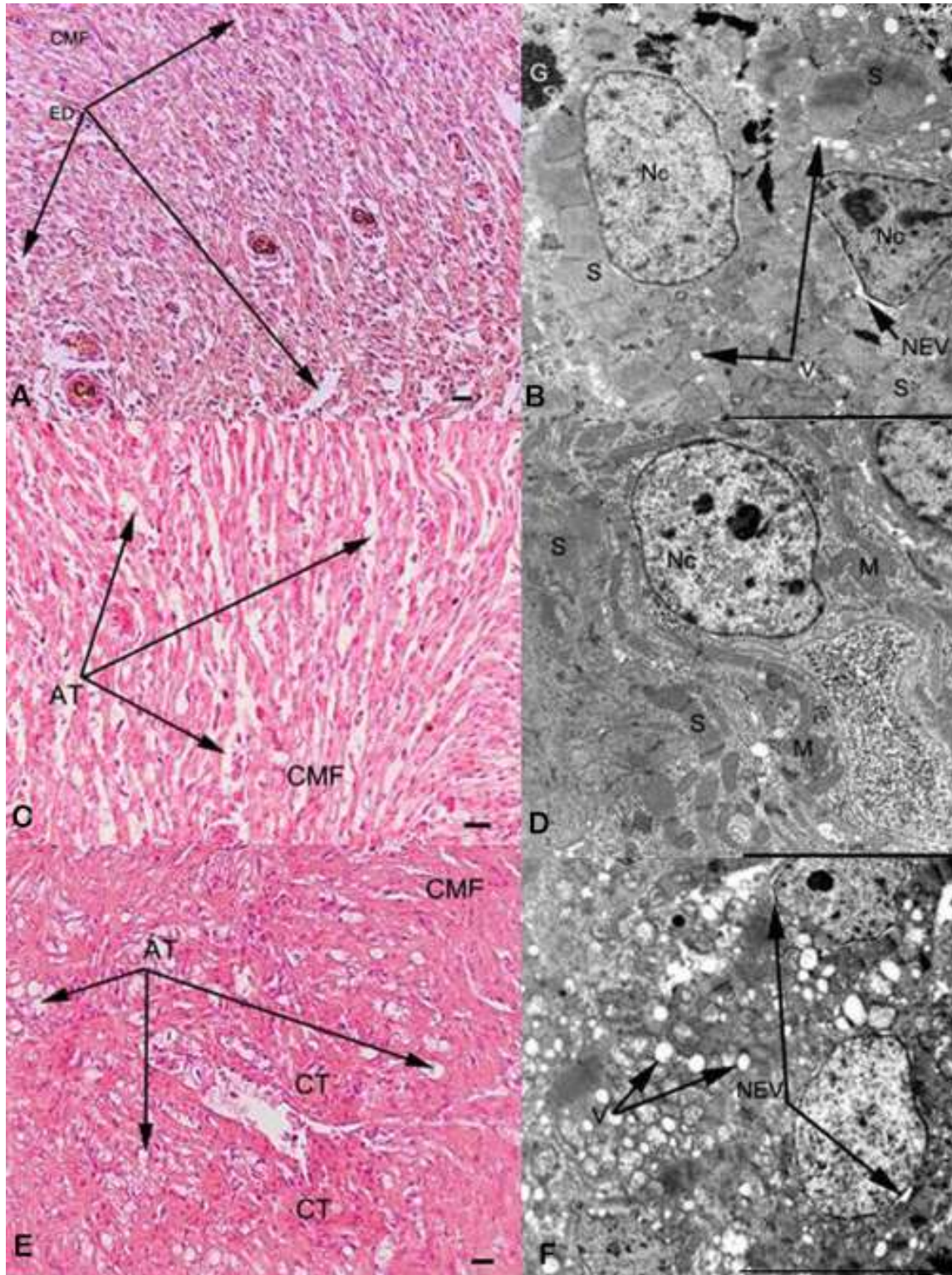


Figure 2: Heart samples collected from the ephedrine experimental groups. **A & B:** Eph 0.5 LM and TEM, respectively; **C & D:** Eph 5.0 LM and TEM respectively; **E & F:** Eph 10 LM and TEM respectively. LM scale bar = 20 μ m, TEM = 5 μ m. (CMF = cardiac muscle fibres; ED = oedema; Ca = capillaries; AT = adipose tissue; N_c = nucleus of cardiac muscle cells; G = glycogen; S = sarcomere; V = vacuoles; NEV = nuclear envelope vacuolisation; CT = connective tissue)

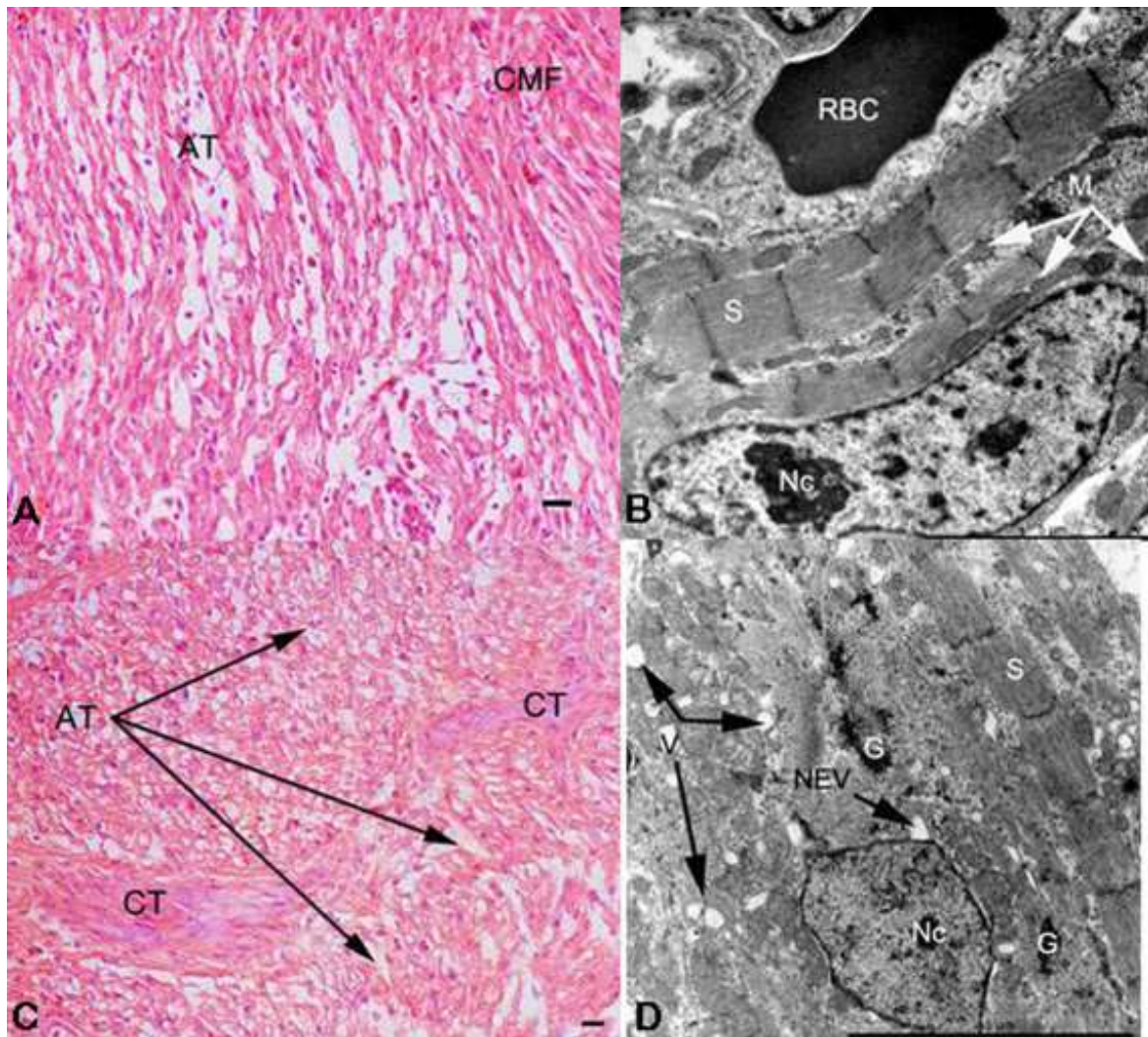


Figure 3: Heart samples collected from the sibutramine experimental groups. **A & B:** Sib 0.5 LM and TEM respectively; **C & D:** Sib 5.0 LM and TEM respectively. LM scale bar = 20 μ m, TEM = 5 μ m. (CMF = cardiac muscle fibres; AT = adipose tissue; N_c = nucleus of cardiac muscle cells; G = glycogen; S = sarcomere; V = vacuoles; NEV = nuclear envelope vacuolisation; CT = connective tissue; M = mitochondria)

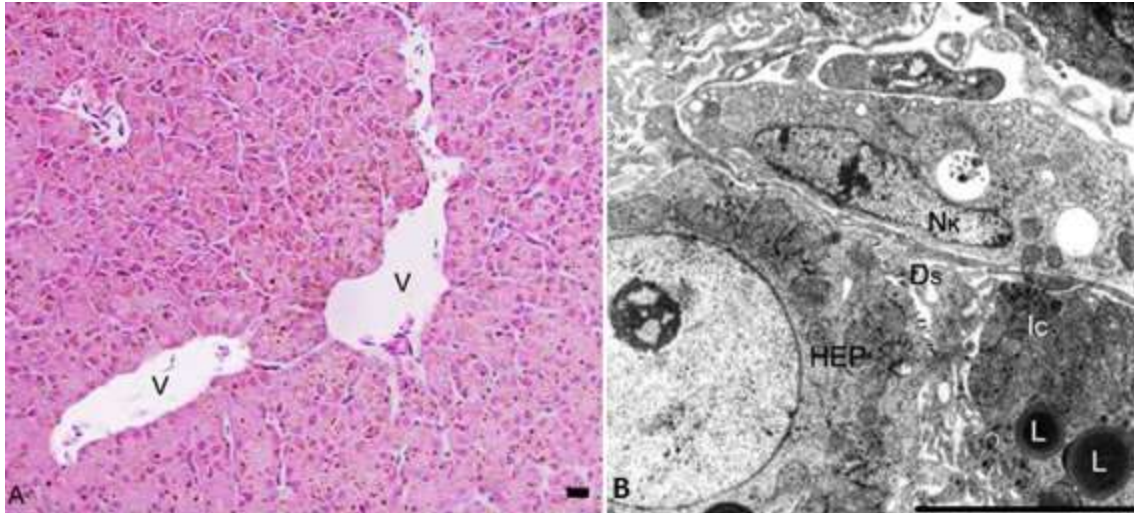


Figure 4: Liver samples collected from the control groups. **A & B:** Control LM and TEM respectively. LM scale bar = 10 μm , TEM = 5 μm . (V = venules; HEP = Hepatocyte; N_k = Kupffer cell nucleus; Ds = Space of Disse; I_c = ITO cell; L = Lipids)

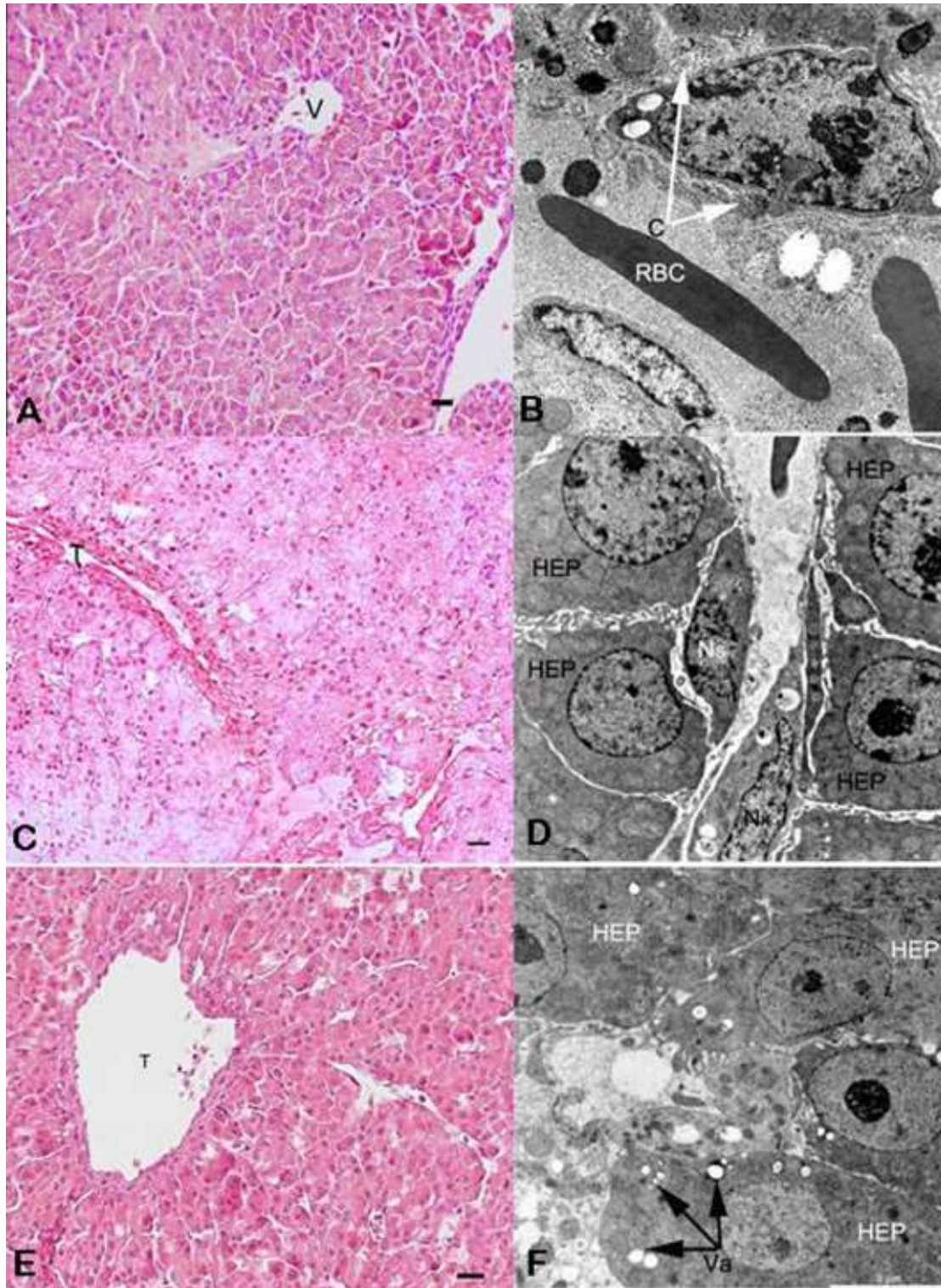


Figure 5: Liver samples collected from the ephedrine experimental groups. **A & B:** Eph0.5 LM and TEM respectively; **C & D:** Eph5.0 LM and TEM respectively; **E & F:** Eph10 LM and TEM respectively. LM scale bar = 10 μ m, TEM = 5 μ m. (T = portal tracts; V = venules; C = collagen fibres; HEP = hepatocyte; L = lipids; Ns = nucleus of sinusoidal lining cell; NK = nucleus of Kupffer cell; Va = cytoplasmic vacuoles)

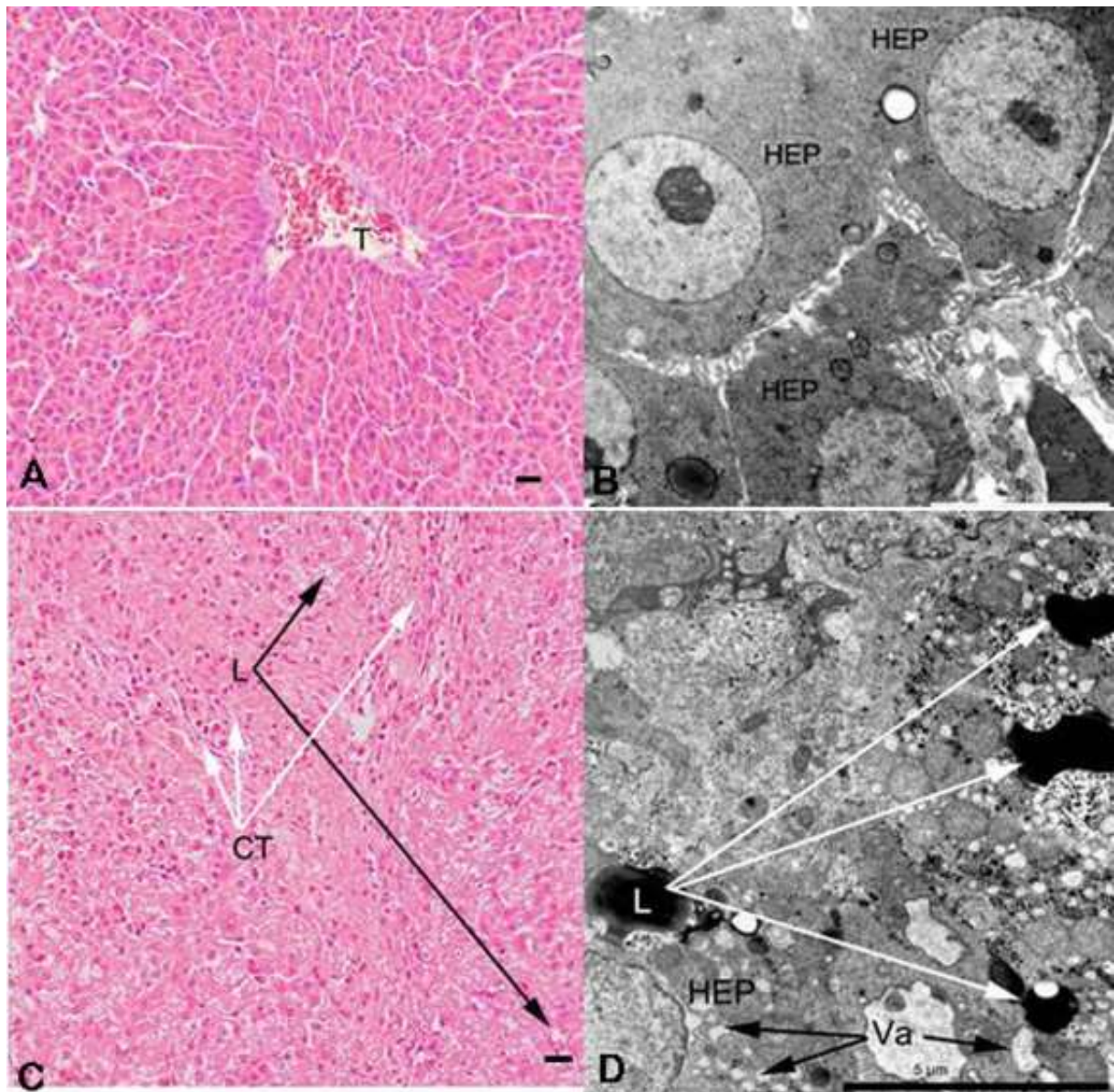


Figure 6: Liver samples collected from the sibutramine experimental groups. **A & B:** Sib0.5 LM and TEM respectively; **C & D:** Sib5.0 LM and TEM respectively. LM scale bar = 10 μm , TEM = 5 μm . (T = portal tracts; L = lipids; CT = connective tissue; HEP = hepatocytes; RBC = red blood cells; Va = cytoplasmic vacuoles)

The possible effects of sibutramine and ephedrine administration on the tissue and cellular structure of the heart and liver samples from the different *in ovo* experimental groups were evaluated. The results are presented in Figures 1 to 6. For LM and TEM analysis, samples were randomly selected from those obtained and processed from the viable embryos.

Table 2: Chick development rates and observed abnormalities noted within the different experimental groups

Experimental Group	Number developed	% Developed	Observed abnormalities
C:Hole	11	84.60	None
C:H₂O	8	61.50	None
Eph0.5	11	68.75	9 embryos with no observable anomalies, 1 with limb abnormality, gastroschisis, 1, underdeveloped
Eph5.0	12	75.00	10 embryos with no observable anomalies, 1 tissue transparency with fluid build-up, 1 with haemorrhaging, gastroschisis
Eph10	10	62.50	7 embryos with no observable anomalies, 2 underdeveloped
Sib0.5	12	75.00	11 embryos with no observable anomalies, 1 with gastroschisis
Sib5.0	4	25.00	2 embryos with no observable anomalies, 1 underdeveloped, limb malformation, haemorrhaging; 1 with gastroschisis, absence of upper limb
Sib10	1	6.25	Severe haemorrhage, underdeveloped, gastroschisis

Heart

Images acquired from the heart tissue of control embryos are shown in Figure 1A and B. Normal myocardial architecture can be observed in Figure 1A whereas Figure 1B shows the ultrastructure of a cardiac myocyte with clear nucleus, sarcomeres, the contractile units of these myocytes, as well as numerous mitochondria.

Figures 2A-F and 3A-D are representative of heart samples obtained from the different experimental groups exposed to the two respective compounds. Compared to the control animals, the histology micrographs revealed increased adiposity as well as chord-like arrangement of muscle fibres, with mild oedema between wavy muscle fibres. Figure 2A and B represent embryos from the Eph0.5 experimental group. Figure 2A shows an increased oedematous appearance (ED) of myocardium with capillaries (Ca) distributed between muscle fibres (CMF) and the corresponding TEM image (Figure 2B) shows loss of structural

integrity as tissue appears less organized. Small vacuoles (V) can be seen and the nuclear envelope appears dilated (NEV). Samples obtained from the Eph5.0 experimental groups also showed adipose infiltration (Figure 2C) although TEM images (Figure 2D) did not reflect these findings. LM (Figure 2E) and TEM (Figure 2F) images obtained from embryos exposed to the highest ephedrine dose (i.e. 10 μ mol) also presented with adipose tissue infiltration, increased connective tissue and ultrastructurally showed signs of severe vacuolisation, mitochondrial degeneration, and nuclear envelope vacuolisation. Sarcomeres were not clearly distinguishable.

Images prepared from the Sib0.5 experimental group is shown in Figure 3A and 3B. Again, adipose tissue (AT) can be observed distributed between the thin, wavy cardiac muscle fibres (CMF). TEM images (Figure 3B) showed normal structural arrangement. The general arrangement of tissue in the Sib5.0 experimental group appears disrupted where connective tissue is increased as well as adiposity (Figure 3C). Vacuolisation is also evident in the TEM images (Figure 3D) with nuclear envelope dilations (NEV).

Liver

Figures 4A and B show LM and TEM micrographs prepared from the collected liver samples from the control embryos. Figure 4A shows the normal histology of the control embryos where the normal plate-like arrangement of hepatocytes can be observed as well as some hepatic venules (V) visualised with the H&E stain. Fig 4B shows the normal ultrastructural arrangement typical of control samples. The space of Disse (Ds) is visible as well as a Kupffer cell (N_k) within the sinusoid, surrounded by hepatocytes (Hep). A hepatic ITO or stellate cell (I_c) is also visible containing lipid droplets (L).

Figure 5 shows LM and TEM micrographs acquired from embryo's exposed to different doses of ephedrine. Figure 5A and B are images acquired from embryos exposed to 0.5 μ mol ephedrine (LM and TEM, respectively) and showed little to no morphological

differences when compared to the control embryos (Figure 4A & B). Hepatic venules (V) are visible in Figure 5A as well as sinusoidal structures including a fibroblast (F) surrounded by collagen fibres (C) and red blood cells (RBC) (Figure 5 B). LM images from embryos in the Eph5.0 groups (Figure 5C) show slight tendencies towards tissue degeneration although the TEM image (Figure 5D) do not differ from the control group indicating normal hepatocyte structure with visible sinusoids and microvilli. As the dosage increased to 10 μ mol ephedrine/egg a slight loss in tissue integrity could be observed upon examination of liver parenchyma (Figure 5E) as sinusoidal spaces appear larger than that seen in the control images. TEM micrographs from this experimental group (Figure 5F) also show lipid depositions and vacuolisation (Va) within the hepatic cytoplasm and general disruption of sinusoidal organisation.

LM and TEM images obtained from embryos exposed to different doses of sibutramine are shown in Figure 6A - D. Embryos which were treated with the 0.5 μ mol dose of sibutramine showed no morphological differences when compared to the control group. The plate-like arrangement of hepatocytes can be observed with portal tracts (T) (Figure 6A) and the corresponding TEM image (Figure 6B) also showed normal ultrastructural arrangement of the hepatocytes (Hep). In Figure 6 C and D signs of hepatic steatosis, i.e. lipid accumulation (L) was observed within the liver tissue. In Figure 6 D the formation of cytoplasmic vacuoles (Va) can be observed. The corresponding LM image (Figure 6C) showed increased connective tissue spread throughout the liver tissue and a general degenerated appearance of liver parenchyma. Table 3 provides a summary of the morphological changes observed.

Table 3: Summary of morphological changes observed

Experimental Group	Heart	Liver
Control	Normal histological arrangement	Normal histological arrangement
Eph0.5	Oedema; Dilated nuclear envelope; Vacuolisation	Normal histological arrangement
Eph5.0	Adipose tissue infiltration; Oedema	Slight degeneration in tissue arrangement
Eph10	Adipose tissue infiltration; Increased connective tissue; Severe vacuolisation (possibly due to mitochondrial degeneration); Nuclear envelope vacuolisation	Loss of tissue integrity; Dilated Sinusoidal spaces; Lipid deposition; Vacuolisation
Sib0.5	Adipose tissue infiltration	Normal histological arrangement
Sib5.0	Adipose tissue infiltration; Loss of structural integrity; Vacuolisation; Nuclear envelope dilations	Lipid accumulation; Decreased structural integrity of space of Disse; Increased connective tissue; Vacuolisation
Sib10	Not viable	Not viable

DISCUSSION

In the current study, gastroschisis was observed in embryos exposed to the low and higher concentrations of sibutramine, as well as ephedrine (Table 2). This is characterized by evisceration of abdominal organs through a defect in the abdominal wall without a membranous covering and usually involves the small intestines but may include other structures. Various compounds have been reported which are able to induce this malformation [Van Dorp et al., 2010]. Examples include mercury, cadmium, and retinoic acid. Ventral body wall defects are one of the leading types of birth defects and, among conditions within this class of malformations, the incidence of gastroschisis has been on the

increase, rendering it a particularly important body wall closure defect. The molecular and cellular interactions which lead to the development of these relatively common defects however remain elusive [Williams, 2008].

Ephedrine, in conjunction with other sympathomimetic agents such as phenylephrine and epinephrine, has also been evaluated for teratogenic effects using chick embryos at different stages of development. High rates of cardiac anomalies have been reported [Werler, 2006] and limb abnormalities have also been described in combination with theophylline and phenobarbital. This prompted further investigations in which pregnant rabbits were exposed to ephedrine and again, cardiovascular and limb malformations were seen as well as exencephaly and ventral wall anomalies [Gilbert-Barness and Drut, 2000].

In a study by Zhang *et al.*, (1998) the teratogenic effects of cocaine were evaluated by use of the same methods implemented in the current study. Cocaine, which possesses well defined local anaesthetic properties, also blocks the reuptake of neurotransmitters dopamine (DA), norepinephrine (NE), and serotonin (5-HT), lending to its sympathomimetic and psychoactive properties. Zhang and co-workers specifically evaluated a 5HT-mediated vasoconstrictive cause of cocaine teratogenicity as alterations in haemodynamic processes could result in ischaemia and eventual differences in oxygen balances [Zhang et al., 1998; Konzen et al., 1995; Martinez et al., 1996]. This resultant oxygen imbalance together with deficiencies in nutrient supply and metabolic waste removal due to impaired circulation could result in embryonic developmental disturbances [Rosenak et al., 1990]. These deviations in physiological processes have been attributed to adrenergic activation as the primary source for the pathogenesis and numerous studies employing both human and non-human observations and methods have demonstrated an indirect activation of the adrenoceptors due to cocaine administration [Schindler et al., 1995; Gillis et al., 1995]. Results obtained by Zhang *et al.* (1998) showed an increase in observed herniated umbilici, a ventral wall defect, which was attributed to a possible indirect consequence of umbilical ischemia due to 5-HT-induced vasoconstriction [Zhang et al., 1998].

Sibutramine shares an almost similar mechanism of action when compared to cocaine as it inhibits the presynaptic neuronal reuptake of 5HT and NE, whereas ephedrine increases the action of postsynaptic noradrenergic activity directly by acting on α and β noradrenergic receptors. Both 5HT and NE possess hemodynamic properties which could affect development as described previously. The increased severity of sibutramine teratogenicity compared to ephedrine could be further attributed to the additional alterations in serotonergic pathways, as this has been described to have an effect on embryonic growth.

Furthermore, previous studies have shown that prenatal administration of various selective serotonin reuptake inhibitors (SSRIs) may result in foetal death, defects, or growth suppression [Mello et al., 2005; Ofori et al., 2007; Pedersen et al., 2009]. All members of SSRI and serotonin–norepinephrine reuptake inhibitor (SNRI) drug classes have also been shown to cross the placenta and are also excreted in small amounts into breast milk [Rosenbaum et al., 1998; Newport et al., 2002; Hannerfors, et al., 2015].

Limb abnormalities often appear in conjunction with body wall defects, collectively referred to as the limb-body wall complex. Previous data from animal models suggest that hemodynamic disruption could possibly give rise to limb-body wall complex. Again, cocaine abuse has been implicated in these processes due to its vasoconstrictive effects which could affect uteroplacental blood flow during significant stages of development [Viscarello et al., 1992].

The present study involved a comprehensive morphological investigation of the possible toxic effects of sibutramine on the developing chick embryo. The results obtained were compared to that of ephedrine, a compound which has been described as a teratogen in a similar experimental model [Nishikawa et al., 1985a; Nishikawa et al., 1985b]. Results indicated noticeable differences between the two compounds, and also between different concentrations of the individual compounds.

Apart from the gross morphological abnormalities that were noted which would render the chicks unviable, histological investigation also showed alterations to various organs.

Liver sections revealed signs of severe steatosis, with characteristic lipid depositions throughout the liver tissue. The incidence of hepatic steatosis has been well defined in neonates and is considered a fatal condition in human new-borns as it leads to liver failure, coma, and eventually death [Boison et al., 2002]. This condition is characterized by a rapid infiltration of hepatic parenchyme by microvesicular fat and the subsequent enlargement of the liver [Satran et al., 1969].

The characteristic fat infiltration as observed in this condition is often attributed to severe impairment of mitochondrial function [Burt et al., 1998; Fromenty et al., 1997; Fromenty and Pessayre, 1997; Claycombe, et al., 2015] which has been associated with drug induced inhibition of beta (β)-oxidation [Fromenty et al., 1997; Fromenty and Pessayre, 1997]. Norepinephrine (NE) is known to play a role in regulating the extracellular uptake and release of carbohydrates and fatty acids and this could provide a mechanism in which sibutramine influences these processes, leading to the development of this condition. Both ephedrine and sibutramine's mechanism of action involve key interactions within various noradrenergic pathways and preliminary biochemical data suggest that sibutramine could affect the metabolic processes directly by inhibiting various vital enzymes involved in fatty acid oxidation (results not shown). This topic of research however requires more investigation before possible conclusions can be made. It is clear though that the observed steatosis in the current study would result in severe liver failure and possibly death.

Upon the histological investigation of the cardiac tissue, adipocyte infiltration within the myocardium was seen in the groups exposed to the different concentrations with an increase in severity related to the increasing dosage concentrations. As of yet, this histological feature has not been well defined, or previously described in chick embryos. The observed morphology, which shows substantial adipose tissue infiltration of the myocardium with an evident increase in connective tissue surrounding the remaining cardiomyocytes (especially evident in the Sib10 heart sections), strongly relates to that described in cases of arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) [Fornes et al., 1998]. ARVC/D is a lethal condition of unknown cause which affects the heart muscle and is

distinguished histologically by the fatty, fibro-collagenous atrophy of the right ventricular myocardium resulting in electrical cardiac irregularities [Marcus et al., 1982; Basso et al., 1996]. This process of myocardial loss is also non-inflammatory and occurs via programmed cell death or apoptosis [Mallat et al., 1996]. Various genetic mutations within numerous components of the cardiac desmosome have been acknowledged as key mediators in the pathogenesis of ARVC/D as they exhibit variations in penetrance and expression, which further implicates environmental and other genetic factors in the onset and progression of this disease. This is however, a late-onset disease. Another condition characterized by fatty infiltration of cardiac tissue is known as Uhl's anomaly. Although this is a congenital defect, it is defined by the almost complete replacement of ventricular myocardium with adipose tissue or the complete absence thereof, as well as epicardial and endocardial apposition [Gerlis, 2003]. The current findings do not coincide with those mentioned. Although very little data is available on the observed histological alterations and possible causes thereof within the current results, it is presumably attributable to some form of myocardial dysplasia which is characterized on a histopathological level by fibre size variability, fibre necrosis, regeneration, inflammation and connective tissues deposition, some of which were seen in the current results.

From the results obtained it can be concluded that both compounds exhibited embryotoxicity when compared to the control groups. Livers and hearts of exposed embryos were severely affected by both compounds in a dose related manner with possible fatal consequences. Heart morphology appears similar to muscle dystrophy, more specifically ARVC/D, in that muscle tissue is replaced by adipose and connective tissue. Severe liver steatosis was also noted. Results are based purely on morphological findings, and in depth investigations of the molecular pathways involved, might shed more light on the exact mechanisms of the observed toxicity of this substance.

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REFERENCES

- Andrews R, Chawla P, Brown DL. 2005. Cardiovascular effects of ephedra alkaloids: a comprehensive review. *Prog Cardiovasc Dis* 47(4): 217-225.
- Bae SK, Cao S, Seo KA *et al.* 2008. Cytochrome P450 2B6 catalyzes the formation of pharmacologically active sibutramine (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine) metabolites in human liver microsomes. *Drug Metab Dispos* 36: 1679–1688.
- Basso C, Thiene G, Corrado D, Angelini A, Nava A, Valente M. 1996. Arrhythmogenic right ventricular cardiomyopathy: dysplasia, dystrophy, or myocarditis? *Circulation* 94: 983-991.
- Boison D, Scheurer L, Zumsteg *et al.* 2002. Neonatal hepatic steatosis by disruption of the adenosine kinase gene. *Proc Natl Acad Sci* 99(10): 6985–6990.
- Brender JD, Werler MM, Shinde MU, Vuong AM, Kelley KE, Huber JC Jr., *et al.*, 2012. Nitrosatable drug exposure during the first trimester of pregnancy and selected congenital malformations. *Birth Defects Res A* 94: 701–713.
- Brown CE, Trauth SE, Grippo RS, Gurley BJ and Grippo AA. 2012. Combined effects of Ephedrine-containing dietary supplements, caffeine, and nicotine on morphology and ultrastructure of rat hearts. *J Caffeine Res* 2: 123-132.
- Burt AD, Mutton A, Day CP. 1998. Diagnosis and interpretation of steatosis and steatohepatitis. *Semin Diagn Pathol* 15: 246–258.
- Claycombe KJ, Roemmich JN, Johnson L, Vomhof-DeKrey EE, Johnson WT. 2015. Skeletal muscle Sirt3 expression and mitochondrial respiration are regulated by a prenatal low-protein diet. *J Nutr Biochem* 26: 184–189.
- Dally A .1998. Thalidomide: Was the tragedy preventable? *Lancet* 351: 1197-9
- Doering PL, Boothby LA, Cheek M. 2002. Review of pregnancy labeling of prescription drugs: is the current system adequate to inform of risks? *Am J Obstet Gynecol* 187(2): 333–339.
- Drake VJ, Koprowski SL, Lough JW, Smith SM. 2006. Gastrulating chick embryo as a model for evaluating teratogenicity: a comparison of three approaches. *Birth Defects Res A* 76: 66–67.
- Einarson A, Bonari L, Sarkar M, McKenna K, Koren G. 2004. Exposure to sibutramine during pregnancy: a case series. *Eur J Obstet Gynecol Reprod Biol* 116(1): 112.

Francia-Farje LAD, Silva DS, Volpato GT et al. 2010. Sibutramine effects on the reproductive performance of pregnant overweight and non-overweight rats. *J Toxicol Env Health* 73: 985–990.

Froster UG, Baird PA. 1993. Maternal factors, medications, and drug exposure in congenital limb reduction defects. *Environ Health Perspect* 101: 269-74.

Fromenty B, Berson A, Pessayre D. 1997. Microvesicular steatosis and steatohepatitis: role of mitochondrial dysfunction and lipid peroxidation. *J Hepatol* 26:13–22.

Froment B, Pessayre D. 1997. Impaired mitochondrial function in microvesicular steatosis effects of drugs, ethanol, hormones and cytokines. *J Hepatol* 26: 43–53.

Fornes P, Ratel S, Lecomte D. 1998. Pathology of arrhythmogenic right ventricular cardiomyopathy/dysplasia - an autopsy study of 20 forensic cases. *J Forensic Sci* 43(4): 777-783.

Gerlis L. 2003. Uhl's anomaly. Orphanet encyclopedia. viewed 10 March 2014, <http://www.orpha.net/data/patho/GB/uk-uhl.pdf>.

Gillis RA, Hernandez YM, Erzouki HK et al. 1995. Sympathetic nervous system mediated cardiovascular effects of cocaine are primarily due to a peripheral site of action of the drug. *Drug Alcohol Depend* 37: 217–230.

Hannerfors A-K, Hellgren C, Schijven D, Iliadis SI, Comasco E, Skalkidou A, Olivier JDA, Sundström-Poromaa I. 2015. Treatment with serotonin reuptake inhibitors during pregnancy is associated with elevated corticotropin-releasing hormone levels. *Psychoneuroendocrinology* 58: 104–113.

Hansen DK, Juliar BE, White GE and Pellicore LS. 2011. Developmental toxicity of Citrus aurantium in rats. *Birth Defects Res B* 92: 216–223.

Hirsch KS, Fritz HI. 1981. Teratogenic effects of mescaline, epinephrine, and norepinephrine in the hamster. *Teratology* 23(3): 287–291.

James WPT, Caterson ID, Coutinho W et al. 2010. Effect of Sibutramine on cardiovascular outcomes in overweight and obese subjects. *N Engl J Med* 363 (10): 905-917

Konzen JP, Levin SR, Garcia JH. 1995. Vasospasm and thrombus formation as possible mechanisms of stroke related to alkaloidal cocaine. *Stroke* 26: 1114–1118.

Marcus FI, Fontaine G, Guiraudon G et al. 1982. Right ventricular dysplasia: a report of 24 adult cases. *Circulation* 65: 384-398.

Mallat Z, Tedgui A, Fontaliran F, Frank R, Durigon M, Fontaine G. 1996. Evidence of apoptosis in arrhythmogenic right ventricular dysplasia. *N Engl J Med* 335:1190-6.

Martinez N, Diez-Tejedor E, Frank A. 1996. Vasospasm/thrombus in cerebral ischemia related to cocaine abuse. *Stroke*. 27: 147–148.

Mello FB, Jacobus D, Carvalho K, Mello JRB. 2005. Effects of *Lantana camara* (Verbenaceae) on general reproductive performance and teratology in rats. *Toxicol* 45: 459–466.

Newport DJ, Hostetter A, Arnold A, Stowe ZN. 2002. The treatment of postpartum depression: minimizing infant exposure [review]. *J Clin Psychiatry* 63(7): 31-44.

Nishikawa T, Bruyere HJ, Takagi Y, Gilbert EF, Hideo U. 1985a. Cardiovascular teratogenicity of ephedrine in chick embryos. *Toxicol Lett* 29(1): 59-63.

Nishikawa T, Bruyere HJ, Gilbert EF, Takagi Y. 1985b. Potentiating effects of caffeine on the cardiovascular teratogenicity of ephedrine in chick embryos. *Toxicol Lett* 29(1): 65-68.

Nishikawa T, Takeshi K, Takao K. 1991. Potentiating effects of forskolin on the cardiovascular teratogenicity of ephedrine in chick embryos. *Toxicol Lett* 56(1-2): 145-150.

Ofori B, Rey E, Bérard A. 2007. Risk of congenital anomalies in pregnant users of statin drugs. *Br J Clin Pharmacol* 64: 496–509.

Pedersen LH, Henriksen TB, Vestergaard M, Olsen J, Bech BH. 2009. Selective serotonin reuptake inhibitors in pregnancy and congenital malformations: Population based cohort study. *Br Med J* 339, B3569.

Reddy V, Adams FH, Baird C. 1963. Teratogenic effects of serotonin. *J Pediatr* 63(3): 394–397.

Rosenak D, Diamant YZ, Yaffe H, Horstein E. 1990. Cocaine: maternal use during pregnancy and its effect on the mother, the fetus, and the infant. *Obstet Gynecol Surv* 45: 348–359.

Rosenbaum, JF, Fava M, Hoog SL, Ascroft RC, Krebs WB. 1998. Selective serotonin reuptake inhibitor discontinuation syndrome: a randomized clinical trial. *Biol Psychiatry* 44: 77-87.

Scheen AJ. 2011. Sibutramine on cardiovascular outcome. *Diabetes care*. 34(2): 114-119

Schindler CW, Tella SR, Erizouki HK, Goldberg SR. 1995. Pharmacological mechanisms in cocaine's cardiovascular effects. *Drug Alcohol Depend* 37: 183–191.

Satran L, Sharp HL, Schenken JR, Krivit W. 1969. Fatal neonatal hepatic steatosis: A new familial disorder. *J Pediatr* 75: 39–46.

Van Dorp DR, Malleis JM, Sullivan BP, Klein MD. 2010. Teratogens inducing congenital abdominal wall defects in animal models. *Pediatr Surg Int* 26: 127–139.

Venter C. 2014. An *in ovo* investigation of the cellular effects of the heavy metals cadmium and chromium alone and in combination, Masters thesis, University of Pretoria, Pretoria.

Viscarello RR, Ferguson DD, Nores J, Hobbins JC. 1992. Limb-body wall complex associated with cocaine abuse: further evidence of cocaine's teratogenicity. *Obstet Gynecol* 80(3): 523-526.

Werler MM. 2006. Teratogen update: Pseudoephedrine. *Birth Defects Res A* 76(6): 445–452

Williams T. 2008. Animal models of ventral body wall closure defects: A personal perspective on gastroschisis. *Am J Med Genet Part C Semin Med Genet* 148C: 186–191.

Zhang X, Schrott LM, Sparber, SB. 1998. Evidence for a serotonin-mediated effect of cocaine causing vasoconstriction and herniated umbilici in chicken embryos. *Pharmacol Biochem Be* 59(3): 585–593.