Micronucleated Erythrocytes in Peripheral Blood of Newborn Rabbits after Exposure to Cyclophosphamide during Pregnancy

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Summary

There are ever increasing numbers of new chemicals and pharmaceutical products that have the potential to induce birth defects or to cause damage during the perinatal period in humans. Many genotoxic compounds possess teratogenic potential and induce the formation of micronuclei (MN). Objective: To demonstrate that the New Zealand rabbit may be a useful animal model for the evaluation of transplacental genotoxicity and potential teratogenicity of compounds by quantifying micronucleated erythrocytes (MNE) in the peripheral blood of newborn rabbits following maternal exposure. Method: For each dose, a single pregnant rabbit was injected daily on the 25th to 30th days of pregnancy with 1, 4, or 7 mg/kg cyclophosphamide (CP) with one pregnant rabbit being treated with sterile water as the control. Following the daily intramuscular administrations, a drop of blood was taken from six newborn rabbits from each female for microscopic analyses. Results: When compared with controls, significant differences ($p < 0.002$) were observed in the number of MNE and micronucleated polychromatic erythrocytes (MNPCE) from newborns of females treated with 4 or 7 mg/kg CP, but not from newborns exposed to 1 mg/kg CP. No cytotoxic effects were observed after the treatments. We concluded that the presence of MNE in newborn rabbits suggests that the rabbit may be useful animal model for the detection and prevention of transplacental genotoxicity and/or potential teratogenicity of compounds in the peripheral blood of newborn rabbits following maternal exposure.

Introduction

A wide variety of tests are commonly used to determine the toxicity of compounds, and more than one test is required to evaluate the genotoxic potential of any single compound (*Shelby et al., 1993*).

The micronuclei (MN) test in the erythrocytes of peripheral blood (*Zúñiga-González et al., 2001a, 2003a, 2005*) is an easy, fast and economical assay that gives clear and accurate results. Furthermore, it is possible to apply the test *in vivo* and just a drop of blood is needed to carry out the study.

The reticuloendothelial system (RS) is responsible

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for eliminating erythrocytes with alterations, including micronucleated erythrocytes (MNE; normochromatic and polychromatic erythrocytes). Some species have spleens that remove MN from the peripheral circulation, making such measurements problematical (*Udroiu, 2006*). In contrast, MNE can be observed at any time during the lifetime of species with a less efficient RS (*Heddle et al., 1983; Zúñiga-González et al., 2001b; Gómez-Meda et al., 2004*). In general, mammals have the potential to spontaneously produce MNE and their presence might increase when organisms are exposed to genotoxic compounds (*Zúñiga et al., 1996a; Zúñiga-González et al., 1998, 2001a*). However, they are not detected in the peripheral blood of all species (*Zúñiga et al., 1996b; Zúñiga-González et al., 2000, 2001b*).

In previous studies, we described high values of spontaneous MNE in a total of 10,000 erythrocytes (TE) in newborn or young animals, while in adult members of these same species, low or near-zero values of MNE were observed (*Zúñiga-González et al., 2001b*). These differences in the MNE number reflected the maturity of the RS. Moreover, the peripheral blood of newborn rats has recently been described as a new model to evaluate the genotoxic and teratogenic potential of compounds administered during pregnancy (*Gómez-Meda et al., 2004*). In adult rabbits, MN tests are carried out on bone marrow samples, because the spleen quickly removes damaged erythrocytes from the peripheral blood (*Zúñiga-González et al., 2001b*). This species possesses a sinusal spleen and, because of this, the adult rabbit could be a 'no appropriate mammalian species' for conducting the micronucleus test on peripheral blood (*Udroiu, 2006*). Indeed, even after splenectomy, the MN number in adult rabbits did not reach that of other species (*Zúñiga et al., 1996b; Ramírez-Muñoz et al., 1999*). However, rabbits from 5 days of age, present a mean of 15 spontaneous MNE in 10,000 TE and in rabbits from 13 days old, the mean spontaneous MNE is 6 in 10,000 TE (*Zúñiga-González et al., 2001b*). Thus, the perinatal stages of this species could be a useful biomonitor.

Many genotoxic compounds that are classified as mutagenic have teratogenic potential and different mechanisms of teratogenesis could be involved in MN induction (*Ferguson & Ford, 1997*). MN have been used in a number of studies to evaluate teratogenicity in fetal rodent liver cells (*Alaoui-Jamali et al., 1989; Chorvatovicova & Ujhazy, 1995*), mouse fetal blood (*Abraham, 1995*), lymphocyte cultures from cord blood (*Henderson et al., 1986*), newborn mouse blood (*Balansky & Blagoeva, 1989*), and in studies of teratogenic potential in peripheral blood erythrocytes from newborn rats (*Gómez-Meda et al., 2004*). Any compound that is transferred across the placental barrier and induces the formation of MNE in the fetus may be considered a potential teratogen (*Gómez-Meda et al., 2004*). Thus, the MN test in newborn animals could be useful to identify compounds with teratogenic potential or genotoxicity effects during gestation (*Gómez-Meda et al., 2004*), with the advantage that the MN test is performed *in vivo*, so that any effects due to the metabolism of the original compound to even more toxic substances will also be observed (*Rodríguez-Ariza et al., 1992*).

In the present study, we describe a simple method to evaluate the genotoxic and teratogenic potential of compounds based on counting MNE in the peripheral blood of newborn rabbits following maternal exposure.

Materials and Methods

Animals

All animals used in the study were supplied by the laboratory animal facility of the Centro de Investigación Biomédica de Occidente, Guadalajara, México. Four 18-month-old female white New Zealand rabbits were housed individually in non-oxidizable cages after mating. The animal room was windowless, with automatic controls for temperature (22 ± 2°C) and lighting (lights on at 07:00 and off at 19:00 h), and relative humidity at 50 ± 10%. The rabbits received standard laboratory pelleted food (Purina®, México) and tap water *ad libitum*.

Ethical considerations

The study was approved by the Institutional Research Committee (registry number 2001249002) and by a local Animal Care Committee. All experiments were performed according to the guidelines for the care and use of experimental animals at the Centro de Investigación Biomédica de Occidente, which are in compliance with those approved by National (México; Norma Oficial Mexicana NOM-062-ZOO-1999; DOF 2001) and International Institutes of Health for the humane treatment of research animals (*Poole, 1994, APA, 2007*).

MNE induction

The formation of MNE was induced with cyclophosphamide (CP; Sigma, St Louis, MO; CAS registry number 6055-19-2) (*Porter & Singh, 1988; Harper et al., 1989*). To minimize toxicity to the developing fetuses, CP and water were given to the pregnant rabbits from the end of organogenesis (*Komae et al., 1998*), at days 25 to 30 of gestation. Four pregnant rabbits (one rabbit per dose) were treated with daily intramuscular (i.m.) injections for six consecutive days as follows: Rabbit 1 received 1.5 ml of water alone and served as a control; Rabbit 2, 1 mg CP/kg; Rabbit 3, 4 mg CP/kg; Rabbit 4, 7 mg CP/kg. The route of administration was chosen according to the ease of management of experimental animals (*Hall et al., 1977; Porter & Singh, 1988; Stanislav et al., 2000*). All doses were adjusted to a final volume of 1.5 ml with sterile water. Six newborn animals were selected at random from each mother for MNE analysis.

Sample preparation and MNE analysis

A drop of peripheral blood was taken from the tail of each newborn rabbit immediately after birth. Two smears were made on pre-cleaned and pre-coded microscope slides. The smears were air-dried, fixed in absolute ethanol for 10 min and stained with Acridine Orange (Sigma, CAS registry number 10127023) (*Zúñiga-González et al., 2003b*).

All samples were scored manually using an OLYMPUS CX40 microscope equipped with epifluorescence and an oil-immersion objective (100x). To detect accumulated and recent damage, the numbers of MNE (normochromatic and polychromatic erythrocytes) in 10,000 TE and micronucleated polychromatic erythrocytes (MNPCE) in 1,000 polychromatic erythrocytes (PCE) were counted respectively. The proportion of PCE/1,000 TE was also determined.

Statistical analysis

The pregnant female was used as the treatment unit and the litter was used as the statistical unit. The results were expressed as mean ± standard deviation of MNE, MNPCE, and PCE from the newborn rabbits per mother. Results were evaluated using the SPSS software (version 11.0) for Windows® Medical Pack (SPSS, Chicago, Ill) by means of one-way ANOVA. Tukey's b-test was employed to correct the significance values for multiple *post-hoc* pair wise comparisons. A p-value of < 0.05 was considered significant.

Results

The means, standard deviation, and significance of the MNE, MNPCE, and PCE frequencies are given in Table 1.

When the treated animals were compared with the controls, significant differences ($p < 0.002$) were observed in the number of MNE and MNPCE from newborns of females treated with 4 or 7 mg/kg CP, but not from newborns exposed to 1 mg/kg CP. No significant differences were observed in the PCE values.

Discussion

In the present study, significant differences were observed in the number of MNE and MNPCE from control animals when compared with the animals exposed to 4 or 7 mg/kg CP. However, no such differences were found between the control animals and those treated with 1 mg/kg CP. No significant dose-response relationship was observed since at the dose of 7mg/kg CP, there being no increase in

Table 1. Transplacental effect of cyclophosphamide on the MNE and MNPCE induction in newborn rabbits.

Rabbit treatment	*n*	MNE/10,000 TE	MNPCE/1,000 PCE	PCE/1,000 TE
Sterile water	6	18.0 ± 8.1	2.0 ± 2.1	278.5 ± 49.1
1 mg CP/kg	6	29.0 ± 5.1	3.2 ± 0.4	246.7 ± 38.3
		NS	NS	NS
4 mg CP/kg	6	59.0 ± 23.4	7.8 ± 2.3	281.2 ± 71.7
		$p < 0.001$	$p < 0.001$	NS
7 mg CP/kg	6	48.8 ± 14.2	7.7 ± 2.0	217.7 ± 15.1
		$p < 0.002$	$p < 0.001$	NS

One pregnant rabbit per treatment; data are expressed as mean ± standard deviation.
n: number of pups evaluated per female; CP: cyclophosphamide; MNE: micronucleated erythrocytes; TE: total erythrocytes; MNPCE: micronucleated polychromatic erythrocytes; PCE: polychromatic erythrocytes; NS: not significant.

MNE and MNPCE at that level. This could be explained by the possible cytotoxicity of such quantities, cytotoxicity that was evaluated in the peripheral blood as the proportion of PCE/TE (*Zúñiga-González et al., 2003a*). No significant differences were observed in the PCE values in control animals (278.5 ± 49.1) or in animals treated with 7 mg/kg CP (217.7 ± 15.1). However, a decrease in cell division was observed, possibly indicating that CP is slightly cytotoxic for rabbits.

CP is a potent inducer of MN that requires hepatic activation (*Krishna et al., 1995*). In the present study, it was administered to pregnant rabbits after embryos had completed organogenesis in order to limit its developmental toxicity because of its known teratogenic effects (*Hayashi et al., 1983; Porter & Singh; 1988, Harper et al., 1989*). The administration of CP, or other compounds, to the mother at an earlier stage of gestation could provoke developmental toxicity in the pups and that could have lethal effects and thus, the induction of MNE in peripheral blood might not be observed.

Genotoxic drugs that are administered to pregnant dams could affect the erythrocyte precursor cells of their pups (*Gómez-Meda et al., 2004*). Depending on the species, these erythrocyte precursors enter into circulation approximately after 24 h in the form of PCE (*Schmid, 1975, Zúñiga-González et al., 2001a*). Furthermore, if we take into account that erythrocyte production is continuous, it is easy to identify the immature or young erythrocytes in circulation due to their larger size and their different staining (*Schmid, 1975; Hayashi et al., 1983; Hayashi et al., 1990*). In contrast to mature erythrocytes, PCE do not lose their ribosomes for approximately 24–48 h after enucleation (*Heddle et al., 1983*). Thus, these cells stain red or orange with Acridine Orange (*Hayashi et al., 1983; Hayashi et al., 1990*), making them easy to detect and differentiate from mature erythrocytes (with stain dark

green) upon microscopic analysis (*Zúñiga-González et al., 2001b; Zúñiga-González et al., 2005*).

The accumulation of MNE and MNPCE are parameters that are related to genotoxicity. Increases in the incidence of MNPCE result from damage that occurred during the 24 to 48 h after treatment (*Heddle et al., 1983*) and as such, MNPCE frequencies allow us to evaluate short periods of exposure. In contrast, MNE frequencies can be used to evaluate chronic exposure, because this parameter measures both mature and immature erythrocytes, since significant increases that can initially be observed by determining the MNPCE frequency are reflected 24 h later in the MNE frequency, when the immature erythrocytes (PCE) becomes mature cells (normochromatic erythrocytes) and the cumulative damage is observed. In the present work, MNE frequencies reflect the cumulative genotoxic damage caused by the administration of 6 consecutive doses of CP, because the MN produced during the first 4 days of CP administration would be observed in the mature erythrocytes at the time of sampling.

The present model detects the teratogenic potential of the compound administered to the mother during gestation, after organogenesis. This effect was observed as an increment of the MN in the peripheral blood of the pups, as was previously demonstrated in other species (*Gómez-Meda et al., 2004*). In general, few spontaneous MNE can be found in the peripheral blood of adult rabbits (*Zúñiga et al., 1996b*). Therefore, the MN assay must be performed using bone marrow and/or by counting MNPCE in peripheral blood. Both neonatal rabbits and premature humans are organisms with relatively high frequencies of spontaneous MNE in their peripheral blood (*Zúñiga-González et al., 2001b; Gómez-Meda et al., 2004)*. In these and other species, the frequency of MNE diminishes with age, becoming virtually undetectable in the adult (*Zúñiga et al., 1996b; Zúñiga-González et al., 2001a, 2001b*). This could be relevant because we previously observed that the neonates of 12 different animal species have relatively high frequencies of MNE (*Zúñiga-González et al., 2001b*), and therefore these species may provide alternative tools for evaluating teratogenic potential and genotoxic effects by counting MNE in the peripheral blood of newborns following maternal exposure, as was corroborated here with the rabbit.

Our results show that newborn rabbits have a relatively high spontaneous MNE frequency. These values were significantly higher than we previously reported in 2-week-old or adult rabbits (*Zúñiga et al., 1996b; Zúñiga-González et al., 2001b*). Therefore, it appears that the developing RS of neonatal rabbits does not efficiently remove MNE or MNPCE from circulation and thus, it is possible to use the MN test directly in the peripheral blood of newborn rabbits.

The purpose of this assay was to determine teratogenic potential and transplacental effects of maternal exposure; this is important because there is no *in vitro* counterpart for this type of investigation. Additionally the advantage of using this, *in vivo,* type of bioindicator is that it provides the opportunity to study the effects of the metabolites of a test compound, as here from the CP, metabolites that may be generated by the organism, and which may be more active and toxic than the original agent. Thus, it is important to employ more bioindicators and to improve the techniques to obtain more precise results given that more than one test may be required to adequately evaluate the teratogenic potential and genotoxicity of any given compound (*Nau, 1986; Shelby et al., 1993; Ferguson & Ford, 1997*).

In conclusion, the present study show the feasibility of using the MN test in newborn rabbits as an alternative method to determine the potential teratogenesis and genotoxicity of compounds administered to the pregnant mother or following maternal environmental exposure by counting MNE in the peripheral blood of newborns.

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