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Determination of Lethal Dose of Disodium 5' Ribonucleotide (E635) on Embryonic Development of *Gallus gallus*

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Abstract

Disodium 5' Ribonucleotide (E635), a food additive, has FDA safety, but concerns about its potential toxicity in developmental biology have been raised due to limited research on its lethal effects. The present research aimed to investigate the lethal dose of E635 when induced into a vertebrate chick embryo model. Pilot doses of E635 ranging from 1 µg- 400 µg per egg were inoculated. Consequently, fertilized chick eggs were randomly assigned to 6 groups, including 1 control and 5 differently intoxicated groups of E635 doses per egg. E635 was induced in eggs by *in vivo* administration and incubated for up to 15 days. LD_{50} was determined by using probit analysis in SPSS. The study showed morphological, physiological and survival alterations. A dose-dependent mortality was observed by E635 induction in the present study. The results were interpreted for functional and biological changes in the developing embryo of *Gallus gallus* to confirm the induced toxicity. LD_{50} of E635 on chick embryo was found to be 0.054 µg per egg.

Keywords: Chick Embryo, Disodium 5' Ribonucleotide, Food Additive, Lethal Dose, SPSS, Toxicity

1. Introduction

In general, food additives are substances or chemicals added to food to improve its taste and appearance with other desirable features[1]. Biologically, food additives bring people sensory enjoyment but on the other hand, if abused can cause histological, histochemical, and functional alterations in humans and other animals[2]. In developing countries like India, the use of food additives in the food industry is found to be one of the important factors to sustain in a competitive global market[3]. Umami is the characteristic taste imparted by Monosodium L-Glutamate (MSG) and Disodium 5'-Ribonucleotides (E635)[4].

E635 is a food additive, used as a flavour enhancer in various food products[5]. It is the product obtained by the crystallization of a mixed crystal of Disodium 5' Inosinate (E631) and Disodium 5' Guanylate (E627) at the molecular level[6]. When E635 are mixed and incorporated into replicating DNA due to deoxyribonucleotide-ribonucleotide similarity, they can bind and change certain properties of the DNA and interfere with the ability of normal DNA to function[7]. When the misincorporated Ribonucleotides remain in DNA, they can distort the DNA helix and stimulate genomic instability by increased mutation and chromosomal alteration[8]. Abundant incorporation of ribonucleotides unpredictably alters the enzymatic reactions and alters genome, function leading

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to several disorders[9]. Acceptable Daily Intake (ADI) of E635 is not specified[10].

The developing chick embryo model is accepted as one of the most extensively used living systems for biological research, due to its availability of fertile eggs, rapid growth of the embryo and elasticity for manipulating it, overall developing chick embryo model proved as a model system for morphological, biochemical, and functional studies about study of growth, differentiation and organogenesis[11]. *In ovo* inoculation minimizes the annoyance of young embryos and may be appropriate to induce teratogen[12]. The chick embryo is a suitable alternative model for acute drug toxicity screening before embarking on full toxicological investigations in rodents in the development of anticancer drugs[13]. In addition to that, the chicken embryo and foetus have features that make the chicken a convenient animal model for nonclinical safety studies in which effects on the entire organ systems can be tested[14].

Taking account of available information and literature, there is a lack of scientific evidence related to toxicity causing lethal dose of E635, so the present investigation was carried out to find out the lethal dose of selected E635 against developing chick embryo *Gallus gallus*, a vertebrate model.

2. Materials and Methods

2.1 Sample Selection

Fresh fertilized chicken (*Gallus gallus*) eggs were collected from a local hatchery located in Miraj, Sangli dist., Maharashtra, India.

2.2 Preparation of Intoxicant and Dose for Induction

The powder form of E635 was obtained from Arochem Private Limited. Different concentrations were prepared for pilot reading in a preliminary study, 0.1 ml of different concentrations was inoculated in each egg. 1 mg of E635 was completely dissolved in 1000 ml of distilled water to prepare a 1 ppm solution for LD_{50} calculation. The freshly prepared 1 ppm solution was ready for induction against the developing embryo of *Gallus gallus*.

2.3 Experimental Procedure

For the pilot study, 1 µg-400 µg of E635 per egg doses were inoculated into each group containing 6 eggs respectively. After the pilot reading, eggs were assigned to 5 different treatment groups with a control group induced by 0.1 ml of distilled water[15]. Each treatment group was included with 6 eggs. On the 0th day of incubation, eggs were cleaned and sterilized using 70% ethanol[16]. E635 solution of 1ppm was inoculated on the 0th day, 5th day and 10th day of incubation for each group as Group 1-0.02 µg/0.02 ml, Group 2- 0.04 µg/0.04 ml, Group 3-0.06 µg/0.06 ml, Group 4-0.08 µg/0.08 ml and Group 5-0.1 µg/0.1ml of E635 each egg. The solution was administered to the eggs via the air chamber using a 31-G sterile BD ultra-fine needle syringe by piercing the eggshell[17,18]. After the injection of respective doses, the eggs were resealed using cotton and sterile surgical tape to prevent contamination. The total procedure was completed in aseptic conditions in the laboratory is shown in Figure 1.

2.4 Observation and Monitoring

The eggs from all experimental groups were incubated under controlled conditions, including temperature which was 37.5 ± 0.5 ºC and relative humidity 75 ± 5 %[19,20]. The embryos were carefully monitored and observed at regular intervals using a candling box throughout the incubation period[21]. Embryos were exposed on the 15th day of incubation for further investigation. After completion of intoxication, the mortality rate was recorded for all the doses. The experiment was repeated in triplicates for confirmation of the LD_{50} dose of E635 against the *Gallus gallus* embryo.

2.5 Data Collection and Analysis

After completion of the toxicity experiment, all data was collected tabulated and subjected to statistical analysis which was performed using Statistical Package for Social Sciences (SPSS). LD_{50} was calculated using probit analysis.

3. Results

Toxicology study in scientific experiments is a standardised protocol to determine dose-dependent alterations in the physiological, biochemical, and overall survivability of the animals. Stepwise induction of any chemical provides a relation of pathetic symptomatic conditions occurring in experimental animals, sometimes measured using the rate of survivability and mortality.

The present investigation aimed and analyse for toxicological profile of E635 against the scientifically

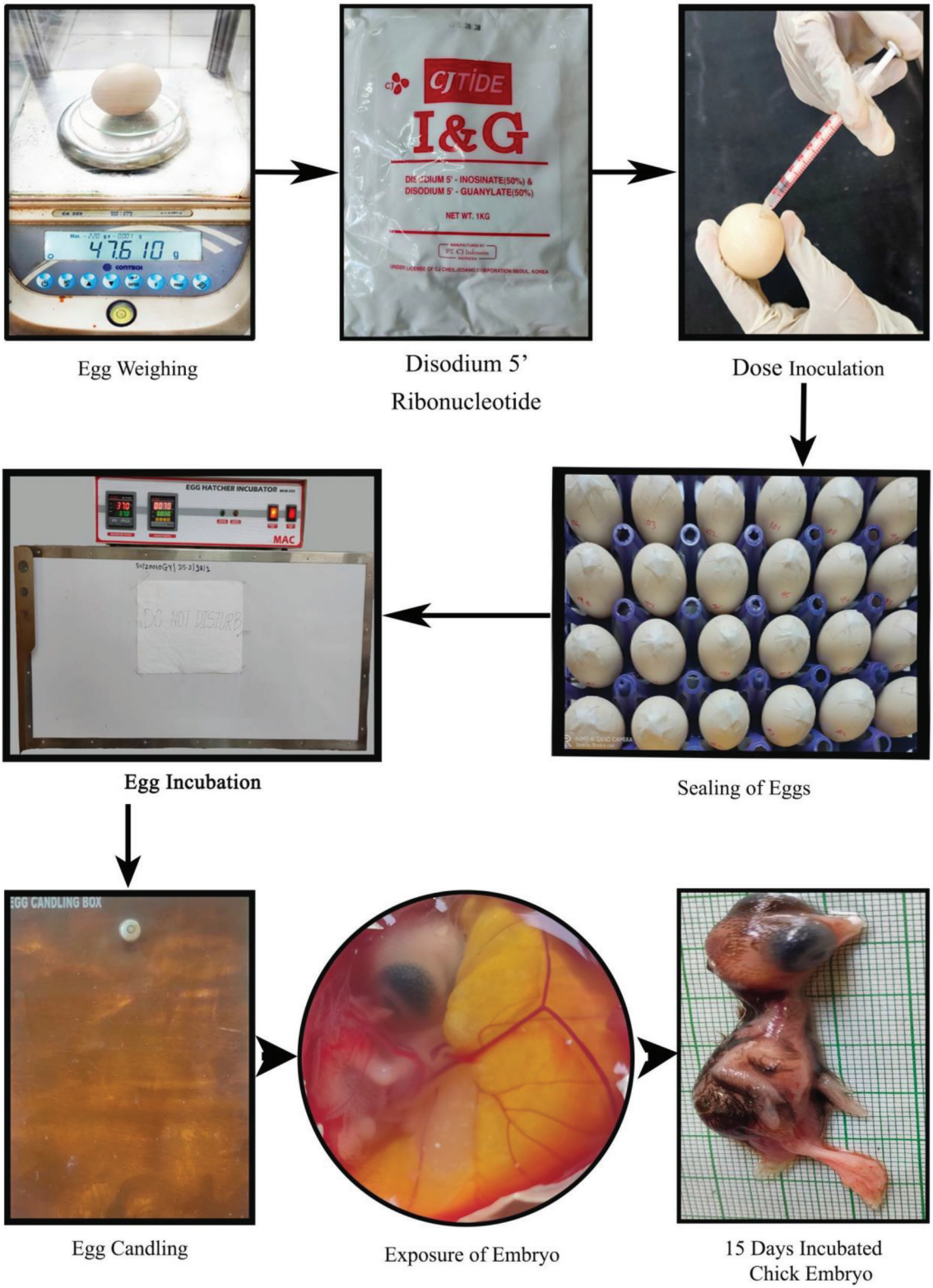


Figure 1. Experimental setup for determination of LD_{50}.

recommended vertebrate model, a developing chick embryo *Gallus gallus*. Critical inoculation of predetermined doses of E635 after inoculation to the developing embryo of a chick from day 0 up to day 15 showed morphological, physiological, and functional changes in the developing embryo. The eggs were divided into six experimental groups.

As a preliminary study, trials were carried out to determine the preliminary toxic doses ranging from 1 µg to 100 µg of E635 per egg. In our pilot study of E635 induction on chick embryos, we found that extra embryonic blood vessels were affected. We found a direct correlation between inoculated dose and angiogenesis pattern (Figures 2 and 3). In 1 µg per egg inoculated with E635, an Anterior Omphalo-Mesentric Vein (AOMV) was not developed (Figure 2(b)). Some of the embryos were abnormally resting on the right side of the body creating disturbance for the heart development (Figure 2(c) and (d)). There was also an underdeveloped embryo and decreased length of all the blood vessels (Figure 2(e)). The Left Omphalo-Mesentric Vein (LOMV) was bifurcated and again rejoined at a node (blue dashed ring) (Figure 2(f)).

In the experiment, as the doses of E635 were increased, the extra embryonic blood vessels decreased. The formation of Secondary blood vessels was in less number. Tertiary blood vessels were completely absent. *Sinus terminalis* was disturbed. There was the absence of the Right Omphalo-Mesentric Artery (ROMA) and AOMV (Figure 3(a)). There was the absence of LOMA (Figure 3(b)). LOMA and POMV were not seen in the group inoculated with 70 µg E635 per egg (Figure 3(c)). There was the absence of AOMV in 80 µg per egg E635 inoculated chick embryos (Figure 3(d)). There was only the presence of LOMA in 90 µg per egg-inoculated embryo (Figure 3(e)). The highest dose of E635 in this experiment which was 100 µg showed incomplete formation of ROMA and no other blood vessels were formed (Figure 3(f)).

After preliminary readings in the toxicological study, 6 groups including the control (6 eggs in each group) were subjected to doses of E635. The experiment was categorized into 5 different doses (Tables 1 to 3). The doses were from 0.02 µg to 0.1 µg per egg. In each experiment, we found mortality among the 6 embryos up to 15 days. The same protocol was repeated thrice concerning the dose-dependent death of the embryo (Tables 1 to 3).

After repeating the experiment thrice, the lethal dose of each set was graphically presented and calculated for confirmation (Graphs 1 to 3). In our meticulous experiment, in all three sets including 5 groups, the calculated LD_{50} dose was 0.053 µg/egg, 0.055 µg/egg and 0.055 µg/egg respectively. To confirm the observed and graphical values of mortality against E635, obtained data was subjected to calculate the mean LD_{50} value by using the SPSS statistical application.

In our study, as per Table 4, all the values were subjected to SPSS to confirm the expected mortality response, observed mortality response, residual values, regression equation and R^2 value (Table 4 and Graph 4).

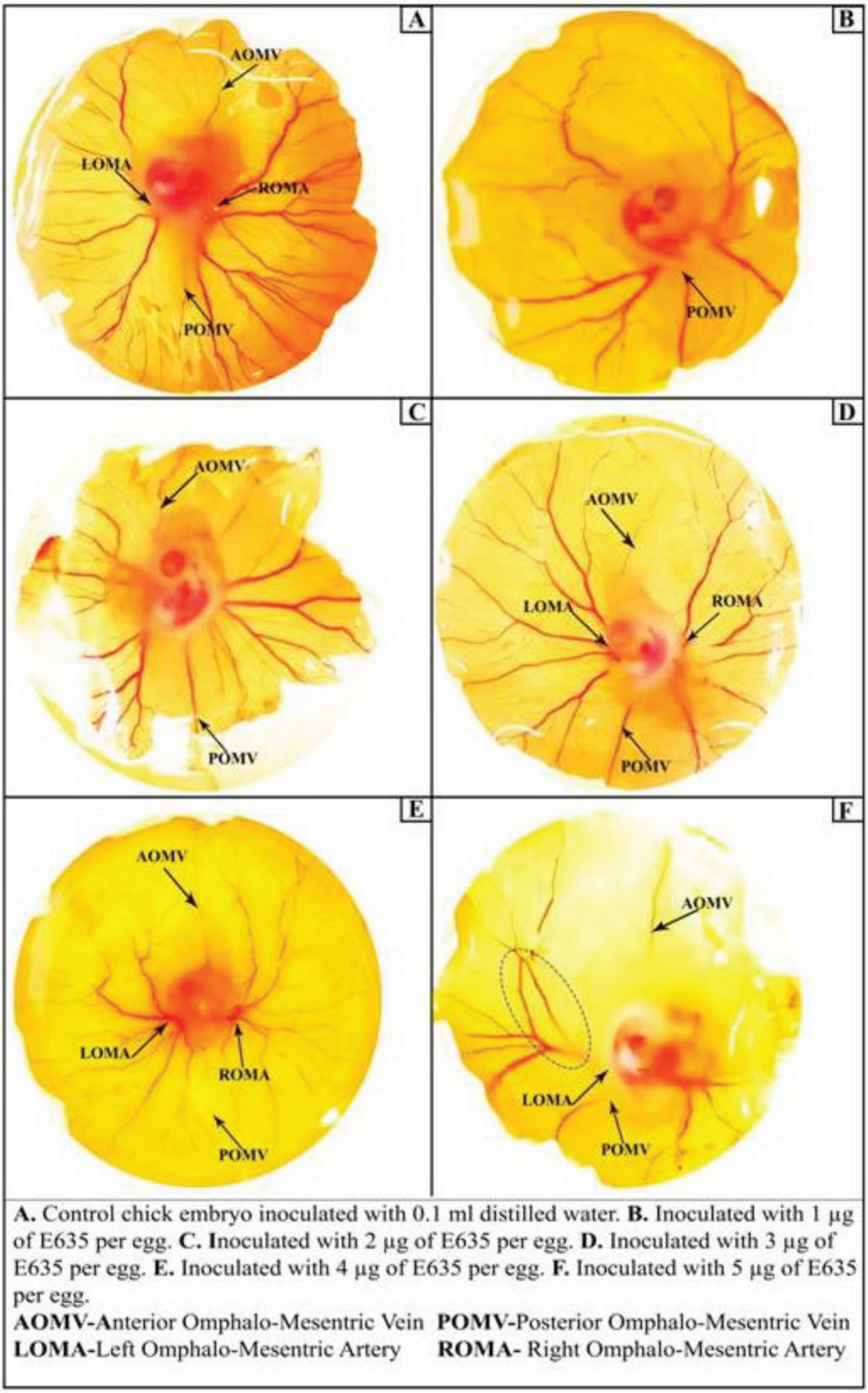
As per the scientific data and available literature, we have confirmed our results specifically the probability of deaths of embryos by calculating 95% confidence limits of the respective dose of E635 against *Gallus gallus* embryo. The data obtained was summarised in Table 5. Where the mean LD_{50} value was experimentally supported and confirmed statistically using a graphical presentation. The whole data was verified by finding the R^2 value which is 0.957 for the regression line y=4.28+3.39x to study the dose-dependent mortality among all 3 sets incubated for 15 days by inoculums of E635 in all eggs.

The results obtained in the critical surveillance of maintained laboratory conditions were discussed with similar kinds of recent references to confirm and strengthen our study.

4. Discussion

The objective of the present study was to determine the LD_{50} value of E635 on chick embryos using probit analysis. The experiment was conducted thrice (Set I, Set II and Set III) to ensure the accuracy and reliability of the results. In all three sets of experiments, the control chick embryos exhibited no mortality, indicating that any observed effects were attributed solely to the exposure of E635. Documented observations support the validity of the experimental model and confirm that E635 was the cause of observed mortality among experimental sets. The LD_{50} values obtained for E635 in each set of experiments were 0.053 µg/egg in Set I, 0.055µg/egg in Set II, and 0.055 µg/egg in Set III. The consistency in LD_{50} values across the three sets of experiments further strengthens the reliability of our findings. It suggests that the toxic effect of E635 on chick embryos was reproducible under the given experimental conditions.

Moreover, probit analysis was performed using SPSS, a similar method was used by Tendulkar and Kamble, and the probit plot against the log of dose for the total of all the



A. Control chick embryo inoculated with 0.1 ml distilled water. **B.** Inoculated with 1 µg of E635 per egg. **C.** Inoculated with 2 µg of E635 per egg. **D.** Inoculated with 3 µg of E635 per egg. **E.** Inoculated with 4 µg of E635 per egg. **F.** Inoculated with 5 µg of E635 per egg.

AOMV-Anterior Omphalo-Mesentric Vein **POMV-**Posterior Omphalo-Mesentric Vein
LOMA-Left Omphalo-Mesentric Artery **ROMA-** Right Omphalo-Mesentric Artery

Figure 2. 5 days incubated chick embryos inoculated with E635 doses ranging from 1 to 5 µg/egg.

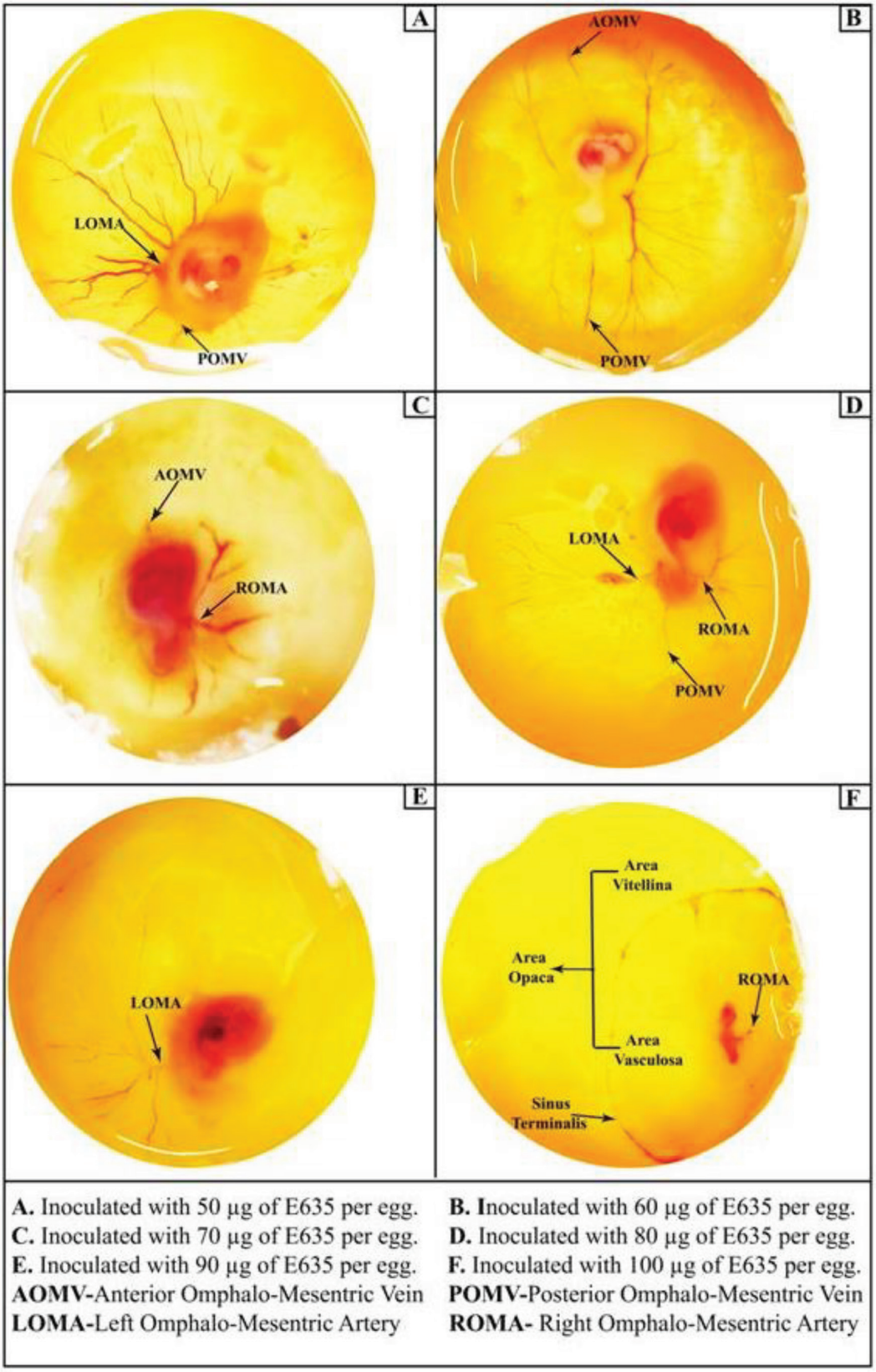
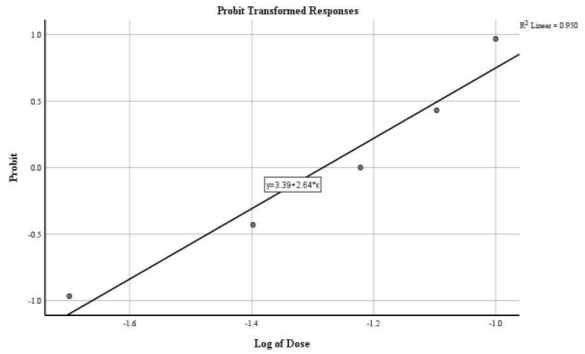


Figure 3. 5 days incubated chick embryos inoculated with E635 doses ranging from 50-100 µg/egg.

Table 1. A lethal dose of E635 on Chick embryo for Set I

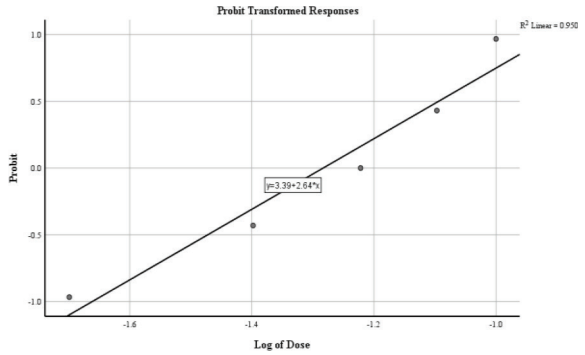
Sr. No.	Dose (µg/egg)	Total No. of Eggs	No. of Dead Embryos	LD_{50} (µg/egg)
1	0.02	6	1	0.053
2	0.04	6	2	
3	0.06	6	3	
4	0.08	6	4	
5	0.10	6	5	



Graph 1. The graph represents probit analysis to find out the LD_{50} of E635 on chick embryos for Set-I of the experiment.

Table 2. A lethal dose of E635 on Chick embryo for Set II

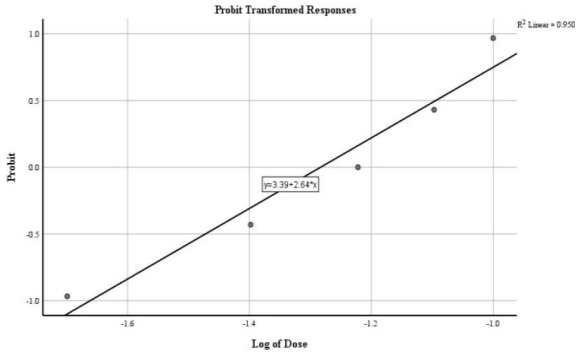
Sr. No.	Dose (µg/egg)	Total No. of Eggs	No. of Dead Embryos	LD_{50} (µg/egg)
1	0.02	6	0	0.055
2	0.04	6	3	
3	0.06	6	3	
4	0.08	6	4	
5	0.10	6	5	



Graph 2. The graph represents probit analysis to find out the LD_{50} of E635 on chick embryos for Set-II of the experiment.

Table 3. A lethal dose of E635 on Chick embryo for Set II

Sr. No.	Dose (µg/egg)	Total No. of Eggs	No. of Dead Embryos	LD_{50} (µg/egg)
1	0.02	6	0	0.055
2	0.04	6	3	
3	0.06	6	3	
4	0.08	6	4	
5	0.10	6	5	



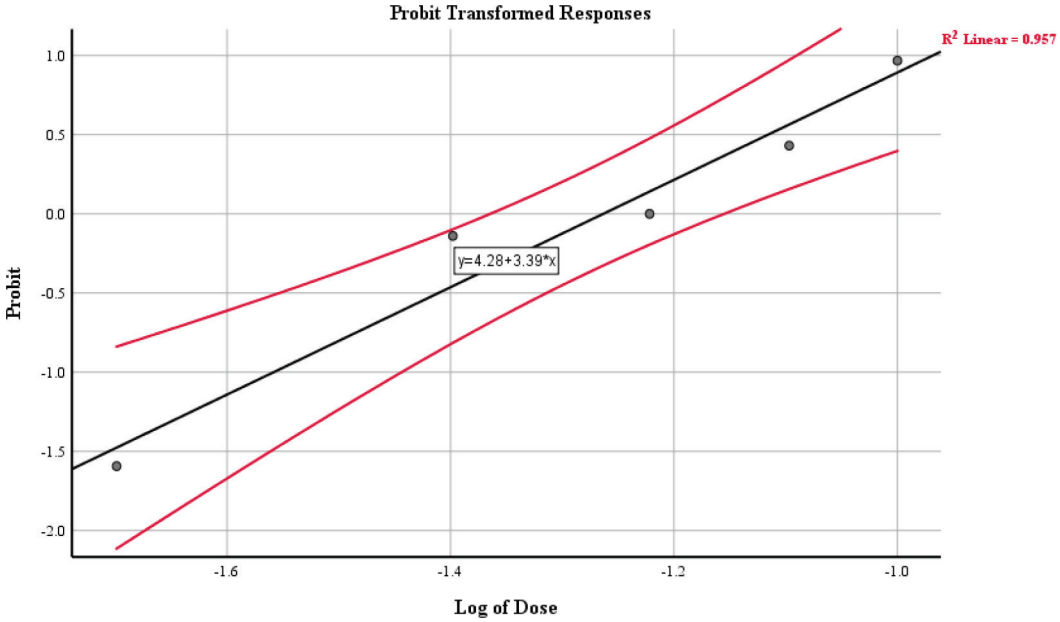
Graph 3. The graph represents probit analysis to find out the LD_{50} of E635 on chick embryos for Set-II of the experiment.

sets was constructed[22]. The graph showed a high R^2 linear value of 0.957, indicating a strong correlation between the log dose of E635 and the observed responses regarding per cent mortality. The R^2 values were high for all growth models indicating a significant relationship between age and weight in two breeds of chicken studied by Norris[23]. In the present investigation, the regression equation y=4.28+3.39x further supports the dose-response relationship between E635 and its toxic effect on developing chick embryos.

The obtained LD_{50} values for E635 in this study were significantly lower than the doses typically encountered in the mortality study of the vertebrate model used in the toxicity study. The present study concludes that even in low doses of E635 developmental stages of *Gallus gallus* show morphological and functional changes leading to death. Similar kinds of morphological and physiological changes were observed and documented by several scientists where sodium 5' ribonucleotide, now called E635 may be responsible for an initiation of carcinogenicity or mutagenic feature in growing embryo[24]. The combination of insecticides cypermethrin and chlorpyrifos was induced in the chick embryo. Pathetic symptoms like

Table 4. Data for 3 Sets of experiments were analysed by the probit method

Sr. No.	Dose Inoculated (µg/egg)	No. of Eggs Dose Inoculated	Observed Responses (No. of Dead Embryos)	Expected Mortality Responses	Residual	Probability
1	0.02	18	1	1.520	-0.520	0.084
2	0.04	18	8	6.112	1.888	0.340
3	0.06	18	9	10.066	-1.066	0.559
4	0.08	18	12	12.749	-0.749	0.708
5	0.10	18	15	14.482	0.518	0.805



Graph 4. The graph represents Probit analysis for induced does of E635 against *Gallus gallus* embryo to find out LD_5 of a total of Set-I, Set-II and Set-III of the experiment.

Table 5. LD_{50} with 95% confidence limits for dose calculated by subjecting data to an SPSS analyzer

Probability	95% Confidence Limits for Dose		
	Estimate	Lower Bound	Upper Bound
0.010	0.010	0.003	0.017
0.020	0.012	0.004	0.020
0.030	0.014	0.005	0.022
0.040	0.015	0.006	0.023
0.050	0.016	0.006	0.025
0.060	0.018	0.007	0.026
0.070	0.019	0.008	0.027
0.080	0.020	0.009	0.028
0.090	0.021	0.009	0.029
0.100	0.021	0.010	0.030
0.150	0.026	0.013	0.034

Table 5 continued...

Probability	95% Confidence Limits for Dose		
	Estimate	Lower Bound	Upper Bound
0.200	0.029	0.017	0.038
0.250	0.033	0.021	0.042
0.300	0.037	0.025	0.046
0.350	0.041	0.029	0.050
0.400	0.045	0.033	0.055
0.450	0.049	0.038	0.061
0.500	**0.054**	**0.042**	**0.067**
0.550	0.059	0.047	0.075
0.600	0.065	0.053	0.085
0.650	0.071	0.058	0.097
0.700	0.079	0.064	0.113
0.750	0.088	0.070	0.133

Table 5 continued...

Probability	95% Confidence Limits for Dose		
	Estimate	Lower Bound	Upper Bound
0.800	0.099	0.077	0.162
0.850	0.114	0.086	0.203
0.900	0.136	0.099	0.273
0.910	0.142	0.102	0.294
0.920	0.148	0.106	0.317
0.930	0.156	0.110	0.346
0.940	0.165	0.115	0.381
0.950	0.176	0.120	0.425
0.960	0.190	0.127	0.484
0.970	0.209	0.136	0.568
0.980	0.237	0.149	0.704
0.990	0.288	0.172	0.985

protrusion of the heart, liver and bowel without any covering sac were observed in the treated embryos[25].

During the digestion process, disodium guanylate and disodium inosinate, which are part of the E635, are catabolised in the human body in the form of uric acid, causing circulatory and nephritic problems[26]. The content of E635 was profoundly steady, did not denature under heating conditions and was also freely dissolvable in water[27]. The mortality of fruit flies which were fed by disodium guanylate, disodium inosinate, Mono Sodium Glutamate (MSG) and glycine exhibited more than 90% mortality[28]. Dose-dependent skeletal abnormalities were also observed in the litters of CD strain rats when fed with 0.1, 1.0 and 2.0 % of E635 in the diet[29]. Glycine and disodium guanylate showed the strongest toxicities by causing 100% mortality in fire ants *Solenopsis invicta* after 84 hr by giving LC_{50} value for disodium guanylate as 0.004 g/ml[30].

5. Conclusion

Based on the study related to the intoxication of E635 on chick embryos, our findings revealed a clear dose-dependent relationship between E635 inoculation and angiogenic pattern in developing embryos. We found significant morphometric changes in the vascularization of the embryo, including its quality and quantity. Results obtained for the mean LD_{50} value were statistically confirmed with a regression line and R^2 value of 0.957 which indicates a positive correlation for dose-dependent mortality among experimental groups up to 15 days of incubation.

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