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Abstract: The aim of these studies was to evaluate the mechanisms of cellular response to DNA damage induced by BNCT. Thyroid carcinoma cells were incubated with 10BPA or 10BOPP and irradiated with thermal neutrons. The surviving fraction, the cell cycle distribution and the expression of p53 and Ku70 were analyzed. Different cellular responses were observed for each irradiated group. The decrease of Ku70 in the neutrons + BOPP group could play a role in the increase of sensitization to radiation.

National Atomic Energy Commission
Department of Radiobiology

March 18, 2011

.....
Andres Kreiner, PhD
Editor-in-Chief
Applied Radiation and Isotope

Dear Dr Kreiner:

Please find attached the copy of the manuscript: "***In vitro studies of cellular response to DNA damage induced by boron neutron capture therapy***" which has been revised again as it was suggested by the reviewer. It has not been published neither in this nor in a similar form (printed or electronically).
There is no conflict of interest in this paper.

Hoping that you and the Editorial Board will find it suitable for publication, I remain

Sincerely

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Detailed Responses to Reviewer

General Comments:

This revised manuscript has been significantly improved from the original version. As indicated below, there are a number of specific suggestions that would further improve the manuscript.

Specific Comments:

1. Abstract. l.31. "surviving" not survival function here and elsewhere in the text. l.32 "responses", "were"
2. Spellings and syntax errors are noted as follows: "cytotoxic" not citotoxic; "autoradiographs" not autoradiography films; "Two tailed" not tails; "was observed" not can be; "failure" not failed.
3. Regarding a brief description of the RA-3 reactor, please include the two sentences that you have written in your Responses, beginning with "This is" and ending with ($\pm 10\%$).
4. Table 1 was omitted from the revised manuscript. A title for the table and explanatory footnotes should be added.
5. Conclusions. l.52 "NHEJ repairs system, as evidenced by "
6. Acknowledgements. Indicate what the acronyms stand for.
7. Omit the beta-actin controls from the figures since they don't provide any essential information.
8. There are many figures in this short manuscript. If space is a problem, Fig 4a and b could be omitted and the results briefly summarized in the text.
9. References. Replace Barth 2003 with a more recent publication, e.g. Altieri S, Bortolussi S, Barth RF, Roveda L, Zonta A. 2009. 13th International Congress on Neutron Capture Therapy. BNCT: A new option against cancer. 67 (7-8S) S1-S379.

Answer to the reviewer

All the changes suggested by the reviewer have been performed.

In vitro studies of cellular response to DNA damage induced by boron neutron capture therapy

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We declare that there is no conflict of interest in this paper.

Abstract

The aim of these studies was to evaluate the mechanisms of cellular response to DNA damage induced by BNCT. Thyroid carcinoma cells were incubated with ¹⁰BPA or ¹⁰BOPP and irradiated with thermal neutrons. The surviving fraction, the cell cycle distribution and the expression of p53 and Ku70 were analyzed. Different cellular responses were observed for each irradiated group. The decrease of Ku70 in the neutrons + BOPP group could play a role in the increase of sensitization to radiation.

Keywords: damage, DNA, cell cycle, NHEJ.

1. Introduction

Boron neutron capture therapy (BNCT) is a high linear energy transfer (LET) radiotherapy for cancer, based on the nuclear reaction that occurs when ¹⁰B, a non radioactive isotope of natural elemental boron, reacts with low energy thermal neutrons to produce an alpha particle (⁴He) and a ⁷Li nucleus. Both particles have a range comparable to the diameter of a cell causing tumor cell death without significant damage to the surrounding normal tissues, provided that the boron-carrier compound accumulates preferentially in tumor cells (Coderre and Morris, 1999). BNCT have been then used in clinical trials for the treatment of high grade gliomas, cutaneous melanomas and its brain metastases using as boron carriers, p-boronophenylalanine (¹⁰BPA) and sodium borocaptate (¹⁰BSH) (Altieri et al., 2009). Also, in the last years, this therapeutic modality has been applied in the treatment of head and neck tumors (Wittig et al., 2009; Kato et al., 2009).

We have performed studies on a human anaplastic thyroid carcinoma cell line using as a carrier of boron, ¹⁰BPA and also tetrakis-carborane carboxylate ester of 2,4-bis (α,β-dihydroxyethyl)-deutero-porphyrin IX (BOPP), both *in vitro* and after being transplanted to nude

1 mice (Dagrosa et al., 2003; Dagrosa et al., 2007). These studies were very encouraging; however
2 little is known about the mechanisms that play a role in the tumor damage produced by BNCT.
3 The DNA molecule is the principal biological target that conduces to cell death. The high LET
4 radiation produces mostly DNA double strand breaks (DSBs) and is normal to observe
5 chromosomal aberrations and its cytoplasmic derivates, the micronuclei (MN) (Norppa and Falck,
6 2003). Cell cycle regulation and repair mechanisms such as homologous recombination (HR) and
7 non homologous end-joining (NHEJ) are important determinants of ionizing radiation sensitivity.
8 A common cellular response to DNA-damaging agents is the activation of cell cycle checkpoints.
9 One of the key proteins in the checkpoint pathway is the tumor suppressor gene p53, which
10 coordinates DNA repair with cell cycle progression and apoptosis (Pawlik and Keyomarsi, 2004).
11 Normally, DNA double-strand breaks activate Ku proteins, which bind to the area of DNA
12 breakage. Ku70 protein is involved in the repair of DNA double strand breaks via NHEJ pathway
13 and can also play an important role in the apoptosis (Ochi et al., 2010).
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16
17 Previously we have shown that both cytotoxic and genotoxic effects produced in the cells
18 treated by BNCT are different and higher of those produced in cells irradiated with the gamma
19 irradiation. The number of micronuclei per cell increases as a function of the total physical dose
20 following a sigmoidal model (Dagrosa et al., 2010). In the present work we evaluated in thyroid
21 carcinoma cells the mechanisms of cellular response to DNA damage induced by BNCT and
22 compared them with the conventional gamma radiation.
23
24

25 2. Material and Method

26
27 **2.1 Cell line:** Human cell line of follicular thyroid carcinoma (WRO) was grown and
28 maintained in RPMI 1640 medium supplemented with 10% de FBS, under 5% CO₂ atmosphere at
29 37 °C.
30

31 **2.2 Experimental design:** Exponentially growing cells were distributed in the following
32 groups: 1) ¹⁰BPA + neutrons: cells were incubated with ¹⁰BPA at a dose of 10 µg¹⁰B/mL (0.925M)
33 during 16 h before irradiation; 2) ¹⁰BOPP 2,4-bis (α,β-dihydroxyethyl)-deutero-porphyrin IX) +
34 neutrons: cells were incubated with ¹⁰BOPP at a dose of 10 µg¹⁰B/mL (33 µg/mL) during 16 h
35 before irradiation; 3) Neutrons (N) and 4) Gamma. A control group without irradiation for each
36 treatment was added: Control (C), C BPA and C BOPP.
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39 **2.3 Dosimetry:** Cells were gamma irradiated using a ⁶⁰Co source (1Gy/min) and neutron
40 irradiated at the thermal column of the RA-3 reactor (Miller et al, 2009). This is an 8MW reactor,
41 located in Ezeiza, Argentina where a highly thermalized and homogenous irradiation field is
42 available. Thermal flux is near (8.5±0.7) x 10⁹ n/ (cm²s), the cadmium ratio is 4100 for gold foils,
43 which allows neglecting fast neutron dose, and the gamma dose rate is approximately (6.0±0.2)
44 Gy/h. In both cases, different time lapses were selected in order to achieve total physical doses
45 ranging between 0.3 and 5 Gy (± 10%).
46
47

48 **2.4 Cell surviving assay:** For cell viability evaluation, 2000 cells were seeded with 200 µL
49 of RPMI medium and 10% FBS in 96-wells plates. Four different wells were seeded per
50 treatment. After irradiation, the medium was changed and the cells were cultured at 37 °C. The
51 medium was changed again at day 4 after irradiation and 20 µL of vital dye 3-(4,5-
52 dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide (MTT) [Sigma 128] 0.5% w/v in PBS were
53 added. Absorbance was measured at 540 nm.
54
55

56 **2.5 Protein expression assay:** For *Western blot* analysis, the cells were incubated at 37 °C
57 24 and 48 h after irradiation. Cells were washed twice in cold PBS, scrapped and centrifuged. The
58 PBS was removed and the pellet was lysed in 100µL of RIPA buffer (50 mM Tris-HCl, pH 7.2,
59 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing protease
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1 inhibitors. The cell lysate was cleared by centrifugation and the supernatant was used as a total
2 cellular protein extract. Protein concentration was determined by Bradford protein assay (BioRad).

3 From each sample 60 μg of total protein was loaded into a 12% SDS-poliacrilamide gel and
4 electroforesed at 90 Volts for 2 h. The separated proteins were transferred to a *Hybond-P*
5 membrane (Amersham) in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol,
6 0.05% SDS) at 100 Volts during 1 h at 4 °C. The membrane was incubated in blocking solution
7 (5% nonfat milk in TBST: 30 mM Tris-Hcl, pH 7.4; 150 mM NaCl; 0.05% Tween-20) and probed
8 with mouse antibodies raised against p53 (Clone DO-7, Pharmingen) and Ku70 (Calbiochem).
9 Beta-actin monoclonal antibody (Clone AC-15, Sigma) was used as a loading control. The
10 membrane was washed and incubated with the secondary antibody (anti-mouse antibody
11 conjugated to horseradish peroxidase, Promega). The bands were visualized by
12 chemiluminescence after addition of substrate (ECL detection kit, Amersham) and exposed to
13 autoradiographs films. Semi quantitative analysis was obtained after the films were scanned with a
14 densitometer to determine the density of each band. The relative optical density (OD) was
15 obtained by dividing the density of each band by the Beta-actin density.
16
17

18 **2.6 Cell cycle analysis:** For cell cycle, 10^6 cells were washed three times with ice-cold PBS
19 and fixed in absolute ethanol at -20 °C, O/N. Cell pellets were resuspended in a staining solution of
20 PBS containing 60 $\mu\text{g}/\text{mL}$ propidium iodide (PI) and 50 $\mu\text{g}/\text{mL}$ RNAase. The flow analysis was
21 carried out using a flow cytometer (BD FACSCalibur). Processing and analysis of flow cytometry
22 data were performed on a PC-based computer system.
23

24 **2.7 Frequency of cell death analysis:** Briefly, cell pellets (10^6) were resuspended in 100 μL
25 of a staining solution containing PI, 4,5-diaminofluorescein (DAF) and Hoechst 33258 at the
26 concentration of 0.6 mg/mL, 0.1 mg/mL and 0.6 mg/mL, respectively. The stained cells were
27 analyzed using a epifluorescence microscope. Samples of 200 cells were analyzed.
28

29 **2.8 Statistical Analysis:** Data were analyzed according to analysis of variance (ANOVA)
30 and the Tukey-Kramer multi comparison test *a posteriori*. Two tailed Student's t test was used for
31 the analysis of cell death frequency. Values were considered significant when $p < 0.05$.
32
33

34 **3. Results and Discussion**

35
36 The results of the surviving cell fraction as a function of the total physical dose are shown
37 in Figure 1. The neutron irradiation of the cells showed a decrease in the cell viability. This effect
38 was greater in the cells incubated with the boron compounds (BPA or BOPP). In these studies we
39 evaluated the surviving cell fraction using the MTT colorimetric assay after the treatment with
40 different types of radiation. We have obtained previously, similar results between the clonogenic
41 assay and this particular colorimetric assay in the physical doses obtained in these experiments, in
42 agreement with other authors (Watts et al., 1989; Slavotinek et al., 1994).
43
44

45
46 Insert Figure 1
47

48 Figures 2a, 2b and 2c show an increase of p53 at 24 h for the neutrons alone and gamma
49 groups compared to the control group (without irradiation and without boron compounds). Also it
50 was observed that controls of boron compounds (without irradiation) have an increased expression
51 of p53 (Figures 2b and 2c). It was reported that WRO cell line expresses the mutated p53 protein
52 (Namba et al., 1995). It is known that the lack of normal p53 function may cause a failure in the
53 cellular response to DNA damage. The levels of mutated p53 protein were abundant without
54 irradiation and this agrees with other published studies (Torres et al., 2004).
55
56

57 On the other hand, the expression of Ku70 showed an up regulation of 4 fold in the group
58 irradiated with neutrons alone compared to control group (without irradiation) at 24 h. Also at this
59 time the relative amount of Ku70 increased for the BPA + N group, 1.16 times compared to its
60 respective control (CBPA) and decreases 1.6 times for the BOPP +N group compared its control
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(CBOPP). However at 48 h an up regulation of Ku70 for gamma and BOPP+N groups was observed. The relative amount increased 1.3 times for the gamma group and 1.13 times for BOPP + N (Figures 3a, 3b and 3c). Ku70 is known to play a role in double strand break repair by the NHEJ pathway. It was described a correlation between decreased expression of Ku70 and an increase of sensitization to radiation (Vandersickel et al., 2010). At the times analyzed in these studies we only observed a lower expression on this protein for the BOPP + N group at 24 h, but further studies are required in order to establish the role of Ku70 in the BNCT treatment.

Insert Figures 2a, 2b and 2c

Insert Figures 3a, 3b and 3c

Accumulation of cells in G2/M was observed at both time points for all the irradiated groups (Table 1). The arrest in G2/M could be a period of time for DNA repair by the studied mechanism. It is known that tumor cells of thyroid that have mutated p53, arrest in G2 after irradiation, independently of the quality of radiation (Namba et al., 1995). On the other hand, the control groups, those which were incubated with boron compounds and that were not irradiated, showed an arrest at 24 h. However in these groups the number of apoptotic and necrotic cells did not increase (Figures 4a and b) and the ATM (for ataxia-telangiectasia mutated) protein did not show an up regulation at these times, indicating that there was not damage in the cells induced by the boron compounds (results not shown). The ATM protein in the presence of DNA double strand break damage becomes activated and phosphorylates different downstream targets which act as signal transducers and effectors initiating cell cycle arrest and apoptosis (Niida and Nakanishi, 2006).

Insert Table 1

The study of the mechanisms of cell death showed that at 24 h the frequency of apoptosis was increased in all irradiated groups compared to control groups (**p<0.01 for Neutrons vs Control and *p<0.05 for N + BPA and N + BOPP vs CBPA and CBOPP, respectively). The number of necrotic cells was higher only in all BNCT groups compared to gamma and control groups (p<0.05 for all BNCT groups vs Controls). At 48 h it was observed an increase in apoptotic cells in all the groups irradiated (**p<0.01 and *p<0.05 for Neutrons and Gamma groups vs Control, respectively; **p<0.01 for N + BPA vs CBPA). However the necrotic cells only increased in the groups irradiated with neutrons (Groups 1, 2 and 3) (**p<0.01 for Neutrons and N + BPA groups vs Control and CBPA, respectively). These studies showed that necrosis was the principal mechanism of cell death induced by boron neutron capture therapy.

4. Conclusions

These results performed on follicular thyroid carcinoma cells suggest a different cellular response for all the irradiated groups. The results of the surviving cell fraction showed a decrease in the cell viability for the cells incubated with both boron compounds (BPA or BOPP). The protein p53 showed an up-regulation for the gamma and neutrons alone groups 24 hours after irradiation that wouldn't seem to be correlated with the p53-dependent G1/S cell cycle arrest. Cell cycle analysis showed that neutrons, neutrons plus boron compounds and gamma irradiation induced a G2/M arrest 24 and 48 hours after irradiation. We founded that cell death was mostly produced by cell necrosis in groups irradiated with neutrons alone and with neutrons and boron compounds, with higher values at 24 hours. All the groups irradiated with gamma or neutrons seem to induce the NHEJ repairs system, as evidenced by an increase of Ku70 at different times. The decrease of Ku70 in the BNCT (neutrons + BOPP) group could play a role in the increase of sensitization to radiation.

Acknowledgments

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References

- Altieri, S., Bortolussi, S., Barth, R.F., Roveda, L., Zonta, A., 2009. 13th International Congress on Neutron Capture Therapy. BNCT: A new option against cancer. *Appl Radiat Isot.* 67(7-8 S):S1-2.
- Coderre, J.A., Morris, G.M., 1999. The radiation biology of boron neutron capture therapy. *Radiat. Res.* 151 (1), 1-18.
- Dagrosa, M.A., Viaggi, M., Longhino, J., Calzetta, O., Cabrini, R., Edreira, M., Juvenal, G., Pisarev, M., 2003. Experimental application of boron neutron capture therapy (BNCT) to undifferentiated thyroid carcinoma (UTC). *Int. J. of Radiat. Oncol. Biol. Phys.* 57(4), 1084-1092.
- Dagrosa, M.A., Thomasz, L., Longhino, J., Perona, M., Calzetta, O., Blaumann, H., Jiménez Rebagliati, R., Cabrini, R.L., Kahl, S.B., Juvenal, G.J., Pisarev, M.A., 2007. Optimization of the application of boron neutron capture therapy (BNCT) to the treatment of undifferentiated thyroid cancer (UTC). *Int. J. of Radiat. Oncol. Biol. Phys.* 69 (4), 1059-1066.
- Dagrosa, M.A., Crivello, M., Perona, M., Thorp, S., Santa Cruz, G.A., Pozzi, E., Casal, M., Thomasz, L., Cabrini, R., Kahl, S., Juvenal, G.J., Pisarev, M.A., 2011. First evaluation of the relative biological effectiveness of boron neutron capture therapy (BNCT) in a colon carcinoma cell line. *Int. J. of Radiat. Oncol. Biol. Physics.* 79(1), 262-268.
- Kato, I., Fujita, Y., Maruhashi, A., Kumada, H., Ohmae, M., Kirihata, M., Imahori, Y., Suzuki, M., Sakrai, Y., Sumi, T., Iwai, S., Nakazawa, M., Murata, I., Miyamaru, H., Ono, K., 2009. Effectiveness of boron neutron capture therapy for recurrent head and neck malignancies. *Appl Radiat Isot.* 67, S37-42.
- Miller, M., Quintana, J., Ojeda, J., Langan, S., Thorp, S., Pozzi, E., Szejnberg, M., Estryk, G., Nosal, R., Saire, E., Agrazar H., Graiño F., 2009. New irradiation facility for biomedical applications at the RA-3 reactor thermal column. *Applied Radiation and Isotopes* 67, S226-S229
- Namba, H., Hara, T., Tukazaki, T., Migita, K., Ishikawa N., Ito, K., Nagataki, S., Yamashita, S., 1995. Radiation induced G1 arrest is selectively mediated by the p53-WAF1/Cip1 pathway in human thyroid cells. *Cancer Res.* 55, 2075-2080.
- Niida, H., Nakanishi, M., 2006. DNA damage checkpoints in mammals. *Mutagenesis.* 21(1), 3-9.
- Norppa, H., Falck, G., 2003. What do human micronuclei contain? *Mutagenesis.* 18(3), 221-223.
- Ochi, T., Sibanda, B.L., Wu, Q., Chirgadze, D.Y., Bolanos-Garcia, V.M., Blundell, T.L., 2010. Structural biology of DNA repair: spatial organization of the multicomponent complexes of nonhomologous end joining. *J. Nucleic Acids.* 25, 621-695.
- Pawlik, T.M., Keyomarsi, K., 2004. Role of cell cycle in mediating sensitivity to radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 59(4), 928-942.
- Slavotinek, A., McMillan, T.J., Steel, C.M., 1994. Measurement of radiation survival using the MTT assay. *European J of Cancer.* 30, 1376-1382.
- Torres, M., Muneera, A.B., Ghazi, A., 2004. Induction of p53 and p21 proteins by gamma radiation in skin fibroblasts derived from breast cancer patients. *Int. J. Radiat. Oncol. Biol. Phys.* 58(2), 479-484.
- Vandersickel, V., Mancini, M., Slabbert, J., Marras, E., Thierens, H., Perletti, G., Vral, A., 2010. The radiosensitizing effect of Ku70/80 knockdown in MCF10A cells irradiated with X-rays and p(66)+Be(40) neutrons. *Radiat. Oncol.* 5, 30.
- Watts, M.E., Roberts, I.J., Wood Cock, M., 1989. A comparison of colorimetric and clonogenic assay for hypoxic-specific toxins with hamster and human cells. *Int. J. of Rad. Oncol. Biol. Phys.* 16(4), 939-942.

1 Wittig, A., Collette, L., Appelman, K., Bührmann, S., Jäckel, M.C., Jöckel, K.H., Schmid, K.W.,
2 Ortmann, U., Moss, R., Sauerwein, W.A., 2009. EORTC trial 11001: distribution of two 10B-
3 compounds in patients with squamous cell carcinoma of head and neck, a translational
4 research/phase 1 trial. J Cell Mol. Med. 13, 1653-65.

5 Legends

6
7 **Figure 1: Survival of WRO cells under the different treatments.** Data are fitted with a
8 quadratic-linear model. Each point is the average of 6-8 wells \pm SEM of two independent
9 experiments.

10
11
12 **Figure 2a: Expression of p53 was assessed by western blot analysis.** All the cells received 3 Gy
13 of total physical absorbed dose and the analysis was performed at 24 and 48 h. Each displayed
14 value was normalized against the density of the respective band of Beta-Actin and the levels of
15 relative optical density are expressed as the ratio between each treatment and its respective control.
16 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for Neutrons vs Gamma, BPA+N and BOPP+N groups,
17 respectively, at 24 h. ^o $p < 0.05$ for Neutrons vs Gamma and Gamma vs BPA+N and BOPP+N
18 groups; ^{oo} $p < 0.01$ for Neutrons vs BPA+N and BOPP+N groups, at 48 h.

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20
21
22 **Figure 2b:** Quantitative analysis of proteins by densitometric scanning of the membranes was
23 performed at 24 h. The western blot was performed twice. Lane 1: Control (without irradiation);
24 Lane 2: CBPA; Lane 3: CBOPP; Lane 4: Neutrons; Lane 5: Gamma; Lane 6: N + BPA; Lane 7: N
25 + BOPP; Lane 8: Molecular weight marker. Beta- Actin was used as the loading control.

26
27
28 **Figure 2c:** Quantitative analysis of proteins by densitometric scanning of the membranes was
29 performed at 48 h. The western blot was performed twice. Lane 1: Control (without irradiation);
30 Lane 2: CBPA; Lane 3: CBOPP; Lane 4: Neutrons; Lane 5: Gamma; Lane 6: N + BPA; Lane 7: N
31 + BOPP; Lane 8: Molecular weight marker. Beta- Actin was used as the loading control.

32
33
34 **Figure 3a: Expression of Ku70 was assessed by western blot analysis.** All the cells received 3
35 Gy of total physical absorbed dose and the analysis was performed at 24 and 48 h. Each displayed
36 value was normalized against the density of the respective band of Beta-Actin and the levels of
37 relative optical density are expressed as the ratio between each treatment and its respective control.
38 *** $p < 0.001$ for Neutrons vs Gamma, BPA+N and BOPP+N groups; * $p < 0.05$ for BPA+N vs
39 BOPP+N groups, at 24 h.

40
41
42
43 **Figure 3b:** Quantitative analysis of proteins by densitometric scanning of the membranes was
44 performed at 24 h. The western blot was performed twice. Lane 1: Control (without irradiation);
45 Lane 2: CBPA; Lane 3: CBOPP; Lane 4: Neutrons; Lane 5: Gamma; Lane 6: N + BPA; Lane 7: N
46 + BOPP. Beta- Actin was used as the loading control.

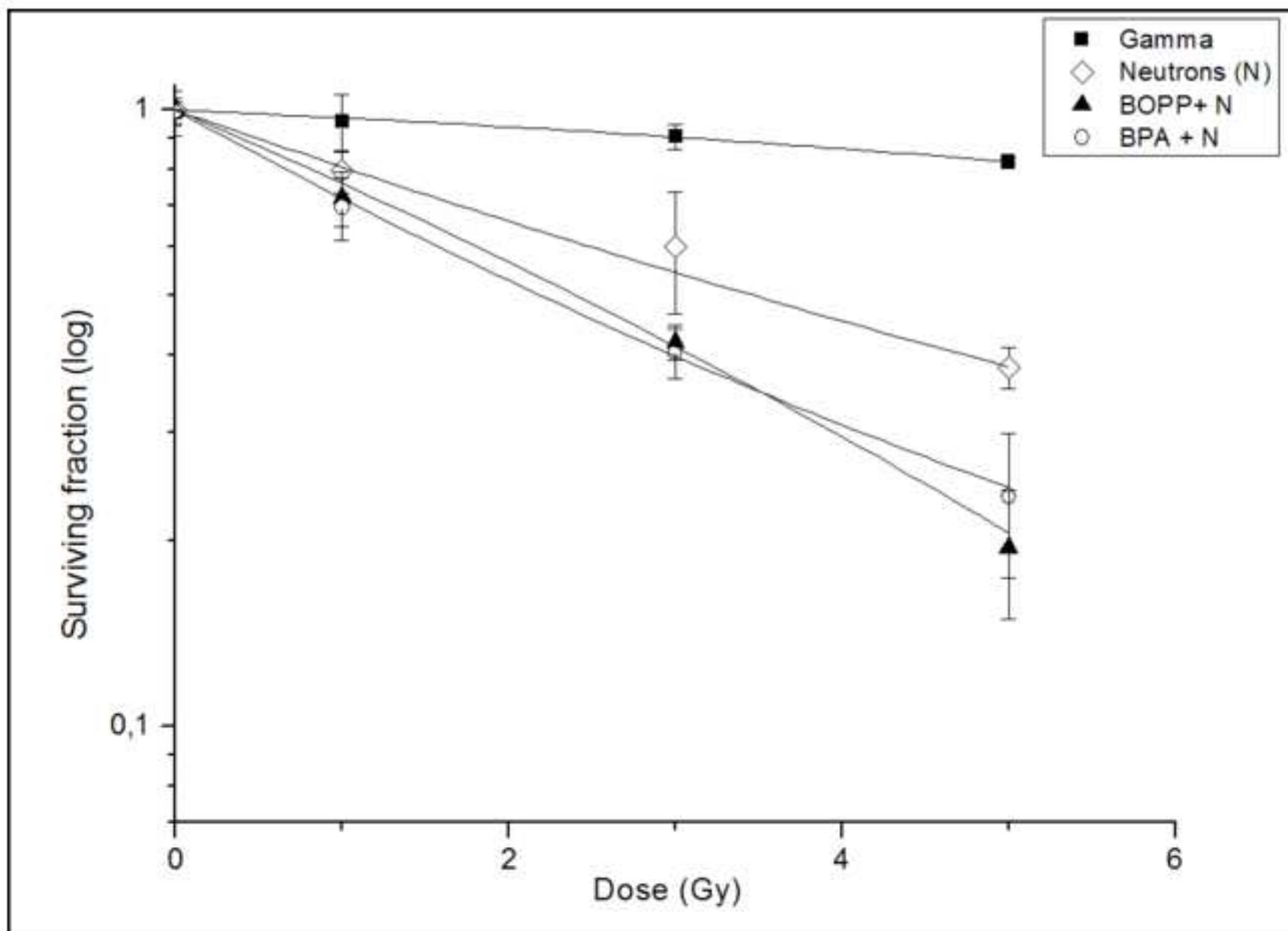
47
48
49 **Figure 3c:** Quantitative analysis of proteins by densitometric scanning of the membranes was
50 performed at 48 h. The western blot was performed twice. Lane 1: Control (without irradiation);
51 Lane 2: C + BPA; Lane 3: C + BOPP; Lane 4: Neutrons; Lane 5: Gamma; Lane 6: N + BPA; Lane
52 7: N + BOPP. Beta- Actin was used as the loading control.

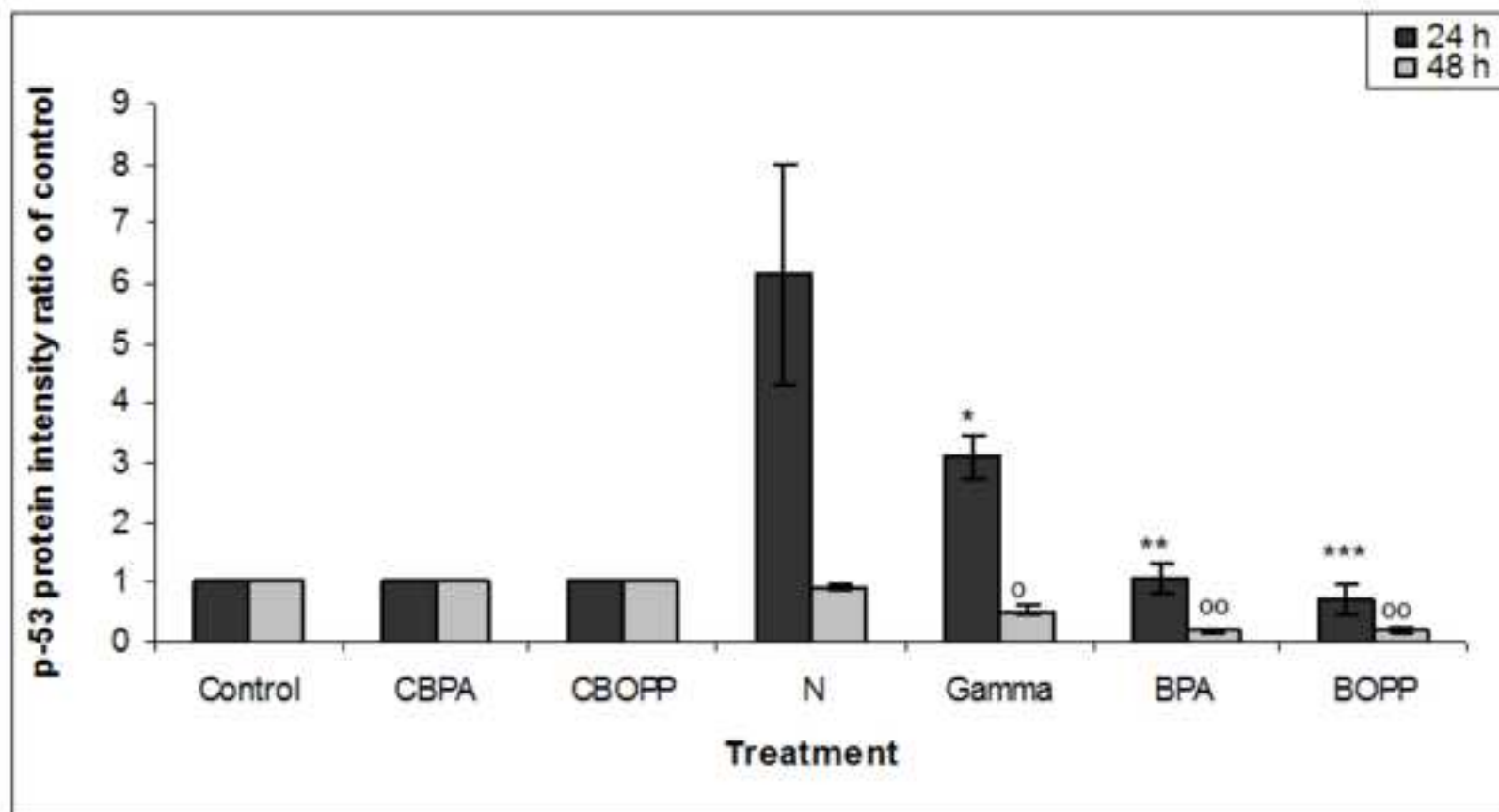
Table 1. Cell cycle analyses

GROUP	TIME (HR)	G1	G2	S
C	24	79.48 ± 14.33	3.31 ± 1.19	17.2 ± 5.55
C	48	77.32 ± 17.39	4.49 ± 0.89	18.17 ± 5.43
C BPA	24	44.07 ± 2.79	19.01 ± 1.6	37.02 ± 4.38
C BPA	48	77.26 ± 4.35	3.09 ± 0.05	19.65 ± 3.2
C BOPP	24	42.75 ± 2.82	16.8 ± 4.03	42.86 ± 1.21
C BOPP	48	66.49 ± 6.23	1.94 ± 0.06	31.57 ± 7.3
Gamma	24	48.38 ± 3.56	17.83 ± 1.55	31.36 ± 2.01
Gamma	48	45.87 ± 15.49	18.72 ± 8.9	33.78 ± 6.57
N	24	53.36 ± 5.18	12.46 ± 1.63	34.17 ± 3.83
N	48	41.54 ± 12.2	16.93 ± 6.89	31.36 ± 11.29
BPA+N	24	63.3 ± 10.18	11.04 ± 0.13	25.53 ± 10
BPA+N	48	67.35 ± 6.87	7.55 ± 3.61	24.81 ± 3.3
BOPP+N	24	61.52 ± 3.85	8.37 ± 2.87	30.01 ± 0.96
BOPP+N	48	54.38 ± 8.18	16.72 ± 5.08	28.83 ± 3.06

Table 1: The percentage of cells in the various cell phases is shown for each sample (values are mean ± SD). Three independent experiments were performed.

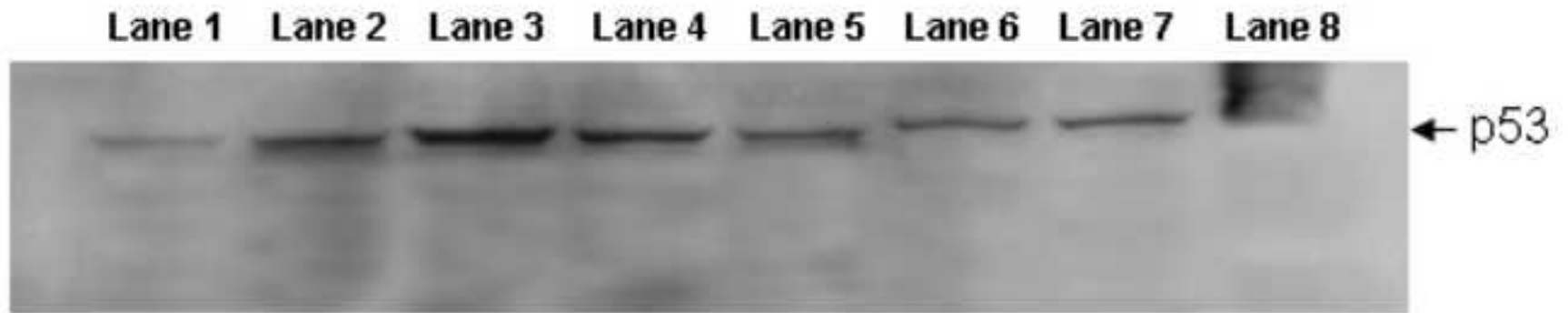
Figure(s)
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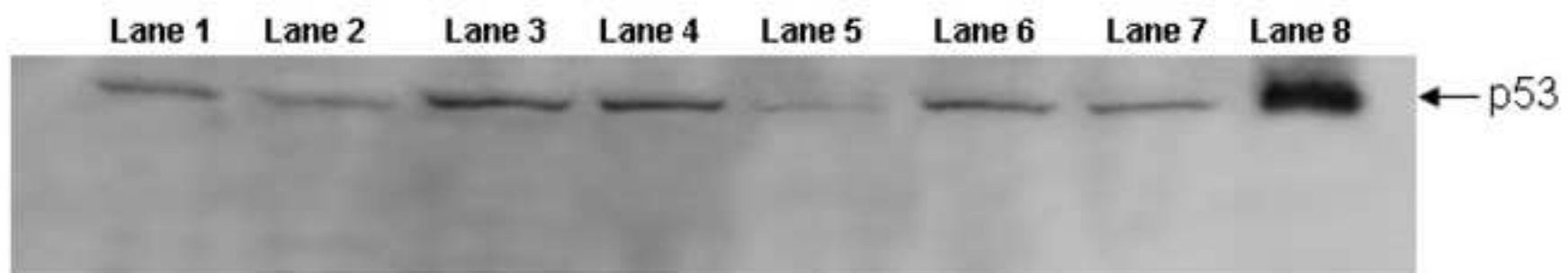
Figure(s)

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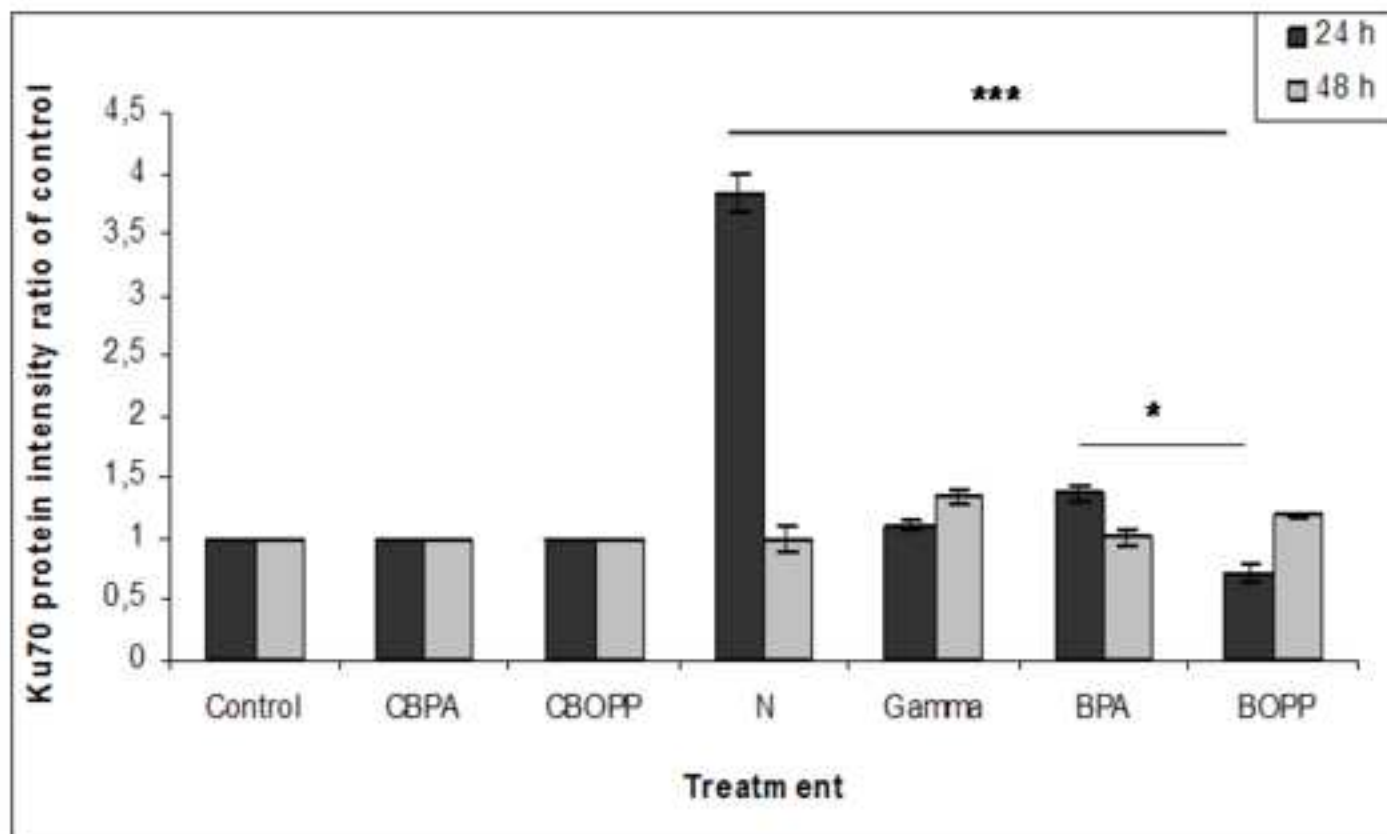
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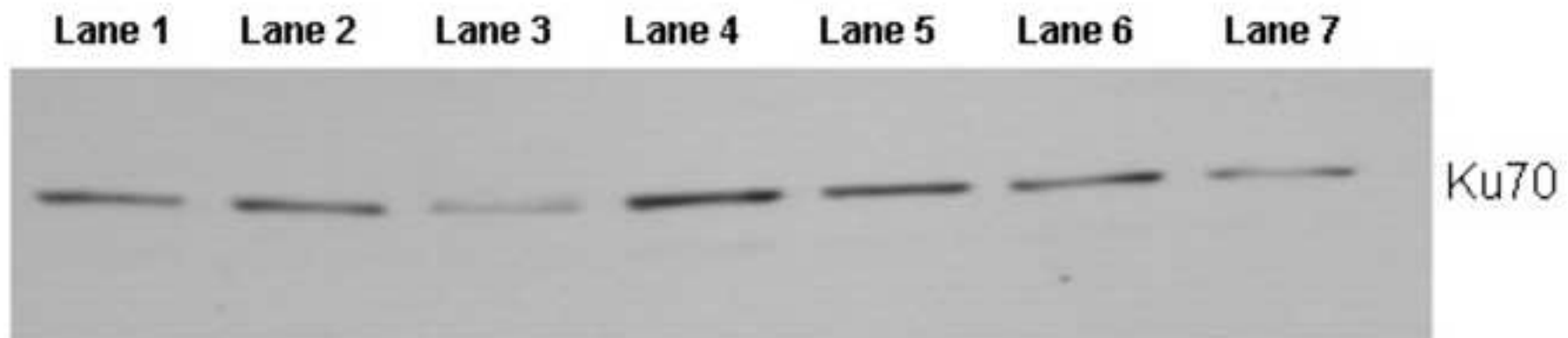
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