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Comparison of the dicentric yield observed in metaphase cells obtained using only Colcemid or Colcemid and Calyculin-A

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Abstract

The objective of Biological Dosimetry is its applicability in radioprotection, quantifying the dose received in individuals who have been exposed to ionizing radiation. For this raison, the analysis of the frequency of chromosome aberrations present in first division metaphases from stimulated lymphocytes is accepted as being the reliable. However, in proliferating cells there are several checkpoints that prevent damaged cells to pass through, and heavily damaged cells show an impossibility to reach mitosis. Concretely the chemically induced premature chromosome condensation by Calyculin-A in combination with Colcemid it is possible to score in the same slide cells that were in interphase and in metaphase. The aim is to determine if Calyculin-A has any effect in the dicentric yield observed in metaphase cells. Peripheral blood samples were irradiated at 1, 4, and 10 Gy of γ-rays and cultured for 48 hours. Colcemid was added 24 or 2 hours before harvesting, and for comparisons cultures were also treated with of Calyculin-A at the last hour. For the three doses the proportion of M cells was always higher after 24 hours in presence of Colcemid than after 2 hours. When the frequencies of dicentrics were compared, there were no major differences between cultures where only Colcemid was used (2 or 24 hours) and those where the Calyculin-A treatment was also done. The results indicate that when PCC-CA is used if metaphase cells are considered similar dose-effect curves will be obtained than those obtained using the conventional mitotic arrest with Colcemid.

Keywords: Biodosimetry, Dicentrics, Premature chromosome condensation, Calyculin-A

Abreveations

CA, chromosomal aberrations; PCC, Premature Chromosome Condensation; LET, linear Energy Transfer; IAEA, International Atomic Energy Agency; Gy, Gray; IR, Ionizing Radiation; PHA, Phytohaemagglutinin; KCl, Potassium Chloride

INTRODUCTION

A key element in the evaluation of the risk associated to an exposure to ionizing radiation (IR) is a precise determination of the received dose. In cases in which there is not physical dosimetry, or that the obtained values are doubtful or little informative, the determination of the dose using a biological parameter, biological dosimetry, is of great interest, for both high and low dose exposures. Nowadays, the analysis of chromosomal aberrations (CA) is considered the more sensitive biological indicator. Concretely the analysis of the frequency of dicentrics present in peripheral blood lymphocytes is considered as "gold standard" and is the most widely used method for dose-assessment [1]. Conventionally, the analysis of radio-induced CA is made analyzing metaphase spreads.

Peripheral blood lymphocytes are non-proliferative cells and to obtain mitotic cells it is necessary to stimulate the cellular division and to arrest the cells using Colcemid (colchicine). Before reach metaphase, cells are passing cell-cycle checkpoints able to arrest damaged cells. However, this approach presents several major limitations due to the low mitotic index of irradiated cells (especially after high doses), the selection of cells harvested at metaphase, and the long time needed for cell cultures to reach metaphase [2], These limitations hinder the application of this approach in cases where the time after exposure needs to be reduced, such as triage after accidental exposure.

It is for this reason that heavily damaged cells are not able to reach mitosis and the use of dicentrics as a biological indicator is restricted to doses up to 4-5 Gy.

Over these doses, the lymphopenia due to cell death reducing the number of lymphocytes available, and the cells showing a greater difficulty or impossibility to arrive at metaphase (M), make it difficult or impossible to obtain chromosome preparations [3]. Therefore other cytogenetic biodosimetry approaches for dose estimation after high exposure irradiation have been applied and reported. [4- 5- 6- 7- 8- 9- 10].

Currently, the application of the premature chromosome condensation (PCC) technique, that compacts genetic material in interphase cells [11], is accepted to realize biological dosimetry after irradiations to doses very lifted [1].

PCC approach is widely used in radiation biology [12-13], and is proposed to be used for biological dosimetry purposes after exposures to high doses [14]. Moreover the analysis of the CA by means of PCC is of particular interest for exposures to high linear Energy Transfer (LET) type radiations, or partial body exposures [14].

PCC can also be induced by poly ethylene glycol (PEG), which helps the mitotic fusion analyzing interphase cells, or as it has been shown recently by phosphatase inhibitors (okadaic acid or calyculin A) [15-16]. The PCC is also used to easily determine the cell cycle stage of lymphocytes the

time of their treatment.

With this method, cells have to be stimulated to growth, and chromosomes from G1 phase, S and G2 phase of the cell cycle can be visualized, although only chromosomes in the G2 phase are easily analyzed using uniform stain. The combination of Colcemid and Calyculin-A permits to analyze in the same slide chromosomes in G2 and M phase [17], It also has the ability to detect the aberrations induced by radiations in over 20% of interphase cells, unlike the conventional method of cultivation of 48 hours based on a treatment with colchicine [18] which allows the detection in only 3% of the cells. However, this technique is particularly used to determine the effects of doses of low energy and high LET radiation [19- 20].The latter is defined as the average energy deposited on the route length of radiation per unit of length. Another application of the PCC is that it also helps to distinguish between total exposure and partial exposure of the individual [21- 22- 23].

Therefore, the search for quick and simple protocols is necessary for the development of its technologies in several laboratories [24-15]. These are easily distinguished because prematurely condensed chromosomes in G2 are morphologically similar to metaphase chromosomes but without a visible centromeric constriction [25].

The percentage increase of dicentric with increasing doses is not accurate beyond a certain threshold [16]. Against by the PCC remains effective in the case where the cells are exposed in vitro at high doses up to 40Gy [26].

The aim of the present study is to determine if Calyculin-A has any effect in the dicentric yield observed in metaphase cells by comparing in M cells if the frequency of dicentrics observed when the conventional method with Colcemid is similar to the one when Caliculyn-A in combination with Colcemid is used.

MATERIALS and METHODS

Blood irradiation

Peripheral blood samples were obtained from a 44 years old healthy male with no history of exposure to ionizing radiation or clastogenic agents with informed consent according to the institutional ethical procedures. Blood samples were kept at 37 °C and irradiated at 1, 4 and 10 Gy of 60Co gamma rays source during irradiation (dose rate 6.30 Gy min−1) using a Cs-137 irradiator (IBL437C, CIS Biointernational, GIF, Yvette, France). During irradiations IAEA recommendations were followed [1].

Lymphocyte culture

For each dose of irradiation, 0.5mL of irradiated blood were cultured during 48 h at 37 °C in 4.5mL of RPMI 1640 medium (Biochrom, Cultek S.L., Madrid, Spain) supplemented with 16% (v/v) foetal calf serum (Biochrom), 27µg of phytohaemagglutinin (PHA) (Biochrom), 2mM of L-glutamine and antibiotics (100 IU·mL−1 penicillin, 100µg·mL−1 streptomycin) (Biochrom), and 12 µg· mL−1 of bromodeoxyuridine (Sigma–Aldrich). Colcemid was added 24 hours before harvesting at a final concentration of 0.1 µg· mL−1 or 2 hours before harvesting at a final concentration of 0.3 µg·mL−1. The treatment with calyculin A consisted in adding a final concentration of 50nM, 1 hour before harvesting. Cultures were harvested, 48 after their set-up, with hypotonic solution treatment (KCl 0.075 M) (Sigma–Aldrich) and fixation with Carnoy's solution (methanol:glacial acetic acid, 3:1, v/v) (Sigma–Aldrich). Finally, the resulting cell suspension was dropped onto slides. Two- to three-day-old slides were stained Fluorescence plus Giemsa (FPG) stain to analyze exclusively cells in their first division (complete metaphase). Metaphases were analyzed in a bright field microscope (Olympus BX-60, Barcelona, Spain) coupled to Cytovision® image analysis system (Applied Imaging, Newcastle Upon Tyne, UK).

Scoring of chromosomes aberrations

Although the study is to compare the frequency of dicentrics obtained using two methodologies, a study analyzing the proportion of cells in G2 and in M phase was also done.

Experimental design and data analysis

After the completion of a study and cytogenetic analysis, of cells from the first cell division, in G2 and M stage, nonetheless, these cells were exposed at different doses of radiation 1Gy, 4Gy and 10 Gy and processed using only Colcemid or Colcemid and Calyculin-A, however this cytogenetic analysis was interpreted statistically by applying the Z test and T-test

RESULTS

The present study comprises a total of twelve different treatments, peripheral blood irradiated at 1, 4 and 10 Gy, and Colcemid incubation during 24 or 2 hours. Parallel cultures were established to add Caliculyn-A, that was added 1 hour before harvesting. With this method, cells have to be stimulated to growth, and chromosomes from G1 phase, S and G2 phase of the cell cycle can be visualized, although only chromosomes in the G2 phase are easily analyzed using uniform stain. The combination of Colcemid and Calyculin-A permits to analyze in the same slide chromosomes in G2 (figure 1) and M phase (figure 2).

As can be seen in figure 3, in the Calyculin-A treated cultures the proportion of M cells decreases clearly as dose increase for both Colcemid treatments (0.48± 0.05, 0.20 ±0.03 and 0.13±0.03 for 24h incubation; and 0.32±0.05, 0.09±0.03 and 0.07±0.01 for 2h incubation. table1.

For all doses the proportion of M cells was higher after 24 h Colcemid incubation respect to 2h incubation (at 1 Gy 0.48± 0.05 vs. 0.32±0.05, $p<0.02$; at 4 Gy 0.20 ±0.03 vs. 0.09±0.03 $p<0.02$; and at 10 Gy 0.13±0.03 vs. 0.07±0.01 $p<0.04$).

These result indicate that 24 hours treatment is able to collect more cells in M phase. All cytogenetics results obtained for each treatment, are shown in tab 1.

When only metaphase cells were considered the cytogenetic results obtained for each treatment are shown in table1.

When the treatment with 24 and 2 hours are considered separately for each irradiation doses, there is no differences in the frequencies of dicentrics with or without Caliculyn-A treatment, On the other hand when the two treatments of Colcemid are compared, after 2 hours treatment the frequency of dicentrics shows a slight tendency to be higher than the one observed after 24 hours treatment, this difference is statistically significant for 4 Gy ($p<0.01$). Similarly when Calyculin-A is also used in the culture, the cultures treated with Colcemid during 2 hours showed higher frequencies of dicentrics, then 24 hours.. In this case the difference is significant for 1 Gy than the others (4 and 10Gy).

DISCUSSION

In the event of a radiological accident, a number of dose assessment techniques are available. Biological dosimetry based on the analysis of cytogenetic damage in peripheral blood lymphocytes provides an approach to estimate the quantity of radiation exposure on the individual level [1-27]. The dicentric assay is generally considered to be the gold standard of biodosimetric methods [28]. The assay can be applied for dose estimation after whole- and partial-body exposure. The disadvantages of the dicentric assay are the time consuming and technically demanding analyses and limitations in assessing doses in excess of about 5–8 Gy due to impaired cell proliferation [29].

The problem of impaired cell proliferation after high doses of radiation can be circumvented by an alternative method the of the premature chromosome condensation (PCC) technique [30]. This method has been widely used, particularly in radiation biology, since it can analyze condensed interphase chromosomes in damaged cells [3- 12-31]. Although the original PCC technique, based on fusion cell, does not require stimulating cells to growth, is laborious and needs especial equipment [31]. PCC is an alternative method that has been proved, at present, be a unique and useful tool in the analysis of radiation-induced chromosome.

In addition, this method allows the interphase chromatin to condense and perhaps as mitotic chromosome, it has opened the way for the analysis of chromosomes not only in metaphase but also in the interphase.

The PCC-CA chemically induced assay was conducted previously [9]; in combination with Colcemid, it is possible to score in the same slide cells that were in interphase and metaphase [17].

This study is presented as a comparison in the frequency of dicentrics present in metaphase spreads obtained by using Colcemid alone or by using Colcemid and Calyculin-A. For this purpose peripheral blood samples were irradiated at 1, 4, and 10 Gy of γ-rays and cultured for 48 hours. To obtain metaphases, Colcemid was added 24 or 2 hours before harvesting, and for comparisons some cultures were also treated with of Calyculin-A during the last hour. For the three doses, the proportion of M cells was always higher after 24 hours in presence of Colcemid than after 2 hours. Moreover, this proportion decreased as dose increased.

This result is in agreement to the major difficulties to reach mitosis for damaged cells, and indicates a strong selection during cell cycle progression. For stimulated lymphocytes a p53/survivin-dependent apoptosis in the G2 to M transition has been reported for those cells bearing dicentrics [27]. Moreover, a strong selection during the same G2 and M transition of unrejoined chromosome damage has been described [13].

The results show that when the treatment with 24 and 2 hours are considered separately, depending on the dose, there is no differences in the frequencies of dicentrics with or without Caliculyn-A treatment, On the other hand when the two treatments of Colcemid are compared,only, after 2 hours treatment the frequency of dicentrics shows a slight tendency to be higher than the one observed after 24 hours treatment. Similarly when Calyculin-A is also used in the culture, the cultures treated with Colcemid during 2 hours showed higher frequencies of dicentrics.

In finishing the results show that Co-treatment with calyculin A modifies the frequency of dicentric. The end of the meiotic division in 24 hours or 2 hours seems to have an effect on the observed average dicentric. This is higher after treatment of 2 hours.

CONCLUSION

The biological effects of ionizing radiation; electromagnetic or particulate nature are multiple and complex. Such ionizing radiation has the ability to bring about damage to the cell constituents principally DNA, it is considered as the preferential target of ionizing radiation.

Most of the induced DNA lesions are repaired without error in order to restore the original structure, and only a small number of them gives rise to chromosomal aberrations that can be analyzed by cytogenetic techniques used principally in biological dosimetry.

For higher doses than 5Gy and 6Gy, the maximum of threshold is reached because the cells in conventional treatment (with colchicine only), are destroyed. For this reason, the PCC technical remains an alternative to circumvent this problem.

The results of the present work show that for the three doses, the proportion of M cells was always higher after 24 hours in presence of Colcemid than after 2 hours. Moreover, this proportion decreased as dose increased. This result is in agreement to the major difficulties to reach mitosis for damaged cells, and indicates a strong selection during cell cycle progression.

In other hand, study has allowed us to compare the frequency of dicentric observed in metaphases's cultures, using the conventional method (only colchicine) and the one using the PCC technique based on the combination of two chemical agents, calyculin A and colchicine.

The results show that Co-treatment with calyculin A modifies the frequency of dicentric. The end of the meiotic division in 24 hours or 2 hours seems to have an effect on the observed average dicentric. This is higher after treatment of 2 hours.

When the frequencies of dicentrics were compared, there were no differences between cultures where only Colcemid was used (2 or 24 hours) and those where the Calyculin-A treatment was also done, indicate that the co-treatment with Calyculin-A does not modify the frequency of dicentrics observed in metaphase spreads.

Overall the results presented here seem to indicate that the traditional method using only Colcemid can be modified by adding Calyculin-A. This will not modify the dose-effect curves obtained for dicentrics in M cells and will permit the analysis of G2 cells more informative after high dose expositions.

Premature condensation of chromosomes (PCC) is an alternative method that has proven, at present, be a unique and useful tool in the analysis of radiation-induced chromosome

In addition, this method allows the interphase chromatin to condense and perhaps as mitotic chromosome, it has opened the way for the analysis of chromosomes not only in metaphase but also in the interphase.

PCC induced by chemical agents has proven useful in cytogenetics and other areas of cell biology

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Conflict of interest

The authors declare that they have no confliet of interest.

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Table 1. Cytogenetic parameters average evaluated.

Dose (Gy)	Treatment	cells	Chromosome type aberrations				
			Dic	**R**	**race**	**ace**	**Other**
1	**24 h Co**	532	54 (0.10±0.01)	3 (0.01±0.01)	4 (0.01±0.01)	49 (0.09±0.01)	6 (0.01±0.01)
	24 h Co - 1h Ca	520	68 (0.13±0.02)	6 (0.01±0.01)	5 (0.01±0.01)	55 (0.11±0.01)	5 (0.01±0.01)
	2 h Co	509	60 (0.11±0.02)	4 (0.01±0.01)	2 (0.01±0.01)	47 (0.09±0.01)	5 (0.01±0.01)
	2 h Co - 1h Ca	500	97 (0.20±0.02)	6 (0.01±0.01)	3 (0.01±0.01)	62 (0.12±0.02)	6 (0.01±0.01)
4	**24 h Co**	108	104 (0.96±0.09)	5 (0.05±0.02)	3 (0.03±0.02)	65 (0.60±0.07)	3 (0.03±0.02)
	24 h Co - 1h Ca	104	102 (0.98±0.10)	1 (0.01±0.01)	2 (0.02±0.01)	71 (0.68±0.08)	8 (0.08±0.03)
	2 h Co	71	104 (1.46±0.14)	8 (0.11±0.04)	1 (0.01±0.01)	73 (1.03±0.12)	16 (0.23±0.06)
	2 h Co - 1h Ca	96	102 (1.06±0.11)	6 (0.06±0.03)	1 (0.01±0.01)	68 (0.71±0.09)	11 (0.11±0.03)
10	**24 h Co**	21	110 (5.24±0.50)	10 (0.48±0.15)	0	66 (3.14±0.39)	9 (0.43±0.14)
	24 h Co - 1h Ca	20	101 (5.05±0.50)	5 (0.25±0.11)	0	61 (3.05±0.39)	15 (0.75±0.19)
	2 h Co	20	110 (5.50±0.52)	6 (0.30±0.12)	0	84 (4.20±0.46)	9 (0.45±0.15)
	2 h Co - 1h Ca	20	120 (6.00±0.55)	10 (0.50±0.16)	0	77 (3.85±0.44)	10 (0.50±0.16)

Values are means ± Standard Error.

Dose in Grays (Gy); treatment with Colcemod (Co) and/or Caliculyn-A (Ca). Chromosome type aberrations scored: dicentrics (dic), centric rings (r), acentric rings (race), extra acentric fragments (ace, not associated to a dicentric or ring). Inversions or translocations, only detected when the morphology of a monocentric chromosome was clearly abnormal were considered as other.

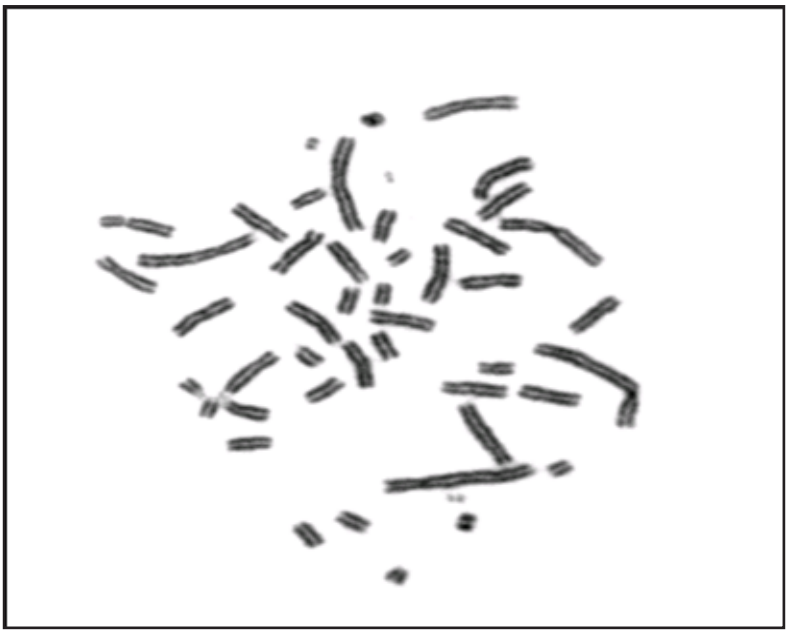

Figure 1. Cell in G2-phase of the cell cycle. Chromosomes have been prematurely condensed and do not show the centromere constriction. The cell contains several extra chromosome pieces and one chromosome ring.

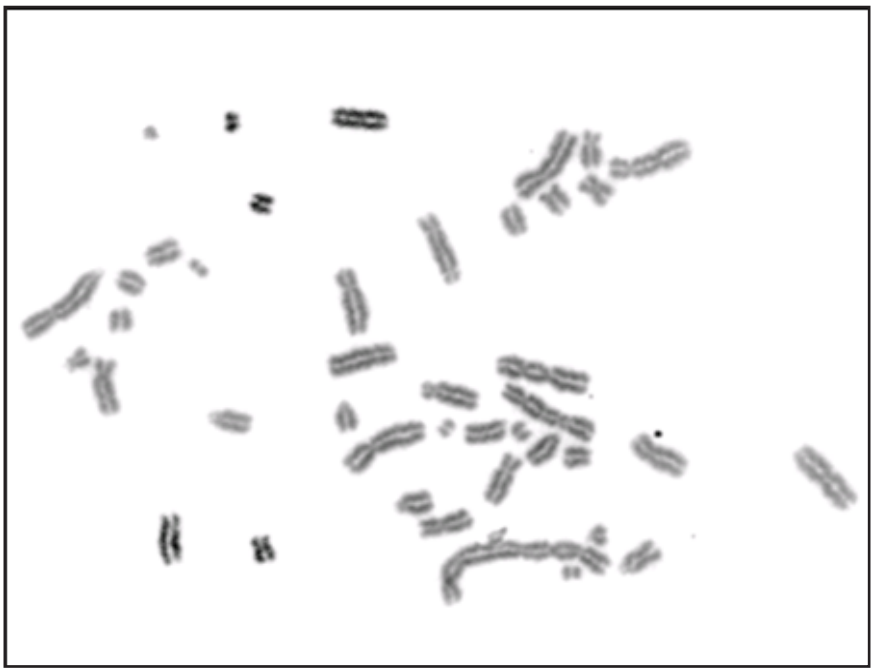

Figure 2. Cell in M-phase of the cell cycle where chromosomes show the centromere constriction. The cell contains several chromosome abnormalities such as dicentric chromosomes and acentric fragments

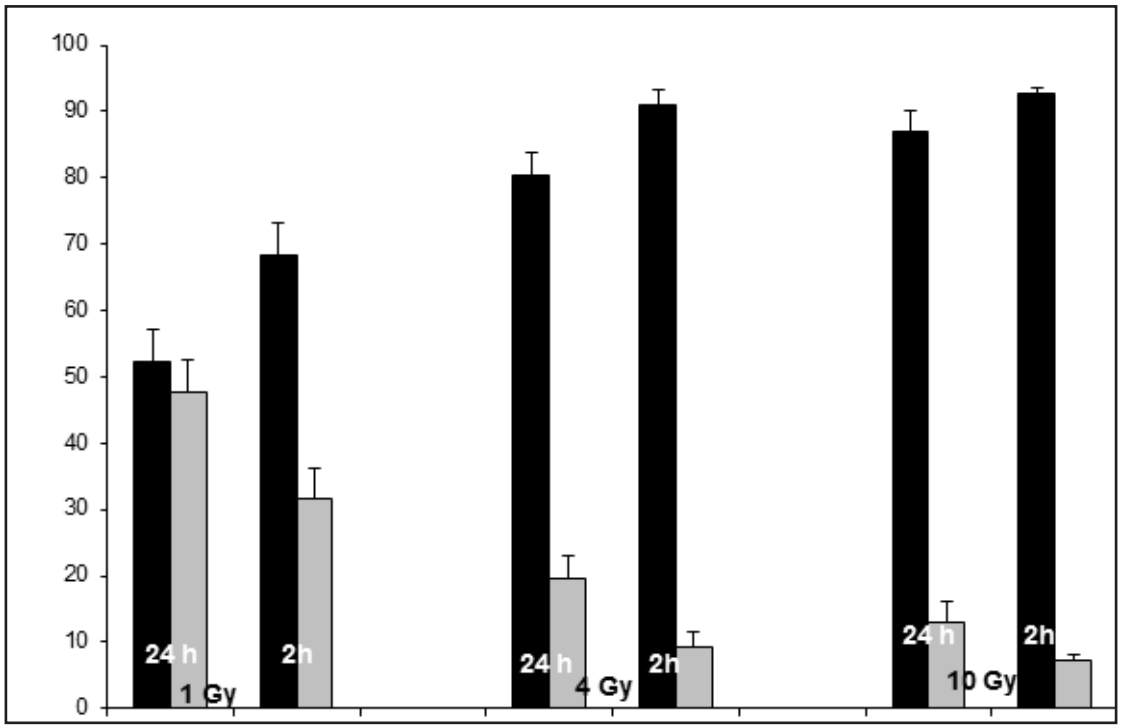

Figure 3. Percentages of cells in G2 (black bars) and M Phase (grey bars) after incubation during 24 and 2 h with Colcemid and 1 hour with Caliculyn-A, for each dose 1, 4 and 10Gy.