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CORRELATIONS BETWEEN GENETIC ANALYSIS OF ANENCEPHALIC FOETUSES WITH SPINA BIFIDA, CLEFT PALATE, SINGLE UMBILICAL ARTERY AND HISTOLOGICAL ARCHITECTURE OF BRAIN, BRAIN STEM AND SPINAL CORD.

Anatomy

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ABSTRACT

Congenital malformations have emerged as the 3rd commonest cause of perinatal mortality in India. 2.5% babies are born with birth defects in India. Among all congenital anomalies, central nervous system anomalies are the commonest. Anencephaly is the most common CNS malformation. The present study was undertaken to evaluate the development of brain and spinal cord in anencephalic human fetuses (specimens). Human fetuses with anencephaly were collected after obtaining written consent from parents and clearance from ethics committee of the institute as per declaration of Helsinki guidelines after spontaneous miscarriages, therapeutic abortions and still births. All the specimens were fixed in formalin and then dissected. Gross examination and histological studies of the brain and spinal cord were performed in each specimen. Genetic analysis by Karyotyping and FISH was done on the foetuses. The brain was observed as a dark brown undifferentiated mass with complete absence of the cerebellum, pons, medulla and midbrain. Spinal cord was normal in appearance in all foetuses with anencephaly. Histological examination of brain showed venous vessels of varying caliber interspersed with connective tissue, similar to an angioma along with islets of nervous tissue which mainly comprised of scattered nerve cells, astroglial cells and cavities lined by ependyma. The spinal cord is normal in fetuses with anencephaly only while it is deformed in anencephalic fetuses with meningomyelocele. Our findings indicate that there is no functional organization of brain in anencephalic fetuses, and the survival of such fetuses is not possible.

KEYWORDS

Spontaneous miscarriages, Therapeutic abortions, Still Birth, Karyotyping, Anencephalic foetuses.

INTRODUCTION-

Neural tube defects (NTDs) such as anencephaly, spina bifida and encephalocele have predisposition to have Meckel syndrome, median cleft face syndrome, Roberts syndrome, Jarchoe Levin syndrome and HARD (hydrocephalus, agyria, retinal dysplasia) syndrome, chromosomal abnormalities of trisomy 18, trisomy 13, triploidy and other structural aberrations, teratogens of valproic acid, aminopterin/ amethopterin and thalidomide, maternal diabetes, family history, and thermol abile mutation in the MTHFR gene.

Anencephaly is a type of neural tube defect characterized by abnormal development of the brain and the bones of the skull. Anencephaly occurs when the 'cephalic' or head end of the neural tube fails to close, causing the absence of a major portion of the brain, skull, and scalp. Anencephaly is one of the most common types of neural tube defect, affecting about 1 in 1,000 pregnancies. Affected babies are usually blind, deaf, unconscious, and unable to feel pain. Almost all babies with anencephaly die before birth, although some may survive a few hours or a few days after birth.

Kalra et al. [1] presented the incidence of anencephaly as 2.57/1000 followed by spina bifida 2.2/1000, hydrocephalus 1.8/1000 and meningocele 1.47/1000. Verma et al. [2] also observed anencephaly 3.6 in 1000 births, followed by hydrocephalus 2.2, meningomyelocele and meningocele 1 and microcephaly 0.2 in 1000 births. Goravalingappa and Nashi [3] however found hydrocephalus and meningocele as the most common CNS malformations (2.0/1000 births) followed by anencephaly (0.83/1000 births). Chaturvedi and Banerjee [4] showed that among CNS malformations, anencephaly and hydrocephalus were the commonest (1.32/1000 births each). Coffey and Jessop [5] observed incidence of anencephaly as 5.9 in 1000 births in Dublin.

Most cases of anencephaly are Sporadic i.e. occur in people with no family history of anencephaly or other neural tube. Familial: in high rates of consanguinity (mating with family members) where it is inherited in an autosomal recessive manner, Multifactorial: (due to interaction of genetic and environmental factors). Genetic (MTHFR Gene): which provides instructions for making a protein that is involved in processing the vitamin folate, shortage of this vitamin is an established risk factor.

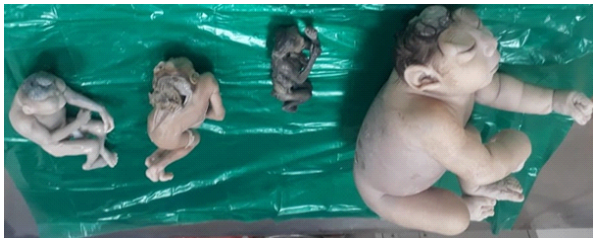
AIMS AND OBJECTIVES-

The present study aims to study the correlations between Genetic Analysis and Histological Architecture of Brain, Brain Stem and Spinal Cord In Anencephalic Foetuses with Spina Bifida, Cleft Lip and Palate.

MATERIALS & METHODS-

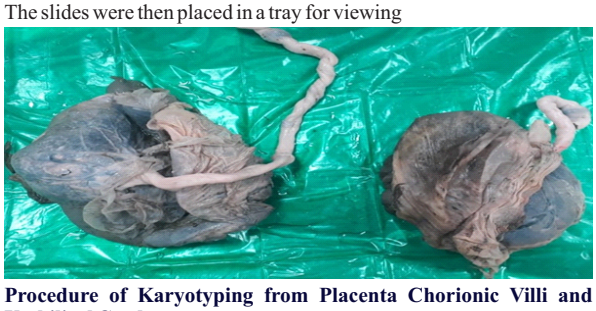
There is very limited data on the actual extent of development of the central nervous system, especially brain in anencephaly fetuses. The study was done to review the extent of development of brain and spinal cord in anencephalic fetuses to deal with ethical issues in defining brain death in such cases as they may be considered as potential organ donors.

In the Department of Anatomy, in a tertiary care hospital in Bhubaneswar 4 Anencephalic foetus of 14 weeks male fetus of 240 gm with Intra Uterine Fetal death (IUFD), 18 weeks female fetus of 70 gm with Intra-Uterine Growth Retardation (IUGR) and Papyraceous, 20 weeks female with Non-Immune Hydrops Fetalis, 36 weeks male fetus of 1.93 kg were procured from Department of Gynaecology and Neonatology. It was observed externally for any obvious congenital abnormalities and then foetal autopsy was carried out. Age estimation was done with the help of menstrual history, crown-rump (CR) length and external appearance of fetus. Cerebellum, Cerebrum, Brain Stem and Spinal Cord were carefully dissected by Dissecting Microscope and the tissues were kept in 10% Formalin for histological processing by H/E stain. The paraffin embedded tissues were processed for Fluorescent in Situ Hybridization (FISH). Conventional cytogenetic analysis of the cultured placental tissues from Chorionic Aminiotic Villus Sampling from Placenta and Umbilical Cord was done for Karyotyping.



Procedure of FISH on Paraffin Sections-

The paraffin embedded slide was placed in an incubator (common lab, hybridization oven, fourth floor) for 2 hours to overnight at 58°C. Seven Coplin jars were filled with the following: xylene – 3, 100% ethanol – 2, sodium thiocyanate – 1, protease solution – 1. The water bath was heated to 80°C, with pretreatment solution (ready prepared sodium thiocyanate from Vysis, fourth floor fridge crisper). The Coplin jar of protease solution (ready prepared from Vysis, fourth floor fridge crisper) at 37°C, 14 minutes was kept. (Protease solution: 250mg protease, 63 ml 0.01 N HCl). The slide was then treated as follows: Xylene 50 ml RT – 4 times; Xylene 50 ml RT – 4 times; Xylene 50 ml RT – 4 times; 100% Ethanol 50ml – RT – 4 times; 100% Ethanol 50ml – RT – 4 times. The air-dried slides were made to stand vertically. The temperature of the water bath was checked which should be 80°C. The dried slides were dropped into the pretreatment solution for 14 minutes at 80°C. The temperature was increased by 1°C for each slide. The slides were removed, rinsed with milliQ water at room temperature for three minutes. The slide should not dry: this can a be holding point. This slide was transferred immediately to protease solution (ready prepared from Vysis, fourth floor fridge crisper) at 37°C, 14 minutes (Protease solution: 250mg protease, 63 ml 0.01 N HCl). The slide was then rinsed in milli Q at RT – 3 times. The slide was air dried, after wiping back of slide and around the tissue. Probe in the marked area was pipetted out. Coverslip was applied to slide and sealed it with rubber cement. The slide was denatured in a 73°C hot plate (digi block heater) for 5min. It was then put in an oven overnight in empty slide box – no humidity at 37°C. All reagents and solutions ready as for standard FISH wash was then kept as: Coplin jar with 2 X SSC with 0.3% NP40, room temp; Coplin jar with 2 X SSC with 0.3% NP40, 73°C; Coplin jar with 2 X SSC with 0.3% NP40, 4 °C. The slides were placed in a Coplin jar which contains 2 X SSC with 0.3% NP40 at RT till coverslips fall off. The coverslip was removed carefully with forceps if they have not fallen off. The slides were then transferred into 2XSSC with 0.3% NP40 at 73°C and kept in this solution for 2 minutes. When the slides are at 73°C, it was transferred to cold (4°C) 2 XSSC with 0.3% NP40 to the work bench. The cold SSC slides were transferred into 2XSSC with 0.3% NP40 at 4°C and kept it in this solution for 2 minutes. It was then air dried, placed at 37°C warmer to dry completely. 8µl DAPI was then added and coverslip was placed. The slides were then placed in a tray for viewing

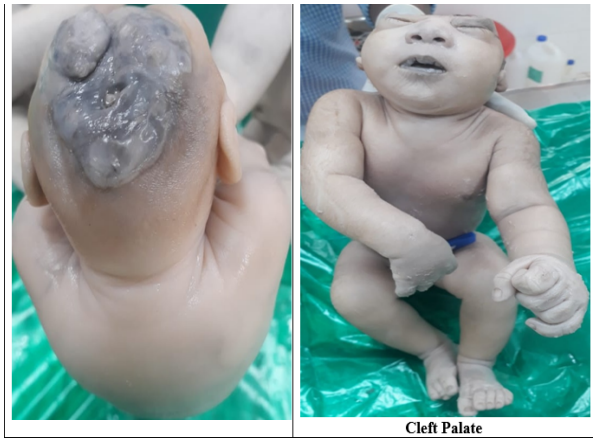


Procedure of Karyotyping from Placenta Chorionic Villi and Umbilical Cord-

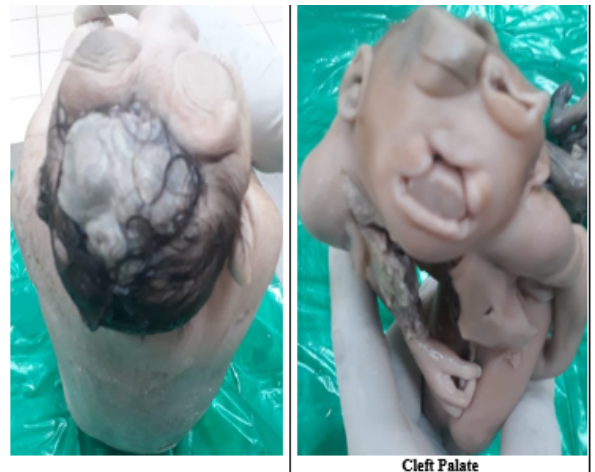
20mg was taken and transferred to petridish. Then it was put in 3-4 ml plain RPMI Medium/ Sterile normal saline 3 times and 2-3 washes were given. Then it was chopped in 1mm diameter and kept in pipette. Typsin was added and EDTA (0.25%) 3-4ml was added to it. It was then incubated 30 min at 37^{o}C. It was then transferred to centrifuge tube for 10 min at 1000rpm. The supernatant was discarded and the tissue was kept at the bottom. 3ml AMINOMAX Medium. Then it was mixed and transferred to culture flask. Then it was kept in 37^{o}C in CO2 Incubator. After 4-5 days days the growth was checked under phase contrast microscope/inverted microscope and AMINOMAX medium 1-2 ml was added to it. Next day the medium was changed and 4-5ml AMINOMAX Medium was added and kept in incubator and then it was left for 2 days. After 10-12 days the tissue was harvested. 30 microlitres of Colchicine was added then it was mixed and placed in Incubator at 37^{o}C and kept for 1 hr. Then it was Centrifuged at 1000rpm for 10 min and the supernatant was discarded and then Vortex was applied to mix. It was then put in Hypotonic 5ml saline (KCl and Tri-sodium citrate) and Vortex was applied to mix for 1-2 min. Then it was put on Incubator for 25 min. Buffy coat and RBC was kept and Supernatant was discarded. Then fixative (Methanol and Glacial acetic acid in 3:1 ratio) 5 ml 20 drops was put and it was placed in Incubator for 1 day at 37^{o}C. Next day it was washed 2 times with fixative and processed for slide making by putting the blood sample at 2 feet distance on the slide. It was placed in dry hot place and put in Incubator at 56^{o}C for 1 day and then at 37^{o}C for 3 days. Then it was processed for Giemsa-Banding (G-Banding). It was put in PBS buffer solution and then in Trypsin for digestion of proteins for 3 sec, then in normal saline and lastly Giemsa stain was done.

OBSERVATIONS-

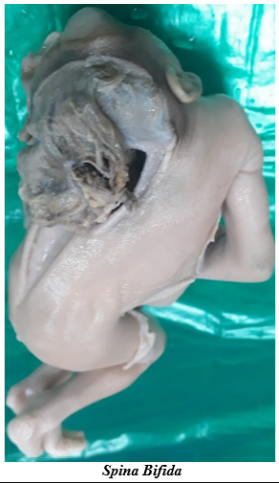
- **External Appearance-** There was no Clubbed Feet and Hand and was without any obvious Pinna or Genitourinary abnormalities. It was also associated with Spina Bifida (Craniospinal Rachichiasis), Cleft Lip and Cleft Palate. There was also a Single Umbilical Artery which is rarely associated in Anencephalic foetuses.

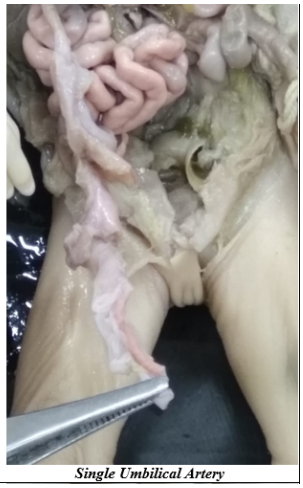


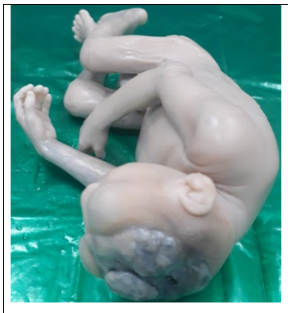
Cleft Palate

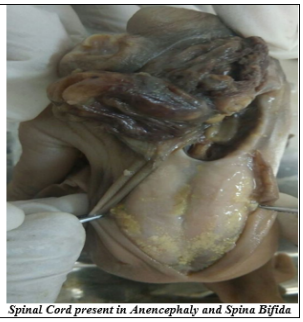


Cleft Palate

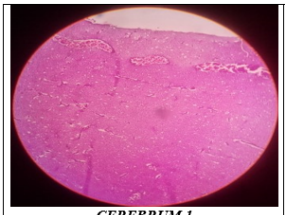

Spina Bifida

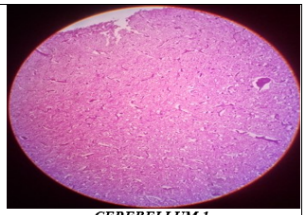

Single Umbilical Artery

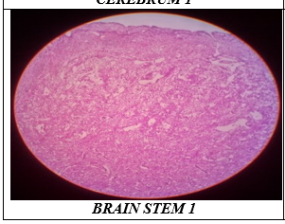


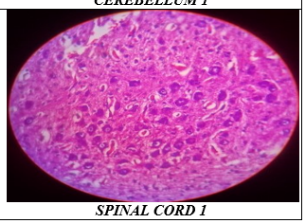

Spinal Cord present in Anencephaly and Spina Bifida

- **H&E stain** of unfused part of neural tube having undifferentiated part of neural tubes shows surface epithelium, degenerated nerve cells and islands of blood vessels of varying caliber interspersed with connective tissue and islets of nervous tissue. The nuclei and motor horn cells were not prominently seen in Spinal Cord. The layers were not prominently distinct in cerebellum and cerebrum.

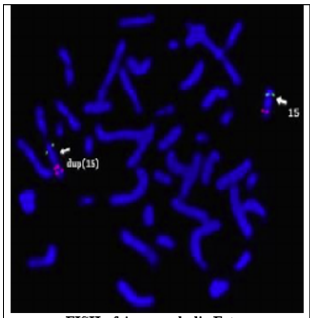

CEREBRUM 1


CEREBELLUM 1

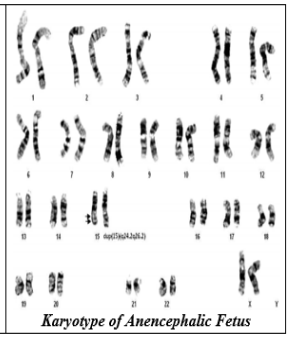

BRAIN STEM 1


SPINAL CORD 1

- FISH Analysis by probes of 5q26.1 Red and 15q11.2 Green showed two red signals and one green signal in the dup(15q) chromosome, and one red signal and one green signal in the normal chromosome 15. Cytogenic analysis revealed karyotype of 46,XX, dup(15) (q24.2q26.2).



FISH of Anencephalic Fetus


Karyotype of Anencephalic Fetus

DICUSSION-

This study was designed to find out the extent of development, organization and differentiation of brain and spinal cord in anencephalic fetuses. The above findings indicate that there is no functional organization of brain and the survival of such fetuses is not possible.

Andersen et al. [6] observed that the brain of anencephaly fetuses mainly consisted of a humpy, dark red mass and its microscopic appearance was reminiscent of an angioma with venous vessels of varying caliber interposed with connective tissue and islets of neurogenic tissue comprising mainly of astroglia and cavities lined with ependyma.

Ashwal et al. [7] in their neuropathological study on anencephaly fetuses, observed unrecognized cerebral structures which was also seen in the present study. This is similar to the observations made by Laurence et al. [8] and Larsen et al. [9] who observed that anencephaly fetuses mostly survive till late gestations in uterus. The findings of predominance of female fetuses with anencephaly in this study are similar to the findings of Menasinki et al. [10]. Brain was seen as a dark brown, undifferentiated mass. This is similar to the findings of Panduranga et al. [11].

However, Vare and Bansal [12] demonstrated presence of rudimentary cerebrum in 53.7% fetuses and rudimentary cerebellum in 14.6% fetuses with anencephaly in their study. In the present study, on histology, scattered neurons were seen with bundles of fibers going in different directions without any organization. Lacro et al. [13] first reported a duplication of 15q22.1-qter and a deletion of 13q32.3-qter in an abortus with an omphalocele and a cephalic defect in neural tube closure. Roggenbuck et al. [14] later reported a duplication of 15q24-q26.3 in a female infant with anencephaly. Putoux et al. [15] reported homozygous deletion in the KIF7 gene in two affected members with anencephaly and the other two affected members with hydrocephalus.

CONCLUSION-

MTHFS is located at 15q25.1 and encodes 5,10- methenyltetra hydro folate synthetase. Elevated MTHFS expression has been shown to increase folate turnover rate or degradation, activity is increased in tumors and MTHFS polymorphisms have been associated with congenital malformations such as congenital heart defects and non-syndromic cleft lip and palate. This study can also be detailed by Using Transmission Electron Microscope (TEM) and Immunohisto chemically by Antibodies and Genetic Analysis by Chromosomal Microarray, PCR and Next Generation Sanger Sequencing.

The correlation between the Histogenesis of Brain Spinal Cord together with the Genetic analysis can help in future Targeted Drug Delivery System In Utero to prevent Congenital Abnormality by Gene Therapy. This may help facilitate harvesting tissues/organ from anencephalic human fetuses for donation for doing research on brain death on these.

It has been found that cartilage homeoprotein (CART1) is selectively expressed in chondrocytes (cartilage cells). The CART1 gene in chromosome 12q21.3–q22 has been mapped. Also, it has been found that mice homozygous for deficiency in the Cart1 gene manifested acromia and meroanencephaly, and prenatal treatment with folic acid will suppress acromia and meroanencephaly in the Cart1-deficient mutants.

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