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Possible radioprotective effect of folic acid supplementation on low dose ionizing radiation-induced genomic instability *in vitro*

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Ionizing radiation (IR) induces DNA damage through production of single and double-strand breaks and reactive oxygen species (ROS). Folic acid (FA) prevents radiation-induced DNA damage by modification of DNA synthesis and/or repair and as a radical scavenger. We hypothesized that *in vitro* supplementation with FA will decrease the sensitivity of cells to genetic damage induced by low dose of ionizing radiation. Annexin V, comet and micronucleus assays were performed in cultured CHO cells. After 7 days of pre-treatment with 0, 100, 200 or 300 nM FA, cultures were exposed to radiation (100 mSv). Two un-irradiated controls were executed (0 and 100 nM FA). Data were statistically analyzed with X^2-test and linear regression analysis (P <0.05). We observed a significantly decreased frequency of apoptotic cells with the increasing FA concentration (P <0.05). The same trend was observed when analyzing DNA damage and chromosomal instability (P <0.05 for 300 nM). Only micronuclei frequencies showed significant differences for linear regression analysis (R2=94.04; P <0.01). Our results have demonstrated the radioprotective effect of folic acid supplementation on low dose ionizing radiation-induced genomic instability *in vitro*; folate status should be taken into account when studying the effect of low dose radiation in environmental or occupational exposure.

Keywords: Apoptosis, CHO cells, Chromosome instability, DNA damage, Occupational/Environmental hazards, Radiation effects

Living beings are permanently exposed to ionizing radiation (IR) emitted either from natural or anthropogenic sources. Such exposure through environmental, diagnostic and occupational sources of radiation often involves low doses (1×10^{-4} Gy per year)[1]. Even so, most of the studies on the effects of IR are usually carried out by exposure to high doses ranging from 0.5-10 Gy as acute biological effects are not easily detected in low dose range[2]. However, experimental results obtained during the past decade seem to indicate that genetic damage induced by either low doses or low rates is higher than expected[3-6]. IR induces DNA damage by producing single-strand breaks, double-strand breaks and reactive oxygen species (ROS); and it is also well documented its ability to induce arrest of the cell cycle at G_1, S and G_2 phases. Folate deficiency induces chromosomal instability of similar type and extent[7].

Folic acid (FA) is an essential B-vitamin, obtained through dietary sources, such as broccoli, cabbage, cauliflower, fruit and nuts. Individuals having a low consumption of fruits and vegetables have plasma folate concentrations of approximately 12 nM while a proper diet leads to near 30 nM plasma folic acid[2,8,9]. Adequate folate status is vital for cell division and homeostasis because it plays an essential role in nucleic acid synthesis, methionine regeneration, and in various one-carbon units required for normal metabolism and regulation[10,11]. FA is required for the synthesis of deoxythymidylate (dTMP) from deoxyuridylate (dUMP), an essential factor for the production of methionine and ultimately S-adenosyl-methionine (SAM), the primary methyl donor for DNA methylation, which determines gene expression and prevents chromosomal fragility in specific regions, such as the centromere and fragile sites[7,12,13].

Folate deficiency reduces SAM and dTMP synthesis, causing hypomethylation in CpG sites and excessive uracil incorporation in DNA, respectively. Excessive uracil incorporation may generate point mutations and can lead to chromosome breakage and associated micronucleus formation[7,14-17]. Results from animal studies suggest that extreme folic acid deficiency causes DNA strands breaks, hypomethylation

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of DNA and increased uracil and apurinic sites in DNA[15,18]. Inadequate intake of FA is associated with increased risk of neural tube defects, Down's syndrome, cardiovascular disease, Alzheimer disease and various types of cancer[7,14,15,19-21].

In vitro studies suggest that FA deficiency increases background levels of DNA damage and can enhance the genotoxicity of chemical and physical agents. Experiments with Chinese hamster ovary cells (CHO) suggest a synergic effect between FA deficiency and γ-radiation with respect to induction of DNA strand breaks and gene mutation[22]. This finding is supported by Blount *et al*.[14] and Beetstra *et al*.[8] who postulated the existence of an interaction between FA deficiency and oxidative stress or genome damage, respectively.

Some reports suggest that folate can act as a radical scavenger, thereby contributing to protection against X-ray-induced chromosomal damage[23-25]. It has been reported that the radical scavenging capacity of folate is equivalent to that of vitamin C[24]. Endoh *et al*.[25] suggested that folate could prevent radiation-induced DNA damage by the modification of DNA synthesis and/or repair and as a radical scavenger that traps oxygen radicals produced by radiation.

Taking into consideration the general knowledge about the relationship between DNA damage prevention and folic acid status and our previous results on low dose radiation effects[26-29], we hypothesized that *in vitro* supplementation with FA will decrease the sensitivity of cells to DNA damage induced by low dose IR. In order to explore this statement, we analyzed the modulation of the low dose radiation-induction of apoptosis, DNA damage and chromosomal instability by the supplementation with different concentrations of folic acid *in vitro* in cultured Chinese hamster ovary cells.

Materials and Methods

Cell culture

CHO cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in Ham's F12 medium (Gibco BRL, Grand Island, NY, USA) containing antibiotics (50 IU penicillin and 50 μg/mL streptomycin) (Bagó Laboratories, Buenos Aires, Argentina). Cells were incubated in Falcon T-25 (Nunc, Denmark) at 37°C in a humidified atmosphere with 5% CO_2.

Experimental design

In order to allow the effect of FA supplementation on apoptosis, DNA damage and chromosomal instability to become evident, CHO cells were cultured for 7 days before harvesting on slides in accordance with an earlier investigation[2]. CHO cultures were set-up in duplicate, at 0.3×10^6 cells/mL and 0, 100, 200 or 300 nM of FA (Sigma, St. Louis, MO, USA) were added. Cultures were sub-cultured after 2, 4, and 6 and at 7 days were exposed to radiation. Two un-irradiated controls were executed: one with non-supplemented medium and another with 100 nM FA. The dose selection for FA was based on studies conducted by Beetstra *et al*. [7] who exposed that given the lack of knowledge of safety of high doses of this critical B-vitamin, more research is required to determine the genomic and epigenetic effect of FA at concentrations greater than 200 nM.

Irradiation treatments were performed with non-dividing confluent cell cultures. They were carried out on ice to prevent the repair of strand breaks during the procedure. The delivered radiation dose of ionizing radiation was 100 mSv (~100 mGy), taking into account previous experiences in our laboratory[26-29] as well as the dosimetry reported in epidemiological exposures[30-32]. The irradiation equipment was provided by Dental San Justo Company (Buenos Aires, Argentina) and operated at 65 kV and 5 mA. Doses were determined by a dosimeter (Keithley Digital 35617 EBS microchamber PTW N 2336/414; C-Com Industries, Robertville, Mo., USA) and administered at a dose rate of 100 mSv/min. Radiation was delivered from above through the medium and exposure times were 60 s. For the irradiation treatment, 10 mL of fresh medium was placed on the attached cells to prevent the presence of detached cells.

After treatment, cells were trypsinized, resuspended and divided into 3 fractions. Two aliquots were obtained for apoptosis analysis and comet assay, and the last fraction was cultured in T-25 flasks for micronucleus analysis. The experiment was repeated 3 times so as to allow an accurate estimate inter-experimental variation.

Analysis of apoptosis by Annexin V/PI staining assay

Early apoptosis, as evaluated by membrane redistribution of phosphatidylserine, was examined by using an Annexin V-FITC. Externalization of phosphatidylserine (PS) to the outer side of the plasma membrane is one of the earliest features of

cells undergoing apoptosis, which can be marked by Annexin V, a calcium-dependent phospholipid-binding protein with a very high affinity for PS[33]. By conjugating fluorescein isothiocyanate to Annexin V, it was possible to identify and quantify apoptotic cells. In short, cells were washed twice with binding buffer, resuspended in 0.1 mL of Annexin V-FITC (1 mg/mL final concentration), incubated 10 min in the dark at room temperature (20ºC), washed twice in phosphate buffer saline (PBS), added 0.1 mL of propidium iodide solution (PI) (1 mg/mL final concentration) and analyzed with a fluorescence microscope (Olympus BX40 equipped with a 515-560 nm excitation filter). About 1000 cells were scored per experimental point.

Single cell electrophoresis

Single cell gel electrophoresis was performed by using the alkaline version described by Singh[3] with some modifications[35]. Briefly, slides were covered with a first layer of 180 µL of 0.5% normal agarose (Carlsbad, Ca, USA). An amount of 75 µL of 0.5% low melting point agarose (Carlsbad, Ca, USA) was mixed with approximately 15000 cells suspended in 15 µL and layered onto the slides, which were then immediately covered with coverslips. After agarose solidification at 4°C for 10 min, coverslips were removed and slides were immersed overnight at 4°C in fresh lysis solution. The slides were equilibrated in alkaline solution for 20 min. Electrophoresis was carried out for 30 min at 25 V and 300 mA (1.25 V/cm). Afterwards, slides were neutralized by washing them thrice with Tris buffer (pH 7.5) every 5 min and subsequently washed in distilled water. Scoring was performed at 400X magnification using a fluorescent microscope (Olympus BX40 equipped with a 515-560 nm excitation filter) connected through a Sony 3 CCD-IRIS color video camera.

Based on the extent of strand breakage, cells were classified according to their tail length in 5 categories, ranging from 0 (no visible tail) to 4 (still a detectable head of the comet but most of the DNA in the tail)[36]. In order to compute DNA damage from the comets, the measuring method of Collins[36] was used. Index Damage (ID) was obtained: if 100 comets are scored, and each comet is assigned a value from 0 to 4 according to its class, the total score for the sample gel will be from 0 to 400 "arbitrary units". Visual scoring (arbitrary units) is not only rapid but also simple and there is very close agreement between this method and computer image analysis (percentage of DNA in tail)[36].

Cytokinesis-block micronucleus assay (CBMN assay)

Chromosomal damage was assessed by the CBMN

$$ID = \frac{(Grade1x1)+(Grade2x2)+(Grade3x3)+(Grade4}{Total} \times 100$$

assay according to the method of Fenech[37]. Briefly, after 7 days in culture cells were resuspended in fresh culture medium in the presence of Cytochalasin-B (3 µg/mL final concentration) (Sigma, St. Louis, MO, USA) for 20 h. Cells were harvested at 30 h and removed by trypsinization and agitation. The cell suspension was centrifuged and the pellet was resuspended in 5 mL of fixative (methanol:acetic acid 3:1). The cells were washed thrice with fresh fixative, they were later resuspended, dropped onto clean slides, and finally stained with 5% Giemsa for 8 min. The chromosome damage biomarkers scored are micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBuds). About 1000 binucleated cells (BN) were analyzed per experimental point. Fenech's[37] scoring criteria for MNi, NPBs and NBuds determinations were used. Cytostatic effects were analyzed through the nuclear division index (NDI), estimated by the ratio of mono-, bi-, and multinucleated cells. 500 viable cells are scored per experimental point to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the formula=$(M_1+2M_2+3M_3+4M_4)/N$; where M_{1-4} represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored[37].

Statistical analysis

Data were statistically analyzed with the X^2-test and linear regression analysis through the Statgraphics® 5.1 software (Manugistics Inc., Rockville, MD) and expressed as mean±standard error with $P<0.05$ considered statistically significant.

Results

Table 1 shows the results of apoptosis analysis, single cell electrophoresis and cytokinesis-block micronucleus assay. Fig. 1 summarizes the effect of folic acid (0, 100, 200 and 300 nM FA) on the irradiated cultures.

A significant increase in apoptosis was observed in irradiated cells 0 nM FA with respect to the un-irradiated controls (0 and 100 nM FA $P<0.001$).

Table 1—Mean frequencies (average±standard error) of apoptotic cells, index damage, micronúcleos (MNi), nucleoplasmic bridges (NPBs), nuclear budding (NBuds) and nuclear division index (NDI) in CHO cells.

		Annexin-V	Comet assay	CBMN			
Treatment	FA (nM)	Apoptotics cells	Index damage	MNi	NPBs	BUDs	NDI
Non-irradiated	0	20 (0.14)***	27 (0.25)***	24 (0.15)*	6 (0.08)	6 (0.08)	1.99 (0.04)
	100	48 (0.21)***	27 (0.25)***	20 (0.14)**	5 (0.07)	6 (0.08)	1.8 (0.04)
100 mSv- irradiated	0	94 (0.29)	56 (0.35)	45 (0.21)	5 (0.07)	4 (0.06)	1.84 (0.04)
	100	64 (0.24)*	65 (0.37)	35 (0.18)	4 (0.06)	5 (0.07)	1.6 (0.04)
	200	53 (0.22)***	43 (0.31)	32 (0.18)	7 (0.08)	6 (0.08)	1.66 (0.04)
	300	59 (0.24)**	31 (0.27)**	27 (0.16)*	7 (0.08)	4 (0.06)	1.78 (0.04)

Folic acid=FA; Cytokinesis-block micronucleus assay=CBMN

X^2-test through the Statgraphics® 5.1 software was used. Significance for the comparison between 100 mSv irradiated cells with all the experimental points was marked with asterisks.

* P <0.05, ** P <0.01, *** P <0.001

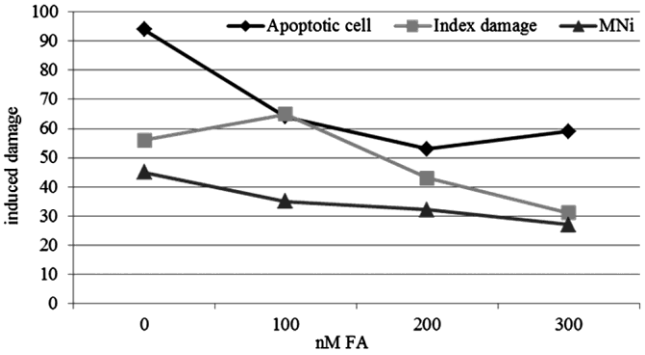

Fig. 1—Relationship between the irradiated cultures supplemented with 0, 100, 200 and 300 nM FA for apoptotic cells, index damage and MNi frequencies were shown. Linear regression analysis was performed with the Statgraphics® 5.1 software.

Irradiated cultures supplemented with 100, 200 or 300 nM FA showed a significantly lower apoptosis percentage than irradiated 0 nM FA (P <0.05; 0.001 and 0.01, respectively). There were no differences among the FA supplemented cultures.

The comet assay values scored in irradiated cells 0 nM FA were significantly higher than those observed in un-irradiated controls (0 nM FA P <0.01; 100 nM FA P <0.005). Cultures supplemented with 200 and 300 nM FA showed lower DNA damage than irradiated 0 nM FA; the differences were statistically significant for the higher FA dose (P <0.01). The successive doses of FA pre-treatment induced index damage decrease, these differences were statistically significant between 100 nM FA and the other two doses (100 nM FA *vs*. 200 nM FA P <0.05 and 100 nM FA *vs*. 300 nM FA P <0.001).

A significant increase was observed in MNi frequency in irradiated 0 nM FA cells with regard to the un-irradiated controls (0 nM FA P <0.01; 100 nM FA P <0.005). The frequency was higher in irradiated 0 nM FA in relation to the supplemented cultures; the differences were statistically significant with 300 nM FA (P <0.05). There was a decrease in MNi frequency in supplemented cultures with increasing FA concentration; the differences were not statistically significant. There were no significant differences in the frequencies of NPBs and NBuds among the experimental points. NDI was higher in control group with respect to the rest of the experimental points. Culture supplemented with 300 nM FA showed a high NDI with regard to the other two doses; the differences were not statistically significant.

The relationship between the irradiated cultures supplemented with 0, 100, 200 or 300 nM FA for apoptotic cells, index damage as well as MNi frequencies, was studied through the linear regression analysis. There was a decrease in the three parameters in the supplemented cultures with increasing FA concentration, even statistical significant differences were only found for MNi frequencies (R2=94.04; P <0.01).

Discussion

Generally, when cells are exposed to low dose IR, double-strand break formation is one of the most important kinds of damage observed[38]. If repair is either wrong or not possible, a cell is meant to die or remain damaged[4,39]. In these sense, we found an increase of apoptotic cells, DNA damage and chromosome instability in irradiated CHO cells

with decreasing folic acid concentration. Several studies have shown that folate affects chromosomal stability playing critical roles in the prevention of chromosome breakage and hypomethylation of DNA[40,41]. Meanwhile, Huang *et al*.[42] has suggested that FA deficiency may block DNA replication and/or mitosis, which may be involved in apoptosis induction[2].

Our results could be contrasted with those obtained by Beetstra *et al*.[7] who found a significant dose-dependent increase in frequency of binucleated cells with micronuclei and/or nucleoplasmic bridges with decreasing FA concentration and IR (1.5 Gy γ-rays) relative to un-irradiated controls. FA deficiency and γ-irradiation were shown to have a significant interactive effect on frequency of cells containing micronuclei such that the frequency of radiation-induced micronucleated cells increased with decreasing FA concentration. In this sense, Crott *et al*.[9] found that folate deficiency (12 nM) causes increase in micronuclei frequency in human lymphocytes when being irradiated with 0.4 Gy of radiation; in another work they found that FA concentration correlated significantly and negatively with all markers of chromosome damage (micronuclei, apoptosis, necrosis, nucleoplasmic bridges and nuclear budding), which were minimized at 60-120 nM FA[43]. Branda and Blickensderfer[22] reported that folic acid-deficient CHO cells manifested strand breaks equivalent to a dose of 0.3 Gy. Meanwhile, Courtemanche *et al*.[2] found that both, radiation exposure and folate deficiency, decreased cell proliferation and induced DNA breaks, apoptosis, and cell cycle arrest in human lymphocytes, but they did not even study the combined effect of both factors. Endoh *et al*.[25] who investigated the influence of supplementation with FA on X-ray induced chromosomal damage *in vivo* in mouse cells and *in vitro* in human lymphocytes also found that the supplementation of FA in a low folate status is beneficial against X-ray-induced chromosomal damage (0.5 Gy), but excess supplementation in a normal folate status has relatively less benefit.

DNA damage reported by all these authors[2,7,9,14,22,25,43] was observed by using various cellular systems, different FA concentration and higher radiation doses than those used in our work. However, we have already reported cytomolecular and cytogenetic damage in CHO and MRC-5 cells exposed to very low doses of X-rays and their progeny by using comet and micronucleus assays indicating that mammalian cells are exquisitely sensitive to this kind of radiation[44].

In our work, the protective role of FA was observed with doses higher than 200 nM, even more the dose of 300 nM showed the minor level of DNA damage and chromosomal instability. These results support the fact that the "normal" human plasma physiological concentration of 20 nM folate is not optimal for minimizing chromosomal instability. In addition, the protective dose we found is higher than those proposed in previous literature[7,9,14,43,45,46]. Fenech and Crott[47] in an *in vitro* study using human lymphocytes found that chromosome damage was minimized at 120 nM FA, which is greater than the concentration of FA normally observed in plasma (20-60 nM) in absence of anemia. In an *in vivo* intervention Fenech *et al*.[45] showed a 25% of reduction in lymphocyte micronuclei frequency following a 3 month daily supplementation with 700 ug folic acid and 7 ug vitamin B12. In the same way, Fenech[15] exposed that the current sufficient levels of folate in plasma (4.9 nM) and red blood cells (298 nM), based on prevention of anemia, are much lower than the averaged concentration levels at which DNA damage is minimized (36 nM for plasma and 938 nM for red blood cells).

Two mechanisms about the influence of folate status on DNA damage induction were proposed: (i) as folate donates methyl groups for DNA synthesis and repair, it could protect against chromosomal damage during both processes[48]; (ii) as folate acts as a radical scavenger removing ROS generated by X-ray irradiation, it has been shown to achieve the same effect that vitamin C[23,24].

Taking into account these considerations and the well-known effect of IR increasing the demand for DNA repair systems and the ROS production, it may be suggested that folate deficiency creates a permissive environment for genomic instability and supplementation with folic acid decreases the sensitivity of cells to radiation-induced DNA damage and chromosomal instability. However, as our study was done using *in vitro* cultured CHO cells, results cannot be directly extrapolated to human cells *in vivo*. Recently, researchers have shown the radioprotective effects of Seabuckthron (*Hippophae rhamnoides* L.)[49] and *Curcuma*[50] against IR-induced DNA damage. Singha and Das[51] have also demonstrated the potential of Grapevine extract in protecting human

lymphocytes against IR-induced oxidative stress and apoptosis. Further research is required to determine the genomic effects of folic acid concentration in irradiated human cells as a result of environmental or occupational exposure.

Conclusion

Our results show that folic acid supplementation has a radioprotective effect on low dose ionizing radiation-induced genomic instability *in vitro*. Therefore, folate status should be taken into account when studying the effect of low dose radiation in environmental or occupational exposure; and also when programming therapeutic irradiations.

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