Original Article

Teratogenic Effects of Different Concentrations of Retinoic Acid on Chick Embryonic Heart Cells Cultured in Vitro

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ABSTRACT

Objective: To detect the possible teratogenic effects of various concentrations of retinoic acid (RA), in chick cardiac cells cultured in micromass culture.

Study Design: Experimental study

Place and Duration of Study: This study was carried out at the University of Nottingham, UK on chick embryonic hearts for a period of six months from Jan. 2010 to June 2010.

Materials and Methods: Embryonic hearts were dissected from 5 day old white leghorn chick embryos to produce a cardiac cell suspension in DMEM culture medium. Cells were either exposed to culture medium or vehicle only or to different concentrations of retinoic acid ranging from1 μM to 100 μM. End points for cellular differentiation were observational scores at 24, 48 and 144 h following explantation. Cell viability was established with resazurin and kenacid blue assays. Statistical analysis of the results was via one way ANOVA and Kruskal Wallis tests and *P*-value < 0.05 was considered significant.

Results: Retinoic acid significantly reduced cellular differentiation at and above 1 μM (*P* value < 0.05), at concentrations 50 μM and above no single focus was observed to be beating. The resazurin assay for viability showed decreasing viability of cardiac cells with increasing concentrations of retinoic acid. Same trend was observed with kenacid blue assay which determined the overall protein content. However the cells exposed to only culture medium or vehicle did not show significant differences in terms of viability, protein content and beating ability.

Conclusion: The potential of retinoic acid as teratogen is proved in this in vitro study and it is recommended that the pregnant ladies should avoid the use of these drugs during early developmental period.

Key Words: Chick Embryonic Hearts, Micromass Culture, Retinoic Acid

INTRODUCTION

Retinoic acid plays an essential role in cell proliferation, differentiation, vision, reproduction and immune functions[1]. Apart from these functions, retinoic acid is used as prevention of acute promyelocytic leukemia [2]. Isotretinoin, a retinoic acid compound, is effective in treating dermatologic disorders, including the treatment of severe, reluctant nodular acne which is refractory to conventional therapy[3].

Retinoic acid also plays an important role during embryonic life, where it influences development of several organs including hind brain, heart, spinal cord, kidneys, and limb buds [4].

Although these compounds are important for adult and embryonic life but, their excess and deficiency results in abnormal homeostasis including birth defects. A combination of birth defects known as retinoic acid embryopathy are produced by excessive intake of retinoids [5] , while its deficiency or absence causes embryonic segmentation, growth failure and eventual resorption. These considerations make it necessary to maintain retinoid homeostasis during developmental processes. The main sources of the preformed vitamin include animal foods (dairy products, liver, eggs etc.), fortified foods and pharmaceutical supplements, while several fruits and green leafy vegetables constitute rich sources for the provitamin form or carotenoids [6]. The major organs affected during developmental process by excessive amounts of retinoids include heart, brain and craniofacial areas. Adverse effects on heart include either lack or fusion of the paired cardiac primordia, impaired or reversed heart looping and truncation of the posterior portion of the heart tube with abnormal expansion of anterior structures. Retinoids exert their pleiotropic effects through binding to two families of nuclear receptors, named as retinoic acid receptors (RARs) and retinoic X receptors (RXRs) [7]. This study is carried out to evaluate the effects of different doses of retinoic acid on micromass culture of chick embryonic hearts.

MATERIALS AND METHODS

This experimental study was carried out at the University of Nottingham, UK on chick embryonic hearts over a period of six months.

Fertile white leghorn chicken eggs were labelled with the date of delivery and stored in a cooled incubator at 12-14ºC until required and used within two weeks of being laid. The eggs were placed onto the automated egg turner and incubated at 38ºC with relative humidity of 100% for 5 days, day zero being defined as the day when the eggs were set in the incubator. A minimum of 24 eggs were incubated for each micromass system to ensure the availability of enough viable embryos. All the embryos were killed by decapitation after being removed from incubator.

Micromass culture:

Eggs were removed from the incubator, six at a time and transferred to the culture hood. Using the blunt end of the curved forceps the broader end of the eggs (near air sac region) was struck until the broken shell could be gently removed and discarded. The shell and all the membranes including vitelline membranes were removed with the help of sterile forceps to expose the embryo. The embryo was lifted from the egg with curved forceps and placed into a petri dish containing Hanks balanced salt solution (HBSS).

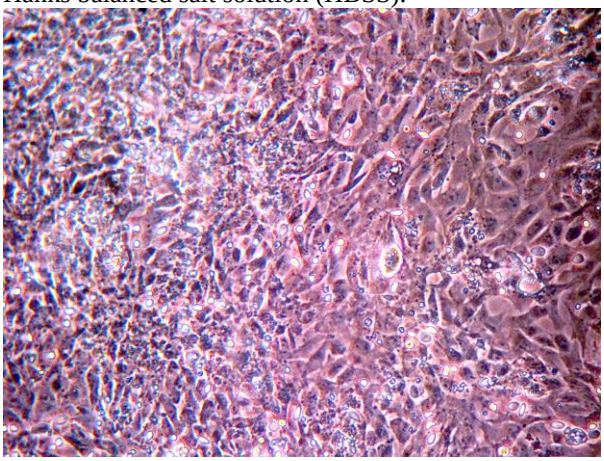

Figure No.1: Light microscopy of micromass. Original magnification x200 (Pic. By Helena Hurst).

After removing the allantois and remaining amnion, under the dissecting microscope the heart was taken out from the embryo and transferred using sterile forceps to a sterile universal tube containing horse serum (50% v/v in HBSS) and stored on ice. Once all the hearts had been collected, the solution of horse serum (50% v/v in HBSS) was removed and tissues were washed twice with HBSS. Heart tissue was incubated in 4ml of trypsin/ETDA (for 24 eggs) at 37ºC in 5 %(v/v) CO_2 in air for 20 minutes, agitating every 5 minutes for further disassociation of tissue. The solution was triturated numerous times in order to break the tissues as much as possible. To inhibit further actions of trypsin 6ml of DMEM culture medium was added. Samples were centrifuged at 1500rpm for 5 minutes, the supernatant was removed and the pellet resuspended in 1ml of warmed DMEM culture medium. Cell density was established using a haemocytometer and the cell suspension diluted to $3x10^6$ cells/ml. A 20µl aliquot of the cell suspension was pipetted on to the centre of the well within a 24 well plate for about two and half hours, to allow them to attach, before being flooded with 500µl of prewarmed culture medium(DMEM) and returned to the incubator for 24 hours to recover.

Scoring method for cardiomyocyte activity: A numerical morphological scoring system was constructed to determine the amount of contractile activity observed for heart micromass (MM) as shown in table 1.

Table No.1: Morphological scoring system to determine contractile activity of cardiomyocytes

Numerical morphological score	Contractile activity
0	No contractile activity
1	Few contracting foci
2	Numerous contracting foci
3	Entire plate contracting
Morphological score	**Pace of contractile activity**
S	Low
M	Medium
F	Fast

Retinoic acid preparation: All-trans retinoic acid (Sigma-Aldrich) is a fat soluble vitamin, so, after weighing out the desired amount in a fume cupboard, the yellow coloured retinoic acid powder was dissolved in 100% ethanol to prepare the stock solution and serially diluted in DMEM culture medium to obtain the test concentrations, ranging between 1 and 100µM. Chemicals were made up at twice the concentration required as 500µl of test solution was added to the 500µl culture medium already present in the 24 well plate, which gave the required concentration. All the test chemicals were made up the day to be used and applied to the MM cultures 24 hours after they were seeded.

Resazurin conversion assay: The resazurin assay was performed on day 6 following explantation. The resazurin stock (100µg/ml) solution was diluted 1:10 in sterile HBSS and warmed to 37ºC in water bath prior to use. The medium was removed from the 24 well plates and replaced with 500µl resazurin solution. The plates were then incubated for one hour at 37ºC and 5% (v/v) CO_2 in air. The optical density was read using a FLUOR star plate reader, excitation wavelength of -530±12.5nm, with a gain of 10. The data was expressed as the increase in optical density above the non-cell blank as a percentage of the untreated cultures. Analyses of data, for statistically significant differences, were performed on the raw data. If repeat assays were performed, once the plate had been read in the spectrofluorimeter, the resazurin solution was

removed and the cells were fixed with 300µl of kenacid blue fixative. The plates were then kept in refrigerator until the kenacid blue assay was performed.

Kenacid blue total protein assay: Wells were aspirated and 300µl kenacid blue fixative was added and allowed to evaporate overnight at 4°C. Kenacid blue working solution (400µl) (Knox et al, 1986) was added to each well and the plate placed on a plate shaker for at least 2 hours. Excess stain was removed and cells were quickly rinsed in 400µl of washing solution before being washed for 20 minutes with agitation. The washing solution was replaced with 400µl of desorb and gently agitated on the plate shaker for one hour. The optical density was read on an ASYS HITEC Expert 96 plate reader with a reference filter of 405nm, and a reading filter of 570nm. The amount of protein per well was calculated from standard curves.

Statistical analyses: The data was calculated in Prism 5 software and analysed using an ANOVA (one way) assay with the post hoc Dunnets test for parametric data. For nonparametric data (scoring of cells), a Kruskal-Wallis test was performed and a Dunn's post hoc test was used to test the significance. In all cases p value < 0.05 was considered significant.

RESULTS

The aim of this study was to detect with a range of retinoic acid concentrations the potential to produce teratogenic effects on cardiomyocytes in micromass culture. Concentrations of altrans retinoic acid tested ranged between 1µM to 100µM. Results for resorufin production, a test for cell viability are shown in fig 3.

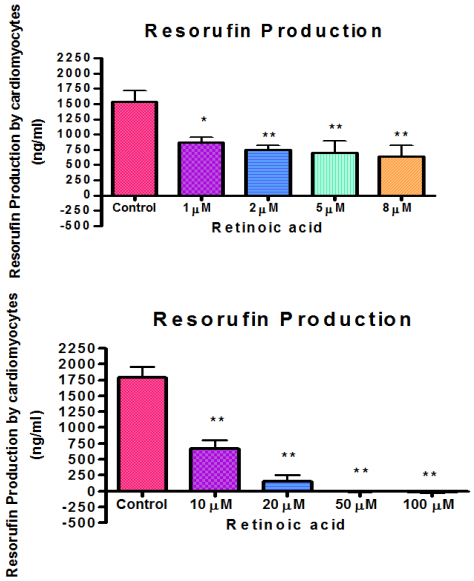

Figure No.2: Showing resorufin content of cardiomyocytes, treated with various concentrations of all-trans retinoic acid

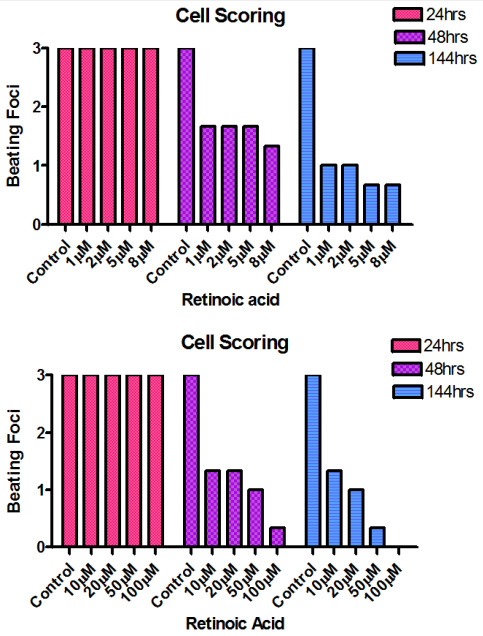

Figure No.3: Showing cell scoring of cardiomyocytes, treated with various concentrations of all-trans retinoic acid.

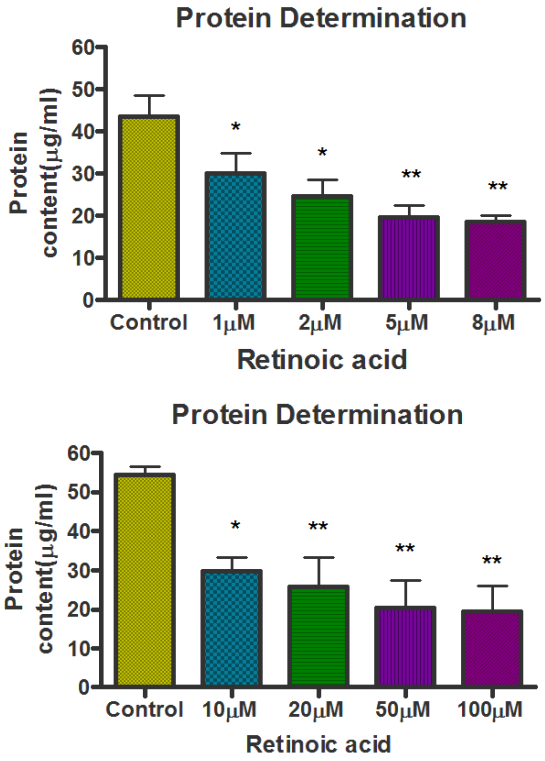

Figure No.4: Showing Protein content of cardiomyocytes treated with various concentrations of all-trans retinoic acid.

DISCUSSION

Retinoids are known human teratogens and therefore have been extensively investigated. This study was aimed to prevent the teratogenic effects retinoic acid by supplementary folic acid.

The teratogenic potential of altrans retinoic acid on chick micromass culture was confirmed by our present work. The effects were consistent for any of the three end points measured. The reliability of chick MM culture was tested by [8], with retinoic acid one of the test chemicals used to treat the limb buds. Results reflected those found in our study, with inhibition of cellular differentiation, cell viability and protein synthesis in chick cardiomyocytes MM cultures exposed to all-trans retinoic acid.

Many animal studies have focused the teratogenic effects of retinoids on developing heart cells. In an investigation on effects of retinoic acid on early heart development by [5],[9] he found that the retinoic acid produced a wide range of heart malformation when applied either to the whole embryo culture or implanting in the form of beads to the precardiac mesoderm same were observed in other species. In both of these experiments the embryos were treated with concentrations of retinoic acid ranging from 1µM to 100µM. Although supporting the current study in some ways, Osmond et al did not find any killing of cells even at higher concentrations of retinoic acid. In contrast our study showed cell death at concentrations ranging from 20µM to 100µM.

In another study conducted on mouse whole embryo culture Zhang et al 2006, found large number of malformation including cranial NTDs,and branchial arch abnormalities were found in 100% of embryos exposed to 0.4µM concentration of retinoic acid. The reason of cell death in our study on micromass system could be due to high concentrations of retinoic acid used or increased sensitivity of the micromass to retinoic acid, because in micromass culture the cells are in direct contact with the retinoic acid whereas in whole embryo culture the embryo is protected by the surrounding membranes and yolk sac.

It is not possible to relate our findings of teratogenic levels of retinoic acid with humans; however, it is apparent from case studies that the human embryos are being exposed to damaging levels of retinoic acid. An epidemiologic study conducted by (1) on women who took an average of >10000 IU of retinol/day in the form of supplements revealed that they had a higher proportion of babies born with birth defects. Another recent study (Barbero et al, 2004) describes a child suffering from congenital anomalies due to retinoic acid embryopathy following treatment of the mother's acne with 10mg/day of acitretin, a retinoid, despite treatment being stopped over a year pre-conception.

CONCLUSION

The potential of retinoic acid as teratogen at higher concentrations when given at early embryonic age is proved in this in vitro cell culture study.

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