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Teratogenic Effect of Disodium 5' Ribonucleotide (E635) on 15th Day Chick Embryo *Gallus gallus* (Research note)

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Abstract

Disodium 5' ribonucleotide (E635), commonly used as a food additive, may pose teratogenic risks. The study aimed to evaluate the teratogenicity of disodium 5' ribonucleotide using chick embryos as an in vivo model. Fertilized chicken eggs were inoculated with LD_{50} dose of 0.05 µg/egg of disodium 5' ribonucleotide on the 0th, 5th, and 10th days of embryonic development. The control group received 0.05 mL of distilled water on the 0th, 5th, and 10th days. After 15 days of incubation, embryos were examined for morphological abnormalities. The disodium 5' ribonucleotide inoculated embryos exhibited significant developmental anomalies, including acrania, monophthalmia, bent toes, curved beak, scissor beak, omphalocele, and hematoma, compared to the control group. These abnormalities were categorized and analyzed through morphometric assessments to evaluate their severity. Data were subjected to statistical analysis using t-test to find out significance of developmental anomalies in dose induced group and control group animals. Anatomical changes indicated that disodium 5' ribonucleotide exposure led to severe teratogenic effects in chick embryos, which has highlighted potential risks associated with its use as a food additive.

Introduction

In general, teratogenicity involves structural abnormalities during embryonic development and found suitable for understanding the potential risks posed by various agents (Calado and dos Anjos Pires, 2018). To find out the impact of external factors, or teratogens, on embryonic development has crucially aided field of teratology, which studies congenital anomalies and their causes (Wachholz *et al*., 2021). Chemicals, drugs, recreational drugs, pollutants, physical agents, and maternal metabolic conditions are all examples of biological and environmental teratogens (Narkowicz *et al*., 2013; Abbey and Kua, 2022). Teratogenicity provides information about substances that cause defects or abnormalities in developing embryos, is an important topic, especially when those substances are linked to commonly consumed foods (Hashem *et al*., 2019).

In the field of toxicology, among vertebrates, avian embryos were used as important laboratory model to study embryological changes and teratogenic alterations induced due to toxicants (Salvaggio *et al*., 2018). Easy availability, stage wise development, clear-cut organization and time bound embryonic features were recommendable scientific advantages to accept chick model for toxicity study (Vergara and Canto-Soler, 2012). Because of these characteristics, the chicken embryo is ideally suited for drug screening, teratogenic evaluations, and research into teratogenicity's underlying mechanisms (Bjørnstad *et al*., 2015). Moreover, the chick embryo organism shares morphological, biochemical, and hereditary similarities with human and other mammals, which enhance its importance for extrapolating discoveries to human teratology (Wachholz *et al*., 2021).

Worldwide among developing and developed countries, the majority population preferentially go for ready-made consumables available all the time in market prepared by addition of some additives and supplementary preservatives, to increase quality and flavor (Thakur *et al*., 2022). Umami taste substances were usually classified into two categories viz., amino acid seasonings, such as glutamate whereas other nucleotide seasonings including disodium 5' ribonucleotide (Hajeb and Jinap, 2015). Most of the processed foods were associated with quantity of disodium 5' ribonucleotide as a flavor enhancer (Jung

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and Koh, 2016). Chemically, it is a combination of Disodium Guanylate and Disodium Inosinate (Campagnol *et al*., 2011).

By taking review of available literature and with the intention of toxicity assessment, present investigation aimed to focus the observing and reporting of disodium 5' ribonucleotide against developmental model of chick embryo up to 15th day of incubation. The obtained results were documented for morphometric alterations and overall physiological changes.

Materials and methods

Animal and Intoxicant under study

Fertilized chicken (*Gallus gallus*) 40 eggs were obtained from a local hatchery situated in Miraj, Maharashtra, India. Prior to incubation, eggs were inspected to ensure viability, and any visibly damaged or cracked eggs were discarded. Disodium 5' ribonucleotide was obtained from Arochem private limited and prepared as a solution in distilled water at a concentration of 1 mg/L.

Experimental Design

The eggs were randomly divided into two experimental groups:

1. Control Group: 20 eggs inoculated with 0.05 mL distilled water on the 0th, 5th, and 10th day of incubation.
2. Dose-Induced Group: 20 eggs inoculated with 0.05 mL of disodium 5' ribonucleotide solution on the 0th, 5th, and 10th day of incubation.

Administration Protocol

Solutions were injected using 31G syringes (Kanagaraju and Rathnapraba, 2019). After each injection, holes were sealed using a cotton and tape to prevent contamination. Selected eggs were incubated at 37.5±0.5 °C and 70±5% relative humidity for the 15 days of the experiment in an Egg Hatcher Incubator (MSW-233) (Nihad *et al*., 2018).

Observation of Teratogenic Effects

Experimental embryos were observed daily using candling method to assess the health of developing embryo. Embryos if found dead were removed from the experimental procedure. Up to 15th day of incubation, all embryos were critically observed for any developmental abnormalities including malformations, vestigial growth, and skeletal deformity. Calibration and documentation were carried out for perfect analysis and interpretation. All developing embryo keenly observed from all sides and images were captured by keeping the embryos on graph paper for further documentation and analysis.

Statistical Analysis

After completion of all the experimental protocol and observation obtained, for statistical analysis, data was subjected to Microsoft excel version 2019 for analysis. *P* value was determined using *t*-test (Andersson *et al*., 2015). Level of significance was considered at $P < 0.05$ (Hussein and Singh, 2016). Numerical data and calibrated count were finally interpreted for stage of malformation among developmental condition of all embryos.

Results

The teratogenic effects of disodium 5' ribonucleotide on 15th day chick embryos, a vertebrate model was extensively studied. Numerous developmental defects were identified. The specific findings for each type of malformation observed were documented as below, with reference to the corresponding figures.

Control Embryos (Figure 1a)

Control embryos inoculated with distilled water developed as expected and served as a base for normal development. These embryos exhibited typical morphological features such as fully developed limbs, proper allotment of wings, and appropriately positioned internal organs. This normal developmental profile was important to compare with the teratogenic effects observed in disodium 5' ribonucleotide inoculated embryos.

Omphalocele (Figure 1b)

Notable teratogenic effects such as omphalocele were found during development of some embryos. In these cases, anatomically the abdominal wall was closed partially, with the heart, liver, and stomach protruding from the body cavity. This condition indicates a significant disturbance in the normal process of abdominal closure during development. The embryo showed scanty dermal derivatives. Cutaneous, specifically skin differentiation and maturation was underdeveloped and showed structural and morphological changes.

General Underdevelopment and Acrania (Figures 1c and 1d)

Embryos intoxicated with disodium 5' ribonucleotide showed signs of gross underdevelopment. These embryos were significantly smaller and had rudimentary limbs compared to controls. The embryo was found featherless. In one particularly severe case, the fetus lacked a skull, leaving the brain exposed, condition called acrania. This severe malformation indicated an impaired development of the skull (cranial box), which is essential for protecting the brain. The presence of red blood spots on the brain of this embryo indicated a compromised vascular integrity and hemorrhage, indicating an underlying systemic developmental problem.

Limb Deformities – Crooked Toes (Figure 1e)

During embryonic development, limb formation is a complex process that requires precise control of gene expression, cell proliferation and differentiation. The particular limb deformity with twisted toes were found in the embryo on its left hind limb. Malformation indicated disturbance of musculoskeletal limb development. Twisted toes indicate misalignment and structural anomaly of the limbs.

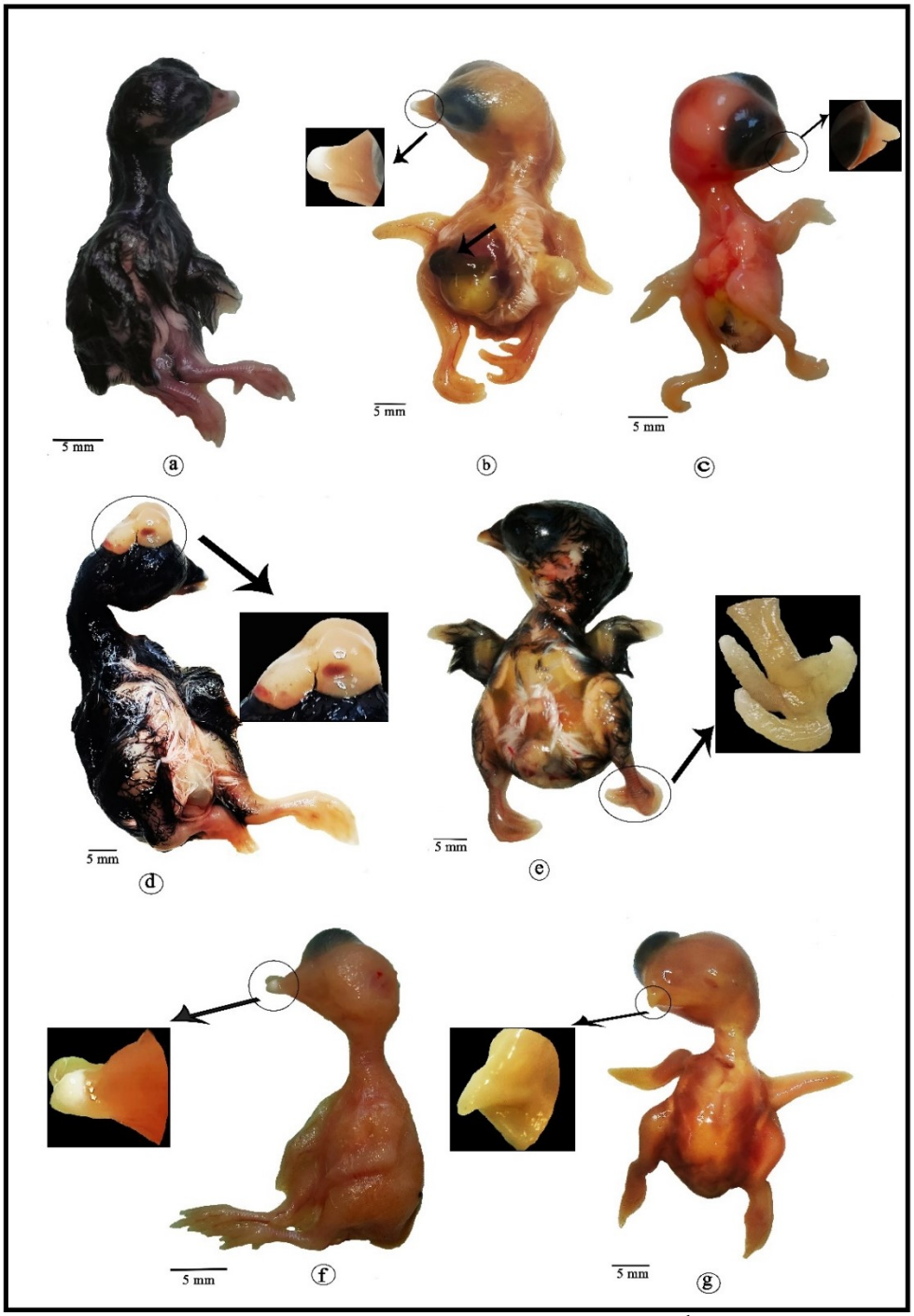

Figure 1. Embryonic development of experimental model *Gallus gallus* at 15th day of incubation. **a.** Control **b.** Disodium 5' ribonucleotide induced embryo with Omphalocele and stunted lower beak. **c.** Disodium 5' ribonucleotide induced embryo showed in general rudimentary and featherless body. **d.** Disodium 5' ribonucleotide induced embryo showed acrania and hemorrhage in brain. **e.** Disodium 5' ribonucleotide induced embryo showed twisted toes. **f.** Disodium 5' ribonucleotide induced embryo with monophthalmia and scissor beak**. g.** Disodium 5' ribonucleotide induced embryo with monophthalmia and parrot (curved) beak.

Monophthalmia and Beak Abnormalities (Figures 1f and 1g)

Severe craniofacial abnormalities were also observed. Embryos exhibited monophthalmia, i.e. presence of only one eye. Underdeveloped condition indicated significant disruption in eye development with craniofacial malformation. Number of embryos had a crossbill or scissor-like beak. Morphological deformity as parrot beak, characterized by a significantly longer upper beak compared to the lower beak, forming a curved structure. In general, craniofacial modifications suggest that disodium 5' ribonucleotide affect molecular pathways involved in craniofacial development and integumentary system differentiation.

Rudimentary lower beak (Figures 1b and 1c)

A number of consistent abnormalities were observed in developing embryos. Particular craniofacial malformation indicated toxic and teratogenic effect on developmental biology of experimental animal. Beak development is a complex and crucial procedure and controlled by multiple signaling pathways, but disodium 5' ribonucleotide has disrupted the beak formation and disproportionate growth of beak structures, leading to facial changes in the embryo.

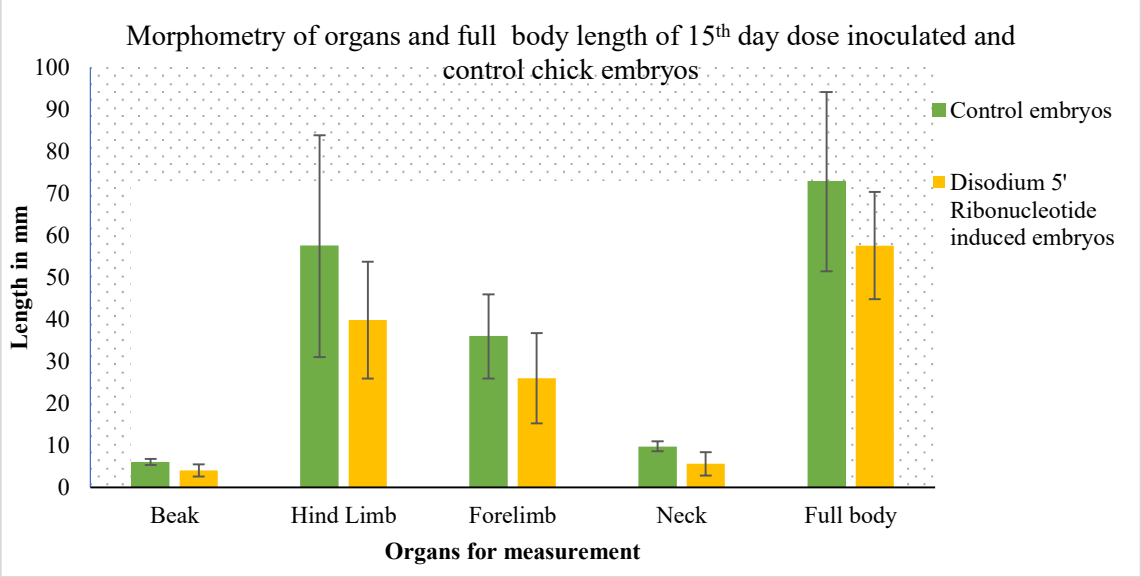

Figure 2. Quantified data of dose induced organogenesis against normal development in chick embryo. Error bars represent standard deviation.

Morphometric analysis for the beak, hind limb, forelimb, neck, and full body length were taken and analyzed. The results are expressed in Figure 2. Our study revealed significant morphological differences between control and disodium 5' ribonucleotide induced chick embryos. Quantified data showed that disodium 5' ribonucleotide induction significantly affected the development of certain organs like beak and neck. The length of beak was significantly reduced in the disodium 5' ribonucleotide inoculated group as compared to the control group with a *P*-value of 0.032. There was also a significant reduction found in neck length in disodium 5' ribonucleotide inoculated group of chick embryos as compared to the control, with a moderately significant *P*-value of 0.002.

Although reductions were also observed in the hind limb, forelimb, and full body lengths, these differences were not statistically significant. These results suggest that disodium 5' ribonucleotide has a noticeable teratogenic effect on the development of the beak and neck in chick embryos.

Discussion

Under the developmental research, our findings revealed significant teratogenic effects of predetermined dose of disodium 5' ribonucleotide by Sharikmaslat and Kamble (2024) on 15th day chick embryos, indicating multiple pathways of developmental disruption. The variety of severe conditions were observed during the embryonic development.

Important signaling pathways regulating embryonic development, such as Sonic Hedgehog (Shh), Wingless-related integration site (Wnt), and fibroblast growth factor (FGF), may have been disrupted by disodium 5' ribonucleotide. The Shh signaling pathway plays a key role in craniofacial development, limb patterning, and organogenesis in chick embryo (Abzhanov and Tabin, 2004). Disruption of this pathway may explain the observed craniofacial deformities (skull and beak abnormalities) and limb defects (curved toes). Acrania was noticed in disodium 5' ribonucleotide induced chick embryo, this type of observation was also observed for chick embryos induced with

Bisphenol A (Zhang *et al*., 2021). Similarly, the Wnt signaling pathway is essential for proper formation of skin (Widelitz, 2008) and feathers (Xie *et al*., 2020), and its disruption may have led to lack of feather development similar to the observation where, Acetamiprid with Thiamethioxam caused scanty feathers in chick (Taha and Mohammed, 2022) .

Teratogens can cause oxidative stress, leading to cell damage and apoptosis (Hansen, 2006). In present study, oxidative stress might have caused malformations in chick embryo as proved by Carmichael *et al*., (2023). High levels of reactive oxygen species can cause DNA damage, protein oxidation, and lipid peroxidation, leading to cell death and developmental abnormalities (Stadtman and Levine, 2000; Gahalain *et al*., 2011; Farag *et al*., 2021). The presence of red blood spots on brain and underdeveloped structures in treated embryos suggests that oxidative stress played an important role in the observed teratogenic effects.

Disodium 5' ribonucleotide may have affected on the expression of important developmental genes. Genes of the Hox family were reported as important for limb and skull patterning (Mallo *et al*., 2010). Misexpression or downregulation of these genes may have led to the observed limb deformities; curled toes were observed in our study, similar observations were found for selenium toxicity on chick embryo (Surai, 2002) and skull abnormalities like monophthalmia were observed similar to the results after inoculation chick embryos with Monosodium Glutamate (Al-Qudsi and Al-Jahdali, 2012) .

Anatomically and morphometrically, cross-beak, parrot-beak and craniofacial developmental deformities were prominent in the dose-induced group, indicating craniofacial developmental disruptions. These findings align with a similar study, which reported beak deformities associated with developmental anomalies in embryos treated with smokeless tobacco (Talukdar *et al*., 2020). Additionally, genes involved in the formation of the abdominal wall might have affected, leading to conditions such as omphalocele, it was also observed after the induction of cadmium in chick embryo (Thompson and Bannigan, 2007). Msx1 and Msx2 genes may be responsible for causing omphalocele (Doi *et al*., 2010). In present study, limb deformities, including crooked and twisted toes, were observed in the hind limb of treated chick embryos, which might be one of the representation of misexpression of vital regulatory genes such as Hoxd-13, which plays a crucial role in development and growth of limb pattern (Fabre *et al*., 2018).

The avian embryo model provides valuable information about the teratogenic effects of chemicals and their mixtures and similarities in to human development (Garcia *et al*., 2021; Wachholz *et al*., 2021). Malformations such as craniofacial defects, omphalocele, and limb deformities observed in chicken embryos resemble human congenital anomalies (Rosano *et al*., 2000). Anatomical anomalies in chick embryos strengthen previous findings regarding teratogenicity in the field. Induction of the chemical led to rudimentary organogenesis, causing an overall impact on the developing embryo. By understanding these mechanisms, regulatory decisions can be made regarding the dose-dependent use and abuse of food additives in various nutritional sources.

The results also indicate that, disodium 5' ribonucleotide has significant teratogenic effects on the chick embryos, particularly in the development of the beak and neck. The beak length and neck length showed a marked reduction in the disodium 5' ribonucleotide inoculated group of chick embryos. Similar findings were reported where malformations in beak observed in copper sulfate immersed chick embryos (Szabó *et al*., 2024). While the measurements of the hind limb, forelimb, and full body length also showed reductions in the disodium 5' ribonucleotide induced group of chick embryos compared to the control group of embryos, these differences were not statistically significant. Similar findings were reported where endosulfan impact was studied on the developing chick (Mobarak and Al-Asmari, 2011). Through a practical approach, observation and documentation, we found that prominent organogenesis of the beak and neck has significant reduction.

These findings align with previous studies of the Monosodium Glutamate indicating that certain food additives can interfere with normal embryonic development, leading to physical malformations (Al-Qudsi and Al-Jahdali, 2012). The significant reduction in beak and neck lengths could imply potential mechanisms where disodium 5' ribonucleotide interfere the molecular pathways essential for anatomical developmental features of embryo. Understanding of these specific pathways disrupted can help for developing strategies to mitigate the risk of congenital abnormalities (Wojcik and Agrawal, 2020). Among vertebrates, chick embryo model accepted as a valuable biological resource for providing scientific insights that can give correct and safety guidelines with proper preventive measures to secure vertebrate life including human being (Smith *et al*., 2012).

Conclusion

Present investigation came up with comprehensive evidence for teratogenic effects of induced disodium 5' ribonucleotide against chick embryos. post inoculation of disodium 5' ribonucleotide, noticeable abnormalities, including omphalocele, acrania, limb deformities, monophthalmia, and beak abnormalities, observed have confirmed the impact of disodium 5'

ribonucleotide on embryological stage. The findings emphasize rigorous evaluation of potential teratogens and their mechanisms of action against developing embryos. Understanding these mechanisms will help to reform and modulate the risk of a food additive pertaining to congenital abnormalities during embryonic development among animals including vertebrate and invertebrates.

Our findings support to restrict the abuse of disodium 5' ribonucleotide in different nutritional sources to increase quality of healthy life.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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