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Adriamycin-induced caudal regression in the chick embryo model by inhibiting the Shh pathway

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Abstract

Adriamycin,an anthracycline antibiotic, is used for certain cancer treatments but is known to be a teratogen for pregnant women, leading to Caudal regression, a rare congenital malformation affecting lower limbs and vertebrae, causing respiratory, renal, reproductive, and anal issues. In this study, we incubated the eggs for about 2days (48h). the embryos were divided into 3 groups, each group containing 10 embryos. The first group (G1) was the control, the second group (G2) was treated with distilled water, and the third group (G3), was injected with a dose of Adriamycin 0.1 mg/kg. Then the egg groups were returned to the incubator again to complete growth. embryos were collected at two different stages HH24 and HH31. The study found that embryos treated with Adriamycin exhibited caudal regression defects exceeding 90%, with secondary malformations appearing in the head and trunk regions. Also this study found, adriamycin could cause caudal regression in chick embryos by inhibiting the shh signaling pathway, there is a

correlation between shh signaling absence and caudal regression. Comparatively, both Adriamycin-treated and cyclopamine-treated embryos exhibited noticeable caudal regression, highlighting the impact of inhibiting the shh signaling pathway.
Keywords: Adriamycin, developing embryo, caudal regression, chick embryo, Shh pathway.

* Introduction

Adriamycin (ADR), also known as doxorubicin, is a class of anthracycline antibiotics that regulate the effects of various antitumor agents(Singh, Singh, & Sharma, 2005) This medication is employed in the treatment of a range of cancer types, including lymphoma, myeloma, and leukemia, as well as solid tumors such as breast cancer, ovarian cancer, and sarcomas (Ogura, 2001). The first trimester of pregnancy is a critical period for the development of organs in both the embryo and placenta. Of particular importance during this stage is blastogenesis, which is integral to the normal growth of the embryos. Essentially, blastogenesis encompasses the first four weeks of a human embryo's life (Singh et al., 2005) . The Adriamycin drug induces teratogenic effects in mice, resulting in a spectrum of defects similar to the VACTERL association in humans, highlighting the potential of using mice as an animal model for studying congenital malformations.(Mc Laughlin, Hajduk, Murphy, & Puri, 2013). Caudal regression represents a rare congenital malformation that emerges in the caudal region at the onset of pregnancy, causing deformities in the lower limbs, lumbar vertebrae, and coccygeal vertebrae. This condition may lead to complications in the respiratory and kidney systems, as well as abnormalities in the reproductive system (Boulas, 2009).

The development of the VACTERL association, specifically caudal regression, is believed to be impacted by the absence of Sonic Hedgehog (shh) at a molecular level. This morphogen is essential for normal embryonic development and is released from the notochord and floor plate of the neural tube (Mortell, Gillick, Giles, Bannigan, & Puri, 2005). The study was conducted to demonstrate that the

administration of Adriamycin to chick embryo models could induce caudal regression by inhibiting the Sonic Hedgehog (SHH) signaling pathway.

* **Material and methods**

Fertile eggs from the Ross strain of Gallus Gallus, obtained from a local hatchery, were incubated in an incubator manufactured by Thermos Electron Corporation, maintaining a temperature of 38°C and a relative humidity of 70%. The administration of ADR at 0.1mg/kg was carried out by injecting the substance into the yolk using a 1ml syringe through a hole created on the upper surface of the egg, followed by sealing the puncture site with tape.

Then, the eggs were incubated for a duration of 2 days. embryos were segregated into three groups, each consisting of 10 embryos. The negative control group (G1) was left without any treatment, the second group (G2) positive control group, wich is treated with distilled water, while the third group (G3) was subjected to an injection of Adriamycin at a concentration of 0.1 mg/kg. Following this, the eggs were placed back in the incubator for further development. The embryos were harvested at two distinct stages, HH24 and HH31, and then fixed in 10 % paraformaldehyde and kept for further analysis.

*** whole embryo culture experiment**

A simple approach to chick whole-embryo culture is detailed, utilizing a filter paper carrier to keep the early blastoderm and vitelline membranes taut as the embryo grows on a base of agar-albumen. This technique is noted for its quick and effective nature in initiating cultures of chick embryos from pre-primitive streak stages through to stage HH 10 (Connolly, McNaughton, Krumlauf, & Cooke, 1995).

*** Culture media Preparation**

Thirty ml of thin albumin was collected from un-incubated eggs, then equilibrated in a water bath at 50°C. A solution containing 1.2mg of agar (CAS: 9002-18-0) dissolved in 30ml of sodium chloride was also placed in a water bath at the same temperature. The albumin was mixed with the agar solution in a 1:1 ratio through swirling for 30-60 seconds, and 1% penicillin/streptomycin (antibiotic) was subsequently added modified from (Chapman,

Collignon, Schoenwolf, & Lumsden, 2001).

*** Preparation of Betri dishes for embryo culture**

In sterile Petri dishes with a diameter of 15 mm, 4 ml of media were added. The dishes were then divided into groups as follows: five dishes containing embryos served as the control group, five dishes containing embryos treated with Adriamycin at a dosage of 0.1mg/kg added to the media, and five dishes containing embryos treated with Cyclopamine at a dosage of 12.25 µM added to the media (Chapman et al., 2001).

*** The whole embryos culture**

After being incubated for 48 hours at 37-38°C with 80% humidity, the eggs were removed and the embryos were cultured as follows: the eggs were broken, and a prepared filter paper was centered over the blastoderm. The vitelline membrane was cut, and then the filter paper was pulled away from the yolk.

The embryo was washed in distilled water and placed onto culture media. The embryos were then incubated for 24 hours, then collected and fixed in 10% formalin for further analysis.

***Results**

*** Effects of Adriamycin on development of embryo**

*** first HH 24 (4 days) of incubation**

Following a 48-hour incubation period, the embryos were administered a dosage of 0.1mg/kg and subsequently harvested at HH24 (4 days post-treatment). The control group (G1)t exhibited a survival rate of 100%, with no observed abnormalities. Furthermore, the group that received treatment with distilled water (G2) similarly demonstrated a survival rate of 100%, with no instances of embryonic mortality and no recorded abnormalities. Conversely, the group subjected to Adriamycin injection (G3) presented a survival rate of 38% and an abnormality rate of 75% among all surviving embryos, as illustrated in figure 1.

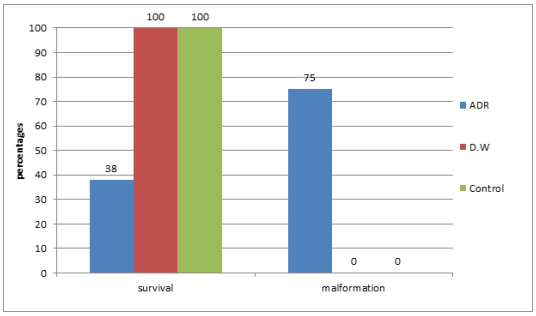

Figure 1: Histogram showed a percentage of survival, death, fertility and malformation in control embryos, embryos treated with D.W, and embryos treated with ADR 0.1mg/kg at HH24.

* Effects of Adriamycin on development on embryo

* Second at HH 31(7 days) of incubation

The secondary collection transpired at developmental stage HH31, which corresponds to a duration of seven days post-incubation. Within the control cohort (G1), the survival rate was observed to be 100%, with no instances of malformations reported. In the cohort receiving treatment with distilled water (G2), the survival rate remained at 100%, and no malformations were noted. Conversely, in the group administered Adriamycin (G3), the survival rate plummeted to 13%, while the malformation rate was documented at 77%, among all surviving embryos as depicted in figure 2.

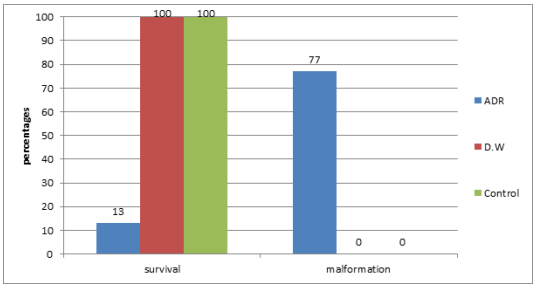

Figure 2: Histogram showed a percentage of survival, death, fertility and malformation in control embryos, embryos treated with D.W, and embryos treated with ADR 0.1mg/kg at HH31.

* Morphological effects of Adriamycin on developing chick embryo

During the HH24 stage, the control group of embryos is denoted by the letter A in figure 3. In this stage, the embryos appear in a normal form with developing organs such as the head parts, eyes, naturally curved trunk area, heart, and forelimbs. The caudal area and hind limbs show normal growth. On the other hand, embryos treated with distilled water are labeled as A1, also developing normally with similar characteristics to the control group. However, embryos treated with Adriamycin, represented as A2, exhibit abnormal development with abnormalities in prosencephalon, mesencephalon,rhombencephalon, microphthalmia, trunk curvature,

ectopic heart, and deformed forelimbs.

Adriamycin treatment at the HH31 stage resulted in the embryos being labeled as B2, and these embryos displayed abnormal morphology. Specifically, the head was larger in size compared to the control group, and there were structural defects in the prosencephalon, mesencephalon, and rhombencephalon. Additionally, craniofacial hypoplasia and cephalomegaly were observed, along with a shortened trunk, absence of curvature, and ectopic features. Furthermore, caudal regression was evident in the caudal region.

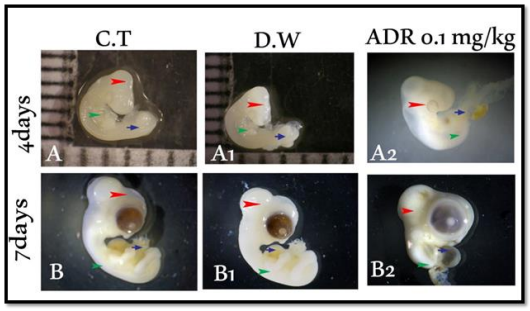

Figure 3: lateral of whole embryo showed the effect of Adriamycin 0.1mg/kg injection after 48h and collection of embryos appears in two different stages. Red arrows point to the head region, blue arrows to the trunk region, and green arrows to the caudal region

Adriamycin induced caudal regression via inhibiting SHH pathway during development.

The experiment aimed to investigate the correlation between the lack of shh signaling and the development of caudal regression. Chick embryo cultures were established, with the control group cultured on albumin agar media, and the Cyclopamine treated embryos cultured on albumin agar media containing 0.5mg/ml Cyclopamine, a known shh inhibitor. Additionally, Adriamycin was introduced to the culture in the Adriamycin treatment group.

The comparison between embryos treated with Adriamycin and those treated with Cyclopamine revealed similar outcomes. The control embryo, denoted by letter A at stage HH16, exhibited normal development with a properly formed head region including prosencephalon, mesencephalon, and rhombencephalon. The lateral body-folds extended to somites 17-20, while the tail-bud appeared as a short, straight cone. In contrast, embryos treated with Adriamycin (letter B) and Cyclopamine (letter C) displayed abnormal features at stage 16. Both showed abnormalities in the head region, with significant distortions in

prosencephalon, mesencephalon, and rhombencephalon. Deformities were also observed in the trunk region, particularly in heart development, and in the caudal region with an opening at the end of the neural tube and somite growth defects. These findings are illustrated in the accompanying figure 4.

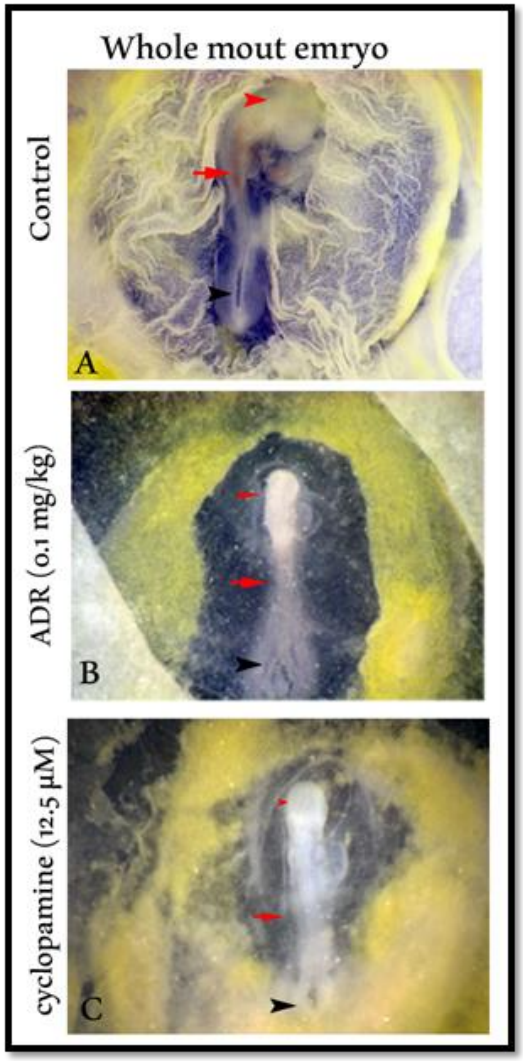

Figure 4: Whole embryos culture shows the effect of Adriamycin and Cyclopamine on shh signaling. Red arrows point to the head region, blue rrows to the trunk region, and green arrows to the caudal region

* Discussion

The study illustrated the impact of Adriamycin on the development of caudal regression during the early stages of chick embryo development, to investigate the disruptions caused by Adriamycin in human

embryonic development. Moreover, the research confirmed the understanding of the molecular mechanism underlying the occurrence of this malformation, underscoring the value of the chicken model in ensuring the precision of the study.

*** Morphological evaluation of Embryos treated with 0.1mg/kg by ADR injected after 48h of incubation**

Results from this study showed that Adriamycin caused caudal regression in over 90% of treated embryos, with the absence of the caudal region observed in both stages HH24 and HH31. These results were consistent with a study (Kotsios, Merei, Hutson, & Graham, 1998) when they got Six of the nine with caudal anomalies also had an imperforate anus. limb anomalies in 35% of embryos

Also These results are consistent with (Naito et al., 2009), who documented caudal regression in more than 90% of Adriamycin-treated of embryos, particularly at stages HH10-11, while Adriamycin was given to embryos in the late stage HH14-16, the incidence of caudal regression only did not exceed 10% in the treated embryos.

In current study, brain malformations that appeared in 99% of all embryos, these results consistent with the results of the research paper by Mortell et al. (2003) reported anophthalmia & exencephaly in chick embryos treated with Adriamycin.

In addition trunk region, we noticed the presence of ectopic heart in most of the embryos, and these results consistent with the results obtained from study by (Merei, Hasthorpe, Farmer, & Hutson, 1999).

The correlation between the lack of shh and the manifestation of caudal regression.

This experiment was structured to validate the hypothesis that the introduction of Adriamycin to chick embryos would result in caudal regression through the inhibition of the sonic hedgehog pathway.

Cyclopamine was used as inhibitor for shh signaling pathway by inhibiting the interaction between shh ligands and their respective receptors located on the cellular membrane as demonstrated by Incardona and others in 2000 (Incardona et al., 2000). Embryos were cultured using media containing either

ADR or Cyclopamine as a suppressor of the Shh pathway, or in the absence of any additional substances as a control.

The findings indicated that 95% of the embryos exhibited anomalous alterations in the caudal region throughout the early embryonic development of the chick.

SHH signals are crucial for cellular differentiation and organ formation. They release a protein from the notochord, bind to receptors, and activate Gli2A and Gli3A, initiating gene expression. It's may Adriamycin alters the notochord's structure, causing alterations that hinder the shh gene's function. This results in inactive receptors for shh, affecting gene expression. Zinc-finger transcription factors Gli2R and Gli3R delay Gli1 activation, leading to a deficiency in organ development (Jeng, Chang, & Lin, 2020). Therefore, it may ADR induces pluripotent differentiation, altering the differentiation potential of tissues such as the central nervous system, formation of various organs, such as the lower limb, is thought to be affected in infancy and can explain the fetal multiple malformations.

* Conclusions

Adriamycin in dose used in this work demonstrates an impact on various regions of the embryo, resulting in over 90% of all embryos displaying caudal regression defects. The validation of the hypothesis regarding Adriamycin's influence on the Shh pathway was substantiated through the using of Cyclopamine as a Shh inhibitor in whole embryo culture. More studies need to be done to more conformations.

* References

Boulas, Mari M. (2009). Recognition of caudal regression syndrome. *Advances in* Neonatal *Care, 9*(2), 61-69.

Chapman, Susan C, Collignon, Jérôme, Schoenwolf, Gary C, & Lumsden, Andrew. (2001). Improved method for chick whole-embryo culture using a filter paper carrier. *Developmental dynamics: an official publication of the American Association of Anatomists*, 284-289.

Connolly, David, McNaughton, Linda A, Krumlauf, Robb, & Cooke, Jonathan. (1995). Improved in vitro

development of the chick embryo using roller-tube culture. *Trends in genetics*, 259-260.

Incardona, John P, Gaffield, William, Lange, Yvonne, Cooney, Adele, Pentchev, Peter G, Liu, Sharon, . . . Roelink, Henk. (2000). Cyclopamine inhibition of Sonic hedgehog signal transduction is not mediated through effects on cholesterol transport. *Developmental biology*, 440-452.

Jeng, Kuo-Shyang, Chang, Chiung-Fang, & Lin, Shu-Sheng. (2020). Sonic hedgehog signaling in organogenesis, tumors, and tumor microenvironments. *International journal of molecular sciences*, 758.

Kotsios, Christine, Merei, Jamal, Hutson, John M, & Graham, H Kerr. (1998). Skeletal anomalies in the adriamycin-exposed prenatal rat: A model for VATER association. *Journal of orthopaedic research*, 50-53.

Mc Laughlin, D, Hajduk, P, Murphy, P, & Puri, P. (2013). Adriamycin-Induced models of VACTERL association. *Molecular Syndromology*, 46-62.

Merei, J, Hasthorpe, S, Farmer, P, & Hutson, JM. (1999). Visceral anomalies in prenatally adriamycin-exposed rat fetuses: a model for the VATER association. *Pediatric surgery international*, *15*, 11-16.

Mortell, Alan, Gillick, John, Giles, Shay, Bannigan, John, & Puri, Prem. (2005). Notable sequential alterations in notochord volume during development in the Adriamycin rat model. *Journal of pediatric surgery*, 403-406.

Naito, Yoko, Kimura, Tokuhiro, Aramaki, Michihiko, Izumi, Kosuke, Okada, Yasunori, Suzuki, Hidekazu, . . . Kosaki, Kenjiro. (2009). Caudal regression and tracheoesophageal malformation induced by adriamycin: a novel chick model of VATER association. *Pediatric research*, 607-612.

Ogura, M. (2001). Adriamycin (doxorubicin). *Gan to*

kagaku ryoho. Cancer & chemotherapy, 1331-1338.

Singh, Santosh Kumar, Singh, Rupa Dalmia, & Sharma, Akhilesh. (2005). Caudal regression syndrome—case report and review of literature. *Pediatric surgery international*, 578-581.