MMSL
Military Medical Science Letters

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2016, vol. 85(3), p. 104-110
ISSN 0372-7025
DOI: 10.31482/mmsl.2016.019

ORIGINAL ARTICLE

EXPRESSION OF p21 AND EXPRESSION AND ACTIVATION OF MAPK REGULATED TRANSCRIPTION FACTORS IN PERIPHERAL BLOOD MONONUCLEAR CELLS IN RATS AFTER WHOLE-BODY γ-RADIATION AND ITS USE IN BIODOSIMETRY

Jaroslav Pejchal[1]✉, Sarka Pruchova[2], Klara Kubelkova[3], Ales Tichy[2], Jirina Vavrova[2]

[1] Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
[2] Department of Radiobiology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
[3] Department of Molecular Pathology and Biology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic

Received 5th September 2016.
Revised 20th September 2016.
Published 27th September 2016.

Summary

The aim of our study was to examine the in vivo expression of p21 and expression and activation of ATF-2, c-Myc, and CREB in rat peripheral blood mononuclear cells (PBMC) after whole body γ-irradiation and to assess its contribution to biodosimetry. For Western blot experiments, male Wistar rats were whole-body irradiated by a single dose of 0, 0.5, 1, 3, and 5 Gy (^{60}Co, 1 m, 0.7 Gy/min). As a positive control, leukaemic MOLT-4 cells were used. For ELISA experiments, male Wistar rats were whole-body irradiated by a single dose of 0, 1, 2, 3, 4, and 5 Gy (^{60}Co, 1 m, 0.6 Gy/min). Blood samples were taken 4 h after the irradiation and PBMC were isolated using centrifugation on Histopaque-1077. Expressions of p21, ATF-2, phospho-ATF-2$^{Thr69/71}$, c-Myc, phospho-c-Myc$^{Thr-58/Ser62}$, CREB, and phospho-CREB^{Ser133} were measured using Western blot method. Expression of p21 was also quantified using ELISA. We observed increase of p21 expression in rat PBMC 4 h after irradiation. According to ELISA, p21 levels increased 2.0-, 3.1-, 5.5-, 3.0-, and 3.1fold after irradiation by 1, 2, 3, 4, and 5 Gy, respectively. We did not detect any expression or activation of ATF-2, c-Myc and CREB. Protein p21 could be considered as a perspective biodosimetric marker of clinically significant irradiation (≥1 Gy) in vivo in unstimulated PBMC.

Key words: p21; ATF-2; c-Myc; CREB; blood; rat; biodosimetry; Western blot; ELISA

✉ University of Defence, Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
jaroslav.pejchal@seznam.cz
☎ +420 973 253 216, +420 777 695 642,
+420 495 513 018

INTRODUCTION

Ionizing radiation causes wide damage to cellular components, which subsequently activates a variety of signalling pathways responsible for maintaining cellular homeostasis and promoting interactions with neighboring cells. Understanding these mechanisms

can provide tools to support effective treatment as well as triage of radiation casualties.

Our previous studies have turned our attention to protein p21 and MAPK signalling pathways [1, 2]. Protein p21 is a potent inhibitor of cell cycle progression. Its expression is regulated mainly by protein p53 and it exerts its function via inhibition of cyclin B1, cyclin/cyclin-dependent kinase complexes and proliferating cell nuclear antigen and by degradation of retinoblastoma protein [3, 4]. In response to ionizing radiation, a massive increase in p21 expression can be observed in non-proliferating (unstimulated) peripheral blood mononuclear cell (PBMC) 24 – 72 h after irradiation by 4 Gy [1].

MAPK signalling pathways are activated in response to many different stimuli including γ-radiation, UV radiation, DNA damaging reagents, osmotic shock, and oxidant stressors [5]. So far, three main groups of MAPK cascades have been identified: ERK pathway, preferably activated by mitogenic stimuli and p38 and JNK pathways regulated by environmental stresses [5, 6]. These cascades modulate function of a wide range of transcription factors, such as ATF-2, c-Myc and CREB, through which MAPK pathways adjust transcription fenotype to the stress stimulation [7]. After irradiation, MAPK and their transcription factors are activated/phosphorylated in tissue-, time- and dose-dependent manner [2, 8]. Low specificity may limit biodosimetric potential of MAPKs and MAPK regulated transcription factors. On the other hand, stress-activated kinases (p38 and JNK), ATF-2, c-Myc, and CREB are activated by very low doses (≤ 1 Gy) of ionizing radiation in intestine and liver and therefore, might seve as markers of low-dose irradiation [2, 8].

The aim of this study is to examine the *in vivo* expression of protein p21 and expression and activation of ATF-2, c-Myc, and CREB in rat peripheral blood mononuclear cells after whole body γ-irradiation and to assess its contribution to biodosimetry.

MATERIAL AND METHODS

Animals

Male Wistar rats aged 12–14 weeks weighing 220–250 g (Velaz, Unetice, Czech Republic) were kept in an air-conditioned room (22 ± 2°C, 50 ± 10% relative humidity, lights from 7:00 to 19:00 h) and allowed access to standard food ST-1 (Velaz) and tap water *ad libitum*. For Western blot experiments, the rats were divided into 5 groups, each group consisting of 6 animals. For ELISA experiments, 6 groups consisting of 6 animals were used. Experimental animals were handled in accredited facility (accreditation number: c.j. 25895/2010-17210) and approved by the Ethics Committee (Faculty of Military Health Sciences, Hradec Kralove, Czech Republic).

Positive control in Western blot experiments

As a positive control, the human T-lymphocyte leukemia cells MOLT-4 obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) were used. The cells were cultured in Iscove‘s modified Dulbecco‘s medium supplemented with 20% fetal calf serum, 0.05% L-glutamine, 150 UI/ml penicillin, 50 μg/ml streptomycin (all from Sigma-Aldrich, Prague, Czech Republic) in a humidified incubator (Galaxy 170R, New Brunswick Scientific, Enfield, CT, USA) at 37 °C and controlled 5% CO_2 atmosphere. The cultures were divided every second day by dilution to a concentration of 2×10^5 cells/ml. The cell counts were performed with a haemocytometer (Baxter Scientific, Markham, Ontario, Canada). The cell membrane integrity was determined by using the Trypan blue (Sigma-Aldrich) exclusion technique. Cell line in the maximal range of up to 20 passages was used for this study.

Irradiation

The animals were irradiated using a ^{60}Co unit (Chirana, Prague, Czech Republic) at a dose rate of 0.7 $Gy.min^{-1}$ (Western blot experiment) or 0.6 $Gy.min^{-1}$ (ELISA experiment) with a target distance of 1 m. Dosimetry was performed using an ionization chamber (Dosimeter PTW Unidos 1001, Serial No. 11057, with ionization chamber PTW TM 313, Serial No. 0012; RPD Inc., Albertville, MN, USA).

Experimental setup

The rats were placed in Plexiglass containers (FVZ, Hradec Kralove, Czech Republic) to assure homogeneous radiation back-front exposure and irradiated by a single dose of 0, 0.5, 1, 3, and 5 Gy and by a single dose of 0, 1, 2, 3, 4, and 5 Gy for Western blot experiments and ELISA experiments, respectively. Rats were humanely euthanized by cervical dislocation 4 h after irradiation. Rat blood was collected into 5 ml tubes (Vitrum, Prague, Czech Republic) heparinised with 0.2 ml 5 kIU heparine

(Zentiva, Prague, Czech Republic) and PMBC were isolated using Histopaque-1077 (Sigma-Aldrich, Prague, Czech Republic) according to the manufacturer´s protocol.

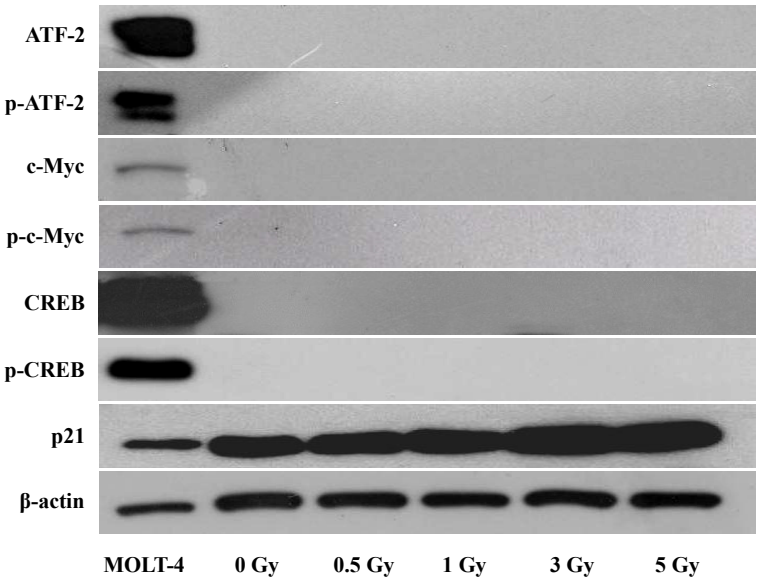
Western blot analysis (WB)

Both isolated PBMC and human MOLT-4 leukemia cells were washed with PBS (Sigma-Aldrich) and lysed. Whole-cell extracts were prepared by lysis in 500 µl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-glucopyranoside, 50 mM NaF, 20 mM Tris, pH 8, 1 mM Na_3VO_4; all from Sigma-Aldrich) and 1 tablet of CompleteTM Mini (Roche, Mannheim, Germany). The lysates containing equal amounts of protein (30 µg) were loaded onto a 12% sodium dodecyl sulphate polyacrylamide gel (Sigma-Aldrich). After electrophoresis (200 V; 50 min), the separated proteins were transferred (100 V; 120 min) to a polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA) and hybridized with antibodies specific for both human and rat proteins at 4°C overnight, including mouse monoclonal anti-p21 (1:500; ITA-interact, Prague, Czech Republic), rabbit monoclonal anti-ATF-2 (1:500; Biotech, Prague, Czech Republic), rabbit polyclonal anti-phospho-ATF-2$^{Thr69/71}$ (1:500; Biotech), mouse monoclonal anti-c-Myc (1:500; Biotech), mouse polyclonal anti-phosho-c-Myc$^{Thr58/Ser62}$ (1:500; Biotech), rabbit monoclonal anti-CREB (1:2,000; Biotech), rabbit monoclonal anti-phospho-CREB (1:500; Biotech), and mouse monoclonal anti-β-actin (1:10,000; Sigma-Aldrich). After washing, the blots were incubated with a secondary antibody. HRP-conjugated-anti-rabbit antibody (DakoCytomation, Prague, Czech Republic) was used for rabbit primary antibodies and biotin-conjugated-anti-mouse antibody (Spinchem, Pilzen, Czech Republic) was used for mouse primary antibodies.

ELISA

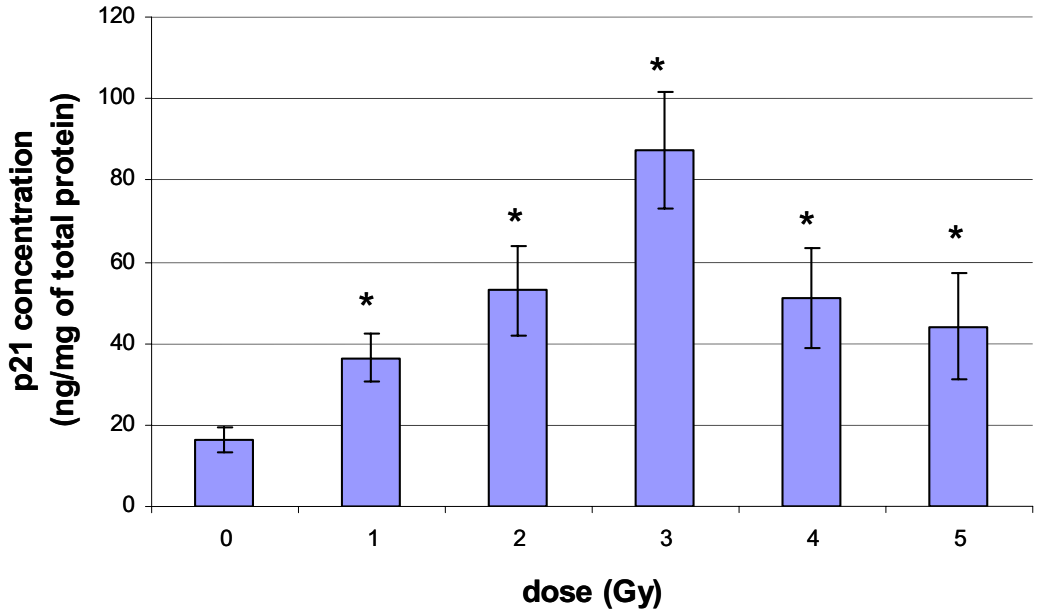
Based on Western blot results, expression of protein p21 was quantified by ELISA method from isolated PBMC. The whole-cell lysates were prepared by adding 500 µl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-glucopyranoside, 50 mM NaF, 20 mM Tris pH 8, 1 mM Na_3VO_4, 1 mM fenylmethylsulphonyl fluoride; all from Sigma-Aldrich). ELISA 96-well plates (Nunc, Langenselbold, Germany) were coated with monoclonal anti-rat-p21-1.1 antibody (50 µl per well, 20 µg/ml; Moravian Biotechnology, Brno, Czech Republic) diluted in coating buffer (12 mM Na_2CO_3, 88 mM $NaHCO_3$, pH 9, all from Sigma-Aldrich) by incubating

Figure 1. Expression of p21 and expression and activation of ATF-2, c-Myc and CREB in peripheral mononuclear blood cells collected from rats 4 hours after whole-body gamma irradiation



Representative Western blots are shown. Increased expression of p21 was found, whereas no expression nor activation of ATF-2, c-Myc and CREB was detected. MOLT-4 cells were used as a positive control.

Figure 2. Concentration of p21 ± 2 × SEM measured by ELISA method.



* statistically significant compared to control group: $p \leq 0.05$

overnight at 4°C. After washing with PBS, non-specific binding was blocked by incubation in blocking buffer [200 µl per well, 3% BSA (Sigma-Aldrich) in PBS] for 2 h at room temperature. Following washes, the plate was incubated with whole-cell lysates (50 µl per well, 1 mg/ml) or purified rat protein p21 HIS (for calibration; Moravian Biotechnology) for 2 h at 4oC. After washing, biotinylated monoclonal anti-rat-p21-118 (50 µl per well, 0.315 µg/ml; Moravian Biotechnology) diluted in PBS was added for 2 h at 4°C. Following further washes, Streptavidin HRP (Sigma-Aldrich) diluted 1:200 in PBS was added for 1 h at 4°C. After final washing, binding was visualized with 3,3', 5,5'-tetramethylbenzidine (Sigma-Aldrich) and immediately detected with a multidetector Magic XBC and Paradigm analysis software (both Beckman Coulter, Prague, Czech Republic). BSA background was subtracted to normalise values.

Statistical analysis

The values represent mean ± 2 × SEM (standard error of mean). The Mann-Whitney U test using SigmaStat 3.1 software (Systat Software Inc., Erkhart, Germany) was used for the statistical analysis. The differences were considered significant at the significance level $2\alpha = 0.05$.

RESULTS

Figure 1 shows the results of Western blotting experiments. In rat PMBC, we observed increased p21 expression after irradiation by 0.5, 1, 3, and 5 Gy. We did not measure any expression nor activation of ATF-2, c-Myc and CREB in control and irradiated PMBC.

ELISA data are in figure 2 showing that expression of protein p21 increased 2.0-, 3.1-, 5.5-, 3.0-, and 3.1fold after irradiation by 1, 2, 3, 4, and 5 Gy, respectively.

DISCUSSION

According to our results, p21 was the only protein detected in rat non-proliferating PBMC. Its expression significantly increased 4 h after irradiation, peaking at the dose of 3 Gy. After irradiation, p21 expression is usually induced by protein p53 [3]. Nevertheless, other members of p53 family (p63 and p73) and several miRNAs and mRNA-binding proteins could participate in p21 transcription and translation [3, 9]. Since p53 is not activated in unstimulated PBMC, p53-independent mechanism seems to regu-

late its expression in non-proliferating PBMC [1]. Protein p21 functions as a regulator of cell cycle progression at different steps in proliferating cells [3]. In G_0 cells, its role is most likely associated with its anti-apoptotic properties and/or DNA damage repair [10, 11]. From this point of view, our results may indicate that ionizing radiation-induced DNA damage is assessed as reparable up to the dose of 3 Gy in PBMC and decrease of p21 expression observed at the dose of 4 Gy might support induction of apoptosis. Mechanisms suppressing p21 could serve as markers of high-dose irradiation (≥ 4 Gy).

Our results also suggest two practical applications. Firstly, protein p21 is a sensitive marker of ionizing radiation. In the 4 hour interval, p21 concentrations range from 13.3 to 20.5 ng/mg and from 25.9 to 103.0 ng/mg of whole cell lysate in non-irradiated and irradiated (1 – 5 Gy) rat blood samples, respectively. The detection of p21 and values up to 20.5 – 25.9 ng/mg may therefore identify worried wells, i.e. non-irradiated casualties as well as victims irradiated by clinically insignificant dose (< 1 Gy). To determine the precise threshold, analysis of non-irradiated human blood and further animal *in vivo* testing will be necessary. Secondly, it is possible to estimate high-dose irradiation using clinical data. Particularly, onset of emesis in ≤ 1 h and body temperature ≥ 38ºC is suggestive of irradiation by the dose of ≥ 4 Gy [12]. After excluding such victims, following equations could be used to estimate radiation dose in the 0 – 3 Gy interval (figure 3).

$D\ [Gy] = (c_{p21}\ [ng/mg] - 16.353) / 21.838$
$Dmax\ (95\%\ upper\ confidence\ limit)\ [Gy] = (c_{p21}\ [ng/mg] - 18.915) / 25.036$
$Dmin\ (95\%\ lower\ confidence\ limit)\ [Gy] = (c_{p21}\ [ng/mg] - 13.792) / 18.641$

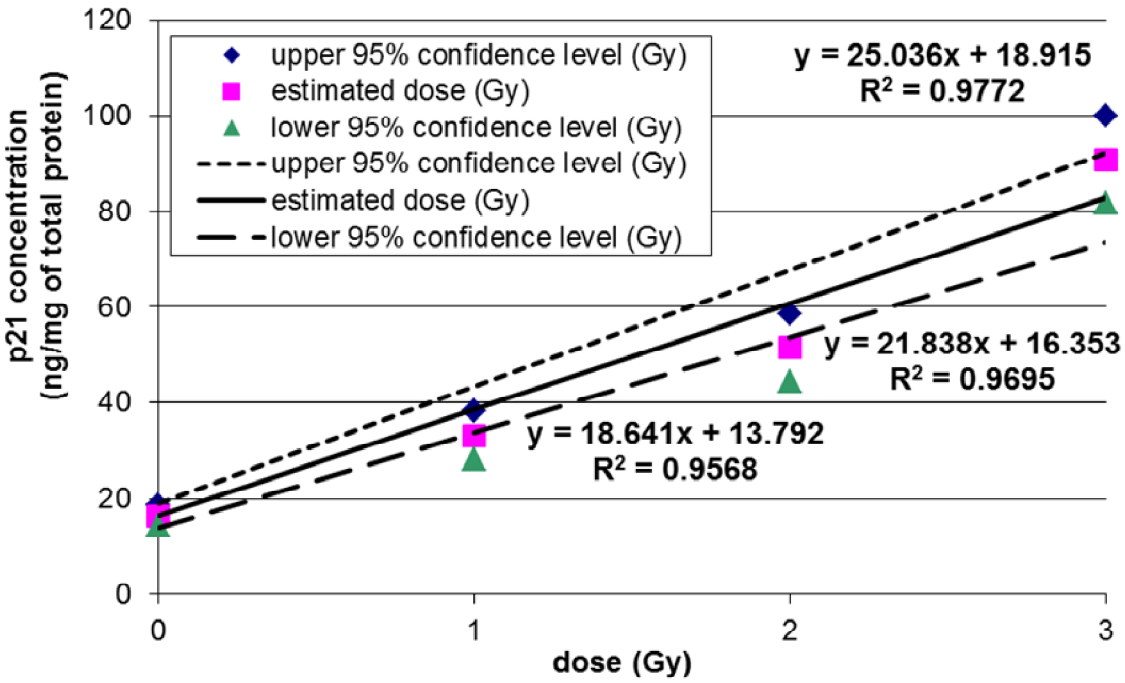


Figure 3. Regression and correlation analysis of 0 – 3 Gy data, where D is the estimated radiation dose and c_{p21} is the concentration of protein p21 measured 4 hours after irradiation.

However, in order to utilize p21 detection for biodosimetric purposes under field condi-tions, further experiments will have to be conducted, including (a) construction of calibration curves in the 24 and 48 h interval, to be able to cover large-scale accidents, (b) confirmation of calibration curves and derivated equations using human blood irradiated *in vitro* and/or blood collected from oncologic

patients undergoing total-body irradiation, (c) assessment of the influence of intrinsic factors (age, sex, *etc*.) on p21 expression, and (d) determination of sensitivity and specificity to C and B agents of operational significance. It is highly probable that radiospecificity will be impaired in infected individuals since stimulation of PBMC using phytohaemagglutinin stimulates p21 expression *in vitro* [1]. On the other hand, infections may also induce expression and activation of ATF-2, c-Myc, and CREB [13-18]. These markers could possibly help to reduce the risk of false p21 positivity in infected subjects.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Defence of the Czech Republic through a Long-term organisation development plan 1011.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Vilasová, Z.; Rezácová, M.; Vávrová, J.; Tichý, A.; Vokurková, D.; Zoelzer, F.; Reháková, Z.; Osterreicher, J.; Lukásová, E. Changes in phosphorylation of histone H2A.X and p53 in response of peripheral blood lymphocytes to gamma irradiation. *Acta Biochim. Pol.* **2008**, 55, 381-390.
2. Pejchal, J.; Osterreicher, J.; Vilasová, Z.; Tichý, A.; Vávrová, J. Expression of activated ATF-2, CREB and c-Myc in rat colon transversum after whole-body gamma-irradiation and its contribution to pathogenesis and biodosimetry. *Int. J. Radiat. Biol*. *2008*, 84, 315-324.
3. Jung, Y. S.; Qian, Y.; Chen, X. Examination of the expanding pathways for the regulation of p21 expression and activity. *Cell. Signal*. **2010**, 22, 1003-1012.
4. Broude, E.V.; Swift, M.E.; Vivo, C.; Chang, B.D.; Davis, B.M.; Kalurupalle, S.; Blagosklonny, M.V.; Roninson, I.B. p21(Waf1/Cip1/Sdi1) mediates retinoblastoma protein degradation. *Oncogene*. **2007**, 26, 6954-6958.
5. Kyriakis, J.M.; Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev*. **2001**, 81, 807-869.
6. Kyriakis, J.M.; Avruch, J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol. Rev*. **2012**, 92, 689-737.
7. Ferrer, I.; Blanco, R.; Carmona, M.; Puig, B.; Domínguez, I.; Viñals, F. Active, phosphorylation-dependent MAP kinases, MAPK/ERK, SAPK/JNK and p38, and specific transcription factor substrates are differentially expressed following systemic administration of kainic acid to the adult rat. *Acta Neuropathol*. **2002**, 103, 391-407.
8. Narang, H.; Krishna, M. Mitogen-activated protein kinases: specificity of response to dose of ionizing radiation in liver. *J. Radiat. Res*. **2004**, 45, 213-220.
9. De Laurenzi, V.; Melino, G. Evolution of functions within the p53/p63/p73 family. *Ann. N. Y. Acad. Sci*. **2000**, 926, 90-100.
10. Piccolo, M.T.; Crispi, S. The dual role played by p21 may influence the apoptotic or anti-apoptotic fate in cancer. *J. Can.Res. Updates*. **2012**, 1, 189-202.
11. Dutto, I.; Tillhon, M.; Prosperi, E. Assessing Cell Cycle Independent Function of the CDK Inhibitor p21(CDKN¹A) in DNA Repair. *Methods Mol. Biol*. **2016**, 1336, 123-139.
12. Fujimoto K. Final report of dose estimation for three victims of JCO accident. National Institute of Radiological Sciences, *Chiba*. **2002**, p 3.
13. Andrade, A.A.; Brasil, B.S.; Pereira, A.C.; Ferreira, P.C.; Kroon, E.G.; Bonjardim, C.A. Vaccinia virus regulates expression of p21WAF1/Cip1 in A431 cells. *Mem. Inst. Oswaldo Cruz*. **2010**, 105, 269-277.
14. Bandobashi, K.; Liu, A.; Nagy, N.; Kis, L.L.; Nishikawa, J.; Björkholm, M.; Klein, G.; Klein, E. EBV infection induces expression of the transcription factors ATF-2/c-Jun in B lymphocytes but not in B-CLL cells. *Virus Genes*. **2005**, 30, 323-330.
15. Dessauge, F.; Hilaly, S.; Baumgartner, M.; Blumen, B.; Werling, D.; Langsley, G. c-Myc activation by Theileria parasites promotes survival of infected B-lymphocytes. *Oncogene*. **2005**, 24, 1075-1083.
16. Li, S.; Bozzo, L.; Wu, Z.; Lu, W.; Romerio, F. The HIV-1 matrix protein p17 activates the transcription factors c-Myc and CREB in human B cells. *New Microbiol*. **2010**, 33, 13-24.

17. Samten, B.; Townsend, J.C.; Weis, S.E.; Bhoumik, A.; Klucar, P.; Shams, H.; Barnes, P.F. CREB, ATF, and AP-1 transcription factors regulate IFN-gamma secretion by human T cells in response to mycobacterial antigen. *J. Immunol*. **2008**, 181, 2056-2064.

18. Yu, Y.; Gong, R.; Mu, Y.; Chen, Y.; Zhu, C.; Sun, Z.; Chen, M.; Liu, Y.; Zhu, Y.; Wu, J. Hepatitis B virus induces a novel inflammation network involving three inflammatory factors, IL-29, IL-8, and cyclooxygenase-2. *J. Immunol*. **2011**, 187, 4844-4860.