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Protective Effects Of Vitamin E On Developmental Toxicity Of Common Environmental Factors: An Evaluation Of Chick Heart Micromass Culture Assay

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Abstract:

This study was aimed to find out whether the chick cardiomyocyte micromass (MM) system could be employed to predict the teratogenecity of common environmental factors and antioxidant (Vitamin E) by blind study. White Leghorn 5-day-old embryo hearts were dissected and trypsinized to produce a cardiomyocyte cell suspension in Dulbecco's Modified Eagle's Medium. The cultures were incubated at 37^0C in 5% CO_2 in air, and observations were made at 24, 48 and 144 hours, for the detection of beating. Cellular viability was assessed by using the resazurin assay and cell protein content was assessed by the kenacid blue assay. These observations were detected by principle investigator as well as another investigator who was unaware about the test chemicals used. The retinoic acid, ethanol and cadmium chloride significantly reduced the beating, cell viability and cell protein content in micromass cultures. It was also observed that vitamin E when added to these chemicals, did not significantly reduced cell activity and beating, whilst not affecting total cell number. The results demonstrate the potential of the chick cardiomyocyte MM culture assay to identify teratogens/embryotoxins that alter functionality, which may result in a teratogenic outcome. This could form part of a screen for developmental toxicity related to cardiac development and function.

Key words: *Chick heart cells, micromass culture, teratogens, vitamin E*

1.Introduction

Vitamin E is a lipid soluble vitamin and includes a group of compounds with similar antioxidant activity. Epidemiological studies have reported a direct relation of vitamin E intake and reduced risks of cardiovascular diseases in dose dependent manner [1]. This could be due to decrease lipid peroxidation of low density lipoproteins [2]. Apart from its antioxidant functions, vitamin E, in particular α-tocopherol, also plays a significant role in cell signalling, it inhibits smooth muscle cell proliferation, increases phosphoprotein phosphatase 2A activity, and decreases protein kinase C activity. It prevents loss of spermatogenesis and thus helps in prevention of male infertility [3]. The deficiency of vitamin E in humans causes abnormalities in neuromuscular systems and is characterized by ataxia, peripheral neuropathies and myopathies [4]. Vitamin A (retinoic acid), a fat soluble vitamin, and its active metabolites play an essential role in various life processes [5]. These compounds are also used in treatment of some cancers e.g. acute promyelocytic leukemia. Isotretinoin, a vitamin A analogue, is effective in a wide variety of dermatologic disorders [6]. Vitamin A also plays an important role during development, where it influences pattern formation of several organs including hind brain, heart, spinal cord, kidneys, and limb buds [7]. Cadmium chloride is a non essential heavy metal with no known biological role in humans. Environmental sources of cadmium include combustion of fossil fuel, certain agriculture fertilizers, mining residues etc [8]. It is also found in tobacco which is a significant source. Cadmium has been implicated in various deleterious effects on different species due to its very long half life. Cadmium exposure gives rise to many developmental defects in chick including limb and anterior body wall defects [9, 10].

The Micromass system involves the culture of primary cells, isolated either from the mesencephalon, heart or the limb buds of developing embryos, plated at high density. The basic principle of assay is the potential of teratogens to disrupt normal differentiation of primary embryonic cells in vitro. Formation of neuronal tissues, chondrocytes or contractile cardiomyocytes is the basis of the endpoints used along with cell viability measurements. Several species have been used for micromass cultures: rat [11-13]; mouse [13, 14], and chick [15-21].

2.Materials And Methods

2.1.Chemical And Solutions

Vitamin E (trolox), all-trans-retinoic acid (tRA), cadmium chloride, Coomassie® brilliant blue R 250 (Kenacid Blue), Hank's balanced salt solution (HBSS), trypsin-EDTA 1mgml^{-1} in 0.4mgml^{-1} EDTA), horse serum, penicillin/streptomycin, resazurin, resorufin, purchased from Sigma-Aldrich (Poole, UK). Dulbecco's Modified Eagles Medium (DMEM) and L-glutamine was purchased from Cambrex Bio Sciences Wokingham, UK, Ltd. Fetal calf serum was purchased from Autogen Bioclear (Wiltshire, UK) and 100% ethanol (EtOH) from Fisher Scientific (Loughbrough, UK). Stock solutions were serially diluted in culture medium to give the desired concentrations. The test chemicals were added within 30 minutes of being prepared, and applied 24 hours after the cultures were seeded.

2.2.Micromass Culture Preparation

White leghorn chicken eggs (Henry Stewart Co., Louth, UK) were incubated on an automatic egg rotator in an incubator at 37°C and 100% humidity for 5 days. The day of the embryo explantation was designated day 0. The embryos were removed with bent forceps, in a Class two laminar flow hood, rinsed and placed into a 90mm sterile Petri dish (Sterilin, Stone, UK) containing approximately 10 ml of HBSS, sufficient to cover the bottom. Once the membranes were removed, with fine forceps the hearts were dissected out and pooled by placing in 5ml of sterile 50% (v/v) horse serum in HBSS at 4°C (on ice) in a bijou bottle (7ml; Bibby Sterilin Ltd , Staffordshire, UK). The cells were dissociated with 4ml 1% trypsin/EDTA at 37°C and shaken by hand every 5 minutes for 20 minutes. The cells were then triturated to give a single cell suspension and culture medium (Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal calf serum, 200mM L-glutamine and 50U/ml penicillin/50µg/ml streptomycin) was added to neutralise the activity of the trypsin, and centrifuged at RT at 1500rpm for 5 minutes. 20 µl of the cell suspension adjusted to 3x10^{6}cells/m was plated into each well of a Nunc 24-well tissue culture plate. The cells were allowed to attach for 2 hours at 37°C and 5% (v/v) CO_2 in air, before careful addition of 500µl culture medium to each of the wells. After 24 hours a further, 500µl of culture medium containing either the diluted chemical or culture medium (containing vehicle) alone was added.

2.4.Cellular Differentiation

A numerical morphological scoring system was constructed to determine the amount of contractile activity observed for heart micromass (MM). Cultures were inspected morphologically prior to chemical exposure and then 24, 48, 120 hour post exposure. The scoring was recorded according to preset characteristics (table 1). To ensure consistency the scoring system was validated by re-scoring sample wells, blind approximately one hour later.

3.Resazurin Reduction 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 37^{o}C in a 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 37^{o}C 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. 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.

5.Kenacid Blue Total Protein Assay

The same cells subjected to the resazurin reduction assay were assayed for total protein using the kenacid blue assay. Wells were aspirated and 300µl kenacid blue fixative was added and allowed to evaporate overnight at 4^{o}C. Kenacid blue working solution (400µl) 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.

5.1.Statistical Analysis

All statistical analysis was performed using Prism 5 (Graph pad Software Inc. San Diego, USA). All results with different dose concentrations were compared using one way ANOVA with Dunnet's multiple comparison post hoc test, with $p < 0.05$ was considered statistically significant.

6.Result

6.1.Vitamin E

The effect of exposure of chick cardiomyocytes to various concentrations of vitamin E (trolox) ranging from 20µM to 200µM were tested over a period of 144 hours. All of these concentrations did not affect cellular proteins or cell viability. The graph for resorufin production shows that the resorufin produced by cardiomyocytes treated with vitamin E is not significantly different to controls at any concentrations used (fig2 a). The protein content of cardiomyocytes is also not significantly different from controls at all concentrations used as shown in fig. 2 b. Statistical analyses of 24 hours post culture scores were not significant because of the reason mentioned earlier. However statistical analyses of day 24 and 120 hours post exposure also showed no significant difference to control for vitamin E treated cells (fig 2 c).

6.2.Retinoic acid with Vitamin E

The retinoic acid was proven to be toxic at concentrations more than 5 µM and above 20 µM majority of the cells died (unpublished data). So the best concentrations chosen in this study were (10-20 µM). Further experiments were performed where 200 µM Vitamin E (trolox) was added to cultures already flooded with retinoic acid (10µM and 20µM) in order to detect any protective effect offered by administration of vitamin E. Cells were scored at 24, 48 and 144 hrs by two investigators (one principle and other with labels hidden). Results show that addition of vitamin E to retinoic acid treated cells can protect the cells from embryotoxic effects of retinoic acid (Fig 3 a). Fig 3b and d, show the resazurin reduction assay. Here again the graph shows that addition of Vitamin E to retinoic acid can protect the cultures from the teratogenic effects of retinoic acid. Cellular protein levels, affected by retinoic acid were also rescued by addition of vitamin E as shown in fig 3c.

6.3.Cadmium chloride with vitamin E

Results show that addition of vitamin E to cadmium chloride treated cells could not protect the cells from its embryotoxic effects. Fig 4 a show resorufin produced by cells. Results shows that addition of vitamin E to cadmium chloride treated cells could not reverse the cytotoxic effects of cadmium chloride. Cellular protein levels, affected by cadmium chloride were also not rescued by addition of vitamin E as shown in (fig 4 b). Similarly addition of vitamin E has no protective effects on contractile activity which is decreased with cadmium chloride as shown in fig 4 c.

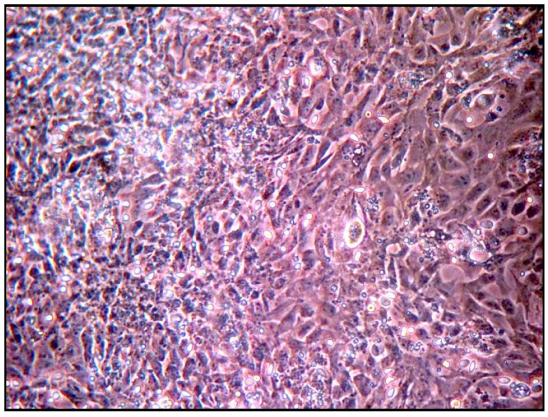


Figure 1: Light microscopy of micromass culture. Original magnification X200

Numerical morphological score	**Contractile activity**
0	No contractile activity
1	Few contracting foci
2	Numerous contracting foci
3	Entire plate contracting

Table 1: Morphological scoring system to determine contractile activity of cardiomyocytes

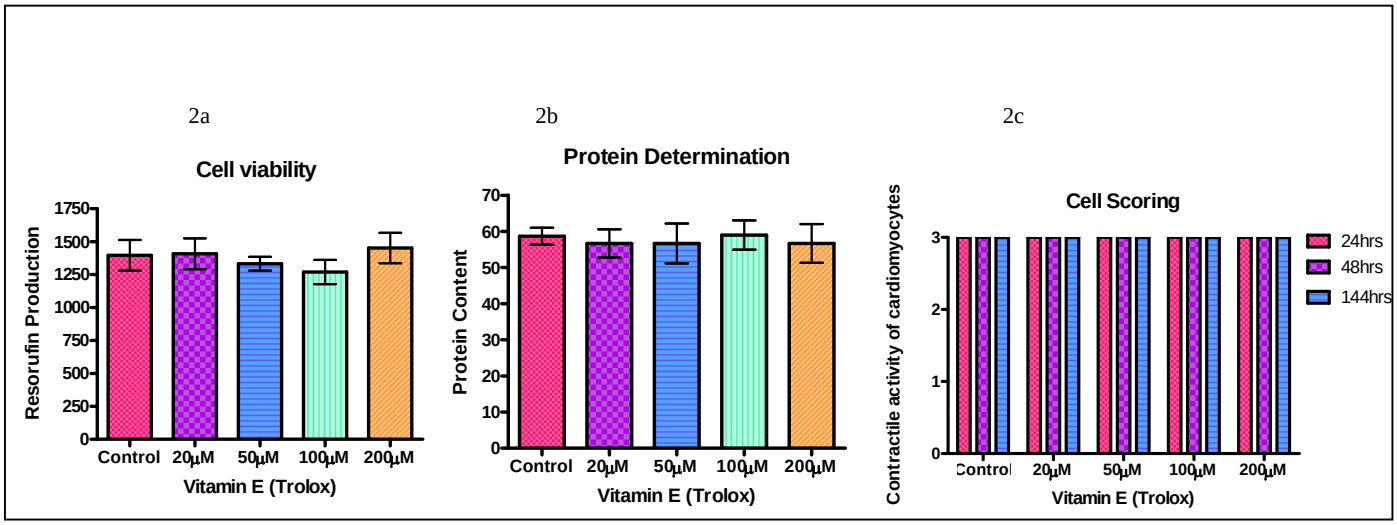


Figure 2: Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of Vitamin E.

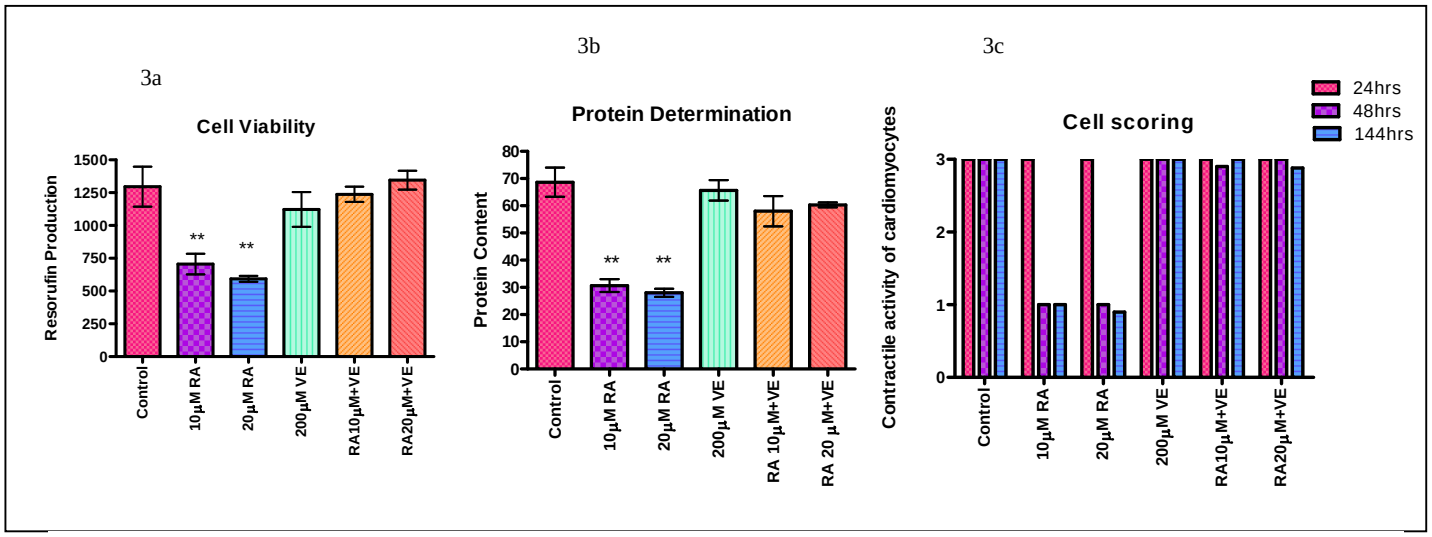
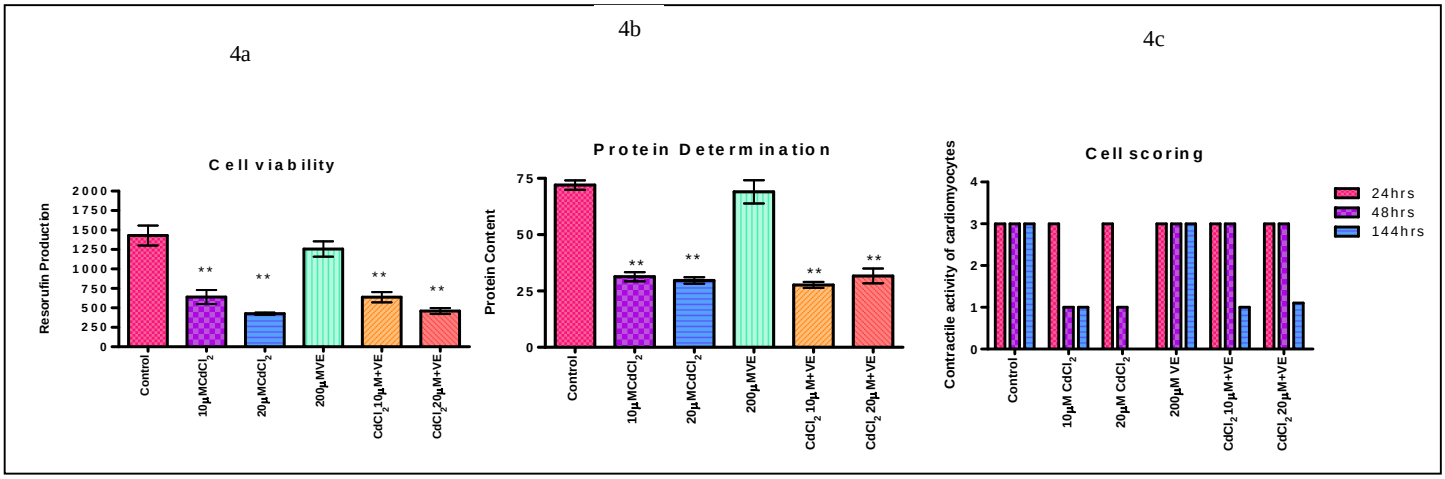


Figure 3: Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of retinoic acid plus 200µM Vitamin E



Figurte 4: Shows resorufin production (a), protein content (b), and contractile activity of cardiomyocytes (c) with different concentrations of cadmium chloride plus 200µM Vitamin E.

4. Discussion

The chick is a very appealing animal for teratogen screening because of its easy storage and handling during embryonic development, the avoidance of the sacrifice of the mother and its rapid development. Vitamin E is a biological antioxidant known to prevent lipid peroxidation of cellular membranes by quenching free radicals thus it functionally protects the cells and tissues from free radical derived damage. These vitamins also required by many animal species such as rats, sheep and chickens to maintain normal growth rate [22]. The range of doses used in this study did not show embryotoxic effects on chick cardiomyocytes. This vitamin acts as antioxidant so the maximum dose which has no toxic effects on ceels was chosen as protective agent in amelioration of toxic effects of certain chemicals used in this study.

Retinoids are known human teratogens and therefore have been extensively investigated. The teratogenic potential of all-trans 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 hurst et al to treat heart cells [15], with retinoic acid as one of the test chemicals used to treat the limb bud cells. 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.

Cadmium chloride is one of the environmental toxins; generated as a result of waste disposal, coal combustion, iron and steel production and phosphate fertilizers

manufactures. Cadmium is known to produce its teratogenic effects in many species. It is used in this study in a range of 1µM to 100µM, which is in line with other cell culture studies, which used cadmium in micro molar concentrations [23, 24]. The chick cardiomyocytes exposed to these concentrations stopped beating and cell viability assays showed a reduction in viability and total proteins. Chow and Cheng [25] studied cadmium's effects on zebra fish embryos at early stages of development and their results showed that cadmium had toxic effects on early development and these effects are due to apoptosis induced by it. In another study Chen and his colleagues found that zebra fish embryos when exposed to more than 100µM concentrations of cadmium chloride during the period of gastrulation showed developmental defects in the head and neck region, heart malformations, and had altered axial curvatures [26]. Similar types of malformations were also observed in xenopus embryos as well [27].

In order to further confirm the reliability of this culture system blind investigation was added and results of both investigators showed same results. This study was further expanded to examine the potential preventive role of vitamin E in teratogenecity induced by retinoic acid and cadmium chloride in chick micromass culture. This study demonstrates a protective effect of vitamin E when added to retinoic acid treated cardiomyocytes. The effective concentration shown to have protective effect is 200µM.

Although vitamin E when added to retinoic acid treated cardiomyocytes offered protection against retinoic acid induced cytotoxicity, results also demonstrate that supplemental vitamin E do not completely overcome the effects of RA particularly at higher concentrations. This may be due to the fact that more than one mechanism of action of RA is involved, as at present mechanism underlying the effects of vitamin E on RA teratogenicity remain unknown [28].

The results in this study suggest no preventive effects of vitamin E against cadmium chloride induced developmental toxicity even at lower concentrations (5µM). There are many mechanisms involved in cadmium induced teratogenicity; one possible mechanism could be the generation of reactive oxygen species as studies conducted on mouse and human promonocytic cells showed that prior treatment with α-tocopherols and N-acetyle-L-cysteine could prevent these cells from cadmium induced toxicity [29]. Another possible mechanism could be due to activation of calcium dependant endonucleases, as cadmium cation may mimic calcium channels and treatment with a calcium channel blocker could protect the cells from cadmium induced cell death [30].

5.Conclusion

This study suggests chick heart micromass culture as a potential alternative method for invitro toxicity assay. Also this study confirmed the role of antioxidant such as vitamin E in prevention of cardiovascular malformations brought about by environmental teratogens.

6.Reference

1. Losonczy, K.G., T.B. Harris, and R.J. Havlik, Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the Established Populations for Epidemiologic Studies of the Elderly. Am J Clin Nutr, 1996. 64(2): p. 190-196.
2. Wolf, G., The Discovery of the Antioxidant Function of Vitamin E: the contribution of Henry A. Mattill. J. Nutr., 2005. 135(3): p. 363-366.
3. Traber, M.G. and J. Atkinson, Vitamin E, antioxidant and nothing more. Free Radical Biology and Medicine, 2007. 43(1): p. 4-15.
4. Bieri, J.G., L. Corash, and V.S. Hubbard, Medical uses of vitamin E. New-Engl-J-Med., 1983. 308(18): p. 1063-1071.
5. Vliet, T.V., et al., Retinoic acdi metabolites in plasma are higher after intake of liver paste compared with a vitamin A supplement in women. Journal of Nutrition, 2001. 131: p. 3197-3203.
6. Robertson, J., et al., A survey of pregnant women using isotretinoin. Birth defects research (part A), 2005. 73: p. 881-887.
7. Mic, F.A., et al., Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. PNAS, 2003. 100: p. 7135-7140.
8. Vahter, M., et al., Integrated personal monitoring of cadmium exposure in Sweden. IARC Sci Publ, 1992. 118: p. 113-119.
9. Gilani, S.H. and Y. Alibhai, Teratogenicity of metals to chick embryos. Journal of Toxicology and Environmental Health, 1990. 30(1): p. 23 - 31.
10. Thompson, J., et al., Effects of cadmium on cell death and cell proliferation in chick embryos. Reproductive Toxicology, 2005. 20(4): p. 539-548.
11. Flint, O.P. and T.C. Orton, An invitro assay for teratogens with cultures of rat embryo midbrain and limb bud cells. Toxicology and Applied Pharmacology, 1984. 76: p. 383-395.
12. Parsons, J.F., J. Rockley, and M. Richold, In vitro micromass teratogen test: Interpretation of results from a blind trial of 25 compounds using three separate criteria. Toxicology in Vitro, 1990. 4(4-5): p. 609-611.
13. Tsuchiya, T., et al., Embryolethality of new herbicides is not detected by the micromass teratogen tests 1991. 65(2): p. 145-149.

14. Doyle, D. and C.M. Kapron, Manganese-induced toxicity in micromass cultures of mouse embryo limb bud cells. Toxicology In Vitro, 2002. 16: p. 101-106.
15. Wiger, R., A. Strottum, and G. Brunborg, Estimating chemical developmental hazard in chicken embryo limb bud micromass system. Pharmacol Toxicol., 1988. 62(1): p. 32-37.
16. L'Huillier, N., M.K. Pratten, and R.H. Clothier, The relative embryotoxicity of 1,3-dichloro-2-propanol on primary chick embryonic cells. Toxicology in Vitro, 2002. 16(4): p. 433-442.
17. Meyer, M.P., et al., The Extracellular ATP Receptor, cP2Y1, Inhibits Cartilage Formation in Micromass Cultures of Chick Limb Mesenchyme. Developmental Dynamics, 2001. 222: p. 494-505.
18. Hurst, H.S., R.H. Clothier, and M. Pratten, An evaluation of a novel chick cardiomyocyte micromass culture assay with two teratogens/embryotoxins associated with heart defects. ATLA, 2007. 35(5): p. 505-511.
19. Memon, S. and M.K. Pratten, Developmental toxicity of ethanol in chick heart in ovo and in micromass culture can be prevented by addition of vitamin C and folic acid. . Reprod Toxicol, 2009. 28(2): p. 262-269.
20. Ahir, B. and M.K. Pratten, Association of anxiolytic drugs diazepam and lorazepam, and the antiepileptic valproate, with heart defects--effects on cardiomyocytes in micromass (MM) and embryonic stem cell culture. Reprod Toxicol., 2011. 31(1): p. 66-74.
21. Memon, S. and M.K. Pratten, Teratogenic effects of diabetic conditions in chick heart in ovo and micromass culture may be prevented by addition of vitamin C and folic acid. Reprod Toxicol, 2013. 35: p. 117-124.
22. Brigelius-Flohe, R., et al., The European perspective on vitamin E: current knowledge and future research. Am J Clin Nutr, 2002. 76(4): p. 703-716.
23. Chan, P.K. and S.H. Cheng, Cadmium-induced ectopic apoptosis in zebrafish embryos Arch Toxicol, 2003. 77: p. 69-79.
24. Yano, C.L. and M.C.C.G. Marcondes, Cadmium chloride-induced oxidative stress in skeletal muscle cells in vitro. Free Radical Biology and Medicine, 2005. 39(10): p. 1378-1384.
25. Chow, H.E.S. and S.H. Cheng, Cadmium Affects Muscle Type Development and Axon Growth in Zebrafish Embryonic Somitogenesis. Toxicol. Sci., 2003. 73(1): p. 149-159.

26. Chan, P.K. and S.H. Cheng, Cadmium-induced ectopic apoptosis in zebrafish embryos. Journal Archives of Toxicology 2003. 77: p. 69-79.
27. Sunderman, F.J., M.C. Plowman, and S.M. Hopfer, Teratogenicity of cadmium chloride in the South African frog, Xenopus laevis. IARC Sci Publ., 1992. 118: p. 249-256.
28. Zhang, Z., et al., Prevention of retinoic acid-induced early craniofacial abnormalities by folinic acid and expression of endothelin-1/dHAND in the branchial arches in mouse. British journal of nutrition, 2006. 96: p. 418-425.
29. Galán, A., et al., Modulation of the stress response during apoptosis and necrosis induction in cadmium-treated U-937 human promonocytic cells. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2001. 1538(1): p. 38-46.
30. Azzouzi, B.E., et al., Cadmium induced apoptosis in a human T cell line. Toxicology, 1994. 88(1-3): p. 127-139.