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Testing genotoxicity and cytotoxicity strategies for the evaluation of commercial radiosterilized fetal calf sera

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Abstract

Effects of 18 commercial lots of fetal calf serum (FCS) after γ-irradiation and their non-irradiated counterparts were comparatively analyzed on CHO-K1 and MDBK MDL1 cells for genotoxicity [sister chromatid exchange (SCE), micronuclei (MNi), and single cell gel electrophoresis (SCGE)], cytotoxicity [cell-cycle progression (CCP), proliferative replication index (PRI), mitotic index (MI), growth promotion (GP), and plating efficiency (PE)], and microbiological properties (mycoplasma and bovine viral diarrhea virus contamination). SCE and SCGE were the most informative end-points for genotoxicity since significant differences were found in 44.4% ($P < 0.05–0.001$, Student's *t*-test) and 61.1% ($P < 0.05–0.001$, χ^2 test) samples, respectively. MI was the cytotoxicity assay revealing the greatest variation, showing differences in 66.7% ($P < 0.05–0.001$, χ^2 test) samples. Thus, these three end-points for screening bioproducts such as FCS were found most suitable for detecting potential geno-cytotoxicants in biological samples; their simultaneous use could be strongly recommended.

Keywords: Gamma irradiation; Comet assay; Commercial fetal calf serum; Mitotic index; Sterilization; SCE assay

1. Introduction

Cell culture system is worldwide employed for different purposes as basic cellular research, protein pharmaceutics, vaccine production, and virus propagations. However, there is one essential requirement within this biological system that should be always present, i.e., the culture must supply the necessary nutrients and growth factors. The chemical composition of the medium is very complex. A large number of artificial culture media are commercially available essentially sharing four groups of components among its formulation, namely inorganic salts, carbohydrates, aminoacids, and various supplements (i.e., vitamins, fatty acids, lipids, and growth factors) [1,2].

The use of tissue culture from animal cells requires a system that should reproduce in vivo condition/s as much as possible. These are defined by physico-chemical factors that influence cellular metabolism parameters such as pH, temperature, osmotic pressure, electrolytes, essential and non-essential metabolites, hormones and specific factors. For most cell lines, the primary media supplement is the fetal calf serum (FCS). It provides several biological molecules such as albumin, antichymotrypsin, apolipoproteins, biotin, and growth supporting factors, which allow optimal cell growth [1]. Different physico-chemical properties of cell growth media depend upon the presence of the serum, which includes viscosity, osmolarity, buffering capacity as well as diffusion rates. These properties contribute to protect cells from enzymatic treatment and/or mechanical damage required for cellular in vitro propagation.

In spite of being one of the major components of cellular culture media, the FCS is also one of the principal bacterial, fungal, mycoplasmic, and/or viral contamination sources. Accordingly, it results evident that sera must be free of these

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contaminating microorganisms before its use in biological studies. Sera sterilization is usually carried out by filtration. However, the use of ionizing radiation for sterilization of medical and biological products/subproducts has become one of the methodologies most employed since while radiation-resistant living organisms such as fungi, bacteria, and viruses are effectively inactivated, no excessive damage to the material is induced [3–5]. Radiation effects on proteins could be related to alterations in either physico-chemical properties, although specific changes in the biological functions of proteins cannot be totally avoided. In most of the cases, if not all, irradiation of biochemical drugs and reagents, including FCS, results more advantageous in dry or frozen state, since most products irradiated in solution have proved to lose their biological activity [6,7].

In the present study the in vitro effects exerted by 18 commercial lots of FCS after γ-irradiation and their non-irradiated counterparts were comparatively analyzed on Chinese hamster ovary (CHO-K1) and Madin Darby bovine kidney (MDBK MDL1) cells by a battery of meaningful bioassays for genotoxicity [i.e., sister chromatid exchange (SCE), micronuclei (MNi), and single cell gel electrophoresis (SCGE)], cytotoxicity [i.e., cell-cycle progression (CCP), proliferative replication index (PRI), mitotic index (MI), growth promotion (GP), and plating efficiency (PE)] as well as for microbiological properties [i.e., mycoplasma and bovine viral diarrhea virus (BVDV) contamination]. The aims of this study were to determine whether γ-irradiated serum behaves differently from, or is comparable to the non-irradiated one, and to determine which in vitro assays are most suitable to detect such differences whether they exist.

2. Materials and methods

2.1. Chemicals

Colchicine (CAS 64-86-8), 5-bromo-2′-deoxyuridine (BrdUrd, CAS 59-14-3), agarose (CAS 9012-36-6), and agarose low melting point (CAS 39346-81-1) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Access RT-PCR Introductory System was purchased from Promega (Madison, WI, USA) while TRIzol® was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Fetal calf sera source and irradiation conditions

A total of 18 samples from FCS lots of a total volume of about 80 L prior and after γ-irradiation were kindly provided by Internegocios S.A. (Mercedes, Buenos Aires, Argentina). Prior to radiosterilization, sera were sterilized by filtration using two sequential 0.1 μm pore-size rate filters. Frozen sera (−20 °C) in 1 L plastic flasks (Nalgene Nunc International Corporation, Rochester, NY, USA) were irradiated with a γ-radiation ^{60}Co source of 18.5 PBq (5×10^5 Ci) at the Industrial Irradiation Installation from the Comisión Nacional de Energía Atómica (CNEA, Buenos Aires, Argentina) distributed in 4 packaging boxes containing 20 individual bottles, and 1 dosimeter was employed per individual packaging box. Irradiation was carried out at 32 kGy during 4.5 h in an irradiation chamber at 20 °C. The serum temperature immediately after irradiation was −10 °C. Afterwards, sera were re-freezed for handling at −20 °C. Samples of non- and γ-irradiated sera were kept frozen at −20 °C until use.

2.3. Cell source

Chinese hamster ovary (CHO-K1, CCL-61™) cells were grown in Ham's F10 medium (Gibco, Grand Island, NY, USA). Madin Darby bovine kidney (MDBK MDL1, CCL-22) were grown in Dulbecco's modified Eagle-MEM (Gibco). All cell lines used throughout all in vitro experiments were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C in a 5% CO_2 atmosphere in the corresponding media supplemented with 100 units/ml penicillin (Gibco), 10 μg/ml streptomycin (Gibco), and 10% of the fetal calf serum (Gibco). No cell adaptation to the 18 FCS commercial lots included in the study was performed before initiation of the different assays as suggested elsewhere [8]. Several reports have demonstrated that CHO-K1 cells represent a suitable target for the accurate detection of genotoxic/cytotoxic compounds of various types in in vitro studies (for review see Aardema and coworkers [9] and references therein). Finally, MDBK MDL1 was selected for its susceptibility to BVDV, among other bovine virus (http://www.atcc.org).

2.4. Sister chromatid exchange (SCE) and cell-cycle progression (CCP) assays

2.4.1. Cell cultures

At the log phase of growth, CHO-K1 cells were seeded in T25 flasks (3.5×10^5 cells per flask) in media supplemented with 10% of the tested FCS. Afterwards, 10 μg/ml BrdUrd were incorporated into cultures and cells were then incubated under a safety light for an additional 24 h period until harvesting. During the last 3 h of culture, cells were treated with 0.2 μg/ml colchicine, then detached with a rubber-policeman, collected by centrifugation, hypotonically shocked (0.075 M KCl, 37 °C, 17 min), and fixed in methanol/acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique.

2.4.2. Fluorescence-plus-Giemsa (FPG) method for sister chromatid differentiation

Chromosome spreads were stained using the FPG technique for sister chromatid differentiation as previously described elsewhere [10,11]. Slides were coded and blindly scored by one researcher.

2.4.3. Cell-cycle progression (CCP) and mitotic index (MI)

A minimum of 100 metaphases per sample were scored to determine the percentage of cells that had undergone one (M_1), two (M_2), and three or subsequent mitoses (M_{3+}). The PRI was calculated for each experimental point according to the formula $\mathrm{PRI} = [(\%M_1) + 2(\%M_2) + 3(\%M_{3+})]/100$, which

indicated the average number of times that cells had divided in the medium since the addition of BrdUrd until harvesting [12]. The MI was determined by scoring 1000 cells from each experimental point and expressed as number of mitoses among 1000 nuclei. Changes in the MI were expressed as a factor (f) of the mean MI from treated cultures (MI_t) over the mean MI from controls (MI_c) ($f = MI_t/MI_c$) [13].

2.4.4. SCE analysis

For the SCE assay, a total of 25 well-spread diploid M_2 cells metaphases were scored per experimental point from each experiment. Data were expressed as the mean number of SCEs per cell ±SE from 75 cells.

2.5. Micronuclei (MNi) assay

2.5.1. Cell cultures

At the log phase of growth, CHO-K1 cells were seeded onto pre-cleaned 22 × 22 mm cover slips in 35-mm Petri dishes (1.2×10^4 cells/dish) in media supplemented with 10% of the tested FCS. Afterwards, cells were incubated at 37 °C in a 5% CO_2 atmosphere under a safety light for an additional 24 h period until harvesting. Cultures were duplicated for each experimental point, in at least three independent experiments.

2.5.2. Slide preparations

After culturing, the cover slips were rinsed with Hank's balanced salt solution (3 times, 2–5 min each) and exposed to a mild hypotonic shock in situ using 0.075 M KCl (37 °C, 5 min). Afterwards, cells were fixed with 100% (v/v) cold methanol and cover slips were stained with 4–6-diamino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). After staining, the cover slips were air-dried and then placed down onto pre-cleaned slides using mounting medium. Coded slides were blindly scored by one researcher at 600× magnification.

2.5.3. MNi analysis

For MNi assay, at least 1000 nucleated cells were scored per experimental point from each experiment. Briefly, criteria applied for MNi identification were: diameter smaller than 1/3 of that of the main nuclei, non-refractability, same staining intensity as or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, MNi boundary distinguishable from main nuclei boundary, and no more than 4 MNi associated with the nuclei [14,15].

2.6. Single cell gel electrophoresis (SCGE) assay

2.6.1. Cell cultures

At the log phase of growth, CHO-K1 cells were detached with a rubber-policeman, centrifuged, and then resuspended in complete culture medium supplemented with 10% of the tested FCS. Afterwards, aliquots containing 2.0×10^4 cells/ml were washed twice with complete culture medium prior to SCGE assay. Cultures were duplicated for each experimental point.

2.6.2. SCGE protocol

The SCGE assay was performed following the alkaline procedure described by Singh and collaborators [16] with minor modifications. Briefly, two solutions containing 0.5% normal melting agarose (NMA) and 0.5% low melting agarose (LMA) solution in Ca^{2+}–Mg^{2+}-free PBS were performed. Seventy-five μl NMA were transferred onto 100% ethanol pre-cleaned slide, spread evenly, and placed at 37 °C for 20–30 min. Afterwards, 95 μl LMA together with 7.0×10^3 cells (20 μl cell suspension +75 μl LMA) were applied, covered with a cover slip and placed at 4 °C for 15 min. After this layer had solidified, a third layer of 75 μl LMA was added and slides were immersed into ice-cold freshly prepared lysis solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10.0, 1% Triton X-100, 10% DMSO) and then lysed in darkness for 2 h (4 °C). After this period, slides were placed in a horizontal electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 20 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 25 V and 250 mA (0.8 V/cm). Finally, the slides were neutralized with a solution containing 0.4 M Tris–HCl, pH 7.5, stained with DAPI (Vectashield mounting medium H1200). Slides were coded and blindly scored by one cytogenetist. Analysis of the slides was performed under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The cellular nucleus diameters plus migrated DNA were individually measured using a calibration scale with a 100X fluorescence objective from 50 randomly selected cells per experimental point for each experiment. Cells were visually graded into four categories as suggested elsewhere depending on DNA damage level as: undamaged, slightly damaged, damaged, and highly damaged, respectively [17,18]. Briefly, cells were classified as undamaged (no tail comet, diameter ≤30 μm), slightly damaged (diameter >30–45 μm), damaged (tail comet length >45 μm), and highly damaged (dying or dead cells).

2.7. Growth promotion (GP) assay

Experiments were set up with CHO-K1 cells at the log phase of growth and seeded in T25 flasks (1.0×10^6 cells per flask) in media supplemented with 10% of the tested FCS. Cells were propagated at 37 °C in a 5% CO_2 atmosphere through five subcultures in the same culture media and the number of total cells was determined every 48 h thereafter. Data were expressed as the mean value of total number of cells from each of the five consecutive subcultures. Cells were examined throughout the subcultures for evidence of morphological changes or cytotoxic effects.

2.8. Plating efficiency (PE) assay

At the log phase of growth, CHO-K1 cells were seeded in media supplemented with 10% of the tested FCS at a concentration of 20 cells/3 ml culture media in 30 mm Petri dishes. Afterwards, cells were incubated at 37 °C in a 5% CO_2

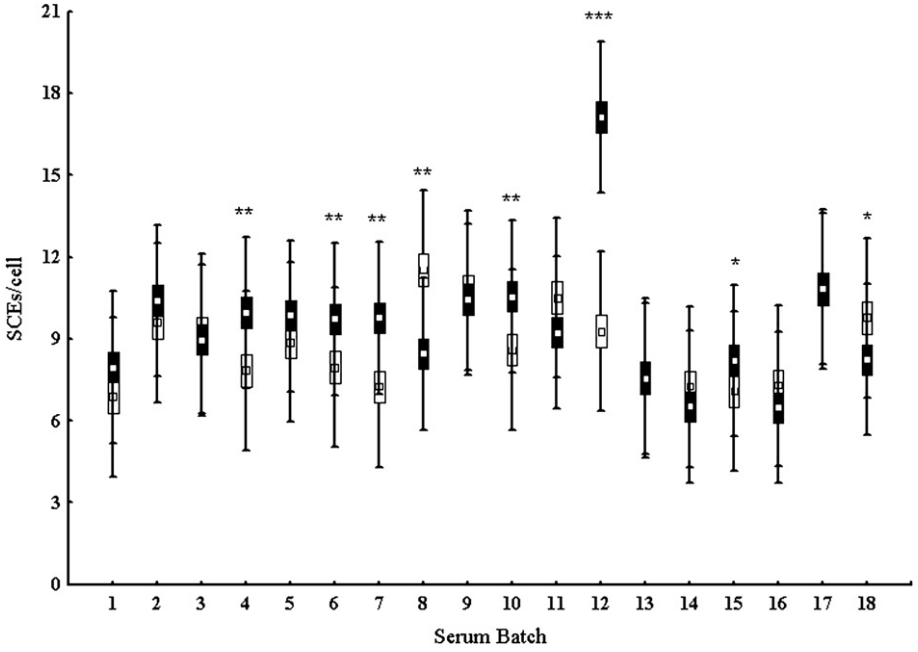

Fig. 1. Frequency of SCEs in CHO-K1 cells cultured with 18 commercial lots of FCS after γ-irradiation (white boxes) and their non-irradiated counterparts (black boxes). Data are displayed as box plots, where the *y*-axis shows the range data from 75 s mitoses analyzed per cell culture. Each box encloses the median value of SCEs/cell (small white square). The top and the bottom of the box mark the SCEs mean value ±SE of the mean. The lines extending from the top and the bottom of each box mark the pooled SD of the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences between pairs of sera.

atmosphere for an additional 7 days period until harvesting. Then, the plates were observed microscopically. PE was expressed as percentage of the total number of non-overlapping colonies/total number of seeded cells. Cultures were duplicated for each experimental point, in at least three independent experiments.

2.9. Mycoplasma detection

Experiments were set up with MDBK MDL1 cells cultured during 7 days in media supplemented with 10% of the tested FCS. At the log phase of growth from the last passage, cells were processed according to the methodology of in situ fluorescent detection of mycoplasma contamination in cell cultures using DAPI staining (Vectashield mounting medium H1200), as described by Chen with minor modifications [19]. A negative control (CHO-K1 mycoplasma free) and a positive control (*M. arginini* contaminated CHO-K1 cells) were run simultaneously with cultured cells for testing FCS.

2.10. Bovine viral diarrhea virus (BVDV) detection

Experiments were set up with MDBK MDL1 cells cultured during 7 days in media supplemented with 10% of the tested FCS. At the log phase of growth from the last passage, $1-2 \times 10^6$ cells were pelleted, and then total RNA was extracted according to the TRIzol® protocol. RT-PCR was performed with the Access RT-PCR Introductory System, following the manufacturer's instructions. RT-PCR was conducted with primers encoding regions 188–205 and 368–384 from the target 5′-UTR region of the BVDV genome, the most conserved portion of the pestivirus' genome [20]. The reactions were carried out in a MJ Research PTC-100. The amplification program included an initial incubation period of 45 min at 48 °C followed by 39 cycles with denaturation at 93 °C with 30 s hold, annealing at 60 °C with 1 min hold, and extension at 72 °C with 1 min hold. The reaction products were analyzed by electrophoresis in 2.0% agarose gel with ethidium bromide, and analyzed under UV-light for the presence of a 197 pb BVDV specific amplicon from either live virus from infected MDBK MDL1 cells or residual viral RNA present within the sample. A negative control (MDBK MDL1 BVDV-free cells) and a positive control (BVDV contaminated MDBK MDL1 cells) were run simultaneously with cultured cells for testing FCS.

2.11. Statistical analyses

The one-tailed Student's *t*-test was used to compare SCE and GP assays data between non- and γ-irradiated sera lots while χ^2 test was employed for CCP, MI, MNi, SCGE, and PE assays. The level of significance selected was 0.05, unless otherwise indicated.

3. Results

3.1. Genotoxicity assays

SCE frequencies are presented in Fig. 1. Results showed statistically significant differences of SCE frequencies in 8 (44.4%) out of the 18 pairs of samples (serum batches 4, 6–8, 10, 12, 15, and 18). Those findings revealed a higher SCE

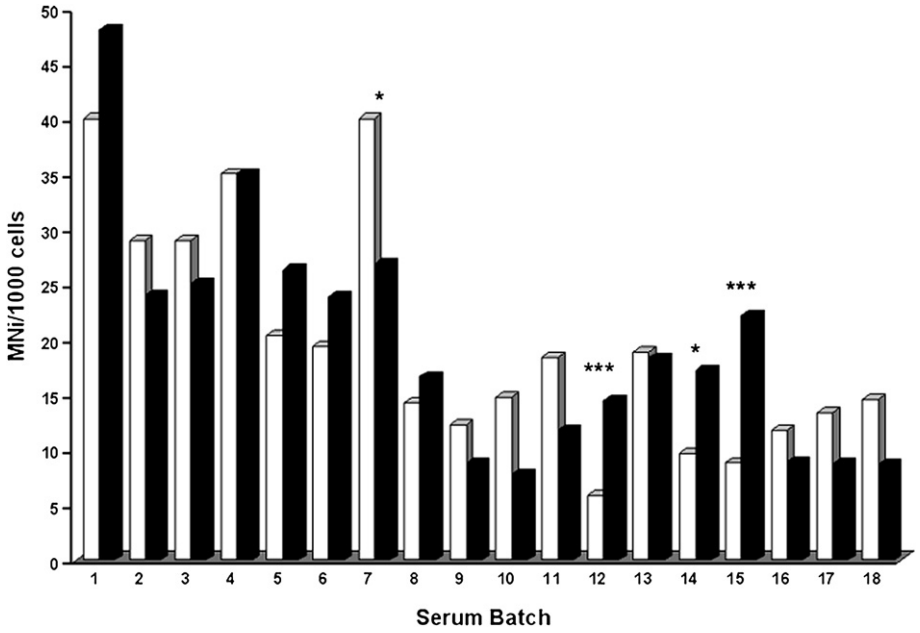

Fig. 2. Frequency of MNi in CHO-K1 cells cultured with different 18 commercial lots of FCS after γ-irradiation (white bars) and their non-irradiated counterparts (black bars). Each bar represents the mean number of MNi among 1000 cells scored per experimental point. *, $P < 0.05$; ***, $P < 0.001$, significant differences between pairs of sera.

frequency in those cells in which the culture medium was supplemented with non-irradiated FCS (6 samples, 75.0%, serum batches 4, 6, 7, 10, 12, and 15) in regard to those cultures in which γ-irradiated FCS were employed ($P < 0.05-0.001$). Finally, in only 2 samples (25.0%, serum batches 8 and 18), a higher SCE frequency was observed when γ-irradiated FCS were employed in regard to those cultures in which non-irradiated FCS were used ($P < 0.05-0.01$).

Results from MNi analysis are shown in Fig. 2. Findings showed statistically significant differences for MNi frequency in only 4 (22.2%) out of the 18 pairs of cultures (serum batches 7, 12, 14, and 15). This revealed a higher frequency of MNi in those cells in which the culture media was supplemented with non-irradiated FCS (3 samples, 75.0%, serum batches 12, 14 and 15) in regard to those cultures in which γ-irradiated FCS were employed ($P < 0.05-0.001$). Finally, in only 1 sample (25.0%, serum batch 7), higher MNi frequency was observed when γ-irradiated FCS was employed in regard to those cultures in which the non-irradiated sample was used ($P < 0.05$).

SCGE results are detailed in Table 1. Statistically significant differences of single strand breaks introduced into the cellular DNA were observed in 10 (55.6%) out of the 18 pairs of cultures when serum batches 2, 6, 8, 11–16, and 18 were used. These differences involved a higher frequency of slightly damaged or damaged cells only in those cultures supplemented with γ-irradiated FCS (5 samples, 50.0%, serum batches 2, 6, 12, 14, and 18) in regard to those cultures in which non-irradiated FCS were employed ($P < 0.05-0.001$). On the other hand, in 3 samples (30.0%, serum batches 8, 11, and 15), a higher comet frequency was found when non-irradiated FCS were employed in regard to those cultures in which γ-irradiated FCS were used ($P < 0.05-0.001$). Finally, in the 2 remaining FCS sample pairs (20.0%, serum batches 13 and 16), an enhancement in the frequency of slightly damaged cells and a significant increase of damaged cells was achieved with the γ-irradiated and non-irradiated FCS, respectively ($P < 0.05-0.001$) (Table 1).

3.2. Cytotoxicity assays

The CCP and PRI rendered a homogeneous outcome, since no alteration of the proliferation rate was observed independently whether γ-irradiated or non-irradiated sera were used. PRI varied from values as low as 1.94 (serum batch 2) to as high as 2.0 (serum batches 3, 6, 9, 14, and 15) ($P > 0.05$) (data not shown).

MI data are presented in Fig. 3. Results showed statistically significant MI differences in 12 (66.7%) out of the 18 pairs of cultures (serum batches 1–3, 6, 8– 13, 17, and 18). Those differences revealed an increased mitotic activity in those cells in which the culture medium was supplemented with non-irradiated FCS (7 samples, 58.3%, serum batches 1, 3, 6, 8, 10, 12, and 18) in regard to those cultures in which γ-irradiated FCS were employed ($P < 0.05-0.001$). Finally, in 5 samples (41.7%, serum batches 2, 9, 11, 13, and 17), a higher MI was observed when γ-irradiated FCS were employed in regard to those cultures in which non-irradiated FCS were used ($P < 0.05-0.001$).

Table 2 summarizes PE analyses. Results showed statistically significant differences in the frequency of colonies in 2 (11.1%) out of the 18 pairs of cultures (serum batches 6 and 7), indicating lower PE in those cultures supplemented with non-irradiated FCS in regard to those cultures in which

Table 1
Frequencies of undamaged, slightly damaged and damaged cells in CHO-K1 cells cultured with different 18 commercial lots of FCS after γ-irradiation and their non-irradiated counterparts.

Serum batch[a]		Number of cells examined	Percentage of cells[b]		
			Undamaged	Slightly damaged	Damaged
1	I	41	48.78	51.22	0.0
	NI	50	46.0	54.0	0.0
2	I	50	46.0	54.0**	0.0
	NI	50	66.0**	34.0	0.0
3	I	50	28.0	66.0	6.0
	NI	50	22.0	72.0	6.0
4	I	50	58.0	42.0	0.0
	NI	50	68.0	32.0	0.0
5	I	50	46.0	54.0	0.0
	NI	49	49.0	49.0	2.0
6	I	50	34.0	64.0***	2.0
	NI	50	62.0***	36.0	2.0
7	I	49	29.0*	69.0	2.0
	NI	50	42.0	58.0	0.0
8	I	50	84.0*	16.0	0.0
	NI	50	62.0	26.0*	12.0***
9	I	50	26.0	66.0	8.0
	NI	50	26.0	68.0	4.0
10	I	50	84.0	16.0	0.0
	NI	50	80.0	20.0	0.0
11	I	50	86.0**	8.0	6.0
	NI	50	58.0	28.0***	14.0**
12	I	50	12.0	22.0	66.0***
	NI	50	72.0***	24.0	4.0
13	I	50	20.0	74.0***	6.0
	NI	50	14.0	36.0	50.0***
14	I	50	14.0	72.0***	14.0
	NI	50	42.0***	42.0	16.0
15	I	50	44.0***	54.0	2.0
	NI	50	12.0	74.0**	14.0***
16	I	50	36.0	54.0*	10.0
	NI	50	46.0	36.0	18.0*
17	I	50	92.0	8.0	0.0
	NI	50	96.0	4.0	0.0
18	I	50	82.0	18.0*	0.0
	NI	50	92.0	8.0	0.0

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences between pairs of sera.

[a] I, irradiated; NI, non-irradiated.

[b] Undamaged cells, diameter ≤30 μm; slightly damaged cells, diameter >30–45 μm; damaged cells, width and length >45 μm; results are expressed as mean values ±SE of the mean.

γ-irradiated FCS were employed ($P < 0.01$ and $P < 0.001$ for FCS 7 and 6, respectively). Table 2 also shows results from GP analysis which revealed statistically significant differences in the GP capability in 1 (5.6%) out of the 18 pairs of cultures (serum batch 2), indicating a higher growth efficiency in those cultures supplemented with non-irradiated FCS in regard to those cultures in which γ-irradiated FCS were employed ($P < 0.01$).

Results from the detection of mycoplasma and BVDV in the 18 samples of γ- and non-irradiated FCS are detailed in Table 2. Presence of mycoplasma and BVDV was only observed in the non-irradiated samples of 1 (5.6%, serum batch 10) and 4 (22.2%, serum batches 8–11) FCS out of the total pairs of samples, respectively.

4. Discussion

In the present report, the genotoxicity and cytotoxicity of 18 commercial lots of FCS γ-irradiated and their non-irradiated counterparts were comparatively evaluated. Eight different end-points, i.e., SCE frequency, MNi frequency, SCGE, CCP, PRI, MI, PE, and GP bioassays were assessed on CHO-K1 cell cultures. Besides, mycoplasma and BVDV contamination were also evaluated on CHO-K1 and MDBK MDL1 cells, respectively.

Our results demonstrated a non-uniform response of each particular batch of serum prior and after γ-irradiation to the battery of tests included in the analyses, highlighting that a single bioassay is not enough to characterize the toxicity of a compound under study, as previously recommended elsewhere [21–23]. However, the presence of some false positive results among our current observations could not be totally ruled out. Recently several reports agree in demonstrating that cell lines widely used for genotoxicity assays, as CHO-K1 cells, have been found to give a high rate of false positive results due to intrinsic cellular deficiencies such as the lack of normal metabolism leading to reliance on exogenous metabolic activation systems, an impaired *p53* function as well as an altered DNA repair capability, among others (for review see Kirkland and collaborators [8], and references therein). In order to clarify this observation, a summary of the results obtained for each particular serum in each bioassay is presented in Table 3. Out of the 18 pairs of sera analyzed, in only one case (5.6%), results revealed no response variations regardless of the end-point assessed (serum batch 5). In the remaining 17 pairs of samples (94.4%), a large degree of variations was found depending not only on the batch of serum tested but also on the bioassay employed.

It seems evident that among all bioassays tested in our study, two closely related end-points, the analysis of the CCP and the PRI could be considered as non-informative. No variations in basal level values estimated by these parameters were found among CHO-K1 cell cultures for any of the batches of FCS tested. The cell culture protocol as well as the labeling system employed in our experiments for analyzing these two end-points lasted 24 h prior harvesting, which approximately covers the time required for CHO-K1 cells to perform two cell-cycles. This assumption is not only revealed by the proportion of M_2 cells and the value of the PRI found in this study (range, 1.94–2.0), but also from previous observations reported elsewhere [24–27]. Similarly, PE as well as GP assays gave little or no information (Table 3). Only differences in PE were found in 2 (11.1%) out of the total number of batches of FCS analyzed while differences in GP activity were observed between non- and γ-irradiated serum-cultured cells in only 1 sample (5.6%). In these 3 cases, the colony formation capability and the growth activity of the cells were found to be higher in non-irradiated FCS than in their γ-irradiated counterparts. Accordingly, it could be suggested that, at least for these particular 3 batches of serum, radiosterilization significantly altered their ability to induce cell growth, most probably by altering or inhibiting some components, e.g.,

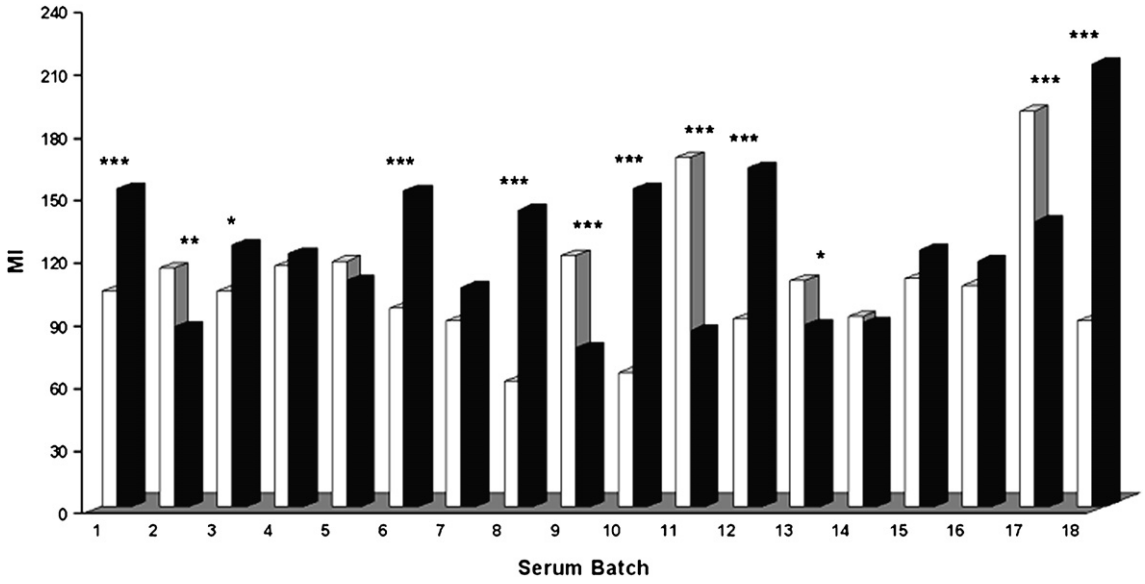

Fig. 3. Mitotic index analyses on CHO-K1 cells cultured with different 18 commercial lots of FCS after γ-irradiation (white bars) and their non-irradiated counterparts (black bars). Each bar represents the mean number of mitotic cells among 1000 cells scored per experimental point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences between pairs of sera.

hormones and/or growth factors, which could be still present or not modified in the corresponding non-irradiated counterparts.

It is worth mentioning here that among all cytotoxicity bioassays included in the present study, MI was the end-point which revealed the wider level variation. Differences in the mitotic activity were found in 12 (66.7%) among all batches assessed, including a higher mitotic activity in those culture media supplemented with either non- or γ-irradiated FCS (Table 3). Thus, radiosterilization could be assumed to significantly inhibit the capability of some components present in some non-irradiated FCS able to induce cellular proliferation, rendering a lower mitotic activity in those cultures where γ-irradiated FCS were used instead. Accordingly, if this assumption is true, the MI of cultures in which γ-irradiated FCS were employed should be higher than in those supplemented with their non-irradiated counterparts.

The analysis of SCE and MNi as well as the introduction of single strand breaks into cellular DNA analyzed by the SCGE assay gave a different scenario. Results revealed MNi differences in 4 (22.2%) out the 18 FCS batches assessed, showing higher frequency of MNi in all but one of those cultures supplemented with non-irradiated FCS (Table 3). Thus, the MNi assay seems to be a not very informative end-point for quantifying the genotoxic damage induced by different FCS batches. Although several reports demonstrate that this assay can be employed as a highly sensitive tool for detecting genotoxicity [28], it cannot be disclaimed.

Bacterial and/or fungal contamination was not observed regardless of the sterilization procedure in any of the FCS, either by filtration or irradiation with 25–32 kGy. This observation seemed of extreme importance since sterility should be the primary condition required for sera when used for tissue or cell culture. Previous reports have demonstrated that a dose 25 kGy is enough to assure the absence of bacterial or fungal contamination of commercial sera [29].

The lack of contamination with mycoplasma in all FCS included in the analysis clearly indicates that the radiation dose employed during radiosterilization of the FCS was effective enough for their denaturation. Previous observations revealed that 3 kGy is able to inactivate mycoplasma present in FCS, at least for *Acholeplasma laidlawii* [29]. It should be noted that in our study this type of contamination was found in only one sample of non-radiosterilized FCS. When Millipore ultrafilters are not used during the sterilization process in the non-irradiated samples of analyzed FCS could lead to mycoplasma contamination within this set of sera, as previously reported elsewhere [30]. No explanation/s for the presence of mycoplasma in this particular batch of serum even after a double 100 nm filtration process can be suggested. However, the possibility of some technical mistake/s during the filtration process cannot be totally ruled out.

Argentina has over 55 million bovines from which around 90% are beef cattle. Its management is extensive, and approximately 70% of the cattle population is concentrated in the central humid and dry region of the country, commonly named as ‘‘Pampas’’. Previous reports employing immunological methods demonstrated the presence of BVDV antibodies in about 70 and 20% of adults and normal fetuses, respectively, regardless of the virus strain involved [20,31–33]. In the present report using RT-PCR we detected BVDV contamination in 22% out of the total non-irradiated FCS samples, value that is in total agreement with that for affected bovine fetuses previously reported for Argentinean cattle [33]. However, disagreement in the results for both types of methodologies for revealing the BVDV infection has been recently reported by Schefers and collaborators [34] for USA cattle. While 2.6% of infected positive calves at birth were

Table 2
Plating efficiency and growth promotion bioassays and presence of mycoplasma (MYC) on CHO-K1 cells and bovine viral diarrhea virus (BVDV) on MDBK MDL1 cells cultured with different 18 commercial lots of FCS after γ-irradiation and their non-irradiated counterparts.

Serum batch[a]		Plating efficiency[b]	Growth promotion[c]	MYC[d]	BVDV[d]
1	I	80	5.18 ± 0.26	−	−
	NI	70	4.94 ± 0.72	−	−
2	I	81	3.91 ± 0.23	−	−
	NI	73	5.59 ± 0.40**	−	−
3	I	90	4.45 ± 0.20	−	−
	NI	100	5.12 ± 0.23	−	−
4	I	85	5.59 ± 0.22	−	−
	NI	76	4.64 ± 0.49	−	−
5	I	70	5.52 ± 0.38	−	−
	NI	70	5.36 ± 0.22	−	−
6	I	100***	4.64 ± 0.21	−	−
	NI	45	5.01 ± .0.67	−	−
7	I	90**	6.63 ± .0.37	−	−
	NI	65	5.15 ± .0.28	−	−
8	I	85	6.61 ± 0.60	−	−
	NI	75	6.88 ± 1.24	−	+
9	I	80	6.30 ± 0.81	−	−
	NI	80	6.92 ± 1.23	−	+
10	I	85	6.68 ± 1.60	−	−
	NI	78	7.18 ± 1.34	+	+
11	I	75	6.73 ± 0.88	−	−
	NI	80	6.74 ± 1.24	−	+
12	I	75	6.69 ± 0.92	−	−
	NI	70	6.95 ± 1.04	−	−
13	I	80	6.10 ± 0.08	−	−
	NI	90	5.91 ± 0.55	−	−
14	I	90	6.59 ± 0.41	−	−
	NI	100	6.70 ± 0.44	−	−
15	I	95	6.44 ± 0.45	−	−
	NI	87	5.52 ± 0.49	−	−
16	I	100	5.90 ± 0.23	−	−
	NI	95	6.18 ± 0.15	−	−
17	I	90	4.73 ± 0.23	−	−
	NI	90	4.28 ± 0.58	−	−
18	I	85	4.88 ± 0.26	−	−
	NI	78	4.60 ± 0.25	−	−

**, $P < 0.01$; ***, $P < 0.001$, significant differences between pairs of sera.
[a] I, irradiated; NI, non-irradiated.
[b] Results are expressed as percentage of colonies after 7 days of culture.
[c] Results are expressed as mean value of number of cells × 10^6 ± SE.
[d] +, presence; −, absence.

Table 3
Summary results of the sister chromatid exchange (SCE), micronuclei (MNi), single cell gel electrophoresis (SCGE), cell-cycle progression (CCP), proliferative rate index (PRI), mitotic index (MI), plating efficiency (PE), growth promotion (GP), mycoplasma (MYC) and bovine viral diarrhea virus (BVDV) detection bioassays in CHO-K1 cells cultured with different 18 commercial lots of FCS after γ-irradiation and their non-irradiated counterparts.

Serum batch[a]		Genotoxicity bioassays			Cytotoxicity bioassays					Contamination bioassays	
		SCE	MNi	SCGE	CCP	PRI	MI	PE	GP	MYC	BVDV
1	I										
	NI						▲				
2	I			▲			▲				
	NI								▲		
3	I										
	NI						▲				
4	I										
	NI	▲									
5	I										
	NI										
6	I			▲							
	NI	▲					▲	▲			
7	I		▲	▲							
	NI	▲						▲			
8	I	▲									
	NI			▲			▲				▲
9	I						▲				
	NI										▲
10	I										
	NI	▲					▲			▲	▲
11	I						▲				
	NI			▲							▲
12	I			▲							
	NI	▲	▲				▲				
13	I			▲			▲				
	NI										
14	I			▲							
	NI		▲								
15	I										
	NI	▲	▲	▲							
16	I			▲							
	NI										
17	I						▲				
	NI										
18	I	▲		▲							
	NI						▲				

[a] I, irradiated; NI, non-irradiated.

detected when using RT-PCR procedures, its frequency reached values as high as 6.8% when immunological procedures were applied [34]. It should be noted that none of the γ-irradiated FCS showed BVDV presence after their radiosterilization, indicating that the dose employed during radiosterilization of the commercial FCS samples was adequate to ensure viral inactivation. It has been previously demonstrated that the D_{10} value (the irradiation dose necessary to inactivate 1 $\log_{10}$ unit of virus) for the frozen samples for BVDV is 4.9 kGy [5] which results 5–6.5 times lower than that used during the radiosterilization of the samples we analyzed.

Finally, the results presented here employing several in vitro bioassays to evaluate the geno- and cytotoxic effects exerted by 18 commercial lots of FCS after γ-irradiation and their non-irradiated counterparts on mammalian cells highlighted that some of the short-term tools employed resulted meaningful end-points for screening FCS. Among them, the SCE, SCGE, and the MI assays were found the most suitable assays for detecting potential geno- and cytotoxicants present in biological samples, as FCS, and that their simultaneous use could be strongly recommended, at least on CHO-K1 cells. Nevertheless, we cannot rule out similar/different results if other cell lines would have been comparatively employed. Overall, it could also be suggested that these end-points were able to reveal that the γ-irradiation process used for radiosterilizing the FCS employed in our study decreased a plausible cellular detrimental capability of the non-irradiated counterparts.

Our results highlight that the geno- and cytotoxicity properties of the majority of the FCS are not increased by the γ-irradiation procedure in the conditions described here. Furthermore, our findings could also suggest that the industrialization procedure including irradiation before commercialization, instead of any traditional filtration method, can assure a more reliable use not only for experimental laboratory research but also in a larger cell culture system/s of longer duration such as a production reactor.

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