International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

Genotoxicity Assessment of Acrylamide in Early Developing Stages of Chick Embryo Using Micronucleus Assay

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Abstract: ***The micronucleus (MN) test in bone-marrow and peripheral blood erythrocytes is one of the best established in vivo cytogenetic assays in the field of genetic toxicology, providing a convenient and reliable index of both chromosome breakage and chromosome loss. Therefore a study was made on micronuclei assay to evaluate the degree of genotoxicity of acrylamide and its toxicologic effects on chick embryo using peripheral erythrocytes. The Acrylamide treatment to chick embryo caused damage not only to peripheral blood cells and reticulocytes. About 3000 erythrocytes were scored for the presence of micronuclei for each dose and time point. The highest frequency of micronucleated erythrocytes (MN-E) was observed in high treated group. In the high treated group, developing chick embryos were treated with 0.6mg of acrylamide (AC). The frequency of MN-E in 24, 48 and 72h of 0.6mg acrylamide treatment 64.27, 68.71, and 204.17% increased were observed compared with 0.1mg acrylamide treatment, respectively. The present results concludes that the idea of using chick embryo as a reliable alternative genotoxicity assay system which is physiologically closer to in vivo conditions than conventional in vitro genotoxicity tests, not conflicting with ethical aspects or regulatory issues of animal protection.***

Keywords: Genotoxicity, Erythrocytes, Acrylamide, Micronucleus, Chick Embryos

1. Introduction

The micronucleus test (MNT) is an effective method for the evaluation of genotoxic or clastogenic effects of physical and chemical agents since the micronuclei (MN) are formed from the condensation of lagging acentric chromosomes, chromatid fragments or entire chromosomes (Uma and Devi *et al.,* 1990). MN is originated from acentric chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during meta or anaphase of cell division (Lindberg *et al.,* 2007). They reflect chromosome damage and may thus provide a marker of early-stage carcinogenesis (Hitoshi *et al*., 2003). The most frequently used genotoxicity test in mammals is the micronucleus test, which provides a simple and rapid indirect measure of induced structural and numerical chromosome aberrations (Heddle *et al*., 1991) and is scientifically accepted by supranational authorities such as the Organization for Economic Cooperation and Development (OECD), International Conference on Harmonization (ICH) and European Union (EU). Although MNT has several advantages over other genotoxicity tests, it can be performed only in dividing cells. In contrast to most *in vitro* genotoxicity assays, the MNT in rats provides a higher systemic complexity. The most important characteristic of the model is its capability to metabolically activate and eliminate promutagens and mutagens.

Measurement of micronuclei frequency in human lymphocytes is one of the most commonly used methods for measuring DNA damage in human populations exposed to genotoxic agents (Bonassi *et al.,* 2007; Zein, 2011). This assay has been also successfully applied to identify occupational, dietary and genetic factors that have a significant impact on genome stability. Micronucleus is originated from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides (Zein, 2011). The formation of MN in dividing cells is the result of chromosome breakage (clastogenesis) due to unrepaired or mis-repaired DNA lesions, or chromosome mal-segregation (aneugenesis) due to mitotic malfunction (Bonassi *et al.,* 2007; Suspiro and Prista, 2011). The most widely used test for the detection of MN is based on the use of cytochalasin B, a fungal metabolite that inhibits cytokinesis, being this assay named the cytokinesis-block micronucleus (CBMN) test (Suspiro and Prista, 2011). Compared to other cytogenetic assays, quantification of MN, using the CBMN assay, confer several advantages, including high reliability and low cost of the technique, no requirement for metaphase cells and reliable identification of cells that have completed only one nuclear division, which prevents confounding effects (Bonassi *et al.,* 2007; Zein, 2011).

Chromosomal aberrations (CA) contribute to cancer development in humans and experimental animals, and elevated lymphocyte CA and MN frequencies have been shown to be biomarkers of cancer risk within a population of healthy subjects. The use of MN as a surrogate for CA is supported by a number of validation studies showing a strong correlation between MN and CA frequencies within the same cell population. In experimental animals, induction of CA or MN in appropriate target cells following defined exposures is considered a biomarker of genotoxic exposure and predictive of an agent's potential to induce cancer (Mateuca *et al*., 2006).

In chick embryos most of the erythroid target cells are formed in the yolk sac. The newly formed erythrocytes appear quickly in the peripheral blood, which consists

almost exclusively from erythroid cells within the time frame of the MNT (Wolf and Luepke, 1997). Nearly all stages of maturing erythrocytes are present. In this manner the composition of the pool of circulating erythrocytes resembles the composition of bone marrow in adult mammals up to a certain way. The fact that the peripheral blood almost completely consists of erythroid cells facilitates the scoring. Artifact producing cell debris, which is common with bone marrow preparations, is rarely encountered. The parameter of genotoxicity is the frequency of all micronucleated definitive erythrocytes independent of their stage of maturity. Wolf *et al.*, 2002 from long-exposure experiments suggests that micronucleated cells are accumulated in the circulating blood since a completely developed spleen, which in most mammals eliminates aberrant and micronucleated erythrocytes is absent at this developmental stage (d11). It is assumed that this might be the reason for the higher sensitivity of the MNT in chick embryo as described earlier (Wolf and Luepke, 1997). Additionally, the egg presents an extremely high rate of erythropoiesis with in the time frame of MNT. On the other hand the target cell population grows very quickly in this way, which additionally might increase the sensitivity of the MNT (Wolf and Luepke, 1997). On the other hand the population of micronucleated cells could be diluted, which might result in a false negative outcome of the assay if the mutagen is eliminated very quickly. To emphasize the difference between conventional *in vitro* assays and the physiologically more complex MNT in chick embryo as an alternative to animal testing, this assay is designated as an *in vivo* assay (Wolf *et al.*, 2002).

The chemical carcinogen used in the present study is acrylamide. The genotoxicity of acrylamide has been studied extensively in *in vivo* and *in vitro* studies. However studies on micronuclei induction in chick embryo have not been carried out. The aim of the present study was to perform the micronucleus assay to confirm the genotoxicity of acrylamide in blood of developing chick embryo.

2. Materials and Methods

Source of Fertilized Eggs and Incubation Conditions

Freshly laid *Bobcock* strain zero day old fertilized eggs were purchased from Sri Venkateswara Veterinary University, Tirupati, and Sri Balaji hatcheries, Chittoor, Andhra Pradesh. They were incubated horizontally at 37.5±0.5˚C with a relative humidity of 65% in an egg incubator, we consider day1 (d1) as an incubation period of 24h. The humidity of the incubator was maintained by keeping the tray full of water inside. The water was replaced every alternate day and the water level was maintained to keep the same percentage of humidity throughout the incubation. Eggs were rotated manually four times a day and were examined through the Candler every day for the proper growth and viability (Ruxana and Kedam, 2010).

Acrylamide treatment

A group of six eggs (n=6) were maintained for each time point and dose. 0.1 to 0.6mg of acrylamide in saline (10mg/1ml (0.1mg)) was administered as with an increment of 0.1mg each single dose (10µl), separately, to fertilized chick embryos on each set of eggs of same group can receive equivalent quantity of acrylamide for 72h, 48h and 24h as single dose.

Blood Sampling

For micronuclei analysis at d11 blood of chick embryo was collected after 24h (d10), 48h (d9) and 72h (d8) initial administration of the test substance. Peripheral blood was also collected by incising blood vessel of the peripheral circulatory system of the chorioallantoic membrane. Approximately 10µl of the obtained blood was spread out on glass slides, immediately, after blood sampling (Wolf and Luepke, 1997).

Micronuclei assay

In this assay, (McCann *et al.*, 1976) substances with mutagenic activity are conveniently tested in a short period of time, and there is a fairly high correlation between the mutagenicity and carcinogenicity of the chemical substances tested. There are a number of short-term mutagenicity testing methods *in vivo* as well as *in vitro*, such as gene mutation assay, chromosome aberration assay and DNA damage assay. These mutagenicity tests have been used for the detection of mutagens, and the results of the tests are sometimes used to understand the fundamental mechanisms of chemical carcinogenesis (Shimoi *et al.*, 1991). The micronucleus assay using immature bone marrow erythrocytes of mice has been widely used as a simple and sensitive short-term screening method *in vivo* for determining the mutagenicity of chemical substances. As this assay uses "whole animals", it has the merits of including such factors as absorption, distribution, and metabolism of the chemical substances in the evaluation. The micronucleus assay using immature erythrocytes in circulating peripheral blood has begun to be used recently.

3. Results

Micronucleus Test (MNT)

The peripheral blood samples of developing chick embryo controls (Saline) and treated with acrylamide (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6mg) were collected at 24, 48, and 72h and smeared on clean microscopic glass slides. After air drying smears were stained with May-Grumwald's- Giemsa stain and micro nucleated RBC were counted as mentioned in the materials and methods.

Micronuclei analysis in peripheral blood erythrocytes of chick embryo treated with different dose of acrylamide

All the slides of MNT were coded before analysis. The modified staining protocol for MNT used in the present study allowed unambiguous identification of the micronucleated erythrocytes (**Figure-3**). About 3000 erythrocytes were scored for the presence of micronuclei for each dose and time point. The highest frequency of micronucleated erythrocytes (MN-E) was observed in high treated group. In the high treated group, developing chick embryos were treated with 0.6mg of acrylamide (AC). The frequency of MN-E in 24, 48 and 72h of 0.6mg acrylamide treatment 64.27, 68.71, and 204.17% increased were

observed compared with 0.1mg acrylamide treatment, respectively (**Fig-1 and 2**). In the control or vehicle control group of developing chick embryo (i.e. (n=6) treated with saline alone), the erythrocytes did not show any induction of micronuclei.

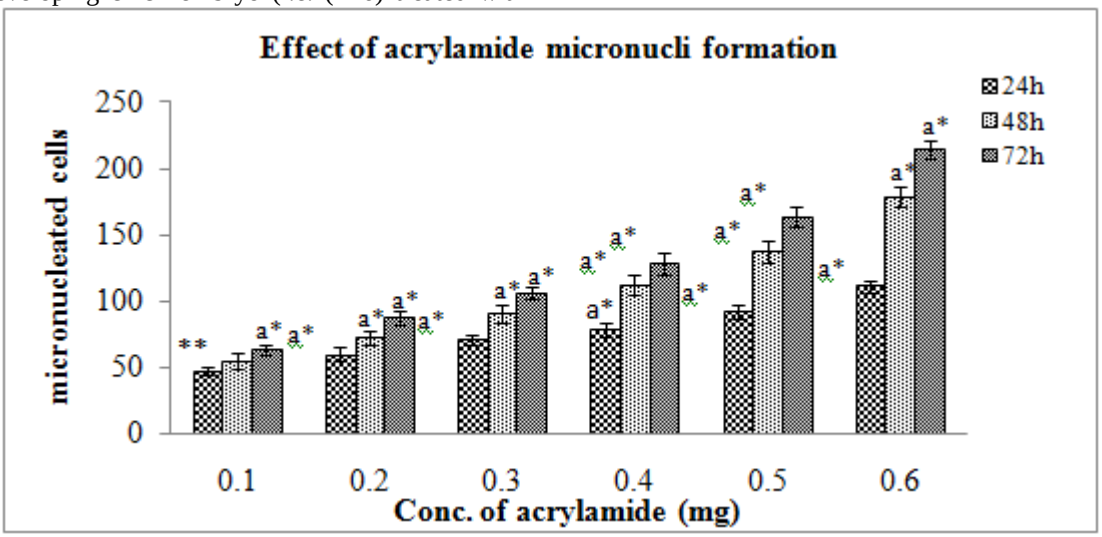


Figure 2: Effect of acrylamide micronuclei formation

Data are expressed as mean± SD

*P ≤ 0.01 Significant, **P ≤ 0.001 More Significant, and a*P ≤ 0.0001 Extremely Significant

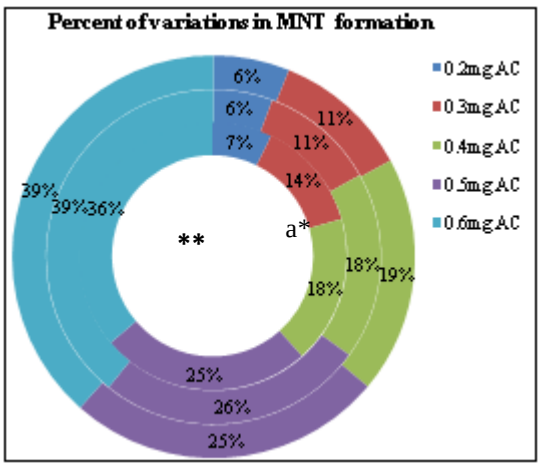


Figure 3: Percent of variations in micronuclei formation

<u>The given values were in the average of percent</u>

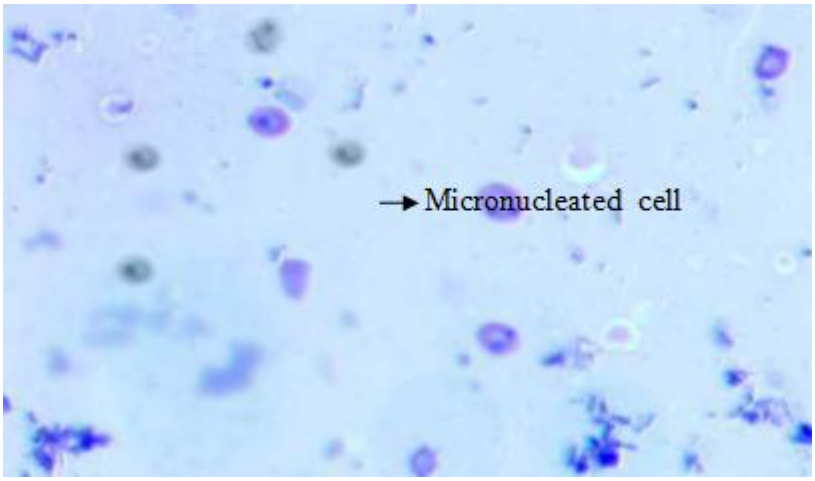
6.33 % variation was found with 0.2mg acrylamide treatment

12.0 % variation was found with 0.3mg acrylamide treatment

18.33 % variation was found with 0.4mg acrylamide treatment

25.33 % variation was found with 0.5mg acrylamide treatment

a*38.0 % variation was found with 0.6mg acrylamide treatment



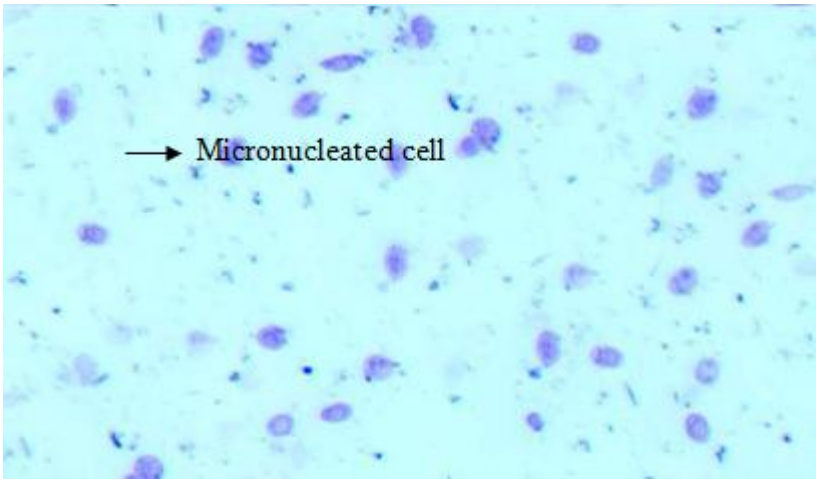


Figure 3.3: Photograph of Peripheral Blood Erythrocytes Showing Micronuclei (400X)

4. Discussion

The micronucleus test (MNT) has been extensively used to detect genotoxic effect of ionizing radiations and environmental pollutants in mammalian system (Muller and Streffer, 1994). Recently, peripheral blood MNT has been reported as a useful technique to study the effects of environmental mutagens and promutagens in the chick embryo (Wolf and Luepke, 1997). In view of the several advantages of the MNT, we have used this assay to measure the genetic damage in peripheral blood erythrocytes in developing chick embryos exposed to different doses of acrylamide. Chick embryos have been used in the past for several years to investigate the effect of environmental chemicals and radiations on developmental effects, morphogenesis, etc**.** (Bloom, 1978).

Acrylamide was found to induce chromosomal alterations (chromosomal aberrations, cell division aberration, chromosome enumeration, polyploidy, spindle disturbances) in a number of in vitro mammalian cell test systems at concentrations as low as 0.01 to 1mg/ml (Adler *et al*., 1993). A test for micronuclei in spermatids collected from Sprague-Dawley rats yielded negative results at concentrations up to 0.05 mg/ml (Lähdetie *et al*., 1994).

The current study on genotoxicity of acrylamide in chick embryonic system using micronucleus test as an end point reveals that there is a significant induction of MN-ES in AC treated chick embryos and it is dose and time dependent. The AC treated chick embryos, the frequency of micro nucleated erythrocytes steadily increased with increase in the concentration and exposure time of the dose. The highest MN frequency in each dose was found when the application took place at d8, which is 72h before blood sampling. In all the treated doses the 24h exposure (d10) application showed minimum induction. The highest MN-Es frequency was observed in 0.6mg AC treatment. These observations suggest the genotoxic effect of AC in chick embryonic system. According to Hart and Engberg-Pederson 1983, a dose related increase in the incidence of MN is the criterion for a positive effect. So based on this finding, AC can be considered to be inducer of micronucleus, which implies cytogenetic damage to peripheral blood erythrocytes.

Acrylamide is a clear germ cell mutagen in experimental animals with the potential to induce heritable genetic damage at gene and chromosomal level (Dearfield *et al.*, 1995). Acrylamide has been reported to be a positive inducer of micronuclei in mice treated *in vivo* (dose around 100mg/kg bw). The results of a series of low doses in the flow cytometer based micronucleus assay in mice have been recently reported (Abramsson-Zetterberg, 2003). Acrylamide induced micronuclei bone marrow reticulocytes of rats (Lahdetie *et al*., 1994). The low DNA content measured in the micronuclei induced by AC indicated an absence of whole chromosomes, i.e. no aneugenic effect of AC, thus suggesting a clastogenic (chromosome –breaking) mechanism. The complex pattern of genotoxicity results indicates that not only acrylamide has activity via Michael-type reactions, but its metabolic product, the epoxide glycidamide also has biological activity via direct nucleophilic substitution. *In vivo* conversion of acrylamide to glycidamide has been shown in rodents and humans. Recent findings suggest that the induction of micronuclei *in vivo* by AC exposure is essentially due to glycidamide by a chromosome breaking mechanism and not by chromosome loss. Both acrylamide and glycidamide appear to, freely distribute systematically in the body. While both compounds react with proteins, form Hb-adducts, they differ markedly in their reactivity with DNA. AC has high affinity to proteins and rather weak capacity to bind DNA. Conversely, glycidamide has strong binding to DNA and relatively weak binding to proteins (Angelo, 2006). Acrylamide-induced increases in micronuclei were seen in bone marrow cells, reticulocytes, spleen lymphocytes, spermatids and splenocytes of mice (Lähdetie *et al*., 1994; Russo *et al*., 1994). Synaptonemal complex irregularities (asynapsis in meiotic prophase) were slightly increased in germ cells of male mice following i.p. injection of AA, without a significant increase in aberrations. Tests for heritable translocations and reciprocal translocations in male mice yielded positive results (Adler *et al*., 1994).

Inhibition of DNA repair has been identified as a critical mechanism contributing to the genotoxic potential of acrylamide. AC is only weak mutagenic as such. It apparently has little direct genotoxic activity and it causes its genotoxic effects through various indirect mechanisms including: the generation of ROS, inhibition of DNA repairs

mechanisms and impairment of the cellular antioxidant defense system.

5. Conclusion

The micronucleus test in chick embryo gave clearly a positive and dose dependent effect of acrylamide. This study has supported the idea of using chick embryo as a reliable alternative genotoxicity assay system which is physiologically closer to *in vivo* conditions than conventional *in vitro* genotoxicity tests, not conflicting with ethical aspects or regulatory issues of animal protection.

6. Acknowledgments

This work was supported financially by University Grants Commission (UGC), New Delhi, India under the Rajiv Gandhi National Fellowship (RGNF) scheme.

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