جامعة السودان للعلوم والتكنولوجيا

Sudan University of Science and Technology

College of Graduate Studies

Evaluation of Biological Effect of Ultrasound in Albino Rat Fetuses in Late Gestation Age

تقويم الأثر البيولوجي للموجات فوق الصوتية علي أجنة فئران الألبينو
في الفترة الأخيرة من الحمل

Thesis submitted For Fulfillment the Required Degree of PhD in Medical Diagnostic ultrasound

Prepared by:

Hussien Abdelrhman Hussien Abdelrhman Ali Dinar

Supervised by :

Prof. Qurashi mohamed Ali

Co-supervisor :

Prof. Mohamed Elfadil Mohamed Garelnabi

2018

بسم الله الرحمن الرحيم

الآية

قال تعالي :

{يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ}

سورة المجادلة الاية (11)

Dedication

This work is dedicated to all these

Candles that glowed up to

Lighten my way

To my father soul

To my mother

To my sisters

To my brothers

To my friends

ACKNOWLEDGMENT

List of Tables

List of Figures

List of abbreviations

3 D: three- dimensional

C°: Centigrade

ICH: International Council for Harmonization

IUGR: Intrauterine growth restriction

SGA: small for gestational age

EFW: estimated fetal weight

2D : two-dimensional

TV : television

PRF :pulse repetition frequency

TGC: time gain compensator

A mode : Amplitude mode

B mode : Brightness Mode

M mode : Movement Mode

I : intensity

W: power

M: Mega

m: Mili

mm : millimeter

hr : hours

Hz : hertz

I_{SP} : spatial peak intensity

I_{SA} : the spatial average intensity

I_{SATA} : spatial average, temporal average intensity

l_{SAPA} : spatial-average pulse-average intensity

I_{SPTA} : spatial peak, temporal average intensity

I_{SPPA} : spatial peak, pulse-average intensity

CRL: crown rump limb

FDA: Food and Drug Administration

Gd : gestation day

HPA: Health protection Agency

TI : thermal index

MI: mechanical index

L: linear array

NBF : neutral buffered formalin

PBS: phosphate-buffered saline

PFA : paraformaldehyde

ID: identification

SPSS: Statistical Package for the Social Sciences

IBM : International Business Machine

10x: magnification by 10

40x:magnification by 40

ALARA : As lows as reasonable achievable

Abstract

This is an In-vivo Experimental (randomized control) study which involves ultrasound wave exposure to pregnant Albino Rat uterus, at late gestation period, to evaluate the bioeffects of ultrasound in fetuses at late gestation age.

Specific objective of this study are to evaluate bio effect of ultrasound as a teratogen in form of intrauterine growth restriction, and see possibility of histology change brain tissue from ultrasound exposure .

The study was conducted at National University –Sudan Radiology Lab from May 2015 to December 2017 using temporary constructed Animal House which follow international guidelines of animal house care with constant external environment and daily monitoring by animal house technician.

Ultrasound machine was calibrated and exposure parameter were fixed as linear array transducer of 12MHz, focal distance was 4.5 cm ,Thermal Index was .5 Mechanical Index was .8 ,duration of exposure was 40 minute.

Pregnant albino rats were grouped to exposed and control, and were sacrificed in the next days of exposure (17, 18, 19, 20, 21) then fetuses were taken out for evaluation of effect, the sample obtained was 55 fetuses. Calibrated weigh scale was used to measure fetal weigh by veterinary specialist, slide brain histopathology done by a specialized technologist and was reported by veterinary histopathologist.

Regarding intrauterine growth , there is reduction in fetal weight through determined study days but more significant at day 19 of rat gestation day P-value < .005.Multiple   histological brain change was observed in different groups, shown as loss of normal pattern formations .

It is concluded that ultrasound exposure to fetal may act as deleterious bio effect in Albino Rat and this may act as same in human subject as some previous studies. This study needs to be replicated with extension and modification   of parameters and using large animal model to mimic humans

ALARA (As Low As Reasonable Achievable) principle is important concept to be adopted by ultrasound medical professional in their practice with update to consensus report from upcoming research that may reveal ultrasound bio effect to humans, especially offspring.

المستخلص

هذه الدراسة التجريبية (العشوائية) عبارة عن تعريض الموجات الفوق صوتية لرحم فئران ألبينو في أواخر فترة الحمل لتقييم التأثير الحيوي للموجات فوق الصوتية في الأجنة في أواخر عمر الحمل.

الهدف المحدد لهذه الدراسة تقييم الأثر الحيوي للموجات فوق الصوتية كاثر ماسخ وكذلك لمعرفة إمكانية تغير نسيج الدماغ جراء التعرض للموجات فوق الصوتية

تم إجراء هذه الدراسة في مختبر الأشعة السينية ـ بالجامعة الوطنية ــالسودان الفترة من مايو 2015 إلى 2017 باستخدام " مربي حيوان مؤقت" الذي اتبع المبادئ التوجيهية الدولية للرعاية الحيوانية مع بيئة خارجية ثابتة و المراقبة اليومية بوسطة فني تربية الحيوان , تم معايرة جهاز الموجات فوق الصوتية وكانت مع تثبيت عوامل التعريض (محول خطي مجموعة من من MHz12 ، كانت المسافة البؤرية 4.5 سم ، وكان MI 5. TI كان 8. ، ومدة التعرض كان 40 دقيقة)

اعتمد تصميم التجربة علي ستخدام جرذان ألبينو الحوامل التي تم تقسيمها الي مجموعة معرضة ومجموعة خالية من ثم تم التضحية بالفئرن بعد اليوم التالي من التعرض وهي (17،18،19،20،21) ثم تم إخراج الأجنة لتقييم التأثير ، وكانت العينة التي تم الحصول عليها 55 جنينًا.

تم استخدام مقياس وزن معياري لقياس وزن الجنين من قبل أخصائي بيطري ، شرائح انسجة دماغ الاجنة تمت معالجته من قبل اختصاصي تقني ، وقراءتها من قبل أخصائي علم الأمراض التشريحي البيطري.

فيما يتعلق بالنمو داخل الرحم ، وجد الباحثون أن هناك نقصًا في وزن الجنين خلال أيام الدراسة المحددة ولكن الأكثر أهمية في اليوم التاسع عشر من الحمل حيث كانت القيمة الاحتمالية عالية ، لوحظ وجود تغيرات نسيجية متعددة في الدماغ في المجموعات المتعرضة المختلفة حيث كانت في كثير من الأحيان فقدت النمط الطبيعي للشكيل .

وخلص الباحث إلى أن التعرض للموجات فوق الصوتية للجنين قد يكون بمثابة تأثير حيوي ضار في الجرذ ألبينو ، وهذا قد يعمل كما هو الحال في حالة الإنسان مثلما اشارت بعض الدراسات السابقة ، فهذه الدراسة تحتاج إلى أن تتكرر مع تمديد وتعديل المعلمة باستخدام نموذج حيواني كبير لتقليد الإنسان من النتائج المكتسبة

مبدأ (ALARA) هو مفهوم مهم يجب اعتماده وتطبيقه من قبل أخصائيي الطب و الموجات فوق الصوتية في ممارستهم مع تحديث توافق الآراء من الأبحاث القادمة التي قد تكشف عن تأثير الموجات فوق الصوتية في الإنسان و ذريته خاصة.

Contents

XV

1. Introduction

Ultrasound has provided a wealth of knowledge in diagnostic medicine and has greatly impacted medical practice, particularly obstetrics. Millions of sonographic examinations are performed each year, and ultrasound remains one of the fastest-growing imaging modalities because of its low cost, real-time interactions, portability, and apparent lack of biologic effects. No casual relationship has been established between clinical applications of diagnostic ultrasound and bioeffects on the patient or operator.(Rumack and Levine, 2010)

Medical diagnostic ultrasound has been use for over 40 years, having found widespread application in anatomic imaging and blood flow velocity measurement in medicine .particularly in Obestrics ,where is radiography is not generally used, ultrasound has provided an important means for fetal dating ,evaluation of fetal development , and diagnosis of fetal , uterine and placental abnormalities. (Merritt et al., 1992)

The physical effects of sound can be divided into two principal groups: thermal and nonthermal. Most medical professionals recognize the thermal effects of elevated temperature on tissue, and the effects caused by ultrasound are similar to those of any localized heat source.

Measurements of temperature rise in tissue under the influence of ultrasound are difficult to make, and relevant data are just beginning to emerge. Calculations of expected temperature rise based on tissue absorption coefficients, taking into account blood perfusion, have been accomplished. The potential for tissue heating depends upon ultrasound frequency, intensity, the area of ultrasound beam ,the duration of

exposure and the rate of removal of heat away from target by blood flow or conduction.the intensity of ultrasound at the target effected by the absorption coefficient of tissue along the path of ultrasound beam and the amount of energy removed by the attenuating pathway from transducer to the site of interest (usually focal zone of transducer).As a consequence of increasing attenuation at higher frequencies, transducer frequency is material factor .the presence of bone in the ultrasound path is also an important factor ,as absorption is significantly increased at soft tissue/bone interfaces ,resulting in greater potential for heating than in soft tissue alone (Merritt et al., 1992)

With ultrasound the heating mainly results from the absorption of the sound field as it propagates through tissue. However, "non thermal" sources can generate heat as well. Many non-thermal mechanisms for bioeffects exist. Acoustic fields can apply radiation forces (non ionizing radiation) on the structures within the body, both at the macroscopic and the microscopic levels, resulting in exerted pressure and torque. The temporal average pressure in an acoustic field is different than the hydrostatic pressure of the fluid, and any object in the field is subject to this change in pressure. The effect is typically considered smaller than other effects because it relies on less significant factors in the formulation of the acoustic field. Acoustic fields can also cause motion of fluids. Such acoustically induced flow is called streaming. Acoustic cavitations is the action of acoustic fields within a fluid to generate bubbles and cause volume pulsation or even collapse in response to the acoustic field. The result can be heat generation and associated free radical generation, micro streaming of fluid around the bubble, radiation forces generated by the scattered acoustic field from the bubble, and mechanical actions   from bubble collapse. The

interaction of acoustic fields with bubbles or "gas bodies" (as they are generally called) has been a significant area of bioeffects research in recent years. (Rumack and Levine, 2010)

The minor effect data on exposure to ultrasound is due to the fact that most studies have been conducted on in vitro animal or plant cells or in insects.(Ali, 1993)

Animal studies were designed to address questions concerning possible deleterious effects of exposure to ultrasound during pregnancy. Use of animal models is of importance since various possible confounding factors could be controlled in such investigations.(Jensh and Brent, 1999)

A large body of literature has investigated and reviewed the embryos of many mammals and found it to be susceptible to heat damage. Embryonic development consists of highly ordered sequences of cell proliferation, cell differentiation, cell migration and apoptosis (programmed cell death) . (Zaiki and Dom, 2014)

The developing brain and nervous system seem particularly sensitive to the effect of the heat. Elevated maternal or fetal temperature can result in spectrum of adverse outcomes that effect many developing tissues .(Swerdlow, 2010)

Consensus and international reports from organization emphasis that more research should be conducted   relating to developing foetuses with more concern to brain since ultrasound effects extend to juvenile and adult life .

1.1.Research problem :

At late pregnancy ultrasound is usually requested by obstetricians for follow-up of pregnancy, abnormal fetuses development and Biophysical fetal profile that imply using of Doppler and 3D ultrasound technique, require high ultrasound power and long duration of exposure and that may affect fetuses..

1.2 Research Objectives

1.2.1 General objectives:

The general objective of the study is evaluate the biological effect of ultrasound on albino rat fetuses in late gestation age

1.2.2 Specific objective:

- To evaluate ultrasound effect on fetuses   albino rat fetuses on different Gestation days (17,18,19,20) using fixed parameters.

- To asses ultrasound affect on   fetus growth (weight) at different Gestation days (17,18,19,20) of exposed and control groups

- To investigate   brain histopathological   changes in exposed versus control groups

2. Study Background

2.1 Rat Embryology

2.1.1 Preimplantaion Embryo

Fertilization

Fertilization is a process by which two gametes fuse together to create a new individual. Fertilization functions to allow genes to be transferred from parent to offspring and the process of development to begin. The rat is an excellent model for the study of fertilization because the timing of events has been well established. In the female rat, the onset of behavioral estrus, or heat, is a suitable reference point to begin the timing of the estrous cycle (figure 2-1)
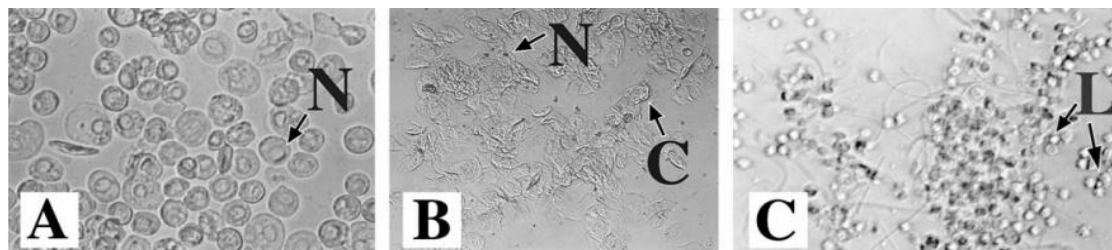
Figure (2-1) Estrus detection in vaginal smears. Panel A shows an unstained vaginal swab during proestrus, panel B shows an unstained vaginal swab during estrus, and panel C shows an unstained vaginal swabs during diestrus. Magnification of all images is 20. N ¼ nucleated cell; C ¼ cornified cell; L ¼ leukocyte. (Photomicrographs courtesy of Dr. Yuksel Agca.) from (Suckow et al., 2005)

The ovum forms the first polar body in the ovarian follicle, although the polar body is lost soon after its formation. The second maturation division begins immediately after completion of the first division.
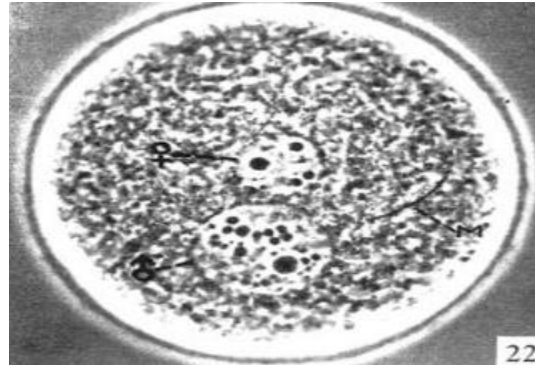
Figure (2-2) Living ovum in which the pronuclei are of maximum size 645. From (Suckow et al., 2005)

In the male, spermatozoa from the testes are stored in the epididymis figure (2-3), where they mature and acquire the ability to move. At the time of ejaculation, sperm still lack the capacity to bind to and fertilize an egg. This final stage of sperm maturation (capacitation) occurs after the sperm are inside the female reproductive tract. Spermatozoa have been found disseminated throughout the uterine cornua and already migrating into the uterine segments of the oviducts within 15 minutes after ejaculation.
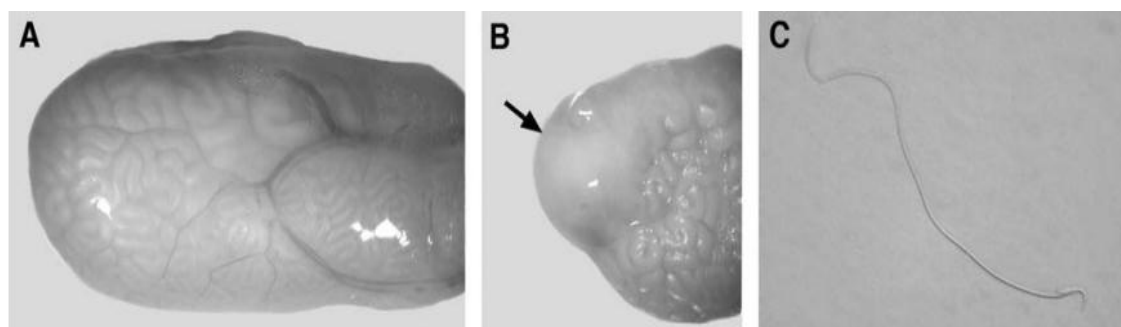
Figure (2-3) Cauda epididymis (A), arrow indicates dense masses of spermatozoa (B), and a single rat spermatozoa (C) from (Suckow et al., 2005)

Fertilization takes place in the ampullae of the oviducts . When the fertilizing spermatozoon comes into contact with the vitelline membrane, the cell membranes of both the spermatozoon and the egg rupture in the area of contact and fuse with each other as the spermatozoon passes into the cytoplasm of the egg . The zona pellucida undergoes a change (zona reaction) after the entry of the first spermatozoon that tends to exclude other spermatozoa. The zona reaction is thus a mechanism that helps to prevent the occurrence of polyspermic fertilization.(Suckow et al., 2005)

Cleavage/Morula Formation

The chromosomes proceed immediately thereafter to become arranged at the metaphase plate of the first cleavage spindle . The first segmentation occurs during the early part of the second day after insemination, and the resulting two-cell stage lasts for a period of about 24 hours .By the end of the second day after insemination, the two-cell embryo has traversed a little over one-half the length of the oviduct. One of the cells usually divides before the other, resulting in a three cell stage. By the end of the third day, only four-cell embryos are found in the oviduct. The first three segmentations are spaced at intervals of about 18 to 24 hours, and by the end of the fourth day, the 12- to 16-cell embryos pass from the oviducts to the uterine horns. In the albino rat, during the first 4 days of the development, there is only a very slight increase on the size of the cell mass compared with the unsegmented ovum with two pronuclei .(Suckow et al., 2005)

Blastocyst

Formation of the blastocoele by cavitation of morula masses composed of 30 to 32 cells begins mid day 5 . The blastocyst is a roughly spherical epithelial structure that ranges in size from 60 to 85 mm and is surrounded by the 2.5 to 3-mm-thick zona pellucida.

It consists of a hollow sphere of cuboidal trophoblast cells with their apical surface facing on the zona pellucid, with an inner eccentrically placed group of large (7–12 mm), roughly spherical, and loosely associated inner cell mass cells, which occupies about one-quarter of the volume of the sphere. The trophectoderm cells have descendants only in placental structures (chorion, ectoplacental cone, and trophoblast giant cells). The trophoblast is continuous, with a massing of epithelial cells at one pole of the blastocyst, the inner cell mass, from which the embryo and its immediate membranes are developed (amnion, yolk sac, allantois, and mesodermal components of the placenta).(Suckow et al., 2005)

Yolk Sac Placenta Development :

That the yolk sac was suggested epithelium of the rat is physiologically a placenta that functions as an organ for maternal and embryo exchange. Over much of its surface, Reichert's membrane is directly bathed by circulating maternal blood that allows for diffusion of materials into the yolk sac cavity, from which they are absorbed into the embryo . Therefore, in the rat there are two placentas that serve as organs for maternal embryo exchange. The first to develop is a villiary highly vascularized yolk sac placenta, which will be followed on day 11.5 to 12.5 by the chorioallantoic placenta . The two placentas are present together throughout most of gestation. The formation of the yolk sac placenta begins on day 7 with the proliferation at the periphery of the inner cell mass of the hypoblast, which will line the blastocoel, converting this chamber into a primitive yolk sac cavity lined with a bilaminar structure of trophoblast and a single layer of hypoblast (endodermal cells); (Figure. 2-4) . By day 8, the amniotic cavity has separated the ectoplacental cone from the inner cell mass. Three cavities eventually form within the ectoderm of the egg cylinder: the amniotic

cavity (most ventral cavity), then extraembryonic cavity (middle), and the ectoplacental cavity that is just under the ectoplacental cone and is transitory. By day 10, the visceral yolk sac mesoderm has split to form an extraembryonic coelom, undergoes angiogenesis and becomes vascularized by the peripheral vitelline circulation. The yolk sac is thus the first hematopoietic organ. Division of the yolk sac into visceral and parietal walls is complete in the rat by gestation day 11 to 12. Meanwhile, the visceral wall of the yolk sac cavity also differentiates into the villous portion, a more highly vascular and more absorptive area and a smooth portion that is less vascular and presumably a less absorptive area.

By day 12, the trophoblastic ectoplacenta on the mesometrial side is vascularized by allantoic capillaries to form a definitive chorioallantoic placenta, with maternal blood sinuses near its fetal surface that lie just external to the placental portion of the parietal yolksac. From day 12 and throughout pregnancy, five distinct morphologic layers are present in the placenta. Starting from the myometrium, there is a strongly decidualized myometrium with many large metrial cells. A stromal layer consisting of moderately large decidual cells is followed by a layer of giant trophoblasts that separates the tissue of maternal origin from that of embryonic origin. A narrow layer follows, which is called thetrophospongium (also called the basal zone, reticular zone, or junctional zone), that consists of highly proliferative cells with rather large syncytiotrophoblasts and lacunae with mature blood cells. Last is a layer called labyrinth, which contains many lacunae; some filled with maternal blood and some with embryonic blood. The last two layers comprise the fetal placenta and occupy about one-third of the thickness of the uterine mesometrial wall .On about day 14, the uterine cavity is re-established ventral (antimesometrial) to the embryo, creating a capsule of

decidua over the implantation site, the decidua capsularis. The remaining uterine lining is the deciduas parietalis.
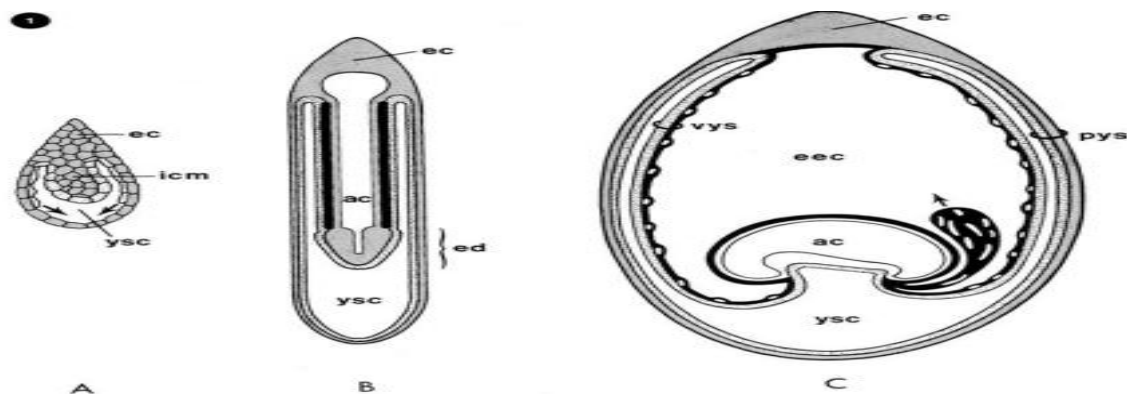
Figure . 2-4. Diagrams of an implanting rat conceptus that illustrate the mode of formation of the yolk sac in myomorphic laboratory rodents. A :At 7 days postcoiturn hypoblast has delaminated from inner cell mass. B:At 8 days an amnionic cavity has separated the ectoplacental cone from the inner cell mass: and the cells of the latter are arranged to form a bilaminar embryonic disc. C:By 10 days visceral yolk-sac mesoderm has split to form an extraembryonic coelom and undergoes angiogenesis to vascularize this portion of the yolk sac . (Suckow et al., 2005)

2.1.2Gastrulation

The period of Gastrulation (days 8.5 to 9.5 in the rat)covers the period of development of the conceptus from a bilaminar germ disc of epiblast and hypoblast that arises from the inner cell mass through the formation of the primitive streak to the trilaminar ectoderm/mesoderm/ endoderm of the embryo. Only in two areas is the original bilaminar condition retained: the prochordal plate, or buccopharyngeal membrane, \and the cloacal membrane . Structures formed during gastrulation include the notochord and neural plate (gestation day 9.5).(Suckow et al., 2005)

2.1.3 Organogenesis

Neurulation and Early Central Nervous System Development
Neurulation is the process of neural tube formation that forms the future brain and much of the length of the spinal cord. The caudal portion of the spinal cord forms during secondary neurulation. The formation of the nervous system depends on cell movements and cell-cell interactions that begin during gastrulation

The development of the notochord in the rat takes place between day 8.5 and 9. Induction is followed by elevation of the lateral margins of the neural plate to form neural folds. On elevation, the neural folds first become bi-convex, then beome concave and finally meet and fuse in the midline. During this time, neuroepithelial cells change in shape from low columnar to high columnar and then to wedge shaped. The initial neural tube fusion occurs in the cervical region of the second and third somites at the seven-somite stage, extending upward into the hindbrain. At the early 10-somite stage, a separate region of fusion begins at the midbrain/forebrain junction immediately above the developing cranial flexure. By the 14-somite stage, final closure of the spindle-shaped opening between these two areas of fusion is complete. The last portion of the cephalic neural fold to close, the anterior neuropore, is located in the future forebrain and closes by the 16-somite stage. This is followed by progressive fusion from the cervical region caudad. The posterior neuropore is found at the caudal end of the embryo in the future lumbosacral region and remains open until about the 21-somite stage. In the rat, the neuropores close between days 10.5 and 11, with the anterior neuropore closing first .

The final processes of neural tube closure are migration of the neural crest cells and individual fusion of the surface ectoderm and neural

ectoderm . The notochord in the tail of the rat develops on day 12 and 13 from a common mass of condensed mesenchymal cells located ventrally to the secondary neural tube, which subsequently splits to form a thin cord (which becomes the notochord) and a thick portion (which gives rise to the tail gut) . Neural crest cells differentiate at the lateral margins of the neural plate and are derived solely from the neural epithelium from the six-somite stage onward . They will migrate away from the neural fold or roof of the incipient neural tube and are induced by structures with which they come in contact to form many cell types, including neurons and glial cells of the sensory, sympathetic, and parasympathetic nervous systems; the medulla cells of the adrenal gland; adrenergic paraganglia; calcitonin-producing cells; melanocytes of the epidermis; endothelium of the aortic arch arteries; septum between the aorta and pulmonary artery; and skeletal and connective tissues of the face and anterior neck region . Neural crest migration occurs in the following sequence: midbrain, rostral hindbrain, caudal otic hindbrain, postotic and finally rostral otic hindbrain, with a general shift in migratory intensity from rostral to caudal regions . In the anterior region, the neural tube balloons into three primary vesicles as the anterior neuropore closes at gestation day 10.5: forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). The optic vesicles extend laterally from each side of the developing forebrain. On gestation day 12, the forebrain becomes subdivided into the anterior telencephalon (cerebral hemispheres) and the more caudal diencephalons (thalami, posterior lobe of the pituitary gland, pineal body and optic stalk, and optic cup, which later generate the optic nerve and retina). The rhombencephalon becomes subdivided into a posterior myelencephalon (medulla oblongata) and a more anterior metencephalon (pons and cerebellum). The cerebellum is formed in the rat on day 14. The remainder of the neural tube forms the

spinal cord. Neurogenesis in the rat spinal cord occurs in a temporal gradient, with neurons in the ventral spinal cord becoming postmitotic before those in the dorsal cord. Most motor neurons are generated between days 11 and 13. Neurons form in the fetal rat brain principally between days 13 and 20—except in the cerebellum, where they do not appear until about day 19—and continue to form during the early postnatal weeks . Naturally occurring neural tube defects have been extensively studied in several strains of mice (curly tail, splotch mutant, loop tail, and those trisomy for chromosomes 12 and 14). (Suckow et al., 2005)

2.2 Teratology

Teratology is the study of birth defects, and its goals are to describe and determine etiology ; to explore mechanisms involved in the production of birth defects and to devise means of prevention. The concept of "birth defects" has evolved beyond the original emphasis on structural congenital malformations. A current operating definition of birth defects is: "Birth defects are structural or functional abnormalities present at birth that cause physical or mental disability. Some may be fatal . More broadly, a birth defect may be considered as any structural or functional anomaly manifesting at any age due to causes acting before birth.

Experimental teratology in the modern sense began in the 1940s when Warkany and his associates first called attention to the fact that maternal dietary deficiencies and X-rays could adversely affect the in utero development of mammals. Three human tragedies resulting from in utero exposure to drugs or chemicals led to the development and revision of testing guidelines. The thalidomide tragedy of 1961 resulted in the birth of infants with rare limb malformations, amelia (absence of the limbs), or

various degrees of phocomelia (absence of the proximal portion of a limb) to mothers who took this sedative. Additional malformations seen with thalidomide treatment included defects of the external ears, facial hemangioma, atresia of the esophagus or duodenum, tetralogy of Fallot, and renal agenesisn. This completely changed the perception regarding the vulnerability of the intrauterine embryo to outside influences.

In the early 1970s, adenocarcinoma of the vagina began occurring in young women whose mothers had been treated with diethylstilbestrol during the first trimester of pregnancy. This raised concerns regarding adverse effects that were not evident until after puberty. The third event was the epidemic of organomercurial poisoning that resulted from the dumping of metallic mercury into Minimata Bay Japan, and its conversion by aquatic plant life into methylmercury. The concern for the potential to affect postnatal development resulted in additional testing for postnatal behavioral changes .

Wilson (1977) formulated six principles of teratology and they remain valid today:

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with environmental factors.
2. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure. Wilson (1965) graphically illustrated this concept, showing periods at which specific organs are more susceptible to teratogenic insult (figure2 -5)
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis (pathogenesis).

4.The final manifestations of abnormal development are death, malformation, growth retardation, and functional disorder.

5.The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).

6.Manifestations of deviant development increase in degree as dosage increases, from the no-effect to the totally lethal level.
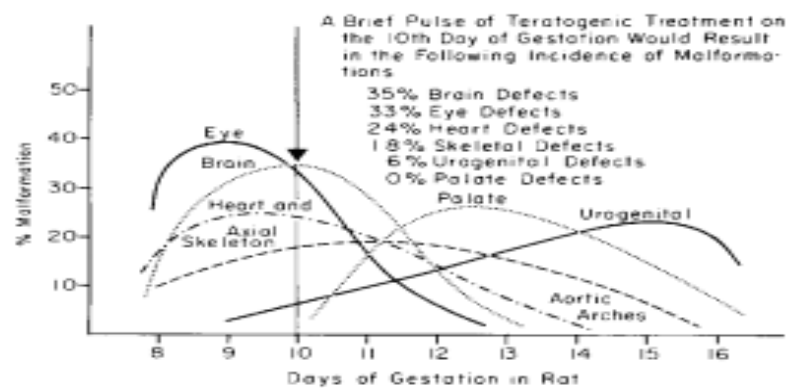
Figure (2-5) Hypothetical representation of how the syndrome of malformations produced by a given agent might be expected to change when treatment is given at different times. The percentage of animals affected as well as the incidence rank of the various types of malformations would be somewhat different from that shown for the tenth day if treatment were given instead on the twelfth or the fourteenth day, for example. From (Suckow et al., 2005)

In addition, Wilson (1977) listed eight mechanisms of teratogenesis: mutation, chromosomal nondisjunction and breaks, mitotic interference, altered nucleic acid integrity or function, lack of precursors and substrates needed for biosynthesis, altered energy sources, enzyme inhibitions,

osmolar imbalance and altered membrane characteristics. The most common treatment period to evaluate teratogenicity requires exposure from implantation to closure of the hard palate or from gestation day 6 through 17 in the rat. Studies to evaluate effects on embryo-fetal development are required in two species: one rodent and one non-rodent, usually the rat and the rabbit. The ICH guidelines state "reasons for using rats as the predominant rodent species are practicality, comparability with other results obtained in this species and the large amount of background knowledge accumulated.

The rat has the following characteristics that make it a suitable species for evaluating effects on embryo-fetal development: it is relatively disease resistant, withstands operative procedures well and has a short reproductive cycle (estrus stage can be determined) and a high breeding rate with a relatively short gestation period and good litter size. The fetuses have a low spontaneous malformation rate and are of sufficient size .(Suckow et al., 2005)

2.3 Breeding

Pregnant females used for embryo-fetal development studies may be obtained in one of three ways: (1) by purchasing timed pregnant females from a commercial supplier, (2) by purchasing male and females from a commercial supplier and breeding them in house and (3) maintaining a breeding colony. The ICH guidelines (1994) state ''that within and between studies, animals should be of comparable age, weight, and parity at the start: the easiest way to fulfil these criteria is to use animals that are young, mature adults at the time of mating with the females being virgin.'' Strains with low fecundity should not be used. The amount of background data available and the experience of the laboratory with specific rat strains will determine the final choice; however, the ICH

guidelines (1994) states that ''it is generally desirable to use the same species and strain as in other toxicological studies.'' If mating is done in house, the stage of the estrus cycle (proestrus, estrus, metestrus, or diestrus) can bedetermined by evaluating vaginal smears of cells from the vaginal . Rats in the proestrus stage are placed for overnight breeding. Rats in estrus are paired with a male if bred for short periods (2 to 4 hours) because ovulation in the rat occurs spontaneously near the end of the estrous period, the period when the female is receptive to the male. The shorter mating period is used to reduce inter litter variability. Males and females are usually cohabited in a 1:1 ratio, but males may be cohabitated with up to five females.

Insemination is confirmed by the presence of spermatozoa in a vaginal smear or the presences of a vaginal plug. The vaginal plug is a coagulated mass of semen, which may be found lying on the bottom of the cage. The day of insemination is usually considered gestation day 0, but day 1 is also used. The gestation period in the rat is 22 days. Care should be taken to avoid excessive stimulation of the vagina when evaluating the female for the estrous cycle stage or for the presence of sperm in the vagina, because this can upset the estrous cycle and produce a pseudopregnancy with a duration of 12 to 16 days before the resumption of the normal cycle .(Suckow et al., 2005)

2.4 Intrauterine Growth restriction (IUGR)

Intrauterine growth restriction (IUGR) refers to a condition in which a fetus is unable to achieve its genetically determined potential size. This functional definition seeks to identify a population of fetuses at risk for modifiable but otherwise poor outcomes. This definition intentionally excludes of fetuses that are small for gestational age (SGA) but are not

pathologically small. SGA is defined as growth at the 10th or less percentile for weight of all fetuses at that gestational age. Not all fetuses that are SGA are pathologically growth restricted and in fact, may be constitutionally small. Similarly, not all fetuses that have not met their genetic growth potential are in less than the 10th percentile for estimated fetal weight (EFW). (Chudleigh and Thilaganathan, 2004)

Maternal causes of IUGR include the following: Chronic hypertension , Pregnancy-associated hypertension ,Cyanotic heart disease ,Class F or higher diabetes ,Hemoglobinopathies ,Autoimmune disease ,Protein-calorie malnutrition ,Smoking ,Substance abuse , Uterine malformations , Thrombophilias ,Prolonged high-altitude exposure Placental or umbilical cord causes of IUGR include the following: Twin-to-twin transfusion syndrome ,Placental abnormalities ,Chronic abruption ,Placenta previa Abnormal cord insertion ,Cord anomalies ,Multiple gestations IUGR occurs when gas exchange and nutrient delivery to the fetus are not sufficient to allow it to thrive in utero. This process can occur primarily because of maternal disease causing decreased oxygen-carrying capacity (eg, cyanotic heart disease, smoking, hemoglobinopathy), a dysfunctional oxygen delivery system secondary to maternal vascular disease (eg, diabetes with vascular disease, hypertension, autoimmune disease affecting the vessels leading to the placenta), or placental damage resulting from maternal disease (eg, smoking, thrombophilia, various autoimmune diseases). Evaluation of causative factors for intrinsic disorders leading to poor growth may include a fetal karyotype, maternal serology for infectious processes, and an environmental exposure history.(Malhotra et al., 2010)

2.5 Ultrasound

Ultrasound is very high frequency (high pitch) sound. Human ears can detect sound with frequencies lying between 20 Hz and 20 kHz. Mechanical vibrations at frequencies above 20 kHz are defined as ultrasound. Medical imaging uses frequencies that are much higher than 20 kHz; the range normally used is from 3 to 15 MHz. These frequencies do not occur in nature and it is only within the last 50 years that the technology has existed to both generate and detect this type of ultrasound wave in a practical way.

Medical diagnostic ultrasound has been in use over40 years, having found widespread application anatomic imaging and blood flow velocity measurement in virtually all medicine .particularly in obestrics where radiography is not generally used; ultrasound has provided an important means for fetal dating, evaluation of fetal development and diagnosis of fetal, uterine and placental abnormalities. During its lifetime as diagnostic procedure, ultrasound has established an enviable safety record, with no evidence of biological effects on patients at intensities typical of instrument in current use. Despite lack of evidence of harm from diagnostic levels of ultrasound, good medical practice requires the physician to consider issues of risk and benefit in performing diagnostic ultrasound examinations, and to take all proper measures to ensure Maximal benefit with minimal risk. Ultrasound is being used by increasing numbers of individuals with limited training and experience, resulting in greater risk of unnecessary or inappropriate fetal exposure .(Pinto et al., 2004)

2.5.1 Interaction of ultrasound with matters

Ultrasound is sound of a frequency higher than humans can hear (above 20 kHz). In medical diagnostics one uses frequencies between 2 and 20 MHz (1 MHz=1 million cycles per second). Only the longitudinal mode of ultrasound vibration is used in soft tissues. The waves propagate in soft tissues at an average speed of 1540 m/s. The wavelength is the distance between two adjacent pressure maxima in space and it is inversely proportional to the frequency .

$$v = f\lambda$$

At 1.5 MHz, the wavelength is 1 mm, at 3 MHz, it is 0.5 mm, at 6 MHz, it is 0.25 mm, etc. The shorter the wavelength the better the resolution of an ultrasound scanner. The intensity of ultrasound waves is measured in watts per square centimetre (W/cm2). The practical consequence of this is that, in order to get the best resolution, one ought to work with the highest practical frequency in any particular case. There are condensation and the rarefactions of the medium as ultrasound passes through it. As ultrasound encounters a boundary between two media of different characteristic acoustic impedances and different propagation speeds, it can be reflected and refracted while crossing the boundary. The ratio of the reflected wave intensity to the incident intensity depends on the difference of the characteristic acoustic impedances of the two media, the angle of incidence and on the propagation speed ratio. At perpendicular incidence, the reflection does not depend on the propagation speeds. Where pr is the pressure amplitude of the reflected wave and pu is the pressure amplitude of the incident wave.

The larger the difference of the acoustic impedances (Z1 and Z2)-the larger the fraction of ultrasound reflected. When crossing through a medium, ultrasound waves are attenuated, i.e. the energy in the beam

decreases due to absorption, scattering and reflection. Attenuation is proportional to frequency—the higher the frequency the less penetrating the ultrasound waves.

Soft human tissues, bones and gases have distinctly different ultrasound propagation properties. Characteristic acoustic impedance is about five thousand times smaller in gases than in soft tissues, and is two to three times higher in calcified bones than in soft tissues. Speed of ultrasound is about 340 m/s in gases, it is on the average 1540 m/s in soft tissues and is between 3000 and 4000 m/s in bones.(Pinto et al., 2004)

2.5.2 Ultrasound Scanner:

An echoscope or an ultrasound scanner is a device, which makes section images of the interior of the body using the information obtained from the echoes of ultrasound pulses transmitted into the body. An echoscope transmits short pulses of high frequency ultrasound (less than 1 microsecond long, frequency between 2 MHz and 10 MHz, about 1000 times per second) into the body via a probe. The system then waits for the echoes to return, and picks them up by the same probe. The direction of transmission-reception is known, because the probe scans in a predetermined way, the round-trip time for the echoes is measured in the echoscope, and so all the data to form a two-dimensional (2D) image are there. An echoscope (sonar, echograph, Ultrasound, scanner) consists of the following parts: - A probe, which contains piezoelectric transducers to generate and detect ultrasound and some scanning, means to direct ultrasound in desired directions. - A microprocessor controlled electronic system, which generates electrical pulses for the probe activation, directs the pulses into the probe for focusing and steering, amplifies the signals obtained from the echoes, and stores the data and displays them on a TV

monitor. The amplifier amplifies the echoes that come later (from deeper structures) more than those coming from shallower structures in order to compensate for attenuation of ultrasound.- The signals, which represent ultrasound echoes, are, as said, processed. Processing before storage in the computer memory is called pre-processing. This includes the TGC (time gain compensator) and other gain and focusing processing. After the data are stored in the memory they can again be processed in order to show them with different shades of gray and different relations of echo intensity to brightness on the screen. This is called post processing.

The echoes from the body can be displayed in three basic ways: the A mode, the B mode and the M mode. In the A mode display (amplitude mode) the echoes from the axis of the beam in front of the probe are shown as peaks proportional to the echo amplitude positioned at their respective distances along the line-of-sight **(Fig. 2-5)**. This is a one-dimensional display. It is used in ophthalmology and sinuses examination, and sometimes in neurology. (Pinto et al., 2004)
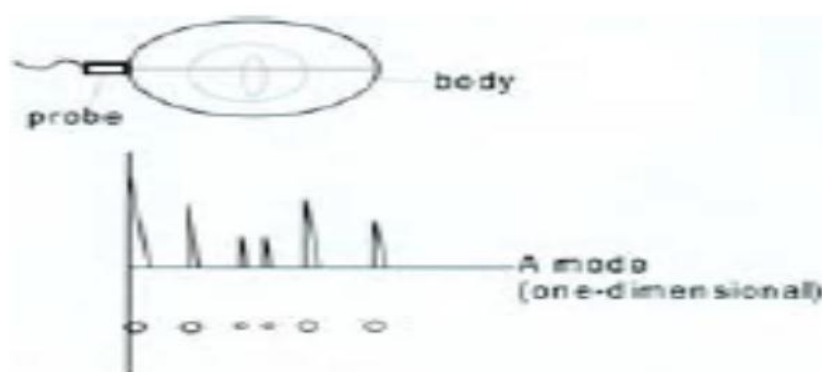
Fig (2-6) : A mode from (Suckow et al., 2005)

In the B mode display (brightness mode), the echoes are shown as bright dots with brightness proportional to the echo amplitudes (**Fig.2-6).** This mode is normally used as 2D scan, in that the interior of the body is scanned with the beam so that the bright dots merge into tomographic contours. (Pinto et al., 2004)

Fig (2-7): B mode from (Suckow et al., 2005)

Focusing :

A narrower beam yields better images. In order to narrow a beam, one focuses it. The beam can be focused with lenses and mirrors as well as electronically with composite transducers **(Fig2-6)**. Ultrasonic lenses act in the same way as optical lenses. They can be focused at different distances. However, when choosing a particular probe, the focus must be chosen at the depth of interest. Electronic focusing is based on using composite transducers, i.e. densely-packed sets of narrow transducers of annular-or strip-shaped arrays. These transducers are activated with

delays chosen such that the waves from each of the transducers reach the intended focus position at the same time. This type of focusing is flexible; i.e. the focus can be set at the desired position. Both fixed and electronic focusing are used in real probes.

Fig (2-8): Focusing Method from (Pinto et al., 2004)

The probes contain one or more piezoelectric transducers. They can basically be divided into sector and linear probes (Fig 2-7). Sector probes look into the body through a small acoustic "window" (1×1 to 2×2 cm), have acoustic lines-of-sight fanning out and yield a nearly triangular (sector) image. The linear probe has a longer front face in contact with the patient's body (5–12 cm), parallel or nearly parallel lines-of-sight and yield a rectangular image. (Pinto et al., 2004)

Fig (2-9): Curved arrays (left and center) suitable for abdominal scanning. A 5 MHz linear array (right) is useful for superficial structures, e.g. gallbladder and anterior abdominal wall. **(Smith, 2010)**

2.5.3 Measurement of Acoustical Output and Bioeffects:

Most clinical applications utilize pulsed ultrasound, in which brief bursts of acoustical energy transmitted into the body are propagated as waves of alternating compression and rarefaction through tissues **(Figure 2-10).** In pulsed operating modes, the pulse duration is the time from the beginning to the end of a pulse, while the reciprocal of the interval between pulses (the pulse repetition period) is the pulse repetition frequency or PRF. The duty factor is the pulse duration divided by the pulse repetition period and is an important determinant of the amount of energy introduced in scanning. Devices operating in pulsed Doppler modes generally have significantly higher duty factors than imaging devices. Which has important consequences in terms of bio-effect production. Each ultrasound pulse results in peak compressional and peak rarefaction pressures within the tissue (**Figure 2-11**). These peak pressure values are of interest with respect to mechanical mechanisms for bioeffects such as cavitations. As pulses of ultrasound are transmitted through tissue, work is performed. From a bioeffects viewpoint, the most important result of this work is the heating of tissues. The capacity to perform work is determined by the quantity of acoustical energy produced. Acoustical

power (expressed in watts or milli- watts) describes the amount of acoustical energy produced in a unit of time. Measures of acoustical power vary, depending on the time interval considered (**Figure 2-12**). For example, the peak power that occurs during the time interval of a single pulse differs considerably from the average power which occurs over a series of pulses. The time-averaged acoustical power is obtained by averaging the energy output from the beginning of one pulse to the beginning of the next pulse. In the case of pulsed ultrasound, the average power is the peak power times the duty factor. Although measurement of power provides an indication of the energy as it relates to time, it does not take into account the spatial distribution of the energy. For bioeffects purposes, a measurement describing the spatial distribution of power is needed. This is particularly true when focused transducers are used. As the power may be concentrated in small areas. Intensity describes the spatial concentration of power and is used to describe the exposure conditions at the target of the ultrasound beam. Intensity (I) is calculated by dividing the power by the area over which the power is distributed.

(Equation l):

$$I \ (W/cm2) \ = \ Power \ (W)/Area \ (cm2) \ (l)$$

In the region of the focal zone of a focused transducer, spatial peak intensities may be considerably higher than at the transducer face. In pulsed modes of operation, intensity may be calculated in several different ways. These take into account different power measurements (e.g. peak or average) and the spatial distribution of the energy. The spatial peak intensity (I_{SP}) is the highest intensity at any point in the ultrasound beam. The spatial average intensity (I_{SA}) is the average value of intensity over some specified area. The spatial average, temporal

average intensity (I_{SATA}) is obtained by dividing the temporal average power by the area of the transducer face. The spatial-average pulse-average intensity (I_{SAPA}) is the spatial average of the intensity during a pulse ($I_{SAPA}= I_{SATA}$/duty factor). Time-averaged intensity is an indicator of thermal effects and is important in safety considerations. This is provided in the spatial peak, temporal average intensity (I_{SPTA}). Which is the time-aver-aged intensity at the position of the spatial peak ($I_{SPTA} = (I_{SATA}\times I_{SP}/I_{SA})$. A final measurement is the spatial peak, pulse-average intensity (I_{SPPA}). This is the intensity that exists during a pulse at the position of the spatial peak and is the highest of the four intensities [$I_{SAPA} = (I_{SATA}\times (I_{SP}/I_{SA})$/duty factor]. Using measurements obtained in vitro and applying certain assumptions regarding attenuation of the ultrasound beam, these intensities may be estimated (with limited precision) for typical clinical conditions. The measurements, which are most relevant to known bio-effects mechanisms, are the I_{SPTA} and the I_{SPPA} the I_{SPTA} is used to estimate thermal bioeffects of ultrasound, while the I_{SPPA} is related to the likelihood of mechanical bioeffects such as cavitations. The I_{SPPA} tends to be higher in imaging than in Doppler instruments. While the I_{SPTA} is generally higher in Doppler modes. The Food and Drug Administration (FDA) have used both of these intensities in the regulation of ultrasound devices. (Merritt et al., 1992)
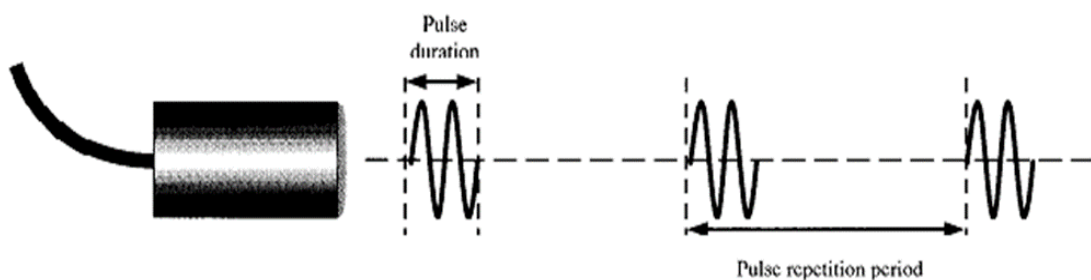

Figure 2-10 With pulsed ultrasound, the pulse duration is the time from the beginning to the end of a pulse. The interval between pulses is the

pulse repetition period and the reciprocal of this is the pulse repetition frequency. The duty factor is the pulse duration divided by the pulse repetition period and is an important determinant of the amount of energy introduced in scanning from (Merritt et al., 1992)
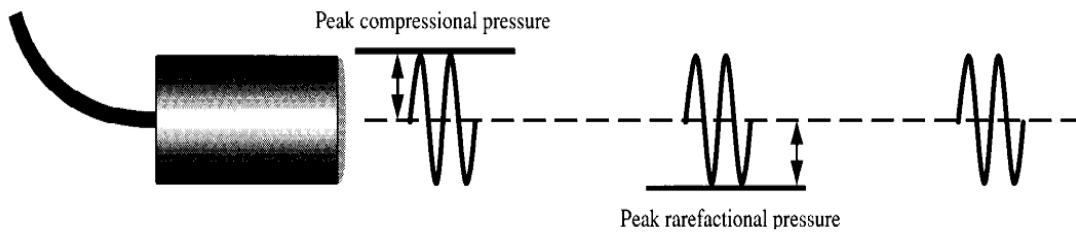
figure 2-11 Each ultrasound pulse results in peak compressional and peak rarefactional pressures. Pressure is expressed in pascals. Unlike the illustration, these are often not identical. Measurements of peak compressional and rarefaction pressures are measured at a specified point, usually whcrc the value would be maximum. Peak pressure values are of interest with respect to mechanical mechanisms for bioeffects such as cavitation from (Merritt et al., 1992)

Figure 2-12 The time-averaged acoustical power is obtained by averaging the energy output from the beginning of one pulse to the beginning of the

next pulse. In the case of pulsed ultrasound, the average power is the peak power times the duty factor. In (a) the pulse repetition factor is high, resulting in a high duty factor and higher average power than in (b) where a longer pulse repetition period results in a lower duty factor and thus a lower average power from (Merritt et al., 1992)
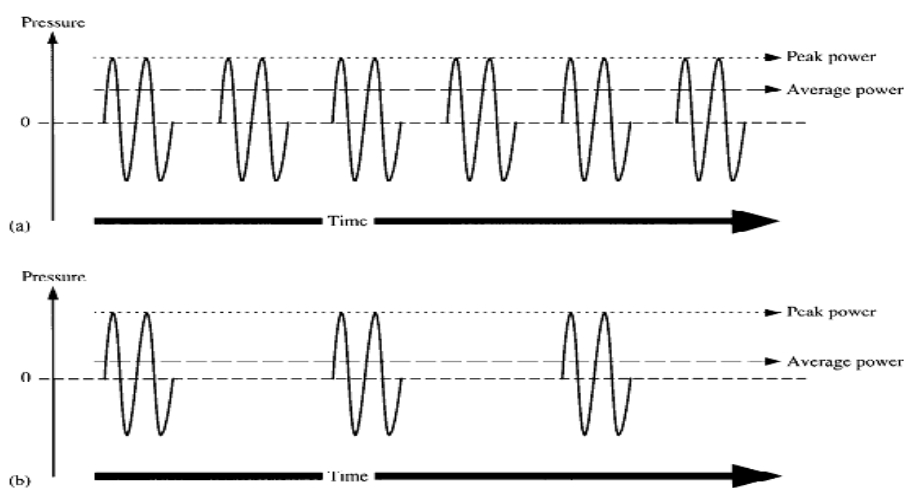
2.5.3.1 Bioeffects Mechanisms

Currently, two potential mechanisms for producing bio- logical effects with ultrasound are recognized. These include thermal mechanisms, which result from tissue heating due to the absorption of ultrasound as it passes through tissue and non-thermal (mechanical) effects such as cavitations and radiation forces. Cavitations refers to the formation and collapse of microbubbles around cavitations nuclei. At present, thermal mechanisms are understood better with respect to their relationship to mammalian bioeffects than are the non-thermal mechanism. (Merritt et al., 1992)

Thermal Mechanism :

Measurements of temperature rise in tissues under the influence of ultrasound are difficult to make, and relevant data are just beginning to emerge. Calculations of expected temperature rise based on tissue absorption coefficients, taking into account blood perfusion, have been accomplished". The potential for tissue heating depends upon the ultrasound frequency, intensity, and the area of the ultrasound beam, the duration of exposure and the rate of dissipation of heat away from the target by blood flow or conduction. The intensity of ultrasound at the target is affected by the absorption coefficient of the tissue along the path of the ultrasound beam and the amount of energy removed by the

attenuating pathway from the transducer to the site of interest (usually the focal zone of the transducer). As a consequence of increasing attenuation at higher frequencies. Transducer frequency is a material factor. The presence of bone in the ultrasound path is also an important factor, as absorption is significantly increased at soft tissue/bone interfaces, resulting in a greater potential for heating than in soft tissues alone. This effect has been shown in recent data for live rat fetuses, for human fetal bone in vitro and for adult rats and fetal mice". For a given frequency and intensity, broader beams produce higher temperatures. In the usual diagnostic ultrasound frequency range of 2-l0 MHz, the estimated temperature rise will not exceed 1C if the in situ spatial-average, temporal-average (SATA) intensity does not exceed 200 or 300 mW/cm^2, depending on beam Width'. All models for estimating the thermal effects of ultrasound make assumptions regarding the attenuating path- way of the ultrasound beam. Conditions of attenuation vary considerably from one ultrasound application to another, and models attempting to predict heating must generalize in making assumptions regarding attenuation. One approach is to assume a uniform rate of attenuation by soft tissue. In some obstetrical applications, however, the path of the beam through amniotic fluid requires different assumptions. Thus a model based on a uniformly attenuating fluid medium would be appropriate for estimating the thermal effects in a first-trimester obstetrical evaluation, but would be inappropriate for an examination of the adult abdomen. On the other hand, in the second and third trimesters, the ossification of fetal bone produces soft tissue/bone interfaces where greater absorption is present, resulting in potential for greater thermal effects under worst-case exposure conditions. (Merritt et al., 1992)

Non-thermal (mechanical) Mechanisms:

Currently, with non-thermal mechanisms, there is much less knowledge regarding the likelihood of clinically significant bioeffects. The occurrence of cavitations in tissues is a variable phenomenon with many contributing factors. Cavitations potential is related to the higher-amplitude short pulses that are typically used with imaging rather than with Doppler modes and with the limited data currently available. It is difficult to specify ultrasound levels at which cavitations will occur in mammals with diagnostically relevant pulse lengths and re- petition frequencies". It is known from observations with lithotripters (for which fundamental frequencies are lower than those for diagnostic instruments) that repeated pulses with peak pressures corresponding to intensities greater than 3300 W/cm^2 can induce cavitation in mammals. With continuous wave exposures, evidence for cavitation in tissues has been reported for physical therapy frequencies and intensities and higher". An approach to predicting cavitational activity involving a range of bubble sizes has been developed. Experimental animal data have provided some early support for this approach. Thermal and mechanical mechanisms are each capable of producing biological effects at high levels. From a mechanistic approach, heat is the more likely mechanism of interest at diagnostic intensity levels. SATA intensities above about 200 or 300 mW/cm^2 (depending on beam width) are in the range that could produce temperature rises of biological significance in soft tissues. (Merritt et al., 1992)

The 'minor effect ' data on exposure to ultrasound is due to fact that most studies have been conducted on in vitro animal or plant Cell or in insects. Although only one study on human being suggests that low birth weight might resulted from ultrasound exposure .unless the experiments

to verify this are well controlled ,perplexity will continue . the only wisdom to draw from this is that patient should only be exposed when there is clinical indication .(Ali, 1993)

2.6 Previous Studies :

Multiple research has been conducted to evaluate ultrasound and its adverse effect toward fetuses, research were different in their prospective and methods. Animal studies were designed to address questions concerning possible adverse effects of exposure to ultrasound through pregnancy The using of Animal subject is of importance since various potential confounding factors could be controlled in such investigations .(Jensh and Brent, 1999)

Effect of ultrasound in Prenatal development of the embryo and foetus have been investigated in many studies (Sikov and Pappas, 1986, Jensh and Brent, 1999), most of these studies have employed teratological techniques and assays to decide endpoints such as the viability of embryos and fetuses ,size, sex ratio of litters , the weight of offspring , the incidence of internal , external abnormalities and malformations .other studies have investigated specific effects on particular fetal tissues , such as the brain or testis.

To evaluate effect of ultrasound different methods have been conducted to study these effects , number of studies focused on developmental effect during embryonic period(McClain et al., 1972, Hara et al., 1977, Norton et al., 1990, Hande and Devi, 1995) other in fetal period (O'Brien, 1983, Sikov and Pappas, 1986, Pizzarello et al., 1978, Fisher et al., 1994, Fisher Jr et al., 1996, Zaiki and Dom, 2014) McClain et al 1972 conducted experiment to evaluate embryonic effects of ultrasound using 2.5 MHz CW for 30 min to 2 hours on rat Days 8, 9,

and 10 or 11, 12, and 13 of gestation age   and observed No malformation increase, but there is some skeletal variation.(McClain et al., 1972)

Hara et al , 1977 investigate embryonic and fetal effects in Rat using pulsed 600 W mW/cm^2 for 5 minute and they observed Reduced maternal weight; fetal abnormalities.(Hara et al., 1977)

 Pizzarello et al.1978 evaluated growth in Rats using 1.5 mWcm2 pulsed for 5 minute and they observed IUGR , same experiment replicated by Child et al 1984 and found no result.(Pizzarello et al., 1978)

O'Brien 1983 using mouse to evaluate fetal weight on day 18 using 1Mhz CW or pulsed wave for 10-300 second and observed that weight decreased dependant on dose .(O'Brien, 1983)

Sikov and Pappas 1986 conducted experiment on Rat at gestational days 9,10,12,15 to evaluate malformation at different Gestational stage growth using .8MHz for 5 minute their result shows increased mortality , malformation depend in gestation stage.(Sikov and Pappas, 1986)

Tarantal and Hendrickx 1989 a,b using monkey Cynomolgus Macaque on days 21-35,36-60 and 61-150 to investite growth behaviour,neonatal and infant observations,apgar scores   Using 7.5Mhz p10 or 20 minute per exam and they observed reduction in weight to 16 weeks ,reduction in CRL to 4 week; lower white blood cells   count on Gd 140.(Tarantal and Hendrickx, 1989)

Norton et al 1990 used Pregnant rats on 16 Gd to study developing cerebral cortical mantle exposed to ultrasound or microwaves from transducers located over one uterine horn using 2.5 Mhz with Ultrasound intensity (SPTA) was 0.78 W/cm2 for 30 min the result show

damage to cortex and that may result to neuron damage.(Norton et al., 1990)

Handi and devi 1992 conducted expirment using swiss Albino mouse on day 3.5,6.5 or 11.5 to evalaute prenatal motality ,body weight and size ,microphtalmia ,sex ratio in day 18 applying 3.5Mhz pulsed waves for 10 minute and rsulted reduction in fetal weight,increased number of growth retarded fetuses day 3.5 ,reductionof weight body length day 6.5; continue with another experiment 1995 investigate prenatal and postnatal motality using 3.5Mhz for 10 minute   on albino mouse and observed increased mortality , transient growth retardation.(Hande and Devi, 1992, Hande and Devi, 1995)

fisher et al (1994,1996) evaluate number of pregnancy ,litter size, gestation length ,sex ratio ,mortality ,fetal weight , total skeletal or visceral malformation using 3Mhz pulsed wave for 10 min and observed no significant effect when analyzed by litter.(Fisher et al., 1994, Fisher Jr et al., 1996)

Horder et al 1998 use guinea pigs to study temperature profile of fetal brain at third trimester gestation during in vivo exposure using 3.5Mhz pulsed for 2 minute and they outcome with increased temperature ,fetal heads clamped during exposure.(Horder et al., 1998)

Timothy A et al 2006 study haemorrhage near fetal bone on fetal Rat at 18-19 day using .9 MHz with different exposure parameter for 60 second and   conclude   that   Haemorrhage   occurrence   increased   slightly   with increasing ITA, as well as peak rarefactional pressure and PRF but does not correlate with exposure parameter.(Bigelow et al., 2007)

F. W. A. Zaiki and S. M. Dom 2014 evaluate fetal weight of rabbit The during gestational day (Gd) 6-7, Gd 17-18 and Gd 28-29 2nd and 3rd stage of pregnancy respectively using 30,60 , 90 minutes from ultrasound power 0.4 W to 0.7 W and I SPTA 0.13 to 0.19 W/cm2 and they observed reduction in fetal weight.(Zaiki and Dom, 2014)

From this reviews it thought to perceive there are agreed of probability of ultrasound to cause potential deleterious effects in Animal subjects. frequency and exposure parameter and duration   used are   low in particular   to advancement   of ultrasound equipment   and application these days , no general concern term to specific organ of   prenatal or postnatal term , though an advisory group of the health protection Agency (HPA) released document report on health effects of exposure to ultrasound and infrasound 2010 and its recommended further study of effects with given priority brain and histological level .

3. Materials and Methods

3.1 Material :

3.1.1Ultrasound

This study used a new calibrated portable ultrasonic diagnostic system A6 ultrasound machine from Sonoscape Co., Ltd. Ultrasound parameters such as transducer frequency, thermal index (TI), and mechanical index (MI) were kept constant. A transducer of linear array (L745), L8–12 MHz was used. Focal distance was kept constant at 4.5 cm throughout the experiments. The TI and MI displayed by the output display throughout the experiment showed the value of 0.5 and 0.8, respectively.

3.1.2 Anmial house :

Animal houses was Temporary Constructed using; international guidelines on Radiology Lab at National university basement all environmental factors was kept constant

Animals

Nulliparous female albino rats weighing between 170 and 190 g and male albino rats weighing between 200 and 220 g were obtained from Khartoum University, pharmacology college animal house. All experimental procedures were conducted with the guidance of institutional animal care. Rats were housed four per cage with a 12 h light/dark cycle. They were supplied with standard laboratory chow and tap water *ad libitum*. This *in vivo* experiment used ten females, half of which served as the control group and the rest as the exposed group. The courtship and mating sessions were arranged systematically, with female to male ratio being 2:2. Mating was confirmed through vaginal smear examination by the presence of vaginal copulation plug, i.e., day 0

of pregnancy. Gestational period of albino rats usually varies between 21 and 22 days.(Suckow et al., 2005)

 All external environments were kept constant (temperature: 28°C and humidity: 60%−65%).

Restraint

None of restraint or anaesthesia used during experiment

3.2 Method :

3.2.1Study design:

In-vivo Experimental (randomized control) study involving ultrasound wave applied against pregnant Albino Rat species at late trimester.

3.2.2Study period and Area :

This study was conducted from may 2015 to 2017 at   National university Sudan   temporary Animal house constructed at Radiology Lab

3.2.3 Study population:

Albino rats females who get pregnant after arranged systemic mating session ,and reaches late trimester

3.2.4Sampling and Sample size

 Sampling:

Randomize sampling fetuses of pregnant Albino Rat gated after exposure with ultrasound beam during different period in different trimester.

Sample size and type :

Fifty five fetuses of pregnant albino rats have been obtained from this study

3.3Experimental design

Pregnant albino rats were captive in the next day of exposure (17,18,19,20,21) and fetuses were taken out and then fetal body weight was measured. A total of 55 fetuses were analyzed for fetal body weight using a calibrated electronic balance from Brother. An average of three readings was recorded.Fetuese then immersed in labelled container contain concentration forma aldhyde 10% and sent to specialized histopathology lab for Brain histology analysis .

Histology processing:

Histological process for rat fetus & brain
Fixation :-
The best fixative for fetus brain is 10% neutral buffer formalin

- The usual fixative for paraffin embedded tissues is neutral buffered formalin (NBF).

- We put all the fetus in a container full of fixative 20 time of the specimen size .

- Fixative volume was 20 times that of tissue on a weight per volume; use 2 ml of formalin per 100 mg of tissue.

- Due to the slow rate of diffusion of formalin (0.5 mm hr), tissue was sectioned into 3 mm slices on cooled brain before transfer into formalin. This will ensure the best possible preservation of tissue and offers rapid uniform penetration and fixation of tissue within 3 hours.

- Tissue should be fixed for a minimum 48 hours at room temperature.

Grossing:-

Fetus size 3.5cm 2.3 cm

Brain selection

firstly make a intracardiac perfusion animal, washing first with PBS and then with PFA solution. That will allow to extract the brain in an easy way and it will be strong and will not break when we extract.

begin dissection from the occipital bone to the frontal bone. Eliminating all the occipital, parietal, frontal bone of the skull, start removing the brain from the frontal lobe backwards, cutting each cranial nerve from . Entire dissection was performed carefully.

No more than 3 mm thick.

Equipment uses :-
1-Dissection set
Labelling :-
Label containers with:
a-PI name

b-Animal ID

c-Collection Date

2-Cassette for processing

Put tissues in a labelled (usually with pencil, as solvents dissolve the ink) cassette

3- Mould for embedding

Processing in processing machine
protocol :-
Once fixed, tissue is processed as follows, using gentle agitation, usually on a tissue processor:-

This protocol is for fetus brain tissue & will be acceptable for fetus rat specimen also:-

Program :-10 hours

Beginning by :-

1-70%alcahole   1hr

2-80%alcahole   1hr

3-90%alcahole   1hr

4-95%alcahole   ½ hr

5-100%alcahole   ½ hr

6-100%alcahole   1 hr

7- 100%alcahole   1hr

8- xylene   1hr

9-xylene   1hr

10- paraffin wax   ½ hr

11- paraffin wax   ½ hr

12-parrafin wax   1hr

Dehydration :-By alcohol in different concentration

Clearing by xylem, Size of specimen is too small decrease time to avoid hardening

Impregnation :-In paraffin wax in temperature **58C°** ,that brain tissue is fragile and fatty tissue

Embedding:-

In medium size mould.

Cutting section

by rotary microtome (thermo)

Water bath temperature is fine (40C°),floating section .

dry section in hot plate (40C°) for 5min.

staining :-

We preferred for routine stain mayer Heamatoxylin as progressive stain fresh prepared using eosin for background .

3.4 Data analysis:

Statistical Package for the Social Sciences, SPSS version 20 from IBM was used to analyze the data. Data were expressed as means ± standard deviation. Statistical differences between the groups were evaluated by paired independent sample   t test. Differences yielding P-values < 0.05 were considered statistically significant. along with used of Microsoft Excel 2007 to draw plot of linear regression and formula .

3.5 Ethical consideration:

The ethical approval obtained from of Sudan university research committee council   all experimental procedures were conducted with follow the guidance of institutional animal care.

Summary :

 In order to determine bio effect of ultrasound research use albino rat fetuses at late pregnancy after being exposed to ultrasound for 40 minute , pregnant scarified and fetuses were taken for measurement as described for result

4. Results

From this studies of fifty five albino rats fetuses have been obtained from systemic mating of five groups of pregnant rat t. Statistical analyses of data were performed using Excel Microsoft office 2007 Data were expressed as means ± standard deviation. Statistical differences between the groups were evaluated by paired Student's t test, Differences yielding P-values < 0.05 were considered statistically significant. For histopathological change data was finding was figured with description of finding .
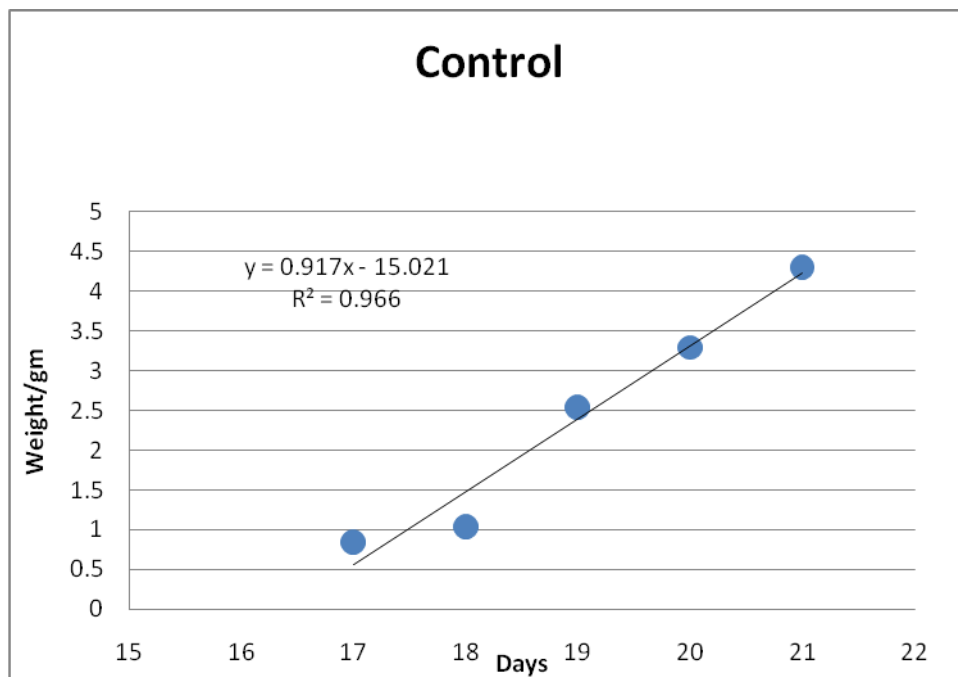

Figure 3-1 Scatter plot show a direct linear increases of fetal weight in control group versus days. where the fetal weight increased by 0.92 g/day.
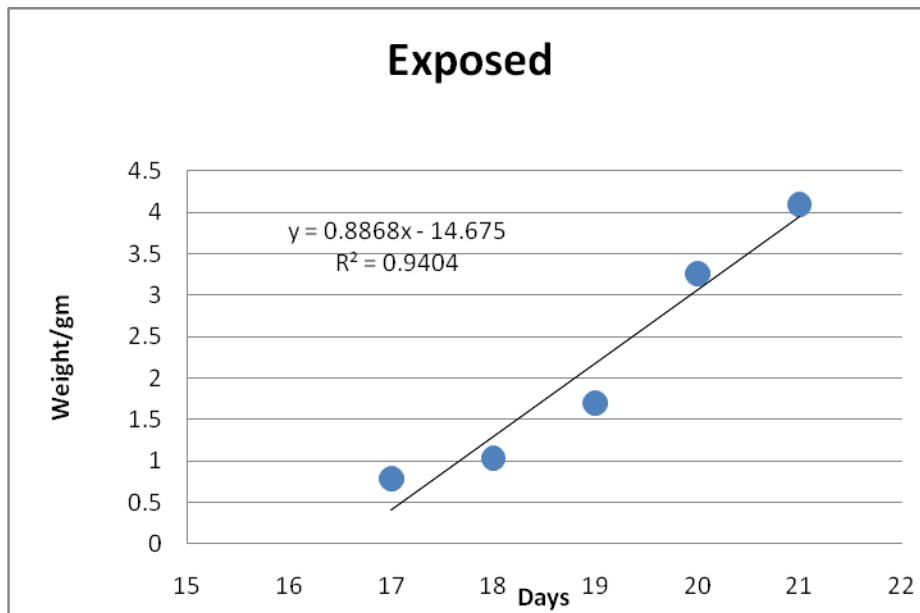
Figure3-2 Scatter plot show a direct linear increases of fetal weight in exposed group (test) versus days. Where the fetal weight increased by 0.89 g/day.
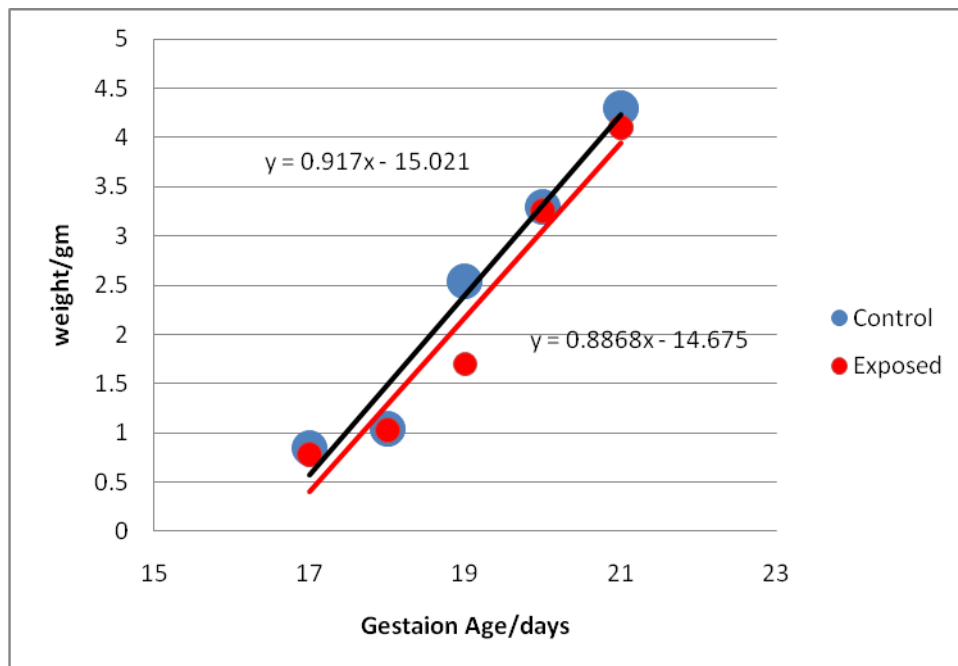

Figure 3-3 Scatter plot show a direct linear increases of fetal weight for both control (black line) and exposed (red line) versus days. where the trend of the two lines were parallel and day 19 is separable day for weight likeness.

Table 3 -1 The average weights of fetal (albino rats) for control and exposed groups and their significance differences using *t*-test

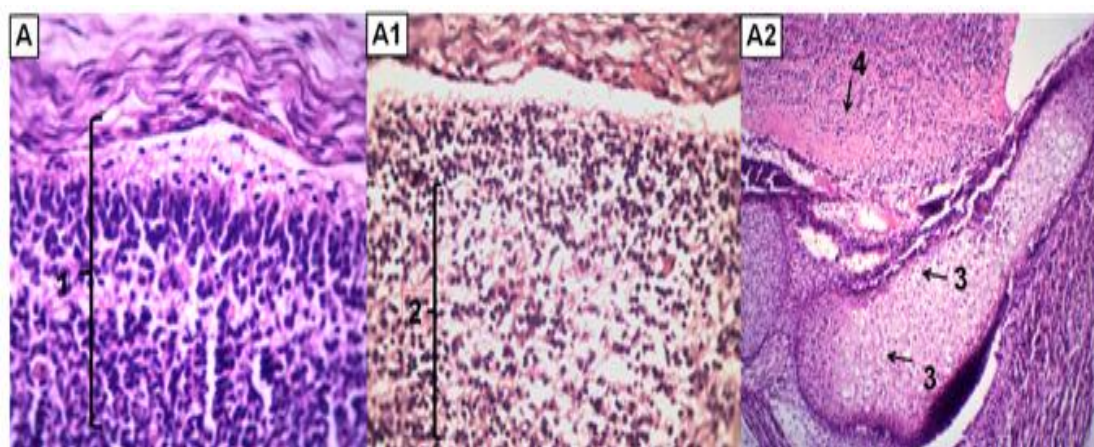| Days	Average weight/g(control)	Average weight/g (exposed)	*t*-value	*p*-value
17	0.84±0.07	0.78±0.029	1.99	0.073
18	1.04±0.08	1.03±0.06	0.12	0.908
19	2.54±0.15	1.7±0.12	11.93	**0.000**
20	3.29±0.35	3.26±0.20	0.19	0.848
21	4.3±0.46	4.1±0.27	2.09	0.066

Figure 3-4   Microphotograph of day 17. A 40x control group show normal, intact cerebral cortex and medulla A1 40x -A2 10x exposed group showing disorganization of cortex with numerous of supporting cells (2) swelling of chondrocytes lacunae (3) loss of arrangement of white matter (4).
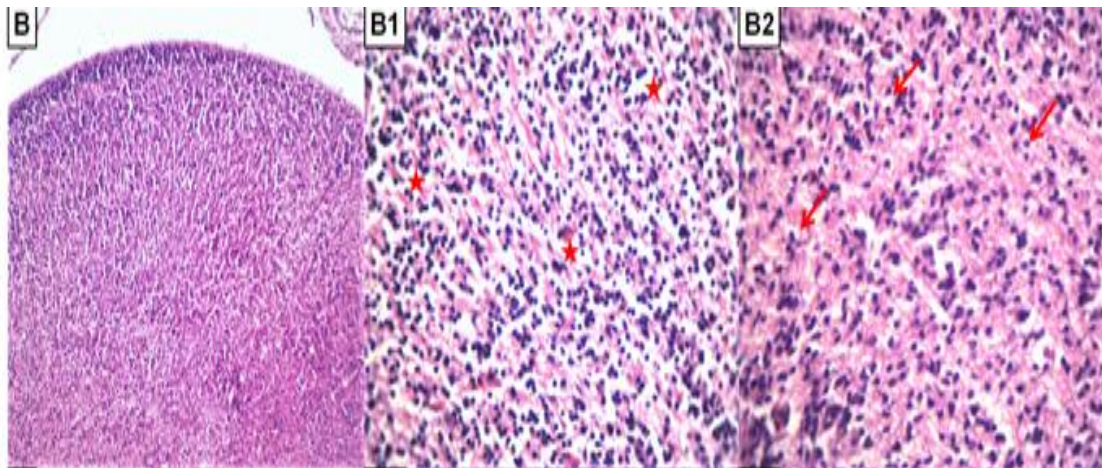
Figure 3-5 Microphotograph of day 18. B 10x control group show normal, intact cerebral cortex and medulla B1 40x -B2 40x exposed group showing neuronal spongiosis, with disorganization of neural cell (★) with vacuoles around glia cell increased sized (red arrows).
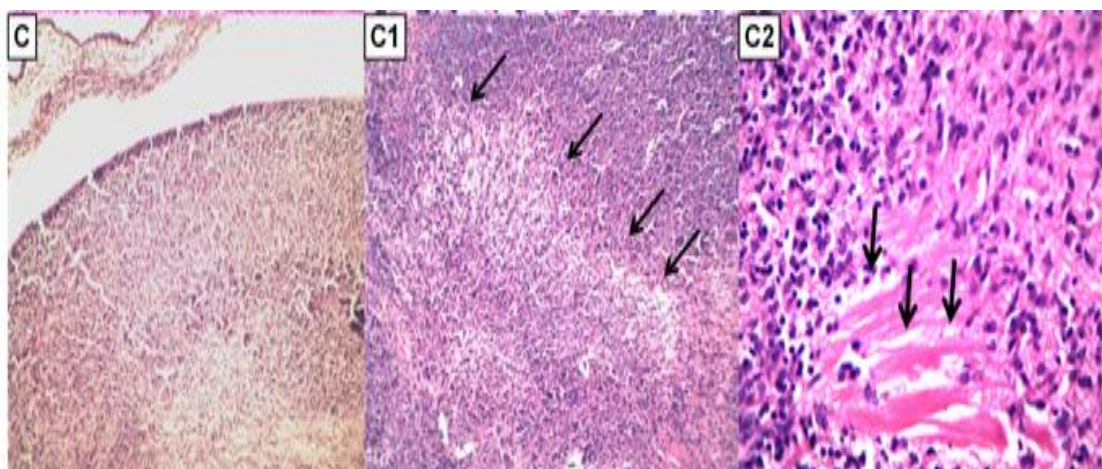

Figure 3-6 Microphotograph of day 19. C 10x control group show normal, intact cerebral cortex and medulla C1 10x -C2 40x exposed group showing slightly vacuolations in brain tissues were recognized specially within the white matter with slight oedema (black arrows)

Figure 3-7 Microphotograph of day 20 D 10x control group show normal, intact cerebral cortex and medulla D1 10x -D2 10x exposed group showing loss of organization of cerebral layers (5)

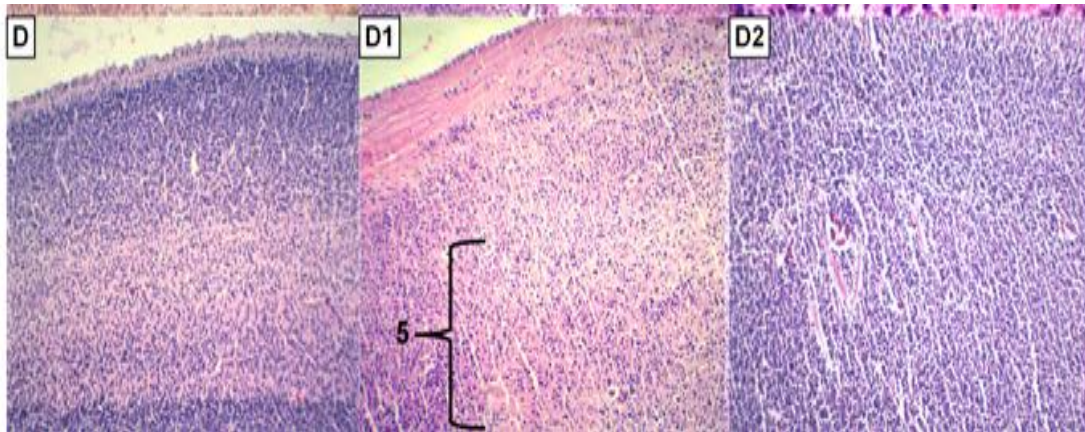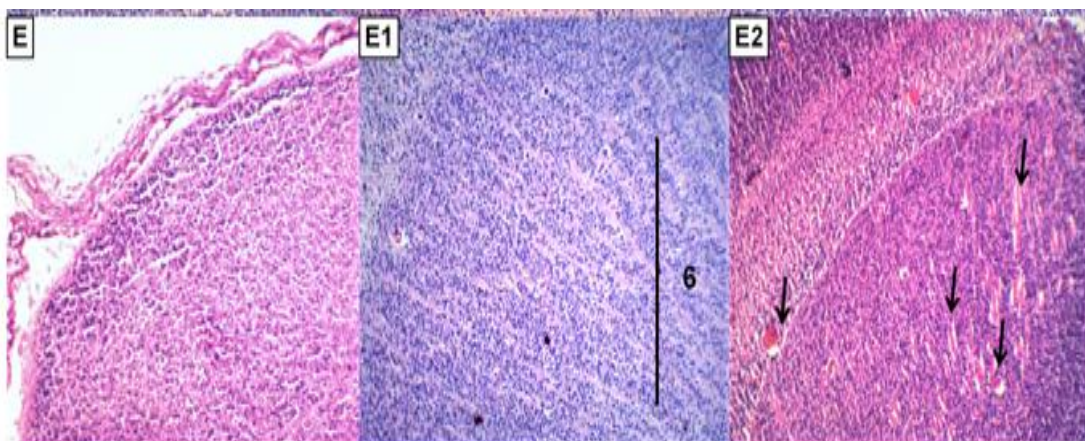

Figure3-8 Microphotograph of day 21 . E 10x control group show normal, intact cerebral cortex and medulla E1 10x -E2 10x exposed group showing multiple change disarrangement of white mater with slight oedema (6) and dilated congested blood vessels different sized of vacuoles between cells (black arrows).

5. Discussion, Conclusions and Recommendations

5.1 Discussions:

Animal research has been important in order to study the effects of various exposures of ultrasound at all stages of pregnancy; since the clinical use of Ultrasonography can take place during the pre-implantation, organogenic, and fetal stages. Animal experiments uses various mammalian species have been able to determine no-effect detected from exposure levels as embryonic defects .(Jensh and Brent, 1999).

Occasional studies report an association between diagnostic ultrasound and some specific abnormalities such as lower birth weight, (Newnham et al., 1993). In our study weights of exposed fetuses showedreductionon17-21 days of gestation age in regards to control group(Figure 3-1,3- 2 and 3-3).This result is in accordance with study done by F. W. A. Zaiki and S. M. Dom using rabbit in different Gestation stages and different exposure time showed that ultrasound exposure might act as agent that promotes intrauterine growth restriction in developing fetus (Zaiki and Dom, 2014). Also Tarnatal and Hendricks conduct experiment using cynomolgus macauques monkey and observed that, there is slight decrease of birth-weight (Tarantal and Hendrickx, 1989). The study showed significant reduction mostly in day 19 (Table3-1).Usually day 19 showed rapid increases in fetal weight and therefore mostly affected by exposure to ultrasound. This finding proved by studies done by Graham and Daniel and confirmed by other study done by Schneidereit 1985 (Graham and Daniel Jr, 1984, Schneidereit, 1985) which realized that high fetal weight obtained during day 19.This means that ultrasound exposure might induce intrauterine growth retardation in 2nd and 3rd trimester depending on the

duration of the exposure as experimental suggested (Jensh and Brent, 1999); intrauterine growth restriction might be resulted from hormonal disturbance or hyperthermia mechanism as suggested (Zaiki and Dom, 2014, Miller et al., 2002)

Regarding to histological evaluation of this study ,examined exposed brain tissue showed changes in histological levels in multiple brain regions ,these observations showed different findings dependant to gestations day , most obvious was disorganizations and arrangement of white matter figure (3-4), (3-6), and (3-8) , with other confounding findings including disorganizations of neural cell ,vacuoles, loss organization of cerebral layer figure (3-5) and (3-7)   previous studies shows sensitivity of developing central nervous and brain to effect of heat ,in addition elevated maternal or fetal temperature can resulted spectrum of adverse outcome (Abramowicz, 2005)

Karagoz et al (2007) study the effect of B-mode and Doppler ultrasound on activities of three antioxidant enzymes and lipid peroxidantion end product in fetal rat brain and resulted of evidence that   B-mode ultrasound and Doppler potential to cause harmful effects possibly increase of radical by high temperature (Karagöz et al., 2007),which may cause significant cytotoxiciy of tissues (Jensh and Brent, 1999) and cause consequential destruction and loss of organization of tissue pattern which it may equivalent to The study findings of loss of tissue disorganizations or differentiation from normal

Haemorrhage near fetal Rat bone exposed to pulsed ultrasound was investigated by Timothy et al 2006 and founded is increased occurrence but not correlated to exposure parameter(Bigelow et al., 2007), in our

study we noted separate ventricular haemorrhage on day 17 which may endorse from ultrasound exposure .

Ange et al 2006 suggest exposure to diagnostic ultrasound is capable of altering neuronal migration in the developing mouse cortex with more effect with long exposure (Ang et al., 2006) , process of migration is high sensitive to exposure from external factor and its deleterious outcome   as our conducted study show loss of cortex and medulla organizations that may impact in process of neuronal migration. vis-à-vis to human although it might differ in developing from animal but the same basic developmental stages of brain development and sequencing occurs (Goldman, 1976). however increased of none right-handedness in male fetuses was suggested to ultrasound induced in utero by Salvesen Ka and Eik-Nes 1999 (Salvesen and Eik-Nes, 1999) ,which is possibly will be due to an increased susceptibility of male fetal brains to ultrasound induced disturbances in neuronal migration and development of synapses (Joy et al., 2006) which extension of exposure effect to behavioural and cognitive process from alteration of brain tissue .

5.2 Conclusion:

Investigator conclude from the results of this study demonstrated , the fetal weight for control group (unexposed) increases by 0.91 g/day while for the exposed grouped increased at lower rate of 0.88 g/day which is become significance at day 19. The results also demonstrate that ultrasound exposure with long duration may adversely affect fetal growth and health wellbeing on mid and full terms, therefore neglected bioeffects of ultrasound should be considered in human Obestrics scan, short duration of exposure should be acquainted with AlARA (as lows as reasonable achievable) principle.

Ultrasound exposure to brain tissue show numerous pattern change in tissue structures in this study , which may affect offspring, cognitive behavioural and adulthood as developmental disturbance from ultrasound. prudent use and apply ultrasound is recommended to avoid such harmful result .

5.3 Recommendations:

More experimental research tackle ultrasound bio effect should be conducted .

Evaluation of exposure by using different animal model in behavioural and activity of postnatal.

Exposure parameter and Dose should be measured to put in consideration the mother and fetus absorption dose.

Extension of field of studies using genetic effect and bio informatics .

Sonographer and health provider should aware community about possibility of ultrasound bio effect.

ALARA principle is essential with prudent use of ultrasound, motoring index TI and MI during exam specially on obestrics is recommended

List of Reference

ABRAMOWICZ, J. S. 2005. Ultrasonographic contrast media. *Journal of ultrasound in medicine,* 24**,** 517-531.

ALI, Q. M. 1993. *Routine Ultrasonography,* new york, vantage press.

ANG, E. S., GLUNCIC, V., DUQUE, A., SCHAFER, M. E. & RAKIC, P. 2006. Prenatal exposure to ultrasound waves impacts neuronal migration in mice. *Proceedings of the National Academy of Sciences,* 103**,** 12903-12910.

BIGELOW, T. A., MILLER, R. J., BLUE, J. P. & O'BRIEN, W. D. 2007. Hemorrhage near fetal rat bone exposed to pulsed ultrasound. *Ultrasound in Medicine and Biology,* 33**,** 311-317.

CHUDLEIGH, P. & THILAGANATHAN, B. 2004. *Obstetric ultrasound: how, why and when*, Churchill Livingstone.

FISHER, J. E., ACUFF-SMITH, K. D., SCHILLING, M. A., VORHEES, C. V., MEYER, R. A., SMITH, N. B. & O'BRIEN, W. D. 1994. Teratologic evaluation of rats prenatally exposed to pulsed-wave ultrasound. *Teratology,* 49**,** 150-155.

FISHER JR, J. E., ACUFF-SMITH, K. D., SCHILLING, M. A., MEYER, R. A., SMITH, N. B., MORAN, M. S., O'BRIEN JR, W. D. & VORHEES, C. V. 1996. Behavioral effects of prenatal exposure to pulsed-wave ultrasound in unanesthetized rats. *Teratology,* 54**,** 65-72.

GOLDMAN, P. S. 1976. Maturation of the Mammalian Nervous System and the Ontogeny of Behavior1. *Advances in the Study of Behavior.* Elsevier.

GRAHAM, T. & DANIEL JR, J. 1984. The effect of accelerated lactation on fetal maintenance in the rat. *Laboratory animals,* 18**,** 103-105.

HANDE, M. P. & DEVI, P. U. 1992. Effect of prenatal exposure to diagnostic ultrasound on the development of mice. *Radiation research,* 130**,** 125-128.

HANDE, M. P. & DEVI, P. U. 1995. Teratogenic effects of repeated exposures to X-rays and/or ultrasound in mice. *Neurotoxicology and teratology,* 17**,** 179-188.

HARA, K., MINOURO, S., OKAI, T. & SAKAMOTO, S. 1977. Safety of ultrasonic waves-irradiation experiment in ICR strain pregnant mice. *Supersonic Med,* 4.

HORDER, M., BARNETT, S., VELLA, G., EDWARDS, M. & WOOD, A. 1998. Ultrasound-induced temperature increase in guinea-pig fetal brain in utero: third-trimester gestation. *Ultrasound in Medicine and Biology,* 24**,** 1501-1510.

JENSH, R. P. & BRENT, R. L. 1999. Intrauterine effects of ultrasound: animal studies. *Teratology,* 59**,** 240-251.

JOY, J., COOKE, I. & LOVE, M. 2006. Is ultrasound safe? *The Obstetrician & Gynaecologist,* 8**,** 222-227.

KARAGÖZ, İ., BIRI, A., BABACAN, F. & KAVUTÇU, M. 2007. Evaluation of biological effects induced by diagnostic ultrasound in the rat foetal tissues. *Molecular and cellular biochemistry,* 294**,** 217-224.

MALHOTRA, N., PURI, R., MALHOTRA, J., MALHOTRA, N. & RAO, J. 2010. Maternal-fetal work-up and management in intrauterine growth restriction (IUGR). *Donald School Journal of Ultrasound in Obstetrics and Gynecology,* 4**,** 427-432.

MCCLAIN, R., HOAR, R. M. & SALTZMAN, M. B. 1972. Teratologic study of rats exposed to ultrasound. *American Journal of Obstetrics & Gynecology,* 114**,** 39-42.

MERRITT, C., KREMKAU, F. & HOBBINS, J. 1992. Diagnostic ultrasound: bioeffects and safety. *Ultrasound in Obstetrics & Gynecology,* 2**,** 366-374.

MILLER, M., NYBORG, W., DEWEY, W., EDWARDS, M., ABRAMOWICZ, J. & BRAYMAN, A. 2002. Hyperthermic teratogenicity, thermal dose and diagnostic ultrasound during pregnancy: implications of new standards on tissue heating. *International Journal of Hyperthermia,* 18**,** 361-384.

NEWNHAM, J. P., EVANS, S. F., MICHAEL, C. A., STANLEY, F. J. & LANDAU, L. I. 1993. Effects of frequent ultrasound during pregnancy: a randomised controlled trial. *The Lancet,* 342**,** 887-891.

NORTON, S., KIMLER, B. F., CYTACKI, E. P. & ROSENTHAL, S. 1990. Acute response of fetal rat telencephalon to ultrasound exposure in utero. *Experimental neurology,* 107**,** 154-163.

O'BRIEN, W. 1983. Dose-dependent effect of ultrasound on fetal weight in mice. *J Ultrasound Med,* 2**,** 1-8.

PINTO, V., WANKELMUTH, M. & D'ADDARIO, V. 2004. Donald School Textbook of Ultrasound in Obstetrics and Gynecology. *Asim Kurjak, Frank A Chervenak, First Ed, Jaypee Brothers, New Delhi, India,* 365.

PIZZARELLO, D. J., VIVINO, A., MADDEN, B., WOLSKY, A., KEEGAN, A. F. & BECKER, M. 1978. Effect of pulsed low-power ultrasound on growing tissues. *Pathobiology,* 46**,** 179-191.

RUMACK, C. M. & LEVINE, D. 2010. *Diagnostic Ultrasound* Elsevier Health Sciences.

SALVESEN, K. & EIK-NES, S. 1999. Ultrasound during pregnancy and subsequent childhood non-right handedness: a meta-analysis. *Ultrasound in Obstetrics & Gynecology,* 13**,** 241-246.

SCHNEIDEREIT, M. 1985. Study of fetal organ growth in Wistar rats from day 17 to 21. *Laboratory animals,* 19**,** 240-244.

SIKOV, M. & PAPPAS, R. 1986. Stage dependence of ultrasound-induced embryotoxicity in the rat. *IEEE transactions on ultrasonics, ferroelectrics, and frequency control,* 33**,** 235-240.

SMITH, J. A. 2010. *Abdominal Ultrasound E-Book: How, Why and When*, Elsevier Health Sciences.

SUCKOW, M. A., WEISBROTH, S. H. & FRANKLIN, C. L. 2005. *The laboratory rat*, Elsevier.

SWERDLOW, A. 2010. Health Effects of Exposure to Ultrasound and Infrasound. *Documents for health protection agency*.

TARANTAL, A. & HENDRICKX, A. 1989. Evaluation of the bioeffects of prenatal ultrasound exposure in the cynomolgus macaque (Macaca fascicularis): I. Neonatal/infant observations. *Teratology,* 39**,** 137-147.

ZAIKI, F. W. & DOM, S. 2014. The effect of prenatal ultrasound heating throughout gestation on rabbit fetal weight. *International Journal of Bio-Science and Bio-Technology,* 6**,** 71-79.

Appendices : Image

Image No (1) show Animal House construction in side Lab

\

Image No (2) show rat during their normal day living activity

Image no (3) show ultrasound model A6 from sonoscape

Image no (4) show weight calibrated scale from brother Inc

Image no (5) show automated histology process of brain fetuses

Image (6) show vaginal smear examination by the presence of vaginal copulation plug

Image no (7) show presence of sperm from vaginal smear

Image no (8) show fetuses on day 19 of gestation