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Teratogenicity of Arecoline Hydrobromide on Developing Chick Embryos: A Preliminary Report

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Arecoline is one of the major alkaloids of betel-nut, constituting 85-95% of the total alkaloid content (Boyland and Nery 1969, Wenke and Hoffman 1983). Betel-nut is used as a masticatory agent in various parts of the globe including S.E. Asia, Central Asia and Latin America (IARC 1985) and is usually taken in combination with lime, catechu and sometimes tobacco, wrapped up in leaves of the betel vine. This mixture is referred to as betel quid (BQ). Various other concoctions of the nut have been used in the treatment of diarrhoea, fever, hysteria and as an abortifacient (Nery 1971).

Betel quid chewing exposes people to four N-nitosamines formed from arecoline (areca-derived-nitrosamines). Two of these, (3-methyl-nitrosamino) propionitrile (MNPN) and N-nitrosoguvacoline (NG) are carcinogenic (Hoffman et al. 1994). Moreover, arecoline, being a monofunctional alkylating agent, loses one of its methyl groups during metabolism and binds with proteins and nucleic acids, which may help to explain its chromosome damaging ability (Panigrahi and Rao 1982) and its mutagenic nature, as envisaged in bacteria and mammalian cells (Shirname et al. 1983, 1984). Another facet of this mutagenic activity was seen in HEp-2 human larynx carcinoma cells where arecoline caused unscheduled DNA synthesis (Sharan and Wary 1992).

Sundqvist et al. (1989) investigated the potential cytotoxic effect of arecoline in cultured human buccal epithelial cells and found that it decreased cell survival, thiol content and vital dye uptake. Thus, arecoline/arecoline derivatives possess carcinogenic, clastogenic, mutagenic and cytotoxic properties making it a good candidate for a teratogenic agent. It was previously shown that a crude extract of betel-nut produced teratogenic effects in the chick embryo (Paul et al. 1996). The aim of the present study was to test whether these effects were caused by the arecoline in the betel nut extract.

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MATERIALS AND METHODS

Fertilized eggs (Rhode Island Red) obtained from the local Government poultry at Golapbag, Burdwan were kept horizontally in a thermostatically controlled incubator at a temperature of 38.5±0.Z°C with 60-65% relative humidity and rotated every 8 hrs. until drug administration. They were candled before injection and unfertilized eggs and dead embryos discarded.

Arecoline hydrobromide (Sigma Chemical Corp. USA) was dissolved in distilled water in black-paper-covered vials and injected aseptically into the air-sac of fertile eggs. Doses injected were 0.25, 0.50, 0.75 and 1 mg/egg at 2,3 and 4 d.i. Injection sites were sealed with sterile tape after each injection. Each egg was injected only once. The volume of injection was kept constant at 0.05 ml/egg (Wilson 1978). Solvent controls were injected with 0.05 ml of the solvent vehicle while unopened controls were kept uninjected. Injected eggs were divided into 3 batches according to the day of injection. On the 14th day of incubation, embryos were removed from the shell and cleared of all extraembryonic membranes. Macroscopic abnormalities were recorded and embryos were fixed in 10% formaldehyde. Some embryos were stained with Alizarin red-S and examined for skeletal defects. The collected data was subjected to statistical regression analysis followed by Students' t-test to test the significance of the results.

RESULTS AND DISSCUSSION

There was a prominent dose-response relationship in the case of embryo mortality and a high frequency of abnormalities (Table 1).

Table 1. Effects of arecoline on chick embryos examined on the 14th day of incubation

Dose	Day	No.of Eggs	Live	Dead	% Live	Malformed	% Malformed
0.25	2	17	16	1	94.1	4	25.0
0.50	2	19	11	8	57.9	7	63.6
0.75	2	23	8	15	34.8	4	50.0
1.00	2	17	2	15	11.7	2	100.0
Vehicle control	2	9	7	2	77.7	0	0.0
0.25	3	14	11	3	78.7	4	36.6
0.50	3	19	9	10	47.6	5	55.5
0.75	3	23	5	18	21.7	3	60.0
1.00	3	17	3	14	17.6	2	66.6
Vehicle control	3	8	7	1	87.5	0	0.0
0.25	4	14	11	3	78.7	2	18.8
0.50	4	13	7	6	53.8	3	42.8
0.75	4	15	8	7	53.3	6	75.0
1.00	4	12	5	7	41.6	3	60.0
Vehicle control	4	7	6	1	85.7	0	0.0
Unopened control		20	20	0	100	0	0.0

The rate of abnormalities was greatest for embryos injected on day 2 of incubation. Abnormalities noted were reduced body size (Figs. la and c), scanty feathering (Figs. la and c), generalized oedema with light body colour (Figs. la and c), everted viscera (Fig. 1c), shortened lower beak (Figs. 1c and d) and clubfoot (Fig. lb). One case of exencephaly (Fig. 1c) and haemorrhage was observed. Fetotoxicity in the form of retarded development was also observed.

Figs. 2a-c show 14 day old chick embryos stained with Alizarin red-S demonstrating normal and abnormal skeletal development. Among the skeletal abnormalities observed were clubfoot, unossified vertebrae, missing/unossified rib. shortening of long bones and unossified phalanges.

Form Figs. 3 & 4 it can be seen that the regression analysis revealed a rather significant dose-response for both mortality and malformation , t-statistics of regression data (data not shown) revealed that for mortality a high level of significance was seen for all the day categories, however, for malformations only day 2 and day 4 of administration proved to be significant .

The chick embryo serves, as a useful experimental model for developmental toxicity testing, since assessment may be carried out independent of interference from the maternal component (Heinrich-Hirsch and Neubert 1991). This prompted us to choose the system to determine the developmental toxicity of arecoline hydrobromide.

Among the numerous deformities observed athrogryposis or clubfoot was one of the most consistent. Carbacol chloride and other cholinomimetics (such as physostigmine) also gave rise to arthrogryposis in chick embryos (Landauer 1975). Landuer postulated the cause of this phenomenon as muscular hypoplasia. It was suggested that these substances probably compete with acetylcholine for the possession of the acetylcholine receptors resulting in a pronounced cholinergic effect (Landauer 1975). This could be the mechanism by which arecoline acts in the chick embryo. Later studies (Forsyth et al. 1994) showed that the cholinomimetics nicotine sulphate and coniine produced similar defects in chick embryos.

Other than clubfoot produced by arecoline and a few other cholinomimetic teratogens, additional abnormalities were found. These were skeletal defects such as missing and ill developed vertebrae, shortening of lower beak and general retardation of skeletal growth. Retardation of skeletal growth, marked reduction in body size and sparse feathering suggest that arecoline is a fetotoxic agent acting to retard embryonic development.

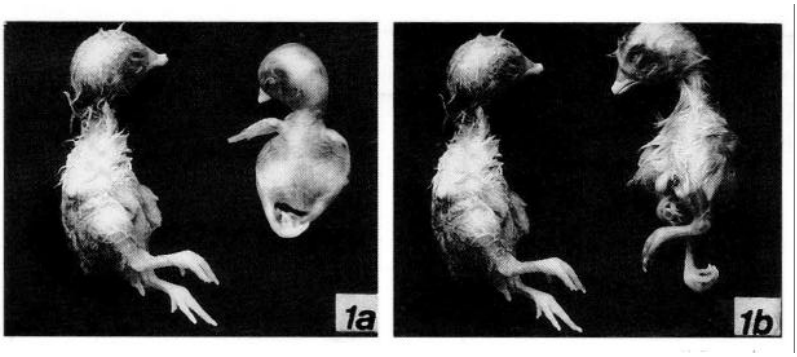


Figure 1a. Control embryo is on the left. Embryo on the right was treated with 0.5 mg/egg of arecoline on the 2nd day of incubation and exhibits reduced body size, oedema, light colouring and reduced feathering.

Figure 1b. Control embryo is on the left. Embryo on the right was treated with 1 mg/egg of arecoline on the 3rd day of incubation and displays clubfoot.

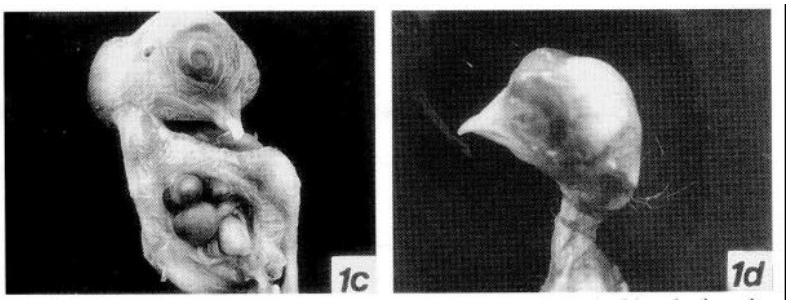


Figure 1c. Embryo treated with 0.75 mg/egg of arecoline on the 4th day of incubation showing excecephaly, reduced body size, everted viscera, short lower beak and reduced feathering.

Figure 1d. Embryo treated with 0.25 mg/egg of arecoline on the 4th day of incubation displaying

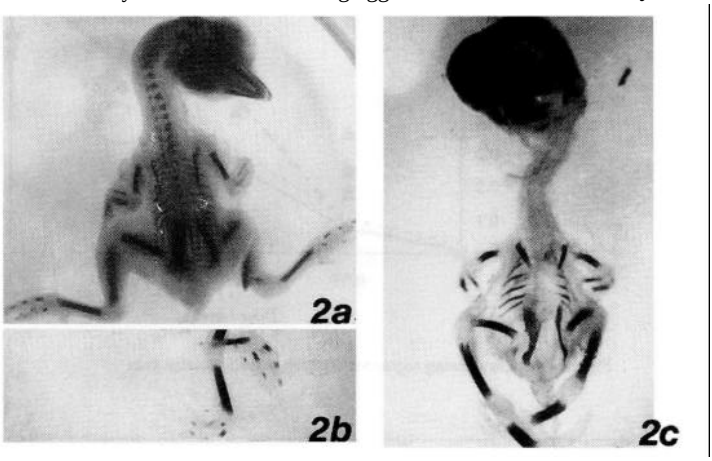


Figure 2a. Control embryo.

Figure 2b. Embryo treated with 1 mg/egg of arecoline on the 3rd day of incubation displaying clubfoot.

Figure 2c. Embryo treated with 0.5 mg/egg of arecoline on the 2nd day of incubation showing retarded skeletal growth characterized by unossified vertebrae, a single unossified rib, shortening of long bones and unossified phalanges.

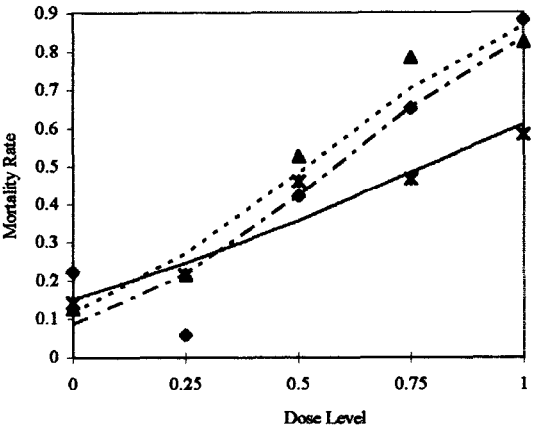


Figure 3. Graph showing regression analysis of mortality data

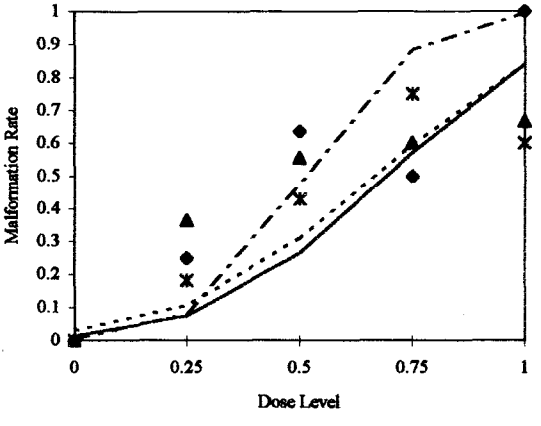
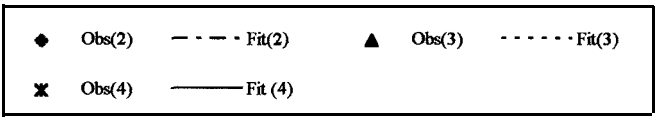


Figure 4. Graph showing regression analysis of malformation data



The generalized oedema observed points towards a possible osmoregulatory distubance resulting in an influx of fluids to the embryo from the yolk-sac and albumen (Grabowski 1977). However, haemorrhages or haematomas typical of the so-called 'oedema syndrome' were only observed in one instance.

Thus, we conclude that arecoline hydrobromide acts in a manner quite similar to other cholinomimetic teratogens and possibly has a similar mode of action. However, several other types of defects observed (i.e. oedema, exencephaly and fetotoxicity) highlight the fact that arecoline may distupt not one, but numerous biochemical pathways leading to the teratogenic effects caused by the drug.

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