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Analysis of Micronuclei Formation is an Indicator of Genotoxicity Assessment to Acrylamide in Rats and Developing Chick Embryos

Venkatasubbaiah, K[1] ., Divya, K[2] ., Pallavi, C[2] ., Kamala, K[2] , Swamy, MV[2] ., Rao, KJ[1] ., and Thyaga Raju, K[2]*

Departments of Zoology[1] & Biochemistry[2] ,
Sri Venkateswara University, Tirupathi 517 502, AP., India.
E-mail: thyagarajuk_2002@rediffmail.com

ABSTRACT

The formation of micronuclei (MN) is a widely used and accepted endpoint of genotoxicity testing. This assay provides a simple and direct measure of the induction of structural or numerical aberrations to chromosomes. In this study we describe about acrylamide treated rats and chick eggs with different doses like 0.1, 0.2 and 0.3mgs to eggs and 16, 32, 48 mgs to rats for 24,48 and 72hrs, for the detection of micronucleus formation in reticulocytes. The Acrylamide treatment to rats and chick embryo caused damage not only to peripheral blood cells and also to reticulocytes. The MN-test in chick embryo gave positive and dose-dependent induced results for acrylamide. The data presented in this study, chick embryos and rat reticulocytes have shown significant induction in micronuclei formation in both the systems considering the fact of retention of Acrylamide from 24hrs to 72hrs. The percent variation analysis and Radar data analysis for chick embryos and rat, respectively, indicated an induction of 20% in 24hrs, 32% in 48hrs and 47% in 72hrs at concentrations of lower, middle and higher doses of Acrylamide. Therefore our micronuclei analysis studies on chick embryo and rat have revealed that acrylamide retention, based on concentration and time, in reticulocytes causes variation in chromosomes so that our results on acrylamide suggest that it can serve as a mutant in *in vivo* and *in vovo*..

Key words : Acrylamide, Micronuclei. Radar data analysis, doughnut data presentation, rat, embryo, reticulocytes.

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 (Heddle *et al*., 1973; and 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

The bone marrow micronucleus test has been a most suitable genotoxicity test along with simultaneously used tests like peripheral blood micronucleus sperm count test and sperm morphology tests. This battery of tests would ensure a 100% accurate genotoxicity testing (Muller and Strffer 1994). 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 (Shelby and Zeiger 1990; Mateuca *et al*., 2006).

In chick embryos most of the erythroid target cells are formed in the yolk sac (Dieterlen-Lievre, 1988), which is together with the embryonic liver, the metabolically most important organ observed using MNT. In this way a quick first pass elimination of potential mutagenic xenobiotics can be avoided.

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.*, 2003).In rats, MN have traditionally been evaluated in bone marrow RET (reticulocytes) due to the rapid and efficient removal of MN-RET from the peripheral blood by the rat spleen (Morita *et al.*, 1997 and Torous *et al.*, 2003). However, results of recent studies have suggested that the peripheral blood MN assay can be used in place of the slide-based bone marrow assay in rats (Hayashi *et al*., 2007 and Cammerer *et al.*, 2008). Data from the tests conducted with rats in this study revealed generally higher frequencies of MN-RET detected in bone marrow slide preparations than peripheral blood.

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. (IARC, 1994). However studies on micronuclei induction in chick embryo have not been carried out. To investigate the genotoxicity of acrylamide in chick embryo and rat using micronucleus test experiments were conducted and the results are discussed.

MATERIALS AND METHODS

Source of Fertilized Eggs and Rats:
Freshly laid *Bobcock* strain zero day old fertilized eggs were purchased from Sri Venkateswara Veterinary University, Tirupati, and Andhra Pradesh. They were incubated horizontally at 37.5±0.5˚C with a relative humidity of 65% in an egg incubator.

Albino rats were acclimatized for seven days after arrival from the supplier (Sri Venkateswara enterprises, Bangalore). Control and treatment groups consisted of six animals. Animals were maintained in constant temperature (71 ± 3°F) with relative humidity of 30–70% on 12:12 hr (5 am–5pm) light: dark cycle.

Treatment
Acrylamide Treatment:
A group of six eggs (n=6) were maintained for each time point and dose. 0.1, 0.2 and 0.3 mg of acrylamide in saline was administered as single dose separately to fertilized chick embryos on day8 (d8), day9 (9) and day10 (d10) of incubation.

Treatment of rats with acrylamide:
The animals were dosed daily for six consecutive days with acrylamide concentrations of 16 mg, 32 mg and 48 mg. Sampling of blood was collected and performed for micronuclei assay after 24 hrs of each dosage.

Blood Sampling:
At d11 blood was taken after 24hrs (d10), 48hrs (d9) & 72hrs (d8) initial administration of the test substance. Peripheral blood was collected by incising blood vessel of the peripheral circulatory system of the chorioallantoic membrane. 10µl of the obtained blood was spread out on slides immediately after blood sampling (Wolf and Luepke, 1997).

Micronucleus Test:
Micronucleus test was carried out using the standard protocol (Chaubey et al., 1993).

The blood smears were air dried and stained using the following procedure:

- 3min in undiluted May-Grunwald,
- 5min in diluted may-Grunwald (1:1, May-Grunwald: distilled water),
- Rinsed in distilled water thrice (5min each) and stained with diluted Giemsa (1:6 of the Giemsa stock: distilled water) for 10min, rinsed in distilled water thoroughly.
- The slides were air dried, cleared for 5min in Xylene; the slides were mounted with cover slips using cedar wood oil.

***Scoring of Micronuclei*:**
The examination of the stained slides was done using an Olympus BX60 microscope under bright field illumination [objective: 40x and 100x (oil immersion)].

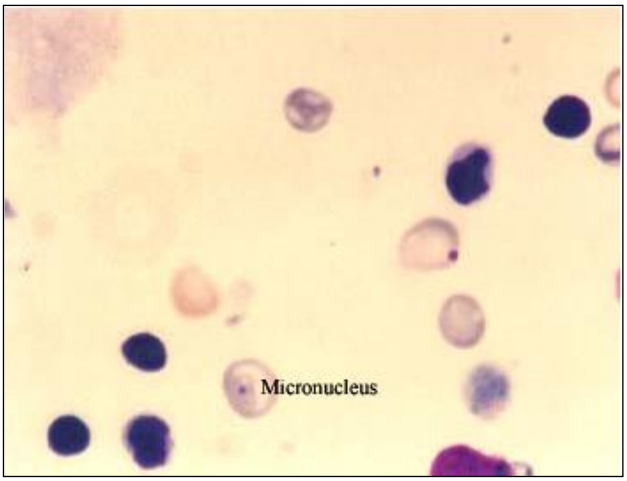
Approximately 1000 erythrocytes and the corresponding number of erythrocytes with or without micronuclei were scored from each slide. Mean frequency of total micronucleated erythrocytes was calculated for control and treated chick embryos. Altogether for each group 6000 erythrocytes were scored. The data was expressed in fold of induction and percentage of variation using data distribution graphs.

RESULTS

Micronucleus Test (MNT)
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**.** About 6000 erythrocytes were scored for the presence of micronuclei for each dose and time point (Fig 1). The frequency of MN-Es in 0.1mg acrylamide

(AC) treated chick embryos was 0.7, 1.3 and 2.0% in 24, 48 and 72 hrs treatment. There were a 2.25 and 1.71 fold increases in the frequency of MN-Es in 72 hr treatment compared to 24 and 48 hrs in the 0.2mg.

Figure-1: Photomicrograph showing micro nucleated reticulocytes in bone marrow 400X



AC treated embryos was 1.6, 2.3 and 3.7% in 24, 48 and 72 hrs treatment. There were a 2.52 and 1.67 fold increases in frequency of MN-E in 72 hr treatment compared to 24 and 48 hrs. There was a significant increase ($p<0.05$) in the induction of MN-Es frequency in 0.2mg AC treated embryos compared to 0.1mg AC treatment. In 0.3mg AC treated chick embryos; the frequency of MN-Es was 2.6, 3.6 and 4.6% in 24, 48 and 72 hr treatment. A 1.94 and 1.42 fold increase was observed in 72hr treatment compared to 24 and 48 hr with a significant increase ($p<0.05$) compared to 0.1 and 0.2mg AC treated embryos.

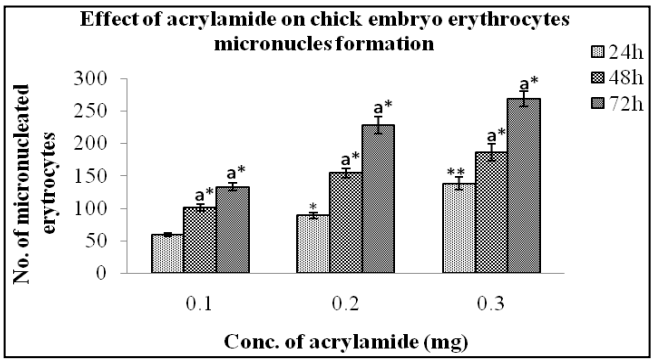
In another way of understanding purpose the data is further given in the form of Table 1.2, Fig a & b of MN as radar type and doughnut type graph analysis in variation for time of incubation and doses of acrylamide. At each stage of concentration on average 20%, 32% and 48% enhancement of formation of micronuclei was observed in embryo erythrocytes by acrylamide treatment when compared to controls

Table-1: Micronuclei analysis in peripheral blood erythrocytes in developing chick embryo treated with different doses of acrylamide.

Note: No. of Erythrocytes scored for each dose and time point: 6000. The statistical differences were assayed between the control and acrylamide treated chick embryo by an independent-sample t-test, $P\geq0.01$ not significant, $^{*}P\leq 0.01$ significant, $^{**}P\leq 0.001$more significant and $^{a*}P\leq 0.0001$ extremely significant.

AC treatment (mg)	Sampling time (h)	No. of erythrocytes containing MN	Increased % of MNE
Control	24	00	00
	48	00	00
	72	00	00
0.1	24	$9\pm2.8^{a*}$	-
	48	$101\pm4.6^{a*}$	71.18
	72	$133\pm6.2^{a*}$	125.42
0.2	24	$9\pm4.6^{a*}$	-
	48	$154\pm7.1^{a*}$	73.03
	72	$228\pm13.5^{a*}$	128.00
0.3	24	$138\pm9.8^{a*}$	-
	48	$186\pm12.9^{a*}$	86.00
	72	$268\pm11.8^{a*}$	168.00

Figure-2: Frequency of Acrylamide Induced Total Micronucleated erythrocytes (MN-Es) in chick embryo (n=6).



Note: The statistical differences were assayed between the control and acrylamide treated chick embryo by an independent-sample t-test, $P\geq0.01$ not significant, $^{*}P\leq 0.01$ significant, $^{**}P\leq 0.001$more significant and $^{a*}P\leq 0.0001$ extremely significant.

Micronuclei analysis in bone marrow reticulocytes of rats treated with different dose of acrylamide:

The frequency of MN-Es in 16mg acrylamide (AC) treated rats were 1.33, 2.26 and 2.78% in 24, 48 and 72 hrs treatment. There were a 1.91 and 1.50 fold increases in the frequency of MN-Es in 72 hr treatment compared to 24 and 48 hrs.

Table-2: Effect of acrylamide on micronucleus formation in rat bone marrow reticulocytes

AC treatment (mg)	Sampling time (h)	No. of reticulocytes containing MN	Increased % of MNR
Control	24	00	-
	48	00	-
	72	00	-
16	24	46±1.8	-
	48	69±3.7^{a*}	50.00
	72	88±5.4^{a*}	91.30
32	24	57±3.8*	-
	48	86±6.6^{a*}	50.87
	72	126±6.8^{a*}	121.05
48	24	82±3.4**	-
	48	141±8.1^{a*}	71.95
	72	186±8.7^{a*}	126.82

Table -3: MNEs formation due to acrylamide effect- determination of %change Percent variation among incubation periods

MNEs formation	24hr	48hr	72hr
Control	0	0	0
0.1	0.99	1.48	2.3
0.2	1.68	2.57	3.1
0.3	2.21	3.8	4.46

Figure-3A: Radar data type of percent of variation analysis among each dose and time of acrylamide treatment

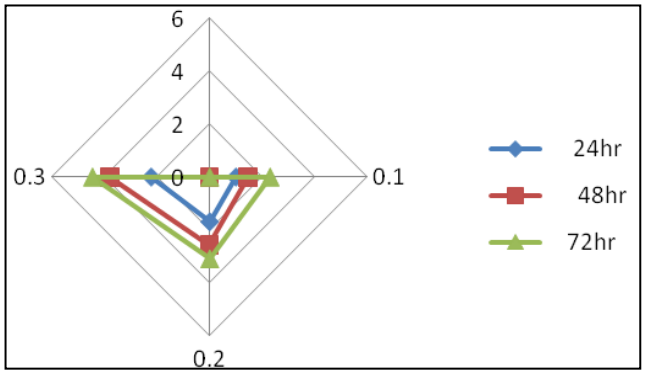
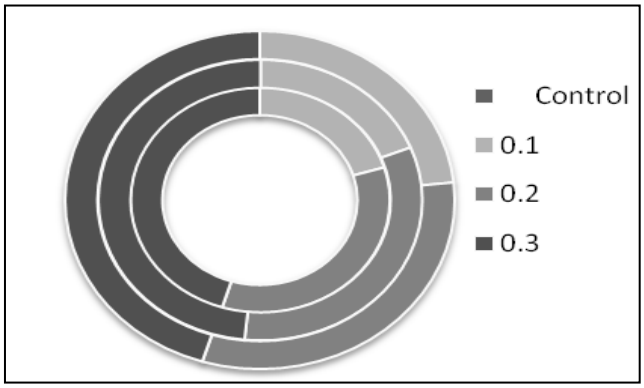
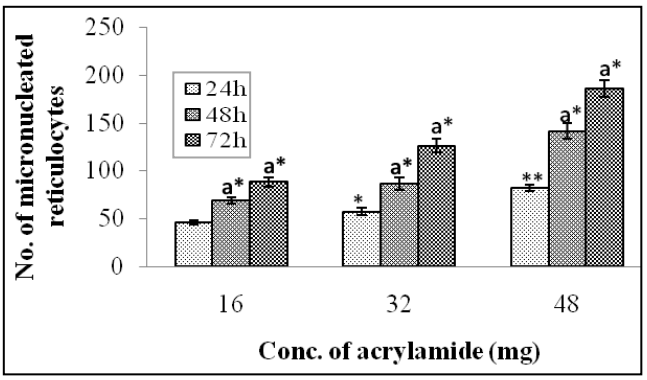


Figure -3B: Comparison data representation in percentage among each treatment of acrylamide



20% variation was found with 0.1mg AA; 32% variation was found with 0.2mg AA; 48% variation was found with 0.3mg AA

Figure-4: Effect of acrylamide on micronucleus formation in rat bone marrow cells.



The statistical differences were assayed between the control and acrylamide treated chick embryo by an independent-sample t-test, P≥0.01 not significant, *P≤ 0.01 significant, **P≤ 0.001more significant and ^{a*}P≤ 0.0001 extremely significant.

A 2.21 and 1.50 fold increase was observed in 72hr treatment compared to 24 and 48 hr in 32mg acrylamide treated rats. The reticulocytes of control group rats (i.e. 6 rats treated with saline alone), did not show any induction of micronuclei. A 2.02 and 1.71 fold increase was observed in 72hr treatment compared to 24 and 48 hr in 32mg acrylamide treated rats.

Figure-5A: Radar distribution data comparison on MNE variation in four subsets (top ½ control, right ½ 16mg, down ½ 32mg and left ½ 48 mg; center pt Zero, middle line right portion 24hrs, center portion 48hrs and left middle line 72hrs; distance among the dots is an indication of percent variation in hrs)

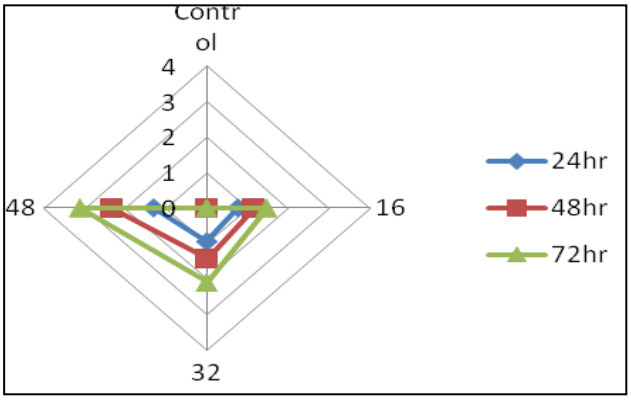


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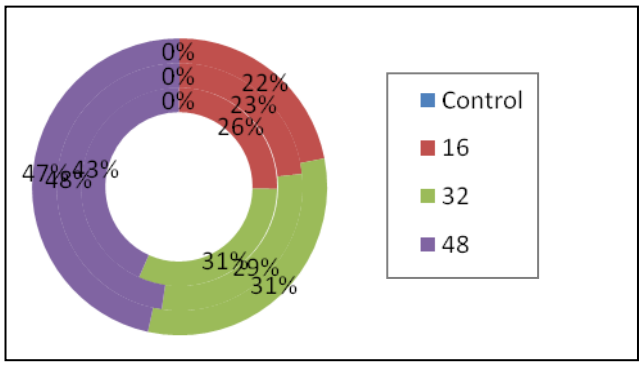
Table-4: Percent of MNE increased in rat erythrocytes due to acrylamide treatment

Treatment	24hr	48hr	72hr
Control	0	0	0
16	0.76	1.15	1.46
32	0.94	1.43	2.1
48	1.3	2.35	3.1

For better understanding purpose the data is further given in the form of Table-3; Fig 5A & B of MN as radar type and doughnut type graph analysis in variation for time of incubation and doses of acrylamide. At each stage of concentration on average 24%, 30% and 47% enhancement of formation of micronuclei was observed in rat erythrocytes by acrylamide treatment when compared to controls.

Figure-5B: Doughnut representation in data variation in percentage of each time and dose (lower circle 24hrs, middle circle 48hrs and outer circle 72hrs of Acrylamide treatment)



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**.** (Stearner and Tyler, 1957; Bloom, 1978).

Metaphase chromosome analysis has been used as one of the most accepted methods by regulatory authorities to measure genotoxicity of physical and chemical agents. Though this technique has several advantages, it is tedious, time consuming, requires small number and good chromosome morphology as well as great deal of expertise on the part of the scorer. Cytogenetic effects of various physical and chemical mutagens on chick embryo have been restricted to chromosomal aberrations, sister chromatid exchanges and chromosomal banding

analysis (Bloom, 1978; Lahijani and Ghafoori, 2000; Wilmer and Bloom, 1991).

There are two erythropoietic cell lineages in the chick embryo, the primitive and the definitive erythrocytes. The erythropoiesis in chick embryo starts in the blood island from 36h of incubation and primitive erythrocytes are released into circulation. The definite erythrocytes of the blood island origin come into circulation by the 5^{th} day of incubation. Bone marrow erythropoiesis starts by the 10^{th} day of incubation and it releases only definite erythrocytes in the blood circulation. The erythrocytes from the bone marrow also enter the peripheral circulation by 10-12 days of incubation. The spleen does not contribute to the pool of blood erythrocytes upto 11 days of incubation and even if it does, it is quite less (Wolf and Luepke, 1997). Hence, most of the erythrocytes observed in the embryos by the 11^{th} day of incubation are mostly of the yolk sac origin, which is the most metabolically active tissue (Wolf *et al.,* 2003). As has been reported earlier, by the 11 days of incubation the spleen is not a functional organ and hence the micronuclei in the cells get accumulated with a long-term exposure.

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 animals and it is dose and time dependent. This treatment not only generates malformations of chromosomes and can produce some chromosomes without genes (knock out). However further studies are necessary to determine on which chromosome they work.

In 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 0.3mg 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).

In *in vitro* mammalian assays acrylamide induced micronuclei in the absence of metabolic activation. Acrylamide has been reported to be a positive inducer of micronuclei in mice treated *in vivo* (dose around 100mg/kg bw) (IARC, 1994; FAO/WHO, 2002). 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; Xiao and Tates, 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. Glycidamide is the predominant genotoxic factor in acrylamide exposure (Angelo, 2006). Glycidamide is clearly positive in the micronucleus assay in mice and with lower potency in rats.

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 (Backer *et al*., 1989). Tests for heritable translocations and reciprocal translocations in male mice yielded positive results (Shelby *et al*., 1987 and Adler *et al*., 1994).

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 1 mg/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 rats using micronucleus test as an end point reveals that there is a significant induction of MN-E in AA treated animals and it is dose and time dependent. These treatments not only generate malformations of chromosomes and can produce some chromosomes without genes (knock out). However, further studies are necessary to determine on which chromosome they work and convert into micronuclei. In AA treated rats, the frequency of micronuclei in erythrocytes, reticulocytes 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 72hr samplings. In all the treated doses the 24h exposure showed minimum induction.

CONCLUSION

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.

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