Chapter 1

Micronuclei Analysis in Assessing Genotoxicity of Acrylamide and Cadmium chloride in Chick Embryo
CHAPTER - I
MICRONUCLEI ANALYSIS IN ASSESSING GENOTOXICITY OF ACRYLAMIDE AND CADMIUM CHLORIDE IN CHICK EMBRYO

Introduction

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 (Boller and Schmid, 1970; 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). Micronucleus test (MNT) in chick embryo utilizes the formation of micronuclei (MN) in erythrocytes of the peripheral blood of incubated hen’s egg as an end point of genotoxicity testing (Wolf and Luepke, 1997). 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 chick embryo provides a higher systemic complexity. The most important characteristic of the model is its capability to metabolically activate and eliminate promutagens and mutagens. The metabolic profile comprises phase I and phase II reactions and the system is sensitive for ah-receptor-type and Phenobarbital type inducers (Wolf and Luepke, 1997). In contrast to conventional in vitro assays and in analogy to in vivo renal elimination, xenobiotics are detoxified and excreted in to the allantoic bladder (Romanoff,
1960), from there the allantoic fluid can be obtained easily for chemical analysis (Wolf and Luepke, 1997).

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 (Heinrich-Hirsch et al., 1990) 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 within 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.

The MNT in chick embryo is not considered as an animal test, since the examinations are terminated at d11 of incubation, which marks the end of the first half of the incubation period. At this developmental stage even countries that regulate the use of avian embryos (HMSO, 1986), do not consider incubated hen’s egg as protected by animal protection regulations.

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 ovo assay (Wolf et al., 2003).

The two chemical carcinogens used in the present study are acrylamide and cadmium chloride. The genotoxicity of acrylamide and cadmium chloride has been studied extensively in in vivo and in vitro studies. (IARC, 1994; Besaratinia and Pfeifer, 2003; Abramson-Zetterberg, 2003; Jagetia and Adiga, 1994; Fahmy and Fawzia, 2000; Han et al., 1992). However studies on micronuclei induction in chick embryo have not been carried out.
Objectives:

1. To investigate the genotoxicity of acrylamide and cadmium chloride in chick embryo using micronucleus test and to explore the possibility of using chick embryo as an alternative model for genotoxicity study.

Materials and methodology of this chapter were mentioned in the chapter "Materials and Methods."

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 (Fig. 10). About 6000 erythrocytes were scored for the presence of micronuclei for each dose and time point.

The highest frequency (9.96%) of micronucleated erythrocytes (MN-Es) was observed in the positive control group. In the positive control group, embryos were treated with 0.05mg of cyclophosphamide. The frequency of MN-Es in 24, 48 and 72 hrs treatment was 1.26, 7.36 and 9.96%, respectively (Table 4). In the negative control or vehicle control group embryos (i.e. embryos treated with saline alone), the erythrocytes did not show any induction of micronuclei (Table 4).

The frequency of MN-Es in 0.1mg acrylamide (AC) treated chick embryos was 0.9, 1.6 and 2.1% in 24, 48 and 72 hrs treatment. There were a 2.3 and 1.3 fold increase in the frequency of MN-Es in 72 hr treatment compared to 24 and 48 hrs (Table 5).
In the 0.2mg AC treated embryos, the frequency of MN-Es in 24 to 72 hrs treatment ranged from 1.4 to 3.4% and there was a 2.4 fold increase in the frequency of MN-Es from 24 to 72 hrs treatment. 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 (Table 5).

In 0.3mg AC treated chick embryos; the frequency of MN-Es was 2.2, 3.1 and 4.1% in 24, 48 and 72 hr treatment. A 1.8 and 1.3 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 (Fig.11).

The frequency of MN-Es in 0.01mg cadmium chloride (CdCl₂) treated chick embryos was 2.3, 3.7 and 3.9% in 24, 48 and 72 hrs treatment. There was a 1.7 and 1.0 fold increase in the frequency of MN-Es in 72 hr treatment compared to 24 and 48 hrs (Table 6).

In 0.02mg CdCl₂ treated chick embryos; the frequency of MN-Es was 3.6, 4.7 and 5.4% in 24, 48 and 72 hr treatment (Table 6). 1.5 and 1.14 fold increase was observed in 72hr treatment compared to 24 and 48 hr with a significant increase (p<0.05) compared to 0.01mg CdCl₂ treated embryos (Table 6).

In the 0.03mg CdCl₂ treated embryos, the frequency of MN-Es in 24 to 72 hrs treatment ranged from 4.3 to 6.2% and there was a 1.4 fold increase in 72 hrs exposure compared to 24hrs treatment (Table 6). There was a significant increase (p<0.05) in the induction of MN-Es frequency in 0.03mg CdCl₂ treated embryos compared to 0.01mg and 0.02mg CdCl₂ treatments (Fig.12).
Figure 10: Photograph of Peripheral Blood Erythrocytes

Showing Micronuclei
Table 4: Micronuclei Analysis in Peripheral Blood Erythrocytes of Developing Chick Embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose /Egg (mg)</th>
<th>Day of administration</th>
<th>Day of Sampling</th>
<th>No. of erythrocytes containing MN</th>
<th>% MNE (Mean ±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (saline)</td>
<td>-</td>
<td>d 10</td>
<td>d 11</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control (Cyclophosphamide)</td>
<td>0.05mg</td>
<td>d 10</td>
<td>d 11</td>
<td>76</td>
<td>1.26±0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>442</td>
<td>7.36±0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>598</td>
<td>9.96±0.16</td>
</tr>
</tbody>
</table>

No. of Eggs examined for each dose and time point: 6

No. of Erythrocytes scored for each dose and time point: 6000
Table 5: Micronuclei Analysis in Peripheral Blood Erythrocytes in Developing Chick Embryos Treated with Different Doses of Acrylamide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose /Egg (mg)</th>
<th>Day of administration</th>
<th>Day of Sampling</th>
<th>No. of erythrocytes containing MN</th>
<th>MNE (Mean ±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>0.1mg</td>
<td>d 10</td>
<td>d 11</td>
<td>54</td>
<td>0.9±0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>96</td>
<td>1.6±0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>126</td>
<td>2.1±0.16</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.2mg</td>
<td>d 10</td>
<td>d 11</td>
<td>84</td>
<td>1.4±0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>138</td>
<td>2.3±0.14</td>
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<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>204</td>
<td>3.4±0.13</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.3mg</td>
<td>d 10</td>
<td>d 11</td>
<td>132</td>
<td>2.2±0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>186</td>
<td>3.1±0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>246</td>
<td>4.1±0.39</td>
</tr>
</tbody>
</table>

No. of Eggs examined for each dose and time point: 6

No. of Erythrocytes scored for each dose and time point: 6000
Table 6: Micronuclei Analysis in Peripheral Blood Erythrocytes in Developing Chick Embryos Treated with Different Doses of Cadmium chloride

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose /Egg (mg)</th>
<th>Day of administration</th>
<th>Day of Sampling</th>
<th>No. of erythrocytes containing MN</th>
<th>% MNE (Mean ±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium chloride</td>
<td>0.01mg</td>
<td>d 10</td>
<td>d 11</td>
<td>138</td>
<td>2.3±0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>222</td>
<td>3.7±0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>234</td>
<td>3.9±0.15</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>0.02mg</td>
<td>d 10</td>
<td>d 11</td>
<td>216</td>
<td>3.6±0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>282</td>
<td>4.7±0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>324</td>
<td>5.4±0.24</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>0.03mg</td>
<td>d 10</td>
<td>d 11</td>
<td>258</td>
<td>4.3±0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d 9</td>
<td></td>
<td>348</td>
<td>5.8±0.34</td>
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<tr>
<td></td>
<td></td>
<td>d 8</td>
<td></td>
<td>372</td>
<td>6.2±0.16</td>
</tr>
</tbody>
</table>

No. of Eggs examined for each dose and time point: 6

No. of Erythrocytes scored for each dose and time point: 6000
Figure 11: Frequency of Acrylamide Induced Total Micronucleated erythrocytes (MN-Es) in chick embryo

![Bar chart showing the frequency of micronucleated erythrocytes (MN-Es) at different concentrations of acrylamide (mg) and time points (24, 48, and 72 hours).]
Figure 12: Frequency of Cadmium chloride Induced Total Micronucleated Erythrocytes (MN-Es) in chick embryo

![Graph showing frequency of micronucleated erythrocytes in chick embryos with different concentrations of cadmium chloride over time.](image)

- **Y-axis**: Micronucleated Erythrocytes (MN-Es) measured in terms of "yes" (presence or absence)
- **X-axis**: Concentration of Cadmium chloride (mg)
- **Time Points**: 24 hrs, 48 hrs, 72 hrs
Discussion:

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. This technique allows detection of different kinds of chromosomal abnormalities, viz. symmetrical translocations and asymmetrical chromosomal abnormalities, euploidy and aneuploidy. 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).

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 and cadmium chloride.

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 5th day of incubation. Bone marrow erythropoiesis starts by the 10th 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; Bruns and Ingram, 1973). Hence, most of the erythrocytes observed in the embryos by the 11th 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 (Wolf et al., 2003).

The current study on genotoxicity of acrylamide and cadmium chloride in chick embryonic system using micronucleus test as an end point reveals that there is a significant induction of MN-ES in AC and CdCl2 treated animals and it is dose and time dependent. These treatments not only generates malformations of chromosomes and can produce some chromosomes with out
genes (knock out). However further studies are necessary to determine on which chromosome they work.

In both AC and CdCl₂ treated chick embryos, the frequency of micronucleated 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 (Fig. 11, 12). The highest MN-Es frequency was observed in 0.3mg AC and 0.03mg CdCl₂ treatment. These observations suggest the genotoxic effect of AC and CdCl₂ 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 and CdCl₂ can be considered to be inducer of micronucleus, which implies cytogenetic damage to peripheral blood erythrocytes.

Acrylamide does not cause mutations in bacterial test systems but does cause chromosomal damage to mammalian cells both in vitro and in vivo (Shiraishi, 1978). Acrylamide was a weak yet distinguishable mutagen in the in vitro test system. Acrylamide is clearly a direct acting clastogen in mammalian cells.

Acrylamide showed equivocal, negative or weakly positive results in mammalian gene mutation assays; while it induced chromosomal aberrations, micronuclei, SCEs, polyploidy, aneuploidy and other mitotic disturbances (IARC, 1994; FAO/WHO, 2002). 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 in spermatids in 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 indicate that not only acrylarnide 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).

There was a dose and time dependent increase in the induction of MN in peripheral blood erythrocytes in chick embryos treated with CdCl$_2$. Cadmium is non-mutagenic in bacterial tests and only weak mutagenic in mammalian cells in vitro (IARC, 1993).

Jagetia and Adiga, 1994; Maha and Fawzia, 2000 reported that CdCl$_2$ induced dose dependent increase in the frequency of micronuclei in mice bone marrow. Maha and Fawzia, 2000 reported that CdCl$_2$ is a potent inducer of chromosomal aberrations in mice bone marrow cells; chromosome abnormalities were also observed in mouse spermatocytes.

Cadmium was also reported to increase the chromosomal aberrations in lymphocytes after occupational exposure (Bauchinger et al., 1976). CdCl$_2$ is a potent inducer of chromosomal aberrations in cultured mouse spleen cells (Maha and Fawzia, 2000).

An increase in frequency of SCEs in cultured mouse spleen cells with CdCl$_2$ treatment were reported (Maha and Fawzia, 2000). Cd was also reported to induce SCE in human lymphocytes (Saplakoglu and Iscan, 1998; Han et al., 1992). My results on CdCl$_2$ has been shown to have clastogenic effects in somatic cells (Fig. 10; Table 6).
Inhibition of DNA repair has been identified as a critical mechanism contributing to the genotoxic potential of cadmium (Hartwig and Schwerdtle, 2002). Cadmium 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 repair mechanisms and impairment of the cellular antioxidant defense system.

The MN-test in chick embryo gave clearly positive and dose-dependent results for acrylamide and cadmium chloride, which are both well-characterized but weak mutagens.

The MNT in chick embryo is 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.
Chapter II

Acrylamide and Cadmium chloride-Induced Oxidative Stress in Chick Embryo